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The Ethiopian Veterinary Journal (*Ethiop. Vet. J.*) is a multidisciplinary peer-reviewed journal intended to promote animal health and production of national and regional/international importance. The journal publishes review articles, original research articles, short communication as well as technical notes in English. Under special circumstances, articles in Amharic may be considered for publication.

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Knowledge, attitude, and practices (KAP) of University students regarding antimicrobial use (AMU) and antimicrobial resistance (AMR) in Ethiopia

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Abstract

A cross-sectional questionnaire-based study on 1252 University students was conducted in Ethiopia to assess their knowledge, attitude, and practices (KAP) towards antimicrobial usage (AMU) and antimicrobial resistance (AMR). Verbal consent was obtained from randomly selected students to participate in this study. A logistic regression analysis was used to assess the association between the socio-demographic profiles of the students against their knowledge, attitude, and practices toward antimicrobial resistance and antimicrobial usage. The Kruskal-Wallis and chi-square tests were used to examine how the median scores in each of the knowledge, attitude, and practice categories varied across study participants. A p-value of less than 0.05 was considered significant. The overall median AMR and AMU knowledge score was 13 (IQR=11, 14). The median AMR and AMU attitude score was 15 (IQR: 13, 15). The median score of AMU and AMR practice was 10 (IQR: 10, 11). Logistic regression analysis revealed that the students' birthplace was found to be a significant factor ($p < 0.01$) regarding the knowledge of students. The analysis further revealed that students' birthplace, field of studies, and good knowledge were significant factors ($p<0.01$) affecting their attitudes. Students with good knowledge had 3.9 times more positive attitudes than those with poor knowledge (OR = 3.9, CI = 3.0 -5.2, $p < 0.01$). Students from VM had 1.6 times better attitudes than students from HS and NHS (OR = 1.6, CI=1.2- 2.1, p = 0.002). Finally, students in the field of veterinary medicine and those having good knowledge had 1.4 and 0.5 times better practice than their counterparts (OR=1.4; CI=1.2, 2.1, $p<0.01$ and OR= 0.5, CI=0.4, 0.6, $p<0.01$, respectively). In conclusion, there were critical gaps in knowledge, attitudes, and practices

among University students regarding antimicrobial usage and antimicrobial resistance. Hence, students are encouraged to exhaustively utilize the digital era to advance their knowledge. Interventions to raise awareness should also target students majoring in fields other than health sciences.

Keywords*:* AMR and AMU; KAP; students; Ethiopia.

Introduction

Antimicrobial resistance (AMR) is a multifaceted global public health issue affecting both human and animal populations. Antibiotic resistance must be carefully monitored, yet in the vast majority of developing nations; it is minimal to nonexistent (WHO, 2014). Additionally, most developing countries lack the necessary quality control practices to ensure that the antibiotics given are of the highest quality (Obodozie *et al.,* 2006). Antibiotic overuse, underuse, and misuse will result in the emergence of antimicrobial resistance (Ventola, 2015) and manifest in an attempt to treat infectious diseases in humans and animals as well as when used as a growth promoter in animals (Robinson *et al*., 2016). In addition to other equally significant social and cultural factors, self-medication, erroneous prescription, inappropriate intake, and excessive use of these antimicrobial medications are the main causes of the development and spread of AMR. Antibiotic self-medication has emerged as a significant issue and a major contributor to antibiotic resistance. It can be caused by a variety of issues, including a lack of awareness regarding appropriate antibiotic usage policies, a lack of public understanding and attitude toward antibiotics, and easy access to antibiotics in many locations (Jairoun *et al*., 2019).

Sunusi *et al.* (2019) reported inappropriate antibiotic usage among college students is documented as a result of self-medication and inadequate antibacterial agent understanding; particularly, their indications, their pathogen specificity, and the adherence to dosing schedules. Taking antibiotics for a short period can lead to drug resistance development in pathogenic bacteria. Stopping a course of antibiotics early may lead to a relapse of illness for the patient and increase the risk of infected individuals passing drug-resistant pathogens to others. Some people stop taking prescribed antibiotics early due to unpleasant side effects because they are feeling better, or because they want to save the remaining antibiotics for later use or to share with others (Mallhi *et al*., 2019).

Future prescribers, such as human and animal health students, must be well prepared throughout their training to contribute to the prevention of AMR. As demonstrated previously, the information learned during training will aid in forming future prescribers' proper attitudes and views regarding the usage of antimicrobials and antimicrobial resistance (Heaton *et al*., 2008). Therefore, it is undeniable that educational interventions, particularly those at the undergraduate level are still essential and should receive special emphasis in the effort to combat antimicrobial resistance. If educational interventions are not properly managed, AMR will continue to be a significant public health issue; with microbes gaining resistance to almost all antimicrobials (Ross and Maxwell, 2012). Antibiotic misuse and the pace of resistance development are closely related, therefore, it is crucial to use antibiotics responsibly in both animal and human health systems to stop the emergence of resistance (Hockenhull *et al*., 2017).

Knowledge, attitude, and practice (KAP) surveys are representative of a specific population to collect information on what is known, believed, and done about a particular topic, and are the most frequently used study tool in health-seeking behavior research (WHO, 2008). Knowledge is usually assessed to see how far students' knowledge corresponds to biomedical concepts (Perring, 1994). Attitude has been defined as "a learned predisposition to think, feel and act in a particular way towards a given object or class of objects" (Ribeaux *et al*., 1978). As such, attitude is a product of a complex interaction of beliefs, feelings, and values. Practices in KAP surveys usually enquire about the use of antimicrobials. Finding out what the public knows, thinks, and does about using antimicrobials helps to plan effective AMR prevention initiatives. As far as we are aware, no research has been conducted in Ethiopia to highlight an in-depth KAP among wider University students. Hence, this study aims to ascertain KAPs toward antimicrobial usage and antimicrobial resistance among Ethiopian public University students.

Material and methods

Description of the study area

The study was carried out at purposely selected 10 public Universities located in different regional states of Ethiopia: University of Gondar (Northern), Metu, and Dambi Dolo Universities (Western), Jimma University (Southwest), Adama Science and Technology and Addis Ababa Universities (central Ethiopia),

Arbaminch, Wolaita Sodo, and Hawassa Universities (Southern), and Haramaya University (Eastern). The Universities were selected purposively based on their geographical location, accessibility, and willingness to participate in this study (Figure 1).

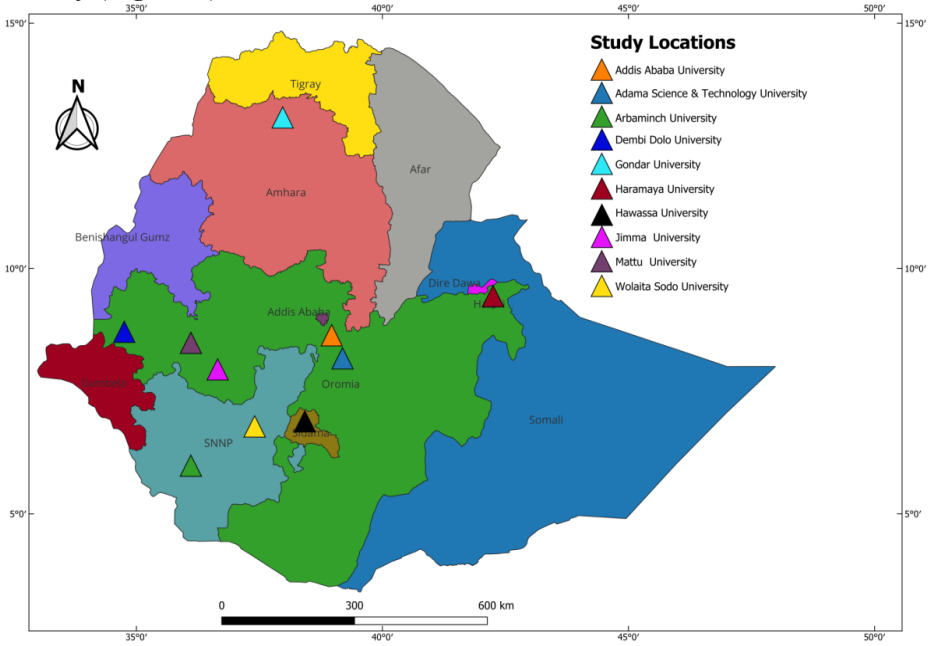


Figure 1. Map of the study area

Study design

A University-based cross-sectional survey was conducted among randomly selected students of human health-related sciences (medicine, pharmacy, nursing, public health, and midwifery), non-human health-related sciences (engineering, computational, social, and business and agriculture), and veterinary medicine students to assess their KAP towards antimicrobial usage and antimicrobial resistance.

Sample size determination

The calculation for the single population proportion was used to determine the sample size (Thrusfield, 2005) by using the following assumptions: a 95% level of confidence, a 5% margin of error, a 50% proportion of low knowledge,

and a 10% non-response rate. A 10% non-response rate was included in the minimum estimated sample size of 423*3=1269 (Bolarinwa, 2020). Of 1269 students, only 1,252 students filed the questionnaire for participation (the response rate was 98.7%).

Data collection

Based on a review of the literature, a structured questionnaire was developed and modified to address all of the major themes of the research issue of antimicrobial usage and antimicrobial resistance (Jairoun *et al*., 2019; Sylvia, 2019; WHO, 2015; Huang *et al*., 2014). The questionnaire's content, design, relevance, readability, and comprehension were then examined and evaluated by subject-matter experts. Ten students and 10 subject-matter specialists participated in pilot research to evaluate the validity and reliability of the questions. Data from the pilot study were not included in the findings but were used to make minor changes to the questions based on the review of the comments that were gathered.

Data management and analysis

Data were entered into a Microsoft Excel spreadsheet and the analysis was made using SPSS version 26.0. To summarize the data, descriptive statistics including frequency, percentages, and median (IQR) were generated. Each respondent's responses to questions on their knowledge, attitude, and practices received a score. Participants received a score of "0" for each question with a 'No' response and a score of "1" for each question with a 'Yes' response for all knowledge, attitude, and practice questions. Following the addition of all the scores, those with scores equal to and above the median were classified as having "good" knowledge, a 'positive' attitude, and 'good' practice, respectively (Kassahun and Mekonen, 2017). The Kruskal-Wallis test and chi-square tests were used to examine how the median scores in each of the knowledge, attitude, and practice categories varied across study participants. To identify the factors that are associated with good knowledge, positive attitudes, and good practices of antimicrobial usage and antimicrobial resistance, multivariate logistic regression was used in conjunction with the independent variables of socio-demographic profiles (Fetensa *et al*., 2020). In the final model, variables with a p-value of 0.05 or lower were deemed significant.

Ethics approval and consent to participate

This research was reviewed and approved by the ethical committees of Addis Ababa University, College of Health Science, IRB (Protocol No.: 031/21), and the University of Liverpool (Reference No.: 9935). A verbal consent was obtained from the students and thereafter they were requested to finish a self-administered survey during their free time.

Results

Socio-demographic profiles of participants

Based on their fields of study, students were divided into three groups: 33.0% (n = 413) from human health-related sciences (HS) (medicine, pharmacy, nursing, public health, midwifery, and biomedical sciences); 27.9% (n = 349) from non-health related sciences (NHS) (engineering, computational, social, business administration, agriculture, arts, and education); and the remaining 39.1% (490) were majoring in veterinary medicine (VM). Overall, more than half of the participants 760 (60.7%) were males, and the mean age of the respondents was 22.31 ± 1.4 years.

Knowledge of antimicrobial usage and antimicrobial resistance

The overall median antimicrobial usage and antimicrobial resistance knowledge score was 13 (IQR=11, 14). Table, 1 shows Ethiopian public University students' knowledge of antimicrobial resistance and antimicrobial usage. The percentage of students in the VM, HS, and NHS fields of studies who correctly answered antimicrobial resistance and antimicrobial usage knowledge questions was 72.0%, 70.0%, and 15.8%, respectively. When asked if they had heard of antimicrobial resistance, 83.3%, 82.9%, and 72.2% of HS, VM, and NHS students responded that they had heard and are aware of antimicrobial resistance ($p<0.01$). A large proportion of students from HS (79.9%), NHS (55.6%), and VM (62.0%) stated that treatment of sick animals should be performed by both human and animal health professionals ($p<0.01$). The interesting point is that 89.2%, 70.0%, and 66.2% of VM, HS, and NHS students correctly answered that antibiotics should be purchased from drug stores or pharmacies based on a prescription ($p<0.01$).

Table 1. Summary of some of the knowledge about antimicrobial usage and antimicrobial resistance among University students in Ethiopia (n= 1252)*

Questions regarding AMU and AMR knowledge	Study discipline	No n (%)	Yes n (%)	X^2	*P value*
Microbes can become resistant to antimicrobials	Human health sciences Non-health science Veterinary medicine	28 (6.8) 199 (57.0) 49 (10.0)	385 (93.2) 150 (43.0) 441 (90.0)	345.8	<0.01
Are there bacteria in the human body that are good for our health?	Human health sciences Non-health science Veterinary medicine	16 (3.9) 152 (43.6) 154 (31.4)	397 (96.1) 197 (56.4) 336 (68.6)	169.6	<0.01
Can antibiotics be used to cure infections caused by viruses?	Human health sciences Non-health science Veterinary medicine	132 (32.0) 213 (61.0) 95 (19.4)	281(68.0) 136 (39.0) 395 (80.6)	157.8	<0.01
Antibiotics shouldn't be obtainable without a prescription at pharmacies.	Human health sciences Non-health science Veterinary medicine	245 (59.3) 206 (59.0) 181 (36.9)	168 (40.7) 143 (41.0) 309 (63.1)	59.1	<0.01
Have you heard of antimicrobial resistance?	Human health sciences Non-health science Veterinary medicine	69 (16.7) 97(27.8) 84 (17.1)	344 (83.3) 252 (72.2) 406 (82.9)	18.6	<0.01
Frequent use of antimicrobials will lead to a decrease in the number of treatment options	Human health sciences Non-health science Veterinary medicine	88 (21.3) 94 (26.9) 66 (13.5)	325 (78.7) 255 (73.1) 424 (86.5)	24.1	<0.01
Is efficacy better if the antibiotics are newer and the price is higher?	Human health sciences Non-health science Veterinary medicine	171 (41.4) 157 (45.0) 183 (37.3)	242 (58.6) 192 (55.0) 307 (62.7)	5.0	0.08
Amoxicillin is an antibiotic?	Human health sciences Non-health science Veterinary medicine	38 (9.2) 119 (34.1) 40 (8.2)	375 (90.8) 230 (65.9) 450 (91.8)	123.2	<0.01
Penicillin is an antibiotic	Human health sciences Non-health science Veterinary medicine	19 (4.6) 67 (19.2) 33 (6.7)	394 (95.4) 282 (80.8) 457 (93.3)	54.0	<0.01
Tetracycline is an antibiotic	Human health sciences Non-health science Veterinary medicine	33 (80.0) 85 (24.4) 60 (12.2)	380 (92.0) 264 (75.6) 430 (87.8)	44.1	<0.01

Questions regarding AMU and AMR knowledge	Study discipline	No n (%)	Yes n (%)	χ^2	*P value*
Bacteria that developed resistance in animals can become resistant in humans through the consumption of food of animal origin	Human health sciences Non-health science Veterinary medicine	216 (52.3) 174 (49.9) 100 (20.4)	197 (47.7) 175 (50.1) 390 (79.6)	119.3	<0.01
Treatment of sick animals should be carried out by both human and animal health professionals	Human health sciences Non-health science Veterinary medicine	83 (20.1) 155 (44.4) 186 (38.0)	330 (79.9) 194 (55.6) 304 (62.0)	56.0	<0.01
Antibiotics should be purchased from drug stores or pharmacies based on prescription	Human health sciences Non-health science Veterinary medicine	121 (22.3) 118 (33.8) 53 (10.8)	292 (70.0) 231 (66.2) 437 (89.2)	72.6	<0.001
It is good to use antimicrobials as additives with animal feeds	Human health sciences Non-health science Veterinary medicine	103 (24.9) 197 (56.4) 235 (48.0)	310 (75.1) 152 (43.6) 255 (52.0)	85.7	<0.01
Antimicrobials used to treat animals can remain within their tissues	Human health sciences Non-health science Veterinary medicine	265 (64.2) 213 (61.0) 156 (31.8)	148 (35.8) 136 (39.0) 334 (68.2)	114.6	<0.01

**' Yes or no' is exclusively the response of the respondents*

Attitudes on antimicrobial resistance and antimicrobial usage

Students were given various statements about their attitudes toward the use of antimicrobials and antimicrobial resistance and asked how many of them they thought were correct or incorrect (Table 2). The overall median antimicrobial usage and antimicrobial resistance attitudes score was 15 (IQR: 13, 15). A higher proportion of students from veterinary medicine (95.9%), human health-related sciences (89.0%), and non-human health-related sciences (NHS) (83.1%) agreed that antimicrobial resistance affects animal health and production (χ^2=38.2, p = 0.01). When students were asked about the inappropriate use of antibiotics, human health-related science students have a positive feeling that inappropriate use of antimicrobials is one of the reasons for the occurrence of antimicrobial resistance (93.5%), followed by veterinary medicine (92.0%), and non-health-sciences (84.5%) (χ^2=31.6, p< 0.01).

Table 2. Summary of the attitude of University students towards AMU and AMR in Ethiopia (n= 1252)*

Questions regarding attitude towards AMU and AMR	Study discipline	No n (%)	Yes n (%)	X^2	*p-value*
Antimicrobial resistance will affect your and your family's health	Human health sciences Non-health science Veterinary medicine	92 (22.3) 60 (17.2) 35 (7.1)	321 (77.7) 289 (82.8) 455 (92.9)	42.3	<0.01
Antimicrobial resistance will affect animal health and production	Human health sciences Non-health science Veterinary medicine	45 (10.9) 59 (16.9) 20 (4.1)	368 (89.1) 290 (83.1) 470 (95.9)	38.2	<0.01
I have sufficient knowledge of antibiotic use	Human health sciences Non-health science Veterinary medicine	300 (76.3) 211 (60.5) 307 (62.7)	102(24.7) 138 (39.5) 183 (37.3)	22.7	<0.01
It is necessary to give more education to clinical-level students about antimicrobial resistance	Human health sciences Non-health science Veterinary medicine	39 (9.4) 54 (15.5) 39 (8.0)	374 (90.6) 295 (84.5) 451 (92.0)	13.3	<0.01
Inappropriate use of antimicrobials causes antimicrobial resistance	Human health sciences Non-health science Veterinary medicine	27 (6.5) 59 (16.9) 32 (6.5)	386 (93.5) 290 (83.1) 458 (93.5)	31.6	<0.01
Poor infection control practices by healthcare professionals will cause the spread of antimicrobial resistance	Human health sciences Non-health science Veterinary medicine	33 (8.0) 34 (9.7) 58 (11.8)	380 (92.0) 315 (90.3) 432 (88.2)	3.7	0.16
Clinical-level students should get special training on the appropriate ways of prescribing antimicrobials before graduation	Human health sciences Non-health science Veterinary medicine	99 (24.0) 63 (18.1) 63 (12.9)	314 (76.0) 286 (81.9) 427 (87.1)	19.0	<0.01
Currently, antimicrobial resistance is a major problem in the world as well as in Ethiopia	Human health sciences Non-health science Veterinary medicine	46 (11.1) 46 (13.2) 25 (5.1)	367 (88.9) 303 (86.8) 465 (84.9)	18.1	<0.01
Prescribing antibiotics should be more closely controlled	Human health sciences Non-health science Veterinary medicine	29 (7.0) 68 (19.5) 37 (7.6)	384 (93.0) 281(80.5) 453 (92.4)	39.0	<0.01
Dispensing antibiotics without prescription or over-the-counter should be more closely controlled	Human health sciences Non-health science Veterinary medicine	49 (11.9) 92 (26.4) 98 (20.0)	364 (88.1) 257 (73.6) 392 (80.0)	25.8	<0.01

**'Yes or no' is exclusively the response of the respondents*

Practice on AMU and AMR

Students were given various statement practices about AMU and AMR and asked how many of them responded as 'yes' or 'no' (Table 3). The overall median AMU and AMR practice score was 10 (IQR: 10, 11). A higher proportion of NHS (81.1%), HS (72.2%), and VM (62.0%) students stated that they terminate antibiotics as soon as their complaints subside (χ^2=32.3, p<0.01). A higher proportion of HS students (71.9%) testified that they use antibiotics without a doctor's prescription, as do (66.8%) of NHS and 48.6% of VM (χ^2=57.5, p<0.01). Overall, a large proportion of students reported using antibiotics to treat a common cold (69.4%), sore throat (76.1%), when coughing up yellow-green (71.7%), and flu (76.4%).

Table 3. Summary of practice towards AMU and AMR among University students in Ethiopia (n= 1252)*.

Questions regarding practice towards AMU and AMR	Study discipline	No, n (%)	Yes, n (%)	X^2	P value
I stop antibiotics as soon as complaints lessen	Human health sciences	115 (27.8)	298 (72.2)	36.3	<0.01
	Non-health science	66 (18.9)	283 (81.1)		
	Veterinary medicine	186 (38.0)	304 (62.0)		
I use antibiotics prescribed by doctors	Human health sciences	15 (3.6)	394 (95.4)	3.2	0.20
	Non-health science	14 (4.0)	335 (96.0)		
	Veterinary medicine	10 (2.0)	479 (98.0)		
I follow the prescription after choosing an antibiotic	Human health sciences	32 (7.7)	381(92.3)	0.6	0.74
	Non-health science	23 (6.6)	326(93.4)		
	Veterinary medicine	32 (6.5)	458(93.5)		
I use antibiotics without the doctor's instructions	Human health sciences	116 (28.1)	297 (71.9)	57.5	<0.01
	Non-health science	116 (33.2)	233 (66.8)		
	Veterinary medicine	252 (51.4)	238 (48.6)		
I ask the doctor to prescribe antibiotics for the common cold	Human health sciences	214 (51.8)	214 (51.8)	68.1	<0.01
	Non-health science	267 (76.5)	267 (76.5)		
	Veterinary medicine	362 (73.9)	362 (73.9)		
I use antibiotics for the common cold	Human health sciences	173 (41.9)	240 (58.1)	37.1	<0.01
	Non-health science	89 (25.5)	260 (74.5)		
	Veterinary medicine	121 (24.7)	369 (75.3)		
I use antibiotics for acute bronchitis	Human health sciences	130 (31.5)	283 (68.5)	22.6	<0.01
	Non-health science	114 (32.7)	235 (67.3)		
	Veterinary medicine	97 (19.8)	393 (81.0)		
I use antibiotics when coughing up yellow/ green phlegm	Human health sciences	158 (38.3)	255 (61.7)	31.1	<0.01
	Non-health science	92 (26.4)	257 (73.6)		
	Veterinary medicine	104 (21.2)	386 (78.8)		
I use antibiotics for a sore throat caused by any microbe	Human health sciences	105 (25.4)	308 (74.6)	29.0	<0.01
	Non-health science	113 (32.4)	236 (67.6)		
	Veterinary medicine	81 (16.5)	409 (83.5)		
I use antibiotics for flu with a fever	Human health sciences	259 (62.7)	154 (37.3)	64.1	<0.01
	Non-health science	288 (82.5)	61 (17.5)		
	Veterinary medicine	409 (83.5)	81 (16.5)		

' Yes or no' is exclusively the response of the respondents

Factors associated with good knowledge, positive attitude, and practice on AMR and AMU

Multivariable logistic regression analysis revealed that students' fields of study were closely related to their level of good knowledge. Students from the field of HS had 18.4 times (OR = 18.4, CI =12.1 - 28.0; p<0.01) and those from VM had 12.7 (OR = 16.8, CI = 11.2 – 25.2, p<0.01) times better knowledge of AMU and AMR compared to students from NHS field of studies. Most of the demographic variables were significantly related to attitudes toward AMU and AMR. Moreover, students having good knowledge had 3.9 times more positive attitudes than students with poor knowledge (OR = 3.9, CI = 3.0 -5.2, p<0.01) (Table 4).

Table 4. Multivariable logistic regression analysis of factors associated with AMU and AMR among University students in Ethiopia (n= 1252)*.

Variables	Good knowledge			Positive attitude			Good practices		
	n (%) aOR (95% CI)	p-value	n (%)	aOR (95% CI)	p-value	n (%)	aOR (95% CI)	p-value	
Birth place									
Town	265 (67.9)	Ref		282 (72.3)	Ref		237 (60.8)	Ref	
Rural	593 (91.0)	0.5 (0.4,0.7)	<0.01	541 (62.8)	0.5 (0.4,0.7)	<0. 01	587 (68.1)	0.5 (0.4,0.7)	<0.01
Gender									
Male	511 (67.2)	Ref		516 (67.9	Ref		532 (70.0)	Ref	
Female	347 (70.5)	1.0 (0.8,1.3)	0.20	307 (62.4))	1.8 (1.4,2.3)	<0.01	292 (59.3)	1.8 (1.4,2.3)	<0.01
Year of Study									
3rd	281 (73.6)	Ref		244 (63.9)	Ref		242 (63.4)	Ref	
4th	317 (65.9)	1.4 (1.0, 2.0)	0.05	334 (69.4)	1.0 (0.7,1.3)	0.98	313 (65.1)	1.0 (0.7,1.3)	0.98
5th	260 (90.0)	1.2 (0.9,1.6)	0.43	245 (63.0)	1.3 (1.0, 1.3)	0.60	269 (69.2)	1.3 (1.0,1.7)	0.10
Field of study									

Variables	Good knowledge			Positive attitude			Good practices		
	n (%) aOR (95% CI)	p-value	n (%)	aOR (95% CI)	p-value	n (%)	aOR (95% CI)	p-value	
NHS	101 (28.9)	Ref		186 (53.3)	Ref		230 (66.0)	Ref	
HS	417 (85.1)	18.4 (12.1,28.0)	<0.01	384 (78.4)	0.9 (0.6, 1.2)	0.34	364 (74.3)	0.9 (0.6, 1.2)	0.4
VM	340 (82.3)	16.8 (11.2,25.2)	<0.01	253 (61.3)	1.6 (1.2,2.1)	<0.01	230 (55.7)	1.4 (1.2, 2.1)	<0.01
				Knowledge					
Poor	0(0)			176 (44.7)	Ref		286 (72.6)	Ref	
Good	858(100)			647 (75.4)	3.9 (3.0, 5.2)	<0.01	538 (62.7)	0.5 (0.4, 0.6)	<0.01
				Attitude					
Negative	211 (49.2)	Ref			Ref		315 (73.4)		
Positive	647 (78.6)						509 (61.8)	0.6 (0.5,0.8)	<0.01

**P-values were obtained from regression models. AMR: antimicrobial resistance, OR: odds ratio, CI: confidence interval, aOR: adjusted odds ratio, na: not applicable, NHS: non-health science, HS: human health science, VM: veterinary medicine*

Discussion

Overall, this study showed critical gaps in knowledge and practices among University students regarding AMU and AMR. Accordingly, it is only 15.8% of non-health science students correctly answered knowledge questions about AMU and AMR. Students in the health-related fields (both human and animal health) had better knowledge (higher percentages of correct answers) in almost all knowledge-related questions, and they also had positive attitudes toward AMU and AMR (higher percentages of correct answers in all attitude-related questions). This study revealed that the field of studies has an impact on the level of knowledge among students. Our finding showed that 90.7% of students irrespective of their field of studies were aware that antibiotics are used to treat bacterial infections. This result is consistent with Jairoun *et al.* (2019). However, Chang *et al.* (2021) and Pogurschi *et al.* (2022) have reported that only 49.0% and 22.2% of the respondents claimed that antibiotics kill bacteria, respectively. Hence, our result is very encouraging that almost all students are aware that antibiotics kill bacteria. Strikingly, 80.6% of VM students reported that viral infections can be treated with antibiotics. Pogurschi *et al.* (2022) reported that antibiotics are ineffective against viruses, however, 37.5% of respondents believe that antibiotics treat viral infections. Similar confusions have been reported by previous researchers (Alqarni and Abdulbari, 2019; Yusef *et al.,* 2018). Seid and Hussen (2018) reported that approximately 28.0% of the participants believed that antibiotics could kill both viruses and bacteria. Our findings were consistent with a study conducted in Portugal, in which more than 60.0% of participants agreed that antibiotics should be prescribed for viral illnesses (Azevedo *et al.*, 2009). Encouraging results were also recorded in this study that a large proportion of students had heard about AMR, and frequent use of antibiotics reduces treatment efficacy antibiotics. Jairoun *et al.* (2019) reported that a large proportion of respondents have similar agreements. This implies that a rigorous assessment of University students' knowledge would aid in the development of an educational program.

In terms of attitude, approximately 63.4% of students have correctly responded to questions regarding antimicrobial resistance and antimicrobial usage. Similar results (66.0%) were reported from India (Sadasivam *et al.,* 2016). This result, however, was lower than the report of Seid and Hussen (2018) in which 96.0% of students had a positive attitude toward antimicrobial resistance. The majority of students (85.1%) also stated that improper antibiotic usage can harm their families. Similarly, Seid and Hussen (2018) found that 90.7% of

the students agreed that their health and the health of their families will be impacted by antibiotic resistance. The vast majority of students (80.8%) irrespective of their field of studies agreed that dispensing antibiotics without a prescription should be strictly regulated. However, only a few students (26.0%) had not received instructions on how to take antibiotics or the importance of completing the entire duration of the treatment. It is worth noting that those who did not seek advice are more likely to discontinue antibiotics when they feel better, resulting in the spread of resistance in the community. Similar studies in Ethiopia and India found that 82.4% (Seid and Hussen, 2018) and 65.0% (Sadasivam *et al*., 2016) of participants believed that antibiotics should never be purchased as over-the-counter drugs.

In terms of practice, nearly 54.6% of students have correctly answered AMR and AMU practice questions; however, the appropriate use of antibiotics was not demonstrated in their daily lives. This is demonstrated by the proportion of students (70.7%) who reportedly stopped using antibiotics after symptom improvement; of which 72.2% (HS), 81.1% (NHS), and 62.0% (VM) students feel similarly. Previous studies in the UAE (Jairoun *et al*., 2019) (44.5%), Pakistan (Limaye *et al*., 2019) (28%), and Lebanon (Mouhieddine *et al*., 2015) (51.5%) found similar results. The tendency of stopping antibiotics after symptom improvement might be related to self-medication. Self-medication could be a potential factor in the medication's discontinuance. Medical students appear to disregard proper antibiotic use, which sets a poor example for the general public (Sylvia, 2019). Antibiotics were among the top three medications used for self-medication among undergraduate healthcare students in Nigeria, who believed they had acquired the necessary medical knowledge of what to use for a specific condition treated (Akande-Sholabi and Ajamu, 2021). However, Akande-Sholabi and Ajamu (2021) reported that the majority of participants (91.0%) always completed the entire course of treatment. Saving money on expensive consultation fees and the inconvenience of making a doctor's appointment for a mild illness are two possible justifications for storing antibiotics for potential respiratory illnesses and using leftover antibiotics.

This study also showed critical gaps in AMR and AMU practice among students; a large proportion of students 69.4%, 76.1%, 71.7%, and 76.4%, respectively, claimed using antibiotics to treat the common cold, or sore throat, when coughing up yellow-green, and flu. Similar studies in Nigeria and Rwanda found that 50.0% and 20.0% of study participants had used antibiotics to treat common colds and sore throats, respectively (Akande-Sholabi and Ajamu,

2021; Nisabwe *et al*., 2020). This inappropriate use of antibiotics could be attributed to a lack of knowledge about antimicrobial use. A systematic review of antibiotic use for the common cold and acute purulent rhinitis, on the other hand, found that antibiotics have no benefit for the common cold, sore throat, and cough (Kenealy and Arroll, 2013). Moreover, a majority of students (61.3%) reported using antibiotics without a doctor's prescription. In a similar study from Nigeria, however, 86% of participants always consulted a doctor before beginning an antibiotic regimen. Nonetheless, only 65.0% agreed that antibiotics should never be purchased as over-the-counter drugs, and doctors take the time to explain the dos' and don'ts of antibiotics in detail. According to Kalungia *et al*. (2019) and Fadare *et al*. (2019), reinforcing antimicrobial stewardship in all health facilities would raise antimicrobial resistance awareness among healthcare professionals.

This study also revealed that 61.3% of the students claimed to have obtained antibiotics without a prescription from vendors or drug stores. Reynolds and Rodin (2009) supplemented our finding that due to relatively lax antibiotic regulations, it is not difficult to obtain antibiotics without a proper prescription (over-the-counter acquisition) in China. Personal observation also showed that purchasing antibiotics without a prescription is a common phenomenon in Ethiopia. This indicates a lack of stringent enforcement of laws and regulations governing how antibiotics are prescribed and dispensed in retail pharmacies. Lukovic *et al*. (2014) revealed that the prevalence of non-prescription drug use is quite high and common among medical students. Personal observation also revealed that there is an indiscriminate abuse of prescription drugs for non-prescription drugs among several pharmaceutical shops in different areas of the country. As far as someone can afford the prices, several pharmaceutical shops can sell them to their customers. Alnasser *et al*. (2021) stressed that the public should be educated on the effectiveness of these medications and how they should be used to reduce AMR. Age, educational level, family attitudes, drug manufacturer advertising, laws governing the dispensing and sale of pharmaceuticals, prior experiences with the symptoms or condition, and significance attached to the disease are factors that have been found to influence how often people self-medicate (Zhu *et al*., 2016), prescription medicines kept at home (Klemenc-Ketis and Kersnik, 2010) and financial circumstance of responders (James *et al*., 2006). It has to be noted that there is a risk of overprescribing antibiotics in retail pharmacies and among vendors if antimicrobial susceptibility tests are not performed. Hence, the findings of this study support strict enforcement of laws and regulations governing how antibiotics are

prescribed and dispensed in retail pharmacies. In conclusion, this study has demonstrated there were critical gaps regarding antimicrobial use and antimicrobial resistance among University students. Hence, students are encouraged to use additional online training platforms to advance their knowledge. Moreover, interventions to raise awareness should also target students majoring in fields other than health sciences is recommended.

Limitations of the study

As the measurements were cross-sectional, we cannot document causal relationships. Moreover, the survey was a self-administered questionnaire; thus, the use of antimicrobials was self-reported, and the frequency of antimicrobial use may be overestimated or underestimated due to recall bias. The results obtained in this study were dependent on the recall ability and honesty of the respondents and hence, underreporting or overreporting could not be ruled out.

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Staphylococcus aureus isolates from cow's milk in dairy farms at Shinshicho town, Kembata Tembaro Zone, Southern Ethiopia: Prevalence, risk factors, and antimicrobial susceptibility profile.

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Abstract

Bovine mastitis is an economically important and highly prevalent infectious disease in dairy herds worldwide. *Staphylococcus aureus* is a common microorganism causing infectious mastitis. A cross-sectional study was conducted between December 2018 and September 2019 in Shinshicho town, Kembata Tembaro Zone, Southern Ethiopia, to estimate the prevalence, and assess associated risk factors and antimicrobial susceptibility profile of *S. aureus* isolates from cow's milk in dairy farms at Shinshicho town. Lactating dairy cows were screened for mastitis based on clinical examinations and the California mastitis test (CMT) followed by laboratory identification of *S. aureus*. All the *S. aureus* isolates were subjected to antimicrobial susceptibility tests using a disk diffusion test. Multivariable logistic regression analysis of the effect of different risk factors on the prevalence of mastitis was performed. From a total of 384 lactating cows examined and tested, 41.7 % (n=160) were found positive for mastitis. Out of the occurrences of mastitis, 5% (n=19) and 36.7% (n=141) were clinical and subclinical respectively. *Staphylococcus aureus* was isolated from 36.84% (n=7) and 39.01% (n=55) of the clinical and subclinical mastitis respectively with a total isolation frequency of 38.75 % (n=62). According to the results of this study, greater herd sizes (OR=2.91, 95% CI: 1.62-5.21), higher parity cows (OR=3.91, 95% CI: 1.73-8.82), late lactation stage (OR=3.36, 95% CI: 1.27-8.91), and muddy floor (OR=2.37, 95% CL: 1.31-4.27) are risk variables linked to the occurrence of *S. aureus* mastitis. In addition, *S. aureus* has total

resistance to ampicillin, amoxicillin, penicillin-G, and Polymyxin. Similarly, 53.2% of the isolates proved resistant to three or more of the antibiotics used. Therefore, regular antimicrobial susceptibility testing should be performed to select potent modified antibiotics, and the effects and dynamics of genetic determinants of antibiotics should also be studied using molecular methods.
Keywords: Antimicrobial susceptibility; Milk; Prevalence; Risk factors; Southern Ethiopia; *S. aureus*.

Introduction

Ethiopia has the largest livestock population in Africa, estimated at 70 million cattle (CSA, 2020/21). Cows represent the biggest portion of the country's cattle population with around 21.4 % of the total cattle heads being milking cows (CSA, 2020/21). However, milk production often does not meet national requirements due to the low genetic potential for milk production of indigenous breeds, the extensive and low inputs of animal husbandry practices under which they are reared, and widespread livestock diseases (Ahmed *et al*., 2004). Mastitis is one of the factors contributing to reduced milk production (Biffa *et al*., 2005). Several etiological agents cause mastitis of which *S. aureus* is a major pathogen that also poses food safety and antimicrobial resistance threats (Kumar *et al.,* 2011).

Staphylococcus aureus is the most predominant cause of mastitis in dairy cows in Ethiopia (Seid *et al*., 2015). The Bacterial contamination of milk from an infected udder may render it unsuitable for human consumption by causing food poisoning or providing a vehicle for the spread of zoonotic diseases to humans (Bitew *et al*., 2010). In Ethiopia, *Staphylococcus* species isolated from animal-origin food in central and southeastern parts of the country indicate the presence of an alarming level of resistance isolates (Beyene *et al*., 2016; Tessema *et al*., 2016). Infection with antimicrobial-resistant bacteria has been known to be associated with frequent treatment failure and increased severity of the disease (Finch and Hunter, 2006). Resistant bacteria from food animals may be passed through the food chain to humans resulting in resistant infections (Anderson *et al*., 2003).

Recently, the same studies were conducted on isolation, identification, and drug resistance patterns of *S. aureus* in lactating dairy cows milk in various parts of Ethiopia, such as in Kombolcha town (Tassew *et al*., 2016), Wolaita Sodo (Derib *et al*., 2017), Assosa Town (Tassew *et al*., 2017), and Asella Town

(Seyoum *et al*., 2018). However, the information on the prevalence, risk factors, and antimicrobial susceptibility profile of *S. aureus* isolates from mastitic lactating cow's milk in Shinshicho town is lacking. Therefore, the objectives of this study were:

- To isolate, characterize and estimate the prevalence of *S. aureus* in mastitic lactating cow's milk
- To assess different potential risk factors associated with the prevalence of *S. aureus* and to investigate the antimicrobial susceptibility profiles of the isolates.

Materials and methods

Description of the study area

The study was conducted in Shinshicho town, Kembata Tembaro Zone, Southern Nation Nationalities, and Peoples Regional State, Ethiopia (Figure 1). It is located at a distance of 293 km South of Addis Ababa at 7°12′N and37°46′E with an altitude of 1875 meters above sea level (Fig.1). The annual mean rainfall ranges from1200 to 1500 milmiters with an average minimum and maximum annual temperature of 18°C and 31°C respectively. It has two administrative Kebeles with an estimated human population of 55,563 of which 25025 males and 30538 were females (STAFEDO, 2018). The land coverage of the study area is 1543 hectares, and the agricultural production system is mixed farming. In the study area, dairy farms typically employed intensive system to manage their improved crossbreed animals. They are often provided with a supplementary diet in addition to the natural pasture and agricultural by-products. The livestock population of the town is estimated to be 11739 cattle, 3035 sheep, 3870 goats, 39100 poultry, and 1450 equines (STAFEDO, 2018).

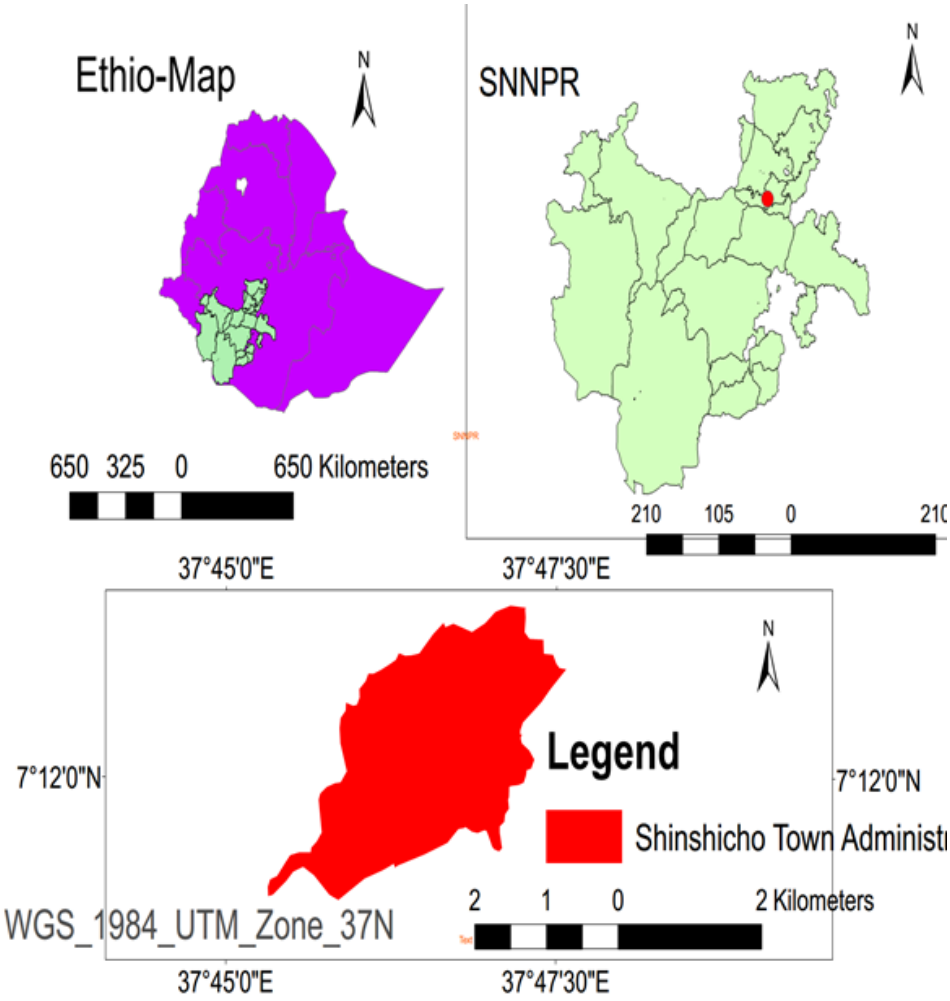


Figure 1. Location map of the study area. Source: (Ethiopia GIS Data Base 1994)

Study design

A cross-sectional study design was conducted between December 2018 and September 2019 in Shinshicho town.

Study animals

The study animals were lactating dairy cows in Shinshicho town (Holstein-Friesian, Jersey, and Zebu cross breeds) in the selected smallholder dairy farms kept under intensive management system. The animals were kept indoor and received concentrated feed in addition to hay and crop residues (such as corn stalks, wheat/barley straw, and other leftovers from grain threshing. The age of the study animals was determined based on birth records, and dentation characteristics and categorized as young (< 4 years), adult (4 to 8 years), and old (>8 years) as described by Torell *et al*. (2003). Parity numbers were categorized as low (1 and 2 calves), moderate (3 and 4 calves), and many (>4 calves). The lactation stage was grouped as early (≤2 months), medium (>2 to 6 months), and late (>6 months). The floor system of the dairy house was classified as cemented and soil or mud types (Tassew *et al*., 2016). The hygienic condition of milking is arranged as good and poor depending upon washing and drying the udder of lactating cows before milking. Previous treatment for mammary gland infection was rated yes or no (Abera *et al*., 2010).

Sample size determination and sampling procedures

The sample size of the study was determined as described by Thrusfield (2018), with a 95% confidence interval and 5% desired absolute precision. Since there was no previous study conducted in the area, the expected prevalence of 50% was assumed and this corresponds to the required minimum sample size by the formula given as follows.

$$n = \frac{1.96^2 pexp\ (1\text{-}pexp)}{d^2}$$

Where: -n=required sample size;
Pexp =expected prevalence;
d2 = desired absolute precision

Therefore, the calculated sample size was 384 lactating dairy cows.
According to Mekonnen *et al.* (2018), the sampling frame of the study area indicated that the farms were smallholder dairy farms having an average of two to three lactating cows each. About 250 smallholder dairy farms with total dairy cows of 674 (199 Holstein-Friesian, 255 Jersey, and 220 Zebu-cross breed) were registered in the Shinshicho town administrative agricultural office. Thus, 146 smallholder dairy farms having a minimum of two and a maximum of five lactating cows were purposely selected based on the availability of lactating dairy cows and the willingness of the owners to participate in the study. Then

focusing on the sampling frame in the smallholder dairy farm, each breed of lactating cow was sampled by simple random sampling techniques using a lottery method. Accordingly, 152 Holstein-Friesian, 117 Jersey, and 115 Zebu-cross cows were sampled.

Questionnaire survey

A semi-structured questionnaire was developed and the data based on the study objective was recorded. Data regarding the different potential risk factors were assessed for each of the 384 lactating cows based on observation and by interviewing the farm owners. The cow's level factors such as breed (Holstein Friesian, Jersey, or Zebu-cross), age (young, adult, or old), parity (few, moderate, or many), lactation stage (early, medium, or late), and pregnancy (pregnant or non-pregnant) were recorded. The farm-level factors such as herd size (≤5 animals or >5 animals), floor types (soil or concreted); previous mastitis infection (present or absent), dry cow therapy (yes or no), and use of the towel (yes or no) were recorded. The hygiene level of milking (good or poor) was assessed by evaluating the hygiene of the udder, which was scored separately according to the methodology outlined by Schreiner and Ruegg (2002). Udder and milk abnormalities (injuries, swelling, milk clots, and abnormal secretion) were also recorded.

Clinical examination and California Mastitis Test (CMT)

Each selected lactating cow was screened for mastitis based on clinical examinations and the California Mastitis Test. Clinical examination of the udder was based on the method previously indicated (Radostits *et al*., 2007). The clinical findings considered include abnormalities of the secretion, abnormalities of the udder and teat, and systemic reaction. The California Mastitis Test was performed to detect subclinical mastitis according to the procedures described by the National Mastitis Council (NMC, 2004). Subclinical mastitis was screened by taking 2 mL of milk sample from each quarter placed into a plastic paddle by adding an equal amount of the CMT reagent to the milk. The paddle was rotated to mix the contents. Positive samples showed gel formation within a few seconds. The CMT result was scored as negative if there was no gel formation and recorded as zero (0) or positive if there was gel formation ranging from trace (T), weakly positive (+1), distinct positive (+2) and strongly positive (+3) as per the recommendation given by NMC (2004). Hence, the cow was considered subclinical mastitis positive if one or more quarters were CMT positive with or without isolation of the microorganisms (Quinn *et al*., 1994).

Milk sample collection

The milk samples were taken from mastitis-positive cows that were not treated with antibiotics recently or currently, and collected according to the earlier protocol (Quinn *et al.*, 2004). Briefly: quarters were thoroughly washed with tap water and wiped dry. The teat ends were then cleaned with cotton soaked with 75% ethyl alcohol and 8-10ml of milk was collected aseptically into a sterile test tube after discarding the first three streams of milk. Finally, milk samples were transported in an icebox with icepacks to the Wolaita Sodo Regional Veterinary Laboratory for bacteriological examination. The milk samples were immediately inoculated in bacteriological media or kept at 4°C overnight until cultured in standard bacteriological media (Quinn *et al.*, 2004).

Bacteriological examination of milk samples

The milk samples were bacteriologically examined according to the procedures employed by Quinn *et al.* (2004). Mastitic positive quarters samples were inoculated separately onto sterile blood agar plates (Himedia, India) enriched with 5% heparinized sheep blood and incubated at 37°C for 24-48 hours under aerobic culture conditions. The plates were examined for the presence of *Staphylococcus* colonies. The identification of bacteria in primary culture as *Staphylococcus* was done according to the procedures described in Quinn *et al.*, 2004. When growth was not observed after incubation for 24 to 48 hours, the milk sample was re-inoculated with enriched tryptone soya broth (Himedia, India) to amplify the bacterial growth. The plates were examined for growth, colony morphology features such as golden, and yellow color, and ß- hemolysis in blood agar within 24-48 hours. A presumptive colony of *Staphylococcus* was selected and subcultured on nutrient agar (Himedia, India) and incubated aerobically at 37°C for 24-48 hours to get a pure culture. After this incubation on nutrient agar, bacteria were identified according to their Gram reaction; morphology, catalase test, and oxidase test (Quinn *et al.,* 2002). *Staphylococcus aureus* was identified by the coagulase test, hemolysis, pigment production (golden yellow), Mannitol, and maltose fermentation (Quinn *et al.*, 2004). Samples were considered positive for *S. aureus* when at least one colony was identified as *S. aureus*.

Antimicrobial susceptibility testing

The *S. aureus* isolates were subjected to antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method on Mueller Hinton agar following

the procedures described by Quinn *et al.* (2011) and CLSI (2016). Antimicrobials of animal health significance were taken into consideration. The following antibiotics discs (Himedia, India) with their corresponding concentration were used for testing: Chloramphenicol (30µg), Gentamicin (10µg), Kanamycin (30µg), Streptomycin (10µg), Cephalothin (30µg), Polymyxin-B (PB 300units), Ampicillin (10µg), Amoxicillin (20µg), Penicillin-G (10 units) and Tetracycline (30µg). In brief: the isolate was inoculated in tryptone soya broth (TSB) and incubated at 37°C for 24 hours. The turbidity of the suspension was adjusted to obtain turbidity visually comparable with that of 0.5 McFarland standards. Muller-Hinton Agar plate (Oxoid, England) was prepared and a sterile cotton swab was dipped into the suspension and swabbed on the surface of the Muller-Hinton agar plate. 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°C under aerobic conditions. The diameter of the zone of complete inhibition was measured in millimeters, interpreted, and classified according to procedures established by CLSI (2016) as susceptible, intermediate, or resistant. Moreover, isolates showing resistance to three or more antimicrobial subclasses were considered multiple drug-resistant (Rota *et al*., 1996).

Data management and statistical analysis

The data was recorded in the Microsoft Excel Spreadsheet 2010 and coded before statistical analysis. All the statistical analysis was performed using Stata 14 statistical software (Stata Corp, 4905 Lake Way Drive, and College Station, Texas 77845 USA). The prevalence of *S. aureus* was calculated as the proportion of *S. aureus*-positive cows (clinical and subclinical) divided into the total number of animals examined. A cow was denoted as positive for mastitis if at least a single teat with clinical mastitis or a CMT-positive result was detected. Accordingly, descriptive statistics such as percentages and frequency distribution were used to describe/present bacterial isolates and antimicrobial susceptibility which was expressed as a percent of susceptible and resistant. In addition, the proportions of bacteria resistant to three or more antimicrobial subclasses were calculated. The association between the dependent variable, *S. aureus* (0=negative and 1=positive), and categorical independent variables were assessed using multivariable logistic regression analysis. The independent variables evaluated were breed, age, parity, lactation stages, previous mastitis treatment, herd size, floor type, milking hygiene, previous infection, and use of a towel for udder cleaning and drying. All independent variables

with p-value <0.25 in the initial univariable logistic regression analysis were checked for multicollinearity using Goodman and Kruskal gamma statistics and those variables whose gamma value ranging between −0.6 and +0.6 were considered in a multivariable logistic regression analysis (Dohoo *et al*., 2009). The final model was built on a backward elimination procedure of the log-likelihood ratio. Values were considered significant at p< 0.05.

Results

Prevalence of *S. aureus*

A total of 384 lactating cows were examined, and the prevalence of mastitis was 41.7% (160/384). Among them, 19 cases (5%) were clinical mastitis, and 141 cases (36.7%) were subclinical mastitis. Milk samples from 160 CMT-positive cows were subjected to microbiological examination, and *S. aureus* was isolated from 7 (36.84%), and 55 (39.01%) of the clinical and subclinical cases, respectively. The overall prevalence of *S. aureus* in this study was recorded to be 62 (38.75%). There was a statistically significant difference (p<0.05) in the prevalence of *S. aureus* between clinical and subclinical mastitis (Table 1).

Table1. Prevalence of *Staphylococcus aureus* in sub-clinical and clinical mastitis

Forms of mastitis	No. of animal's positive	No.of *S. aureus* isolated	Prevalence	χ^2	P-values
Subclinical	141	55	39.01	10300.57	≤ 0.01
Clinical	19	7	36.84		
Total	160	62	38.75		

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

Among the risk factors examined in initial univariable logistic regression, age was withdrawn from further analysis in consideration of collinearity with parity but parity as a risk factor was retained, due to its important implications regarding mastitis based on biological plausibility. Also, the milking hygiene was eliminated from further analysis because of collinearity with floor types of husbandry systems. Likewise, previous mastitis was removed from further analysis due to collinearity with herd sizes, and the use of towels was excluded from further analysis because of collinearity with dry cow's therapy. Therefore, the variables considered for further analysis with multivariable logistic regression were parity, lactation stages, previous infection treatment, herd size, floor

type, and dry cow therapy. Thus, the results revealed that infection with *S. aureus* was more likely to occur in cows with higher parities than cows with few parities (OR=3.91, 95% CI: 1.73-8.82), and cows in the late lactation stage were more likely to be positive to *S. aureus* than cows in early lactation stage (OR=3.36, 95% CI: 1.27-8.91) (Table 2).

Table 2. Multivariate logistic regression analysis of cow's level associated risk factors with the prevalence of *S. aureus* isolates

Risk factors	Category	No. of exa mined	Prevalence of *S. aureus* (%)	Crude odds ratio (95%CI)	Adjusted odds ratio (95%CI)	P-values
Parity in calves	Few (1 and 2)	45	13 (28.9)	1	1	
	Moderate (3&4)	76	29 (38.2)	1.59 (0.79-3.20)	1.88 (0.90-3.94)	0.10
	Many (>4)	39	20 (51.3)	3.25 (1.50-7.04)	3.91(1.73-8.82)	0.00
lactation stage in months	Early (1 & 2)	26	8 (30.8)	1	1	
	Medium (3- 6)	103	40 (38.8)	2.53 (1,14-5.61)	2.65 (1.17-6.02)	0.02
	Late (>6)	31	14 (45.2)	3.00 (1.14-7.87)	3.36 (1.27-8.91)	0.01
previous infection treatment	Yes	79	11 (13.9)	1	1	
	No	81	51 (62.9)	1.56 (0.77-3.14)	1.90 (0.90-4.02)	0.09

1=Reference, CI=Confidence interval

The dairy farms with a larger herd size of more than 5 animals were more likely to be positive for *S. aureus* than small herd sizes less than or equal to 5 animals (OR=2.91, 95% CI: 1.62-5.21), and soil or muddy floor housing were more likely to be positive than cemented floor housing (OR=2.37, 95% CI: 1.31-4.27) (Table 3).

Table3. Multivariate logistic regression analysis of farms level associated risk factors with *S. aureus* isolates

Risk factors	Category	No. of Examined	Prevalence of *S. aureus* (%)	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)	P-values
Herd size	≤5animals	98	24(24.5)	1	1	
	>5animals	62	38(61.3)	2.81(1.61-4.91)	2.91(1.62-5.21)	0.00
Floor types	Concreted	104	34(32.7)	1	1	
	Soil	56	28(50)	2.45(1.40-4.28)	2.37(1.31-4.27)	0.00
Dry cow therapy	Yes	85	11(13)	1	1	
	No	75	51 (68)	0.26(0.13-0.52)	0.27(0.13-0.53)	0.00

1=Reference, CI=Confidence interval

Antimicrobial susceptibility testing

A total of 62 isolates of *S. aureus* were tested against ten antimicrobial agents following CLSI guidelines. From 62 isolates of *S. aureus* showed 95.2% susceptibility to Chloramphenicol, similarly susceptiblities to Gentamicin (91.9%), Cephalothin (90.3%), Kanamycin (88.7%) and Streptomycin (80.6%) were also recorded. However, 100% of the isolates were resistant to Penicillin-G, Polymyxin, Amoxicillin, and Ampicillin while resistance ot Tetracycline was 80.6% (Table 4).

Table4. Antimicrobial susceptibility patterns of *S. aureus* isolates (n = 62).

Antimicrobial agents	Inhibition zone diameter by millimeters																											
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	>29
AMX	0	0	33	6	7	4	5	3	1	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AMP	0	50	6	0	4	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CEP	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	1	2	4	6	17	5	8	6	8	2	0	0	0
CHR	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	5	4	13	5	16	9	7	0	0	0
GEN	0	0	0	0	0	0	0	0	0	1	0	1	3	0	4	5	7	16	6	5	3	6	5	0	0	0	0	0
KAN	0	0	0	0	0	0	0	1	0	1	0	2	1	0	1	0	1	7	10	19	6	13	0	0	0	0	0	0
PG	0	62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PLY	0	62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
STM	0	0	0	2	0	0	1	2	0	1	2	1	2	1	3	4	22	11	1	9	0	0	0	0	0	0	0	0
TTC	0	0	15	11	18	6	0	0	0	0	0	0	0	0	2	1	0	0	2	3	4	0	0	0	0	0	0	0

The black color shaded represents zone diameters in the intermediate and breakpoints range; the gray and light color shaded represent zone diameters in the resistant and susceptibility ranges respectively. AMX=Amoxicillin, AMP= Ampicillin, CEP= Cephalothin, CHR=Chloramphenicol, GEN= Gentamicin, KAN = Kanamycin PG=Penicillin, PLY=Polymyxin, STM=Streptomycin and TTC=Tetracycline.

Multiple antibiotic-resistant phenotypes of *S. aureus* isolates

Multiple antibiotic-resistant phenotypes were determined for the *S. aureus* isolates as depicted in Table 5. Out of the total *S. aureus* isolates recovered from the study area, 53.2 % of the isolates developed multiple antibiotic-resistant phenotypes. Among all multiple antibiotic-resistant phenotypes of *S. aureus* isolates, 24.2% of them were resistant to five or six antibiotics, and 6.4% of the isolates were resistant to seven or eight antibiotics. The percentages of the resistant phenotype were calculated by dividing the number of particular antimicrobial-resistant phenotypes by the total number of isolates identified in the study area.

Table 5. The predominant MAR phenotypes for *S. aureus* isolated from mastitic milk (N=62)

Phenotype	Number observed	Percentage
AMP-PG-PLY	3	4.8
AMX-PG-PLY	2	3.2
AMP-AMX-PG-PLY	6	9.7
AMX-PG-PLY-TTC	3	4.8
AMP-AMX-PG-PLY-TTC	9	14.5
AMP-AMX-KAN-PG-PLY	2	3.2
AMP-AMX-PG-PLY-STM-TTC	3	4.8
AMP-AMX-CHR-PG-PLY-TTC	1	1.6
AMP-AMX-CEP-PG-PLY-STM-TTC	2	3.2
AMP-AMX-KANA-PG-PLY-STM-TTC	1	1.6
AMP-AMX-CEP-GEN-KAN-PG-PLY-TTC	1	1.6

AMP=Ampicillin, AMX=Amoxicillin, PG=Penicillin, PLY=Polymyxin, CEP=Cephalothin, CHR=Chloramphenicol, GEN=Gentamicin, KAN=Kanamycin, TTC=Tetracycline and STM=Streptomycin.

Discussion

The present study showed a 38.75% overall prevalence of *S. aureus* in the mastitic cows in the study area, which is in agreement with the 39.1% prevalence reported from Asella, Oromia Regional state, Ethiopia (Bedada *et al.*, 2009), but lower when compared with the reports of Abera *et al.*, 2010; Kemal *et al.*, 2017; and Seyoum *et al.*, 2018 who reported 42.1% in Adama town and 44.62% and 46.5% *S. aureus* isolates in the town of Asella. The variable prevalence of *S. aureus* in cows with mastitis 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 procedures and contact with animals (Radostits *et al.*, 2007).

Multivariable logistic regression analysis revealed that the prevalence of *S. aureus* isolates was significantly different among higher parity groups. A significant association of higher parity with the prevalence of *S. aureus* isolates was reported by other authors (Seyoum *et al.*, 2018) in and around Asella town Ethiopia. In the present study, cows with higher parity had 3.91 times more risk of infction 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 (Schukken *et al.*, 2011). Higher parity, or the number of times a cow has calved, is inversely associated with cure rates, possibly due to the mammary gland's large volume of specialized secretory cells and supporting tissues, which hinder antibiotic penetration and infection clearance. (Macias and Hinck, 2012).

The current result revealed that cows in late lactation stages were 3.36 times more likely infected as compared with early stages. This result was found consistent with the previous reports of Nibret *et al.* (2012), and Garedew *et al.* (2015) in Hawassa and Addis Ababa towns, respectively. Nevertheless, the report from the Southern part of Ethiopia indicates a higher prevalence in the early stage of lactation which disagrees with this study's results (Kerro and Tareke, 2003; Biffa *et al.,* 2005). The current result might be because chronic mastitis, most often subclinical, is more frequent later during lactation. *S. aureus* is a predominant cause of subclinical mastitis (Radostits *et al.*, 2007).

A significantly higher risk ($p<0.05$) was observed in the herd size of more than 5 animals (OR=2.91, 95% CI: 1.62, 5.21), than in small herd sizes of less than or equal to 5 animals. This result agreed with the finding of Kemal *et al*. (2017) who reported higher risk in the larger herd size from Asella town, Ethiopia. This is due to *S. aureus* having adapted to survive in the udder; known by their contagious nature and are shed in the milk which serves as a source of infection for other healthy cows during the milking process. It is generally observed that larger herds are characterized by increased stocking density and increased risk of exposure to infection (Radostits *et al*., 2007).

The present study revealed that dairy farms with muddy floors were 2.37 times more likely to be infected with *S. aureus* than cemented floor housing systems. The association between soil floor, and the high prevalence of *S. aureus* revealed in this finding agreed with the result of Kemal *et al*. (2017) who reported from Asella town, Arisi Zone, Eastern Ethiopia. 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 mastitis pathogens.

In vitro, antimicrobial susceptibility tests of *S. aureus* isolates revealed that the highest rate of susceptibility among the isolates was recorded against Chloramphenicol, Gentamicin, Cephalothin, Kanamycin, and Streptomycin. The results agree with the finding of Abera *et al*.(2010) who reported susceptibility to *S. aureus* to Chloramphenicol, Gentamicin, Kanamycin, and Streptomycin, Thaker *et al*. (2013) who reported the isolates sensitive to Cephalothin, and Gentamicin, Kemal *et al*. (2017) who reported the isolates susceptible to Chloramphenicol and Gentamicin, and Beyene (2016) who reported the *S. aureus* susceptible to Chloramphenicol Streptomycin and Gentamicin.

On the other hand, *S. aureus* isolates showed the highest resistance to Penicillin-G (100%), Polymyxin-B (100%), Amoxicillin (100%), Ampicillin (100%), and Tetracycline (80.6%). The current investigation was in agreement with the report of Tsige (2018) who reported the resistance of *S. aureus* to Penicillin (100%), Ampicillin (100%), Amoxicillin-clavulanic (82.7%), and Tetracycline (60.49%) in Arsi Negelle, Ethiopia. Moreover, the present report was comparable with the result of Lencho (2015) in Ambo and Gudar town recording 100% for both Penicillin and Ampicillin followed by 90% for Amoxicillin. This is supported by the findings of Tassew *et al*. (2016) from Kombolcha who reported *S. aureus* resistant to penicillin (100%), amoxicillin (100%), and tetracycline

(77.4%). Similarly, *S. aureus* resistance to penicillin (100%), and amoxicillin (100%) were reported from Bangladesh (Jahan *et al*., 2015). The variability in susceptibility results could partly arise from how frequently a drug was in use for dairy cow treatments 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 (Huber *et al*., 2011). Resistant to penicillin-G is used as a marker to assess the susceptibility of *S.aureus* isolates against other ß -lactam antibiotics (Waage *et al*., 2002; Pace *et al*., 2006). In the study area, penicillin was the drug of choice for the therapy of intramammary infections, such that frequent and often inadequate use of these medications has probably contributed to the emergence of resistant bacteria in the herds. A similar finding was reported by Kemal *et al*. (2017) that the resistance of *S. aureus* to penicillin and ampicillin may be attributed to the production of beta-lactamase, an enzyme that inactivates penicillin and closely related antibiotics. It is believed that around 50% of mastitis-causing *S. aureus* strains produce beta-lactamase and there is evidence that these strains are more difficult to cure with all antibiotics (Green and Bradley, 2004).

The current study has demonstrated the existence of alarming levels of resistance to *S. aureus* to commonly used antimicrobials (including penicillin-G, amoxicillin, and tetracycline) in the study farms. Reports from Argentina suggested a possible development of resistance from prolonged and indiscriminate usage of the same antimicrobial (Gentilini, 2000; Edward *et al*., 2002). Therefore, it is important to implement a regular application of an in-vitro antimicrobial susceptibility test before the use of antimicrobials in both therapeutic and prophylactic Staphylococcal mastitis infections. Antibiotic-resistant *S. aureus* isolates pose a challenge to both animal and public health (Kashoma *et al*., 2015)

Multiple antibiotic-resistant in this study was relatively high. Thus, 53.2 % of the isolates develop multiple antibiotic-resistant phenotypes. This result was in line with the findings of Mekuria *et al*. (2013) who reported from Addis Ababa, 50.6%, and Kashoma *et al*. (2015) who reported 43.2% from Tanzania. This result was higher than the finding of Mohammed (2015) who reported 26.09% of multiple drug-resistant *S. aureus* isolated from cow milk in Tanzania. 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

including mastitis. Multiple antibiotic-resistant *S. aureus* strains have been isolated from milk obtained from dairy animals in many parts of the world (Waage *et al*., 2002; Shitandi and Sternesjo, 2004; Ateba et *al*., 2010). The prevalence of antibiotic resistance in *S. aureus* usually varies between isolates from different sample stations and even between isolates from different herds and/or flocks of the same farm (Pace and Guang, 2006). Besides, *S. aureus* has developed multidrug resistance in many regions of the world and the usage of antibiotics correlates with the emergence of antibiotic-resistant traits (Pesavento *et al*., 2007).

Conclusions

The present study revealed that multiple antibiotics-resistant *S. aureus* isolates are prevalent in dairy farms in the study area. Parity, lactation stages, herd size, and floor type were risk factors significantly related to *S. aureus* prevalence. It was observed that *S. aureus* isolates were highly sensitive to Chloramphenicol, Gentamicin, Cephalothin, Kanamycin, and Streptomycin. Whereas, the highest rate of resistance among the isolates was against Penicillin-G, Amoxicillin, Ampicillin, and Tetracycline. *Staphylococcus aureus* from mastitic cows showed multiple antibiotic resistance to a great extent to commonly used antibiotics, ensuring that the right use of antibiotics of choice is very important in the line of treatment and control of the infections caused by *S. aureus*. Hence, regular antimicrobial sensitivity tests to select effective and alteration of antibiotics must be carried out, and the impacts and dynamics of genetic antibiotic determinants should also be investigated using molecular methods.

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Disclosure

The authors report there is no conflict of interest.

Author contributions

Conceptualization, A.M., F.B., Y.A. T.T.A.M., F.B., Y.A. T.T.; analysis, A.M., F.B.; investigation and Lab analysis, A.M.; writing—original draft preparation, A.M.; writing—review and editing, A.M., F.B., Y.A. T.T. All authors have read and agreed to the published version of the manuscript.

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Assessment of dairy farm's hygienic practice and knowledge of farm workers on milk-borne zoonoses in three selected towns of the Wolaita Zone, Southern Ethiopia

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Abstract

Consumption of unhygienic milk is the most common source of milk-borne zoonotic diseases. These zoonoses have public health importance and are a major obstacle to trade in livestock and livestock products. A cross-sectional questionnaire-based study was conducted from December 2021 to June 2022 to assess milk-borne zoonotic diseases, the habit of milk consumption, and the hygienic practices of dairy farm workers in three purposefully selected towns in the Wolaita zone, southern Ethiopia. Dairy farms and farm workers were selected by a simple random sampling technique. A total of 100 respondents, one per farm, were selected and participated in the interview. The result indicated that 41% of farms used individual towels and 21% used common towels to dry their cows' udders; however, the remaining 38% of farms did not use any towels at all. The majority (59%) of farms clean the floor once a day and around 92% remove dung manually. Of total farm workers, 43% had no formal education, 28% had primary-level education, 5% had secondary-level education, 2% were college diploma holders, and 22% were first-degree and above graduate workers. Concerning milk consumption habits, 64% of respondents used raw milk, 29% used raw and boiled milk, 3% of interviewees consumed all types of milk (raw, boiled, refrigerated, and processed milk), and 4% didn't drink milk at all. Furthermore, 50% of respondents were aware of disease transmission through the consumption of raw milk. Regarding respondents' knowledge of milk-borne zoonoses, 51% of interviewees didn't know about zoonotic diseases, while the remaining 4% knew about tuberculosis, 32% were aware of salmonellosis, 5% knew about both tuberculosis and salmonellosis, and 8% were aware of tuberculosis, anthrax, mastitis, salmonellosis, and brucellosis. Concerning the knowledge of respondents on disease transmission, 73% of respondents didn't know that zoonotic diseases can transmit from humans to animals and vice versa. Furthermore, when compared to other educational levels, partici-

pants with a degree or higher (86.7%) had better awareness of disease transmission from raw milk consumption, and there was a statistically significant difference (p-value < 0.05). The farms had poor awareness of dairy farm hygienic standards and milk-borne zoonoses. To lessen the animal and public health concerns associated with milk-borne zoonoses, it is critical to create awareness, provide extension services, and provide training programs.

Keywords: Dairy farm; knowledge; milk-borne; milking hygiene; zoonoses.

Introduction

Globally, the livestock sector is highly dynamic and contributes 40% of the global value of agricultural output and supports the livelihoods and food security of almost a billion people (Thornton, 2010). The Ethiopian total cattle population is estimated to be about 70 million and the agricultural sector engaging 80% of the population, contributes 52% of the gross domestic product (GDP) and 90% of the foreign exchange (CSA, 2021; Stoltenow *et al*., 2013). The livestock sub-sector alone contributes 12% of the total and over 45% of the agricultural GDP, and over 85% and 90% of the farm and pastoral incomes respectively (Amanuel and Ulfina, 2018).

Dairy production, in the livestock production sector, is a critical issue in Ethiopia; because it is among the main sources of food and income (Birhanu *et al*., 2022). In such sceneries, people have close interaction with animals and animal products. In developing countries like Ethiopia, the dairy sector has a greater potential especially in poverty alleviation by increasing the income of dairy producers and improving the living standards of people, improving nutrition arising from milk consumption and creating employment, and transforming the existing largely subsistent type of milk production to commercial level (Njombe *et al.,* 2011; Yilma *et al*., 2011). However, milk is a highly perishable commodity and highly nutritious food and serves as an ideal medium for the growth and multiplication of various microorganisms (Parekh and Subhash, 2008). Infections that are naturally transmissible from vertebrate animals to humans and vice-versa are classified as zoonosis (WHO, 2019). These zoonoses can be transmitted to humans in several ways that include consumption of infected raw or unpasteurized milk and milk products (mostly) and contact with infected dairy animals

and products and infected farm environments (Bertu *et al*., 2010; Birhanu *et al*., 2022).

Considering the high burden of zoonotic diseases in Ethiopian livestock, the community is at risk of zoonotic transmission through inhalation and ingestion of pathogens. Consumption of uncooked or unprocessed food increases the risk of disease transmission (Deneke *et al*., 2022). According to estimates for the burden of zoonotic diseases, infections discovered in milk are responsible for around 61% of human infections and 90% of diseases associated with consuming dairy products. Some of the microbial contaminants are responsible for milk spoilage while others are pathogenic with potential health effects which cause milk-borne diseases (Weldekidan *et al*., 2019).

Contamination of milk and milk products with pathogenic bacteria is largely due to handling, processing, and unhygienic conditions (Maity *et al*., 2010). Animal-source foods have been found guilty of the majority of food-borne diseases (De Buyser *et al*., 2001) and incidences increase with increasing access to such foods, especially without adequate hygiene, inspection for safety, or satisfactory heating for killing pathogens (McCrindle, 2008). The common raw milk zoonotic bacterial diseases are Brucellosis, bovine Tuberculosis, Salmonellosis, Anthrax, Mastitis, Campylobacterosis, etc. (Weldekidan *et al*., 2019).

Milk and other dairy products are produced in unhygienic settings in developing countries, and milk and dairy products, in general, have poor hygiene standards (Yilma *et al*., 2007). According to the National Hygiene and Sanitation Strategy program (WHO, 2019), Ethiopia's poor hygiene and sanitation are responsible for more disease burden. Currently, a large number of dairy productions are operating in and around Wolaita Sodo Zone, using improved dairy breeds. However, knowledge of farm hygiene practices and farmers' awareness of zoonoses carried by cow's milk is still poor. This results in public health risks and economic losses affecting the livelihoods of dairy producers. Hence, an understanding of farmers' knowledge of milking hygiene and cattle milk-borne zoonoses is very important to reduce the risk of cattle milk-borne zoonoses transmission. Therefore, the objectives of this study were to assess the hygienic practice of dairy farms that include dairy house hygiene, udder cleaning, and milk handling; and to assess the awareness of milk-borne zoonotic diseases and the habit of raw or unpasteurized milk consumption and associated risk factors in three selected towns of Wolaita zone, Southern Ethiopia.

Materials and methods

Study area

The study was conducted in three selected towns (Sodo, Boditi, and Areka) of the Wolaita Zone, southern Ethiopia. Sodo town, the capital of Wolaita Zone, is situated 390 kilometers south of Addis Ababa, the capital of Ethiopia. The town is located at a latitude of 8°50°N and a longitude of 37°45°E, with an altitude of 2025 meters above sea level. The town has a mean annual temperature of 20°C, receives rainfall of 450–1446 mm, and has minimum and maximum daily temperatures of 12 and 25°C. On the other hand, Boditi town is found 18 km north of Sodo town, at 37° 52' E and 6.967°N. Boditi has an average rainfall of 1000mm and temperatures ranging from 26°C in January to 11°C in August. Areka town is located 29 kilometers west of Wolaita town at longitude 37° 47' E and latitude 7°4′ N. The average rainfall is 1538.44 mm, and the temperature ranges from 14.48°C to 28.5°C (WZAO, 2017).

Study population

The study populations were dairy farms and farm workers from three purposefully chosen towns in the Wolaita zone. The three towns were projected to have 175 dairy farms available. Each dairy farm employed eight (8) workers on average. The study covered dairy farms of all sizes, including small, medium, and large-scale operations. Only one dairy farm worker per dairy farm was interviewed in each dairy farm about hygienic practices on the farm and knowledge of milk-borne zoonotic diseases. According to Arnett (2007), the respondents' ages were categorized into four groups: 18–25, 26–35, 36–50, and >50 years.

Study design

A cross-sectional questionnaire-based study design was conducted by collecting data through structured questionnaires. From the selected farms, only one farm worker was selected in each farm. The questionnaire was generally divided into two sections: 1) general information and hygiene practices of dairy farms; and 2) specific questions about zoonotic illness, and milk consumption patterns of farm workers. The first section of the questionnaire was supported by another unselected worker who had more information in the case when the selected worker did not have complete information about the farm. But the second part of the questionnaire, which related to personal perceptions about

zoonotic diseases and milk consumption habits, was only filled out by selected individuals.

Sampling technique and sample size determination

Dairy farms and farm workers were chosen using simple random sampling methods, but the three towns were chosen purposefully based on the prevailing numbers of dairy farms. The sample size was calculated according to Yamane (1967). The current study comprises a randomly selected dairy farm that is found in Sodo town, Areka, and Boditi.

$$n = \frac{N}{1+ (N (e^2))}$$

Where n is the sample size of the study; N is the total number of farms; e is the maximum variability or margin of error of 5% (0.05); and 1 is the probability of the event occurring. Therefore, a total farm was selected at a 5% standard error with a 95% confidence interval. However, 100 dairy farms were chosen for this study based on the owners' willingness, time, and resources.

Method of data collection

A questionnaire-based survey was carried out by asking and observing dairy farm characteristics, followed by specific questions related to the assessment of the hygienic practices of dairy farms and their awareness of milk-borne zoonosis diseases. A structured questionnaire was prepared to assess the hygienic measures of dairy farms and their knowledge of milk-borne zoonosis on selected dairy farms in the study areas. During the questioning and distribution of the questionnaire to the dairy farm workers, it was appropriately translated into the local languages of "Wolaitgna" and "Amharic."

Data management and statistical analysis

The collected raw data were stored, coded in a personal computer's Microsoft Excel spreadsheet program, and analyzed using STATA statistical software version 14. Descriptive statistics such as frequencies, distributions, and percentages were used to summarize the data. Pearson's chi-square test was used to detect the existence of an association between different demographic risk

factors such as age, sex, farm owners, and educational status and outcome variables like milk consumption habits and transmission of disease by raw milk consumption. Besides, a p-value of less than 0.05 was considered statistically significant.

Ethical clearance

All interviewees gave their informed consent for inclusion before they participated in the study. The study protocol was approved by the Wolaita Sodo University Research Ethics and Review Committee with reference number WSU 41/22/2241, and the verbally informed consent process was documented in the manuscript. The best practices for veterinary care were followed, and those who owned the farms were informed of the objective of the research and that the protocol had been approved.

Results

Socio-demographic characteristics

The study revealed which categories of dairy farm workers were greater in number at the farm level in terms of gender and level of education. Out of the total farms selected, 48% had more female than male employees, 29% had more male than female employees, and 23% had an equal number of male and female employees. Regarding the educational levels of dairy farm workers, 43% of farms had a majority of workers with no formal education, 28% had a primary education level, 5% had a secondary education level, 2% had college diplomas, and 22% of respondents had a first degree or higher. (NB: Dominance of workers' gender and educational status at the farm level has been addressed even in the case of a plus-one worker). Regarding farm working experience, 10% had less than five years, 31% had between 6 and 10 years, 8% had between 11 and 15 years, 9% had between 16 and 20 years, and 42% had above twenty years of experience (Table 1).

Table 1. Socio-demographic characteristics of respondents and farms

Questions/ variables	Category	No of Farms	Frequency (%)	95% CI
Farm owners	Family	95	95.0	88.38 - 97.93
	Private	3	3.0	0.95 - 9.05
	Government	2	2.0	0.48 - 7.81
Which gender is predominant among the farm's employees?	Female workers	48	48	38.24- 57.9
	Male workers	29	29	20.8 – 38.7
	An equal number of both genders	23	23	15.68 – 32.5
Which educational levels prevail in numbers on the farm?	No formal education	43	43	33.5 - 53.1
	Primary education	28	28	19.9 – 37.7
	Secondary education	5	5	2.06 - 11.61
	Diploma holder workers	2	2	0.48 - 7.81
	Bachelor's degree and above	22	22	14.8 – 31.4
Dairy farm working experiences (years)	< 5	10	10.0	5.40 - 17.76
	6-10	31	31.0	22.59 - 40.87
	11-15	8	8.0	4.02 - 15.35
	16-20	9	9.0	4.69 - 16.56
	>20	42	42.0	32.58 - 52.03
Age of respondents (years)	18-25	21	21.0	14.00 - 30.25
	26-35	17	17.0	10.75 - 25.83
	36-50	60	60.0	49.95 - 69.26
	>50	2	2.0	0.48 - 7.81

CI: Confidence interval; No: number

Dairy farms' breed composition

The majority of farms featured different breed types rather than just one breed of the animal when it came to the breeding makeup of farms. Out of all farms, 22% had a larger percentage of crossbred animals, 52% had a higher percentage of Holstein Frisian, 3% had a higher percentage of Jersey breeds, and the remaining 23% had a local breed predominate.

Hygienic practices of dairy farmhouses

The study showed that 53% of the dairy farms' house floors were made of concrete, which was washable, and 47% were not washable. Regarding house cleaning, 59% of farms cleaned the floor once a day, 21% cleaned twice a day, 15% cleaned thrice a day, and only 5% cleaned more than thrice a day. From the total farms, 92% removed dung manually, 4% used hose water for mixing to drain by itself, and the remaining 2% used both of these methods. Out of all the farms, 49% removed fluid waste manually, 46% utilized hose water to drain by itself, and the remaining 5% employed both methods (Table 2).

Feeding, watering, hygiene, and health care

Regarding feeding methods, 59% of farms used indoor feeding, 5% outdoor feeding techniques, and 39% mixed feeding approaches. Although the majority of the farms used indoor feeding systems, most animals (77%) fed on the ground, while only 23% used feeders. The majority of farms (88%) had access to piped water, while only 1% of farms used groundwater. Some farms (11%) used river water for cleaning and drinking. A total of 61% of farms provided water for each animal separately, while 39% provided water for all animals in common (Table 2).

Table 2. Feeding, watering, and cleaning of dairy cows in the study areas

Variable	Category	No of respondents	Percent (%)	95% CI
Feeding	Grazing	5	5.0	2.06 - 11.61
	Indoor feeding	56	56.0	45.99 - 65.54
	Mixed	39	39.0	29.81 - 49.03
Feeding on	On floor	77	77.0	67.57 - 84.31
	In feeder	23	23.0	15.68 - 32.42
Water source	River	11	11.0	6.13 - 18.94
	Underground water	1	1.0	0.13 - 6.97
	Pipe water	88	88.0	79.87 - 93.12
How water given	Individual	61	61.0	50.96 70 .18
	Common	39	39.0	29.81 49.03
Type of floor	Washable	53	53.0	43.05 62.70
	Nonwashable	47	47.0	37.29 56.94
Frequency of cleaning	Once a day	59	59.0	48.96 68.33
	Twice a day	21	21.0	14.00 - 30 .25
	Thrice a day	15	15.0	9.16 - 23.57
	> Thrice a day	5	5.0	2.06 - 11 .61
Dung removal	Manually	94	94.0	87.12 - 97 .31
	Use of hose water for mixing to drain by itself	4	4.0	1.48 - 10.33
	Both	2	2.0	0.48 - 7.81
Urine and other fluid removal	Manual	49	49.0	39.19 - 58.87
	Use hose water to drain by itself	46	46.0	36.34 - 55.96
	Both	5	5.0	2.06 - 11.61

The findings showed that 68% of farms had cleaners and milkers in different ways, compared to 32% of farms that used the same personnel for both jobs. Out of all farms, 66% of milkers washed their hands with only cold water, 16% with cold water and detergent, 2% with only warm water, and 1% with warm water and detergent. In terms of cleaning the udder and teat, 37% of farms used only cold water, 46% used warm water, and 17% of farms used nothing at all. The study also reported that 41% of farms used individual towels for each cow, 21% used common towels for drying the udder after washing, and the

remaining 38% didn't use towels at all for drying. Only 3% of farms reported using disinfection for udder and teat cleaning after milking cows and the majority of farms (97%) did not practice antiseptic for teat disinfection after milking (Table 3).

Table 3. Hygiene and health care of cows on farms

Variable	Category	No of respondents	Percent (%)	95% CI
Do you wash cows?	Yes	56	56.0	45.99 - 65 .54
	No	44	44.0	34.45 - 54.00
Frequency of dairy cow washing	Once a week	28	28.0	19.96 - 37.74
	Twice a week	16	16.0	9.95 - 24.70
	Thrice in week	7	7.0	3.33 - 14.12
	> Thrice a week	2	2.0	0.48 - 7.81
	Do not wash	47	47.0	37.29 - 56.94
Do you use a towel	Yes	58	58.0	47.96 - 67.41
	No	42	42.0	32.58 - 52. 03
How you use a towel	Individually	41	41.0	27.98 - 47.02
	Commonly	21	21.0	36.34 - 55.96
	Do not use	38	38.0	10.75 - 25 .83
What do you use to clean udder	Cold water only	37	37.0	27.98 -47.02
	Warm water only	46	46.0	36.34 - 55.96
	Do not wash udder	17	17.0	10.75 - 25 .83
Milk stored	Refrigerated	13	13.0	7.62 - 21.28
	As milked	87	87.0	78.71 - 92 .37
Milkers and cleaner	The same	68	68.0	58.08-76.51
	Separate	32	32.0	23.48 -41.91
Protective cloth on the farm	Use	17	17.0	10.75 -25.83
	Do not use	83	83.0	74.16- 89.24
Milkers clean their hands before milking	Yes	68	68.0	58.08-76.51
	No	32	32.0	23.48-41.91

Hygienic practice of milk storage and milking equipment

The results of the study indicated that 51% of farms filtered milk during storage, whereas 49% didn't. 58% of farms used plastic to store milk, 15% used metal, and 27% used traditional equipment. On farms, 22% washed milking equipment using cold water alone, 44% with cold water and detergent, 5% with warm water alone, and 29% with warm water and detergent. Only 13% of all farms kept their milk in refrigerators, while 87% kept it at room temperature.

Milk consumption habits and awareness of milk-borne zoonotic diseases

Concerning milk consumption habits, 64% of respondents reported they consumed raw milk, 29% answered they consumed both raw and boiling milk, 3% claimed they used any kind of milk, including raw, boiled, refrigerated, and processed milk, and 4% stated they did not consume any milk at all. The results of the survey indicated that 50% of respondents were aware that consuming raw milk could transmit disease. In terms of interviewees' perceptions and knowledge of milk-borne zoonotic diseases, only 4% were aware of tuberculosis (TB), 32% were aware of salmonellosis, 5% were aware of both TB and salmonellosis, and 8% were aware of TB, anthrax, mastitis, salmonellosis, and brucellosis. The remaining 51% were unaware of zoonotic diseases. Only 27% of interviewees were aware that zoonotic diseases can be spread from animals to humans, and 73% of respondents were unaware that zoonotic diseases can be spread from humans to animals and vice versa. Only 2% of respondents had a history of milk-borne illness, compared to 98% of respondents who were unaware they had it (Table 4).

Table 4. Respondents' milk consumption habits

Variable	Categories	No of respondents	Percent (%)
Do you consume milk	Yes	97	97.0
	No	3	3.0
Consuming habits	Do not consume milk	4	4.0
	Consume raw milk	64	64.0
	Consume raw and boiled milk	3	3.0
	Consume raw, boiled, refrigerated, and processed milk	29	29.0
Know disease transmitted via raw milk consumption	Yes	50	50.0
	No	50	50.0
Do you know the name of milk-borne zoonotic diseases	Don't know	51	51.0
	Tuberculosis	4	4.0
	Salmonellosis	32	32.0
	Tuberculosis, Salmonellosis	5	5.0
	Tuberculosis, Anthrax, Mastitis, Salmonellosis, Brucellosis	8	8.0
Experience in acute raw milk consumption illness	Yes	2	2.0
	No	98	98.0
Milk of sick animal	Discarded	41	41.0
	Given to calves	11	11.0
	Use after processes	23	23.0
	Use without processing	25	25.0
Milk of drug-treated animals	Discarded	41	41.0
	Given to calves	10	10.0
	Use it after processing	18	18.0
	Use without processing	31	31.0

The association of milk consumption habits with different demographic characteristics like sex, age, educational level, and farm experience was assessed. Accordingly, 81.2% of respondents who had no formal education consumed raw milk as compared to other educational levels (Table 5).

Table 5. Association of socio-demographic factors with milk consumption habits

Variable	Category	Responses in percent (%)				X^2	P-value
		Raw	Not consume	Raw and boiled	Raw, boiled, refrigerated, processed		
Sex	Male	64.0	4.0	30.0	2.0	0.368a	0.947
	Female	64.0	4.0	28.0	4.0		
Age (in years)	18-25	76.2	4.8	9.5	9.5	10.780	0.291
	26-35	64.7	5.9	23.5	5.9		
	36-50	60.0	3.3	36.7	0.0		
	>50	50.0	0.0	50.0	0.0		
Educational status	No formal Education	81.2	6.2	12.5	0.0	19.489	0.077
	Primary Education	67.7	3.2	25.8	3.2		
	Secondary Education	46.7	0.0	53.3	0.0		
	Diploma	57.1	0.0	42.9	0.0		
	Degree and above	40.0	6.7	40.0	13.3		

Comparing disease transmission from raw milk consumption with risk factors

Different socioeconomic characteristics were compared with disease transmission from raw milk consumption; as a result, 86.7% of respondents with a degree or higher had a good perception compared to those with other educational levels, and there was a statistically significant difference (p-value < 0.05). Female respondents had higher knowledge (56%) than male respondents (44%) about disease transmission through raw milk consumption (Table 6).

Table 6. Interviewees' knowledge of disease transmission by the intake of raw milk

Variable	Category	Know disease transmission from raw milk consumption		X^2	P-value
		Yes (%)	No (%)		
Owner	Family	48.4	51.6	2.428a	0.297
	Enterprise	66.7	33.3		
	Government	100.0	0.0		
Sex	Male	44.0	56.0		
	Female	56.0	44.0		
Age (in years)	18-25	38.1	61.9	4.328	0.228
	26-35	35.3	64.7		
	36-50	58.3	41.7		
	>50	50.0	50.0		
Education status	No formal Education	18.8	81.2	30.345	0.000
	Primary Education	41.9	58.1		
	Secondary Education	80.0	20.0		
	Diploma	85.7	14.3		
	Degree and above	86.7	13.3		

Discussion

Milk is a complete food that is high in protein and contains all the required amino acids. Despite this, milk can act as a possible route for the spread of various diseases under certain conditions. A questionnaire-based survey of 100 dairy farm respondents was undertaken. Out of all the farms included in the study, 48% had more female employees than males, 29% had more male employees than females, and 23% had an equal number of male and female employees. The current study was similar to a report from Jinka by Abebe *et al.* (2020) and from Dilla by Hailemariam *et al.* (2022), in which 58.23% and 60.8% of milk-handling participants were female, respectively. Similarly, Bereda *et*

al. (2012) in the Gurage zone's Ezha district found that 48% of respondents were female workers.

The present study, however, did not support data from Addis Ababa (Belay and Geert, 2016) and northwest Ethiopia (Yitaye *et al*., 2008), which found that farms employed more men than women. In a study conducted in Tanzania, 27% of respondents were female (Omore *et al*., 2021), which was lower than the percentage of female workers in the current study. The present study was lower than the previous report by Azage (2004), in which most of the respondents (75.9%) were male as compared to female workers. This discrepancy might be due to females being more prone to milking and caring for animals than males.

In terms of dairy farm worker's educational backgrounds, 43% of farms had a majority of employees without a formal education, 28% had a majority of employees with a primary education level, 5% had a majority of employees with a secondary education level, 2% had a majority of employees with college degrees, and 22% had a majority of employees with a first degree or higher. Due to lower wages, a large number of workers without a formal education were working on the farm, and most farm owners do not welcome educated people to work on their farm.

According to the current study, 53% of the dairy farms' house floors were washable concrete, whereas 47% were not. This finding was consistent with previous reports in Addis Ababa (Bruktawit, 2016), Bishoftu (Lencho and Seblewongel, 2018), and Jimma, eastern Ethiopia (Belay and Geert, 2016), where most cows were confined to concrete floors; however, the study disagreed with a previous report in Dire Dawa, eastern Ethiopia, where the major floor structure of cattle sheds was hardened soil (Emebet and Zeleke, 2008). In terms of housekeeping, 59% of farms cleaned the floor once per day, 21% cleaned it twice per day, 15% cleaned it three times per day, and just 5% cleaned it more than three times per day. This was similar to the report from Mukaturi and Sululta towns (Sema *et al*., 2019), in which 55.9% of farms cleaned the floor once a day and 44.1% cleaned twice a day, but the current study differed from the findings reported in Addis Ababa that the floor or barn on 74.3% of dairy farms was washed daily, and some farms (25.7%) were cleaned three times a week (Fufa *et al*., 2019). This demonstrated a lack of knowledge regarding farm hygiene in the current study farms.

According to the current survey, 92% of farms removed manure manually, 4% utilized deranges, and the remaining 2% employed both deranges and hand removal. In terms of fluid waste, 49% of farms eliminated it manually, 46% used a deranged system, and the remaining 5% used both techniques. This finding contradicted the previous research in Sodo (Enkeshe, 2020), in which dung was completely removed by hand while fluid was eliminated by derangement. This revealed that manually removing dung takes time and puts the person doing the removal at risk.

In terms of feeding systems, the majority of farms (59%) utilized indoor feeding systems, 39% used mixed feeding systems, and only 5% used outdoor feeding systems. Although most farms use indoor feeding systems, this differs from the previous study, which found that 63.3% of farms used outdoor grazing, 30% used indoor feeding systems, and only 6.7% used mixed feeding systems (Oumer *et al*., 2017). This disparity could be attributed to a scarcity of farmland.

According to this study, 77% of farms fed on the floor, while just 23% fed on the feeder. This result was consistent with the findings of Enkeshe (2020) in Sodo, where 77.3% of farms were fed on the floor and 22.6% on the feeder. This could be because dairy production has received a lot of attention and funding. In terms of water supply, 88% of farms had access to piped water, while only 1% used groundwater. The river water was used for cleaning and drinking by 11% of farms. Sixty-one percent of farms provided water for each animal separately, while 39% provided water for all animals collectively. This finding was consistent with the findings of Alebachew and Alemu (2015), who found that 91.7% of commercial dairy farms in Addis Ababa used pipe water as their primary source to clean and supply cow farms, whereas 8.3% used river water. Similarly, a study in Dilla, Gedeo Zone, by Hailemariam *et al*. (2022) reported that dairy farmers used two sources to water their cattle, either from the river (54.3%) or from pipes (45.8%) and also (Tesfaye and Wondossen, 2019) for Gurage Zone and (Bekuma and Addisu, 2021) for Buno Bedele Zone, which have all indicated such water sources. This study contradicted the findings of Bereda *et al*. (2012), who discovered that the majority of dairy cow producers in the Gurage zone used river and hand-dug well water, which may not be of the required standard.

According to our findings, 68% of farms used separate cleaners and milkers, while 32% used the same staff for both tasks. Sixty-six percent of milkers on all

farms used only cold water to wash their hands; sixteen percent used cold water and detergent; two percent used warm water alone; and one percent used warm water and detergent. To clean the udder and teats, 37% of farms used just cold water, 46% used warm water, and 17% used no water at all. According to Duguma and Geert (2015), the majority (96.3%) of Jimma respondents practiced hygienic milking, which included washing hands, milk containers, and udders before milking. The study also found that 41% of respondents used individual towels and 21% used common towels to dry the udder after washing before milking, while the remaining 38% did not. In this study, the majority of farms (97%) did not use antiseptic for teat cleaning after milking, and just 3% disinfected udders and teats after milking cows. This was in line with a study in Mersa Town, North Wollo, in which most dairy farm owners (65%) did not sufficiently perform cleaning of cow's udder and teat even with potable water and did not dry it properly (Oumer *et al*., 2017). This could be because farm owners and staff in the research area are unaware of proper hand washing and udder disinfection. Using a towel to clean the udder is statistically associated with socio-demographic characteristics ($p < 0.05$).

According to the current study, 64% of respondents consumed raw milk, and 29% consumed raw and boiled milk. This was similar to the findings of an earlier survey (Fufa *et al*., 2019), which indicated that 81.25% of respondents consumed raw milk and 18.75% boiled milk. Furthermore, Sema *et al.* (2019) discovered that 60.5% of milk consumers consumed raw milk. In contrast to Lencho and Seblewongel (2018), in Bishoftu, the majority (57.5%) consumed milk after boiling it, 23.4% used raw milk as milk, and 19.1% consumed milk after processing (yogurt), while Duguma and Geert (2015) reported that the majority (92.6%) of Jimma farmers boiled the milk before consumption, and 3.7% consumed raw milk.

In terms of disease transmission, the survey found that 50% of respondents were aware of pathogen transfer from raw milk to humans, while the remaining 50% were unaware. This finding was consistent with the study in Mersa Town, North Wollo (Oumer *et al*., 2017), where 67.5% of respondents used raw milk, as well as the study of Sema *et al*. (2019), which found that 60.5% of milk users consumed raw milk. However, this finding contradicted the findings of Lencho and Seblewongel (2018) in Bishoftu, who reported that the majority (57.5%) of milk was consumed after boiling, and Duguma and Geert (2015) in Jimma, who reported that the majority (92.6%) of farmers boiled milk before

consumption. Among socio-demographic factors, educational level was strongly associated with disease transmission by raw milk drinking ($p< 0.05$). This is due to respondents' ignorance of disease transmission through raw milk intake.

Concerning the knowledge of workers about milk-borne zoonotic diseases, only 4% of workers were aware of tuberculosis, 32% were aware of salmonellosis, 5% were aware of both TB and salmonellosis, and 8% were aware of TB, anthrax, mastitis, salmonellosis, and brucellosis. The remaining 51% were unaware of zoonotic illnesses. This revealed that participants were unaware of zoonotic diseases. The vast majority (98%) of respondents had never had a milk-borne disease, while 2% had a history of milk-borne sickness. This was similar to the findings of Lencho and Seblewongel (2018), who discovered that 91% of respondents had not had any milk-borne disease, while 9% had. However, it contradicted the findings of Oumer *et al.* (2017) in Mersa Town, North Wollo, where 44.1% and 55.9% of respondents reported suffering from milk-borne infections as a result of consuming raw milk. This discrepancy is related to the fact that the majority of people are unaware of the cause of their ailment.

Conclusions

The result indicated that a limited number of farms used individual towels, some used common towels, and others did not use any towels at all to dry their cows' udders. Furthermore, the findings showed that the majority of the farmhouses were cleaned once a day; most farms removed dung manually; raw milk consumption was also high; and awareness of milk-borne zoonotic diseases was poor. The study considered several demographic risk factors, such as gender, age, education level, and farm experiences. Those with a bachelor's degree or higher exhibited significantly greater awareness of the transmission of diseases through raw milk consumption during interviews. This was caused by a lack of knowledge, inadequate awareness-raising initiatives, and a lack of teamwork among public health players. Therefore, the following recommendations were required based on the aforesaid conclusion:

- Regular farm hygiene training and awareness should be provided to all farm personnel of all ages and educational levels.
- Cooperation should be established between human health and animal health offices.

- The milk handling procedure and farm hygiene practices should adhere to the standards.
- On dairy farms, good personal hygiene should be practiced.

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Review on performance responses of dairy cattle against thermal stress

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Abstract

This manuscript is aimed at reviewing the performance responses of dairy cattle against thermal stress resulting from climate change. Climate change is the major factor that largely affects the dairy industry. Thermal stress (TS) is the perceived discomfort and physiological strains associated with exposure to excessive ambient temperature. Temperature and Humidity Index (THI) have been adopted to describe thermal conditions that drive thermal stress in dairy cattle. The effects of TS are devastating in the dairy industry if not managed well. The thermo-neutral zone for dairy cattle and calves in the tropics is THI <72. Milk yield reductions of up to 50% have been reported for Holstein cows due to thermal stress under summer climate conditions. Moreover, thermal stress is associated with alterations in milk composition. TS reduces the length and intensity of estrus manifestation. Moreover, 80% of estrus may be unnoticeable during the summer season in temperate regions, which further reduces fertility. Conception rates of dairy cows may drop up to 20–27% in the summer season. Climate-induced thermal stress resulted in a decrease of 3.5 kg and 25 kg in the birth weight and weaning weight of Fogera calves, respectively. Dry matter feed intake was reduced by 9.6%. Commonly used thermal stress reduction strategies included modification of the physical environment, breeding for heat-tolerant dairy cattle, and nutritional management. The productive and reproductive traits are susceptible to the negative impacts of thermal stress with an increase in THI above 68.0 for Holstein Frisian cattle and their crosses and 72 for tropical cattle under an open shed system. Mitigations of thermal stress by breeding heat-tolerant animals, using a loose house system, and season-based feeding should be considered an integral component of the dairy cattle management system.

Keywords: Thermal Stress; Climate Variable; Performance Response; Mitigation Measures; Dairy Cattle.

Introduction

Climate change is a major concern/issue that largely affects the dairy industry. By the year 2100, the mean global temperature may be 1.1-6.4 °C warmer than in 2010 (Sheikh *et al*., 2017). The impact of climate change can be direct or indirect. Livestock, depending on the species and level of productivity, have an optimal environmental zone and they must be maintained within this zone for optimal growth, lactation, and reproductive functions (Armstrong, 1994). Thermal stress is the perceived discomfort and physiological strains associated with exposure to an extremely hot or cold environment. Thermal stress includes both thermal stress during the extreme summer season as well as cold stress during the extreme winter season. The surplus of produced heat needs to be emitted into the surrounding air. However, this is difficult when the air temperature is already high and relative air humidity is elevated. As a result, the body temperature of animals increases. To prevent overheating, cows consume less feed, which leads to lower milk production. Moreover, thermal stress is most often conditioned by air temperature (Temp), relative air humidity (RH), solar radiation, and air movement velocity (West, 2003; West *et al*., 2003). The temperature and Humidity Index (THI) was first introduced by Thom (1959) to describe the effect of ambient temperature on humans but has been adapted to describe thermal conditions that drive thermal stress in dairy cattle (De Rensis *et al*., 2015). It is divided into categories that potentially indicate the level of thermal stress. Armstrong (1994) used THI <72 [Temp=23.9 °C and RH=65% or Temp=32.2 °C and RH=0%] as a thermal comfort zone, 72 to 78 as mild TS, 79-88 as moderate TS, 89-98 as severe TS and >98 as danger TS. Nevertheless, THI <68 [Temp=22.2 °C and RH=45% or Temp=26.7 °C and RH = 0%] was defined to be outside the thermal danger zone for cows. Mild signs of TS are observed at THI of 68 to 74, and a THI ≥75 will cause drastic decreases in production performance (Collier *et al*., 2009; De Rensis *et al*., 2015). Various authors (Mader *et al.,* 2006; Bohmanova *et al*., 2007) have described the thermo-neutral zone for various mammals, as indicated earlier. Habeeb *et al*. (2018) better described the thermo-neutral zone of cattle in tropical countries like Ethiopia and Egypt. According to his report, cattle were in comfort zone if (THI < 68), mild discomfort zone if (68 < THI < 72), discomfort if (72 < THI < THI < 79), danger (79 < THI < 84), and emergency (THI > 84).

The effects of TS are devastating in the dairy industry unless managed well. Milk yield reductions of up to 50% have been reported for Holstein cows due

to thermal stress during the summer climate condition in the open shed cattle management as compared to the winter (Baumgard and Rhoads, 2013). Moreover, thermal stress is associated with alterations in milk composition, somatic cell counts, and mastitis incidences (Pragna *et al*., 2017). High-yielding breeds are more susceptible than low-yielding breeds (Sunil *et al*., 2011; Pragna *et al*., 2017). The feed nutritional requirements of the high-yield dairy animals are high as they utilize the feeds for maintenance and production. When the external temperature goes beyond its thermal neutral zone, the animal experience thermal stress. As a result, the animal decreases their feed intake dramatically to reduce the internal heat production as a cooling mechanism of their body, and thereby their milk production goes do as compared to the low-yielding dairy animals. TS reduces the length and intensity of estrus besides increasing the incidence of anestrous and silent heat in farm animals (Kadokawa *et al*., 2012; Singh *et al*., 2013). Moreover, 80% of estrus may be unnoticeable during summer, which further reduces fertility (Rutledge, 2001; Singh *et al*., 2013). Conception rates of dairy cows may drop up to 20–27% in summer (Shiekh *et al*., 2017). It also affected testicular volume, hormonal profiles, sexual behavior, and semen quality, which affect the reproductive performance of males (Abera *et al*., 2020). Besides, thermal stress has been also associated with the deterioration of embryo development and increased embryo loss in cattle (Hansen, 2007). Thermal stress also causes severe economic loss in approximately 45%-60% of dairy farms around the world (Bernabucci *et al.,* 2010; Pragna, *et al*., 2017). Commonly used TS reduction strategies included modification of the physical environment, breeding for heat-tolerant dairy cattle, and nutrition management (Armstrong 1994; Silanikove, 2000). Despite various studies conducted at different research centers and universities to examine the impact of thermal stress on cattle performance, there has been insufficient review and publication of these findings. Therefore, the objective is to review the performance responses of dairy cattle against thermal stress.

Thermal stress in Cattle

Responses of Cattle to Thermal Stress

The efficiency of conversion of food energy for maintenance and production while maintaining a reasonable thermal balance of the animals in its environment is seldom achieved in tropical climates for the most highly productive stocks. As animal agriculturalists strive for an increased rate of growth, lactation, and fertility by improved feeding, especially in the more stressful humid

tropics they are immediately confronted with the problems of dissipation of the increased metabolic heat. The genetic blending of heat in addition to nutritional adaptability and disease resistance with productivity is essential for animal production in climate zones where portions of the day, seasons, or year exceed the thermo-neutral zone for production. Stress may be climatic, nutritional, or internal, pathogens or toxins (Stott, 1981). The thermo-neutral zone (TNZ) of dairy animals ranges from 5 °C to 25 °C [THI<68/72], within which they maintained a physiological body temperature of 38.4-39.1°C (Yousef, 1985) (Figure 1). However, air temperatures above 20-25°C in a temperate climate and 25-37°C in a tropical climate, enhance heat gain beyond that lost from the body and induces thermal stress (Vale, 2007; Sunil *et al*., 2011) (Figure 2). As a result, Body Surface Temperature (BST), Respiration Rate (RR), Heart Rate (HR), and Rectal Temperature (RT) increase which in turn affects feed intake (FI), production, and reproductive efficiency of the animals (Figure 3). RT greater than 39.0°C and RR greater than 60/min indicated that cows were undergoing thermal stress sufficient to affect milk yield and fertility (Kadokawa *et al*, 2012). Respiration rates are the recommended measure of heat stress because there is little to no lag time compared to body temperatures and milk production. Normal respiratory rates for adult dairy cattle range from 40 to 60 breaths per minute (bpm). If more than 10% of cows have a respiratory rate exceeding 100 bpm, the situation is considered an emergency, and immediate action should be taken (https://extension.umn.edu/dairy-milking-cows/heat-stress-dairy-cattle).

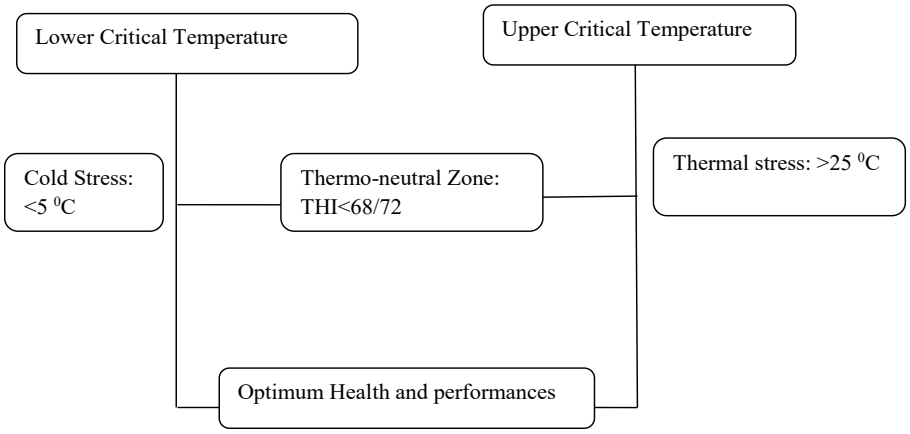


Figure 1. Thermo-neutral zone of dairy cattle. Source: Adopted and modify from Yousef (1985)

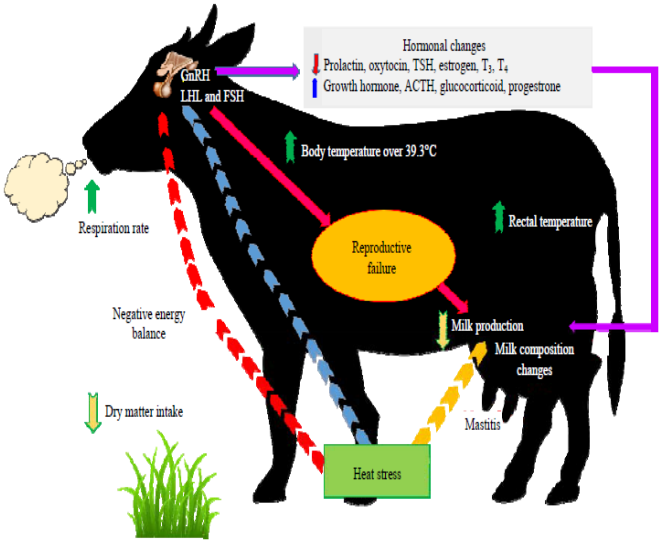


Figure 2. How dairy cattle do experience thermal stress, Source: Atrian and Shahryar (2012)

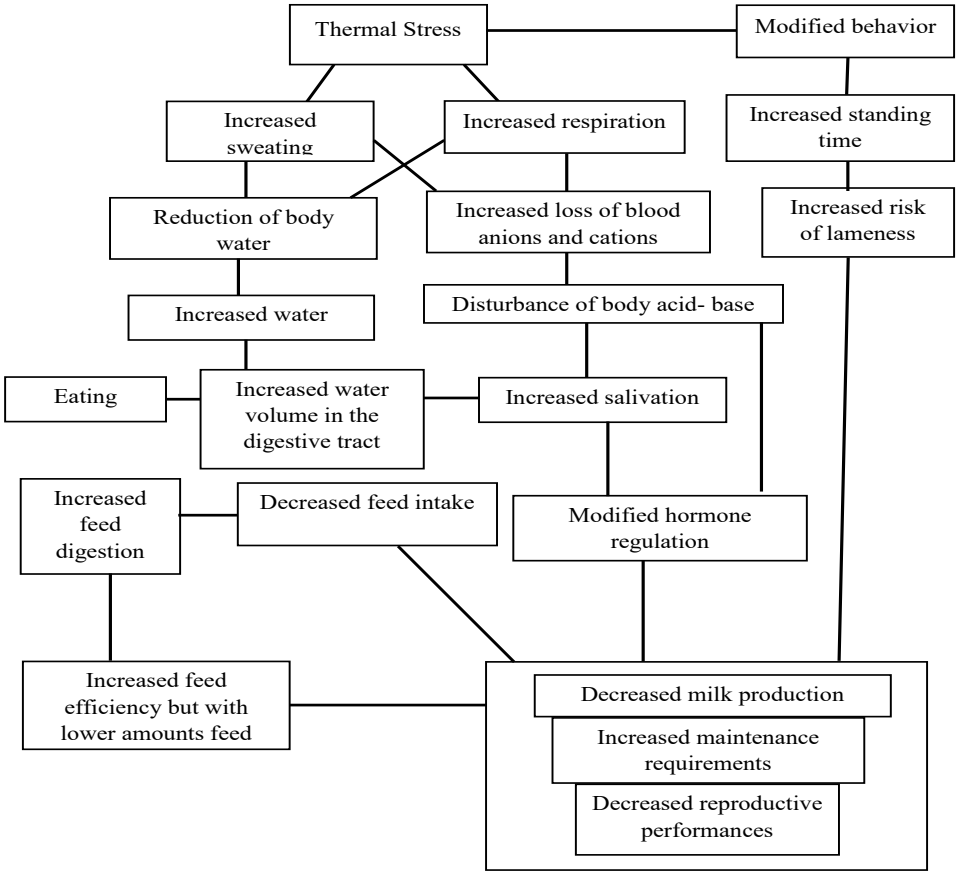


Figure 3. The Responses of dairy cattle to Thermal Stress. Source: Atrian and Shahryar (2012)

THI as a Predictor for Thermal Stress

THI is a single value depicting the integrated effects of air temperature and humidity associated with the level of Thermal stress. It is a useful and easy way to assess the risk of Thermal stress and was first introduced to describe the effect of ambient temperature on humans but later it has been adapted to describe thermal conditions that drive Thermal stress in dairy cattle (De Rensis *et al.*, 2015). This index has been developed as a weather safety index to control and decrease Thermal stress-related losses (Bohmanova *et al.*, 2007).

THI is widely used in hot areas all over the world and is commonly used as a practical indicator for the degree of stress on dairy cattle caused by weather conditions (Hahn and Mader, 1997). However, some studies show that THI values only serve as a rough measure of the HS effect on production (Polsky *et al*., 2017). THI does not account for the effects of wind speed and solar radiation. However, Mader *et al*. (2006) extended the THI to include wind speed and solar radiation, to improve its effectiveness. Solar radiation can greatly influence heat load while changes in wind speed result in altered convective cooling. Different temperature and humidity Index equations (Table 1) have been previously developed by various workers to describe the severity of Thermal Mader *et al*., 2006; Bohmanova *et al*., 2007).

Table 1. THI estimation equations for different classes of dairy cattle

No	THI Equations	Classes of dairy animals	References
1	THI8 = (0.8 × Tdb) + [(RH/100) × (Tdb − 14.4)] + 46.4	For lactating dairy cattle	Mader *et al*., 2006
2	THI = Temperature – (0.55 – (0.55x (RH/100) x (Temperature – 58)	For Pregnant dairy cattle	Amundson *et al*., 2015
3	THI = (0.8 × Tmax) + (% average RH/100) × (Tmax – 14.4) + 46.4	For growing calves	Nienaber *et al*., 1999

NB: Those equations which place more weight on the humidity work better in humid climates, whereas in drier climates, those which place more weight on the temperature work best (Bohmanova *et al*., 2007).

THI classification and levels of Thermal stress

McDowell *et al.* (1976) developed THI and identified three different classes of THI as: comfortable (<70), stressful (71-78), and extreme distress (>78). Moran (2005) described five categories of THI as: no stress (THI<72), severe stress (78-89), very severe stress (89-98), and dead cows (>98). The LWSI described the TS zone into 4 categories with different ranges of THI under each category like normal (≤74), alert (75-78), danger stress (79-83), and emergency stress (THI≥84) in livestock (Hahn *et al*., 2000; Nienaber *et al*., 2003). Bohmanova *et al.* (2007*)* indicated a THI of >65 as an upper-critical THI for lactating cows associated with decreasing milk and protein yields whereas Brugemann *et al.* (2012) indicated a THI of 60 as upper critical THI for lactating HF cows associated with decreasing milk and protein yields. Garcia-Ispierto *et al.* (2006) developed five THI classes as:<70, 71-75, 76-80, 81-85, and >85, and reported that a conception rate declined from 30.6% to 23% when THI>85 for 1-3 days

before insemination. The negative effects of thermal heat stress on growth traits were observed when THI >67-72 (Abera *et al.*, 2021). The symptoms of when animals are exposed to different levels of thermal stress are presented in Table 2 and Figure 4.

Table 2. Influences of TS on performances of cattle at different THI levels.

THI	TS level	Symptoms	References
>72	Mild	Cows likely begin experiencing TS and their calf rates affected	(Bohmanova *et al.*, 2007; Verkerk, 2009; Nidumolu *et al.*, 2010).
>78	Moderate	Cows' milk production is seriously affected	(Bohmanova *et al.* 2007; Verkerk, 2009; Nidumolu *et al.*, 2010).
>82	Severe	Very significant losses in milk production are likely, cows show signs of severe stress and may ultimately die	(Bohmanova *et al.*, 2007; Verkerk, 2009; Nidumolu *et al.*, 2010).

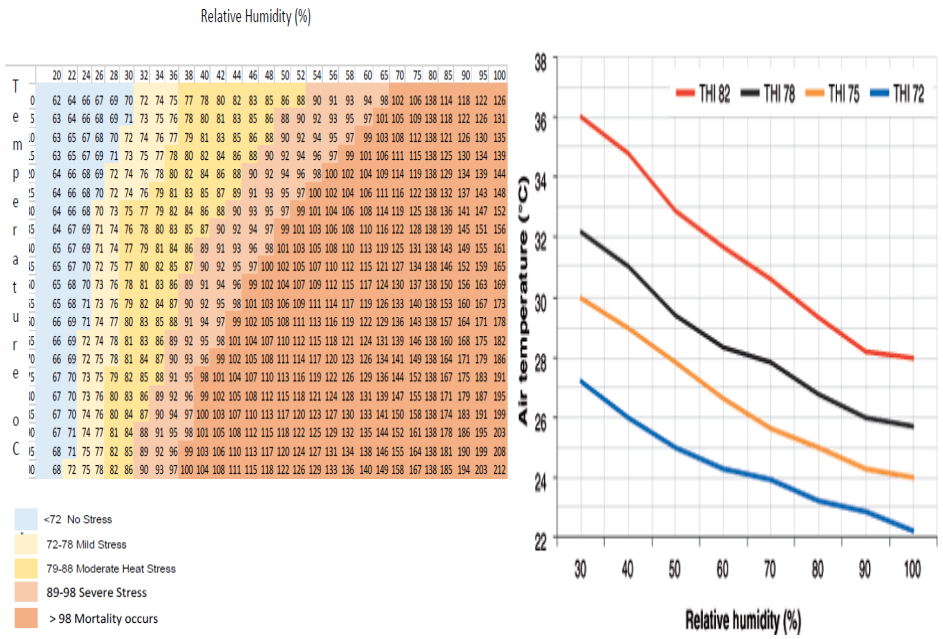


Figure 4: Bovine Temperature-Humidity Index Charts (Armstrong, 1994) (left) and (Nidumolu *et al.*, 2010) (right)

Thermal stress tolerance variations in cattle

The differences among different livestock for heat tolerance can provide the breeders opportunities to plan and perform breeding for heat resistance. Based on Thermal stress tolerance capability differences among different livestock (cattle), the following generalizations were drawn and summarized. Compared to beef cattle, dairy cattle are more vulnerable to thermal stress. Additionally, high-yielding cattle producing over 10 liters of milk per day are less tolerant of heat than low-yielding cattle producing less than 10 liters per day. *Bos taurus* cattle are more affected by thermal stress than *Bos indicus*, but Jersey cattle have a better ability to withstand thermal stress and adverse climatic conditions than other exotic dairy breeds. As a result, they are the preferred breed for tropical and subtropical environments because they sweat and drink more in hot weather (Sharma *et al.,* 1983; Bajagai, 2011). Lactating cows were also less resistant to thermal stress than dry-off cows. The impact of thermal stress on cows varied based on their lactation stage, with mid-lactation cows being more affected than early and late-lactating cows. Multiparous cows were also found to be more susceptible to thermal stress. However, small and young animals were less affected than large and old animals, likely because they are better able to dissipate heat and experience thermal stress less rapidly (Espinoza *et al*., 2009; Verkerk, 2009; Sunil *et al.,* 2011; Pragna *et al*., 2017).

Effects of thermal stress on performance traits

Effects on growth traits

Broucek *et al*. (2008) did a study on the effects of high temperature on the growth, feed intake, and health condition of calves kept in individual hutches in southern Slovakia for 92 days and reported those sixty-six days with a THI value above 72.0 and twenty-six with values higher than 78.0 were found. The calves born during the high-temperature period had the lowest body weight from the first week to the weaning than those born in the low-temperature period. These calves drank water up to the weaning and had the least intake of the starter mixture ($p<0.05$). Lacetera *et al.* (1994) did a study on the effects of temperature on calves and reported that exposure to a hot environment may negatively affect the growth of young calves. He also found lower wither height, oblique trunk length, hip width (−35%, −26%, −29% respectively), and body condition score (0.0 *vs* +0.4 points) in six 5-month-old female Holstein Friesian calves exposed to hot conditions as compared with a control group (the corresponding six sisters of six pairs of twins), kept under thermo-neutrality

conditions. Temperatures between 15-29 ◦C do not seem to exert any influence on growth performance. In temperatures above 30 ◦C, adverse effects were recorded in daily weight gain. Under high ambient air temperature and solar radiation, steers reduce daily dry matter intake, hence average daily gain and carcass weight fall, and fat thickness drop (Mitlohner *et al.*, 2002). The birth weight and weaning weight of Fogera calves managed under Metekel cattle breeding and multiplication ranch, were decreased by 3.5 and 25 kg, respectively when THI increases from 67 to 72 (Abera *et al*., 2021). Abera *et al*. (2021) also reported that at higher environmental THI, the average daily gain and final body weight of the calves were significantly affected in the same breed at Metekel Ranch. However, THI had negative and insignificant influences on different growth traits of Holstein Frisian crosses, maintained under the loose sheds and proper feeding and watering management (Tassew, 2021, unpublished).

Effects on reproduction traits

Thermal stress has adverse effects on the reproduction of dairy cattle (Kadzere *et al.*, 2002; West, 2003; Verwoerd *et al.*, 2006; Hansen, 2007) and conception and mortality rates (Hahn, 1999; Dikmen and Hansen, 2008). Thermal stress does not prevent the occurrence of normal estrus cycles. It does, however, amplify the problem of heat detection by reducing the length of the estrus period, from 18 hours down to about 10 hours, and lowering the intensity of estrus behavior (Shearer, 1990). Thermal stress compromises oocyte growth in cows by altering progesterone, luteinizing hormone, and follicle-stimulating hormone secretions during the estrus cycle (Ronchi *et al.*, 2001), as well as impairing embryo development and increasing embryo mortality (Wolfenson *et al.*, 2000). Moreover, TS may reduce the fertility of dairy cows in summer by poor expression of estrus due to a reduced estradiol secretion from a dominant follicle developed in a low luteinizing hormone environment (De Rensis and Scaramuzzi, 2003). About a 20-27% drop in conception rates (Lucy, 2002) or a decrease in the 90-day non-return rate to the first service in lactating dairy cows (Al-Katanani *et al.*, 1999) can occur in summer. In these situations, the calving interval is longer, the birth rate is lower and farm milk yield per year can be reduced.

Thermal stress during pregnancy slows down the growth of the fetus, although active mechanisms attenuate excursions in fetal body temperatures when mothers are thermally stressed. There are several changes in reproductive

performance (decreased fetal growth and calf size, increased risk of early embryonic death, and increased number of AI per conception) have been reported (Upadhyay *et al*., 2009b). Besides, TS has been also associated with deterioration of embryo development and increased embryo loss in cattle (Hansen, 2007). It also affected testicular volume, hormonal profiles, sexual behavior, and semen quality, which affect the reproductive performance of males (Abera *et al*., 2020). In an unpublished study conducted by Tassew (2021), it was observed that under loose sheds with proper feeding and watering management, the impact of THI on age at first service, age at first calving, and weight at first calving was not significant ($p>0.05$). However, the study revealed a negative and significant effect ($p<0.05$) of THI on the number of services per conception and pregnancy period.

Effects on milk production traits

Thermal stress has adverse effects on milk production and reproduction of dairy cattle (Kazdere *et al*., 2002; West, 2003; Verwoerd *et al*., 2006; Hansen, 2007) (Table 3). Reduced performances under heat and cold stress are due to associated effects on thermal regulation, energy balance, water balance, and endocrine changes among other factors. Reduced milk yield under Thermal stress is caused by associated effects on thermal regulation, energy balance, and endocrine changes (Yousef, 1985; Ominski *et al.,* 2002) and reported a decrease of 4.8 % in milk production when cows were exposed to TS compared to their milk production in the thermal neutral zone. Bouraoui *et al.* (2002) reported that for every degree above the temperature humidity index of 69 in dairy cattle a 0.4 kg decrease in milk production. Berman (2005) estimated that effective environmental heat loads above 35°C activated the stress response system in lactating dairy cows. Milk yield reductions of 10 to 40% have been reported for Holstein cows during summer as compared to winter (Broucek, 2009). Brügemann *et al.* (2012) indicated a milk yield decline between 0.08 and 0.26 kg for every increase in THI units in Germany. When the THI value increased from 68 to 78, milk production was reduced by 21%, and dry matter intake was reduced by 9.6% (Bouraoui *et al*., 2002). The reduction in feed intake could be due to the adaptive mechanism of animals to produce less body heat (Veerasamy *et al*., 2015). In 2017, Tibor Könyves and colleagues conducted a study to investigate the correlation between temperature and humidity index and milk production and feed intake of Holstein-Friesian cows across different seasons. The study revealed that thermal stress had a negative impact on daily milk yield, with reductions of 1.32 kg (9.46%), 0.92 kg (9.62%), and 1.27

kg (9.48%) observed as THI values increased from 64 in spring, 66 in autumn, and 42.34 in winter to 79 in summer.

A study by Robert *et al.* (2014) on analysis of Thermal stress in UK dairy cattle and its impact on milk yields indicated that the number of days where the THI exceeds this threshold (>70) could increase to over 20 days yr−1 in southern parts of England by the end of the century. Forough *et al.* (2017) did a study on the Effect of the temperature and humidity index and lactation stage on milk production traits and somatic cell score of dairy cows in Iran and found that the greatest milk yields were recorded in THI≤60 ($p<0.05$). The highest decrease in milk yield in connection with THI values was recorded in the early lactation (0 to 100 DIM). SCS was positively associated with the THI and increased more in an early period of lactation.

In 2016, Kaiser Parveen conducted a study on the impact of thermal stress on Sahiwal cattle performance in Haryana. The study findings showed that the THI values ranged from 57.13 in January to 81.84 in July. Additionally, the study determined that an increase in THI value above 68 resulted in a sudden decrease in the wet average/daily milk yield of Sahiwal cows within the herd. Furthermore, the study developed a prediction equation for Sahiwal's wet average using the THI value, which was represented as Y = 18.28-0.175Xi. THI significantly affected the daily milk yield (DMY) and peak daily milk yield (PDMY) of Holstein Frisian crosses, maintained in the loose housing system with feeding and watering management (Tassew, 2021, unpublished). According to the same author, DMY and PDMY declined by 0.054 kg and 0.10 kg, respectively for a unit increase in THI from the threshold (68 units).

Table 3. The size of milk production reduction with the unit rise in THI

Breeds	THI Variation	MY Reduction	References
HF cows	Unit rise >66	0.23 Kg/D/C	Santana *et al.* (2016)
HF cows	Unit rise of THI	0.14 - 0.2Kg/D/C	Lombertz *et al.* (2014) Ravagnalo *et al.* (2000)
Dairy cows	Unit rise >69	0.41 Kg/D/C	Spiers *et al.* (2004)
Dairy cows	Unit rise of THI>65	0.23-0.59 Kg/D/C	Bohmanova *et al.* (2007)
HF crossbreds	Unit rise of THI	0.43 - 0.886 Kg/D/C	Das (2009) Upadhyay *et al.* (2009)
Sahiwal cows	Unit rise of THI	0.16 Kg/D/C	Upadhyay *et al.* (2009)
Sahiwal cows	Unit rise>62	0.175 Kg/D/C	Kaiser Parveen (2016)
German Cows	Unit rise of THI>60	0.08-0.26 Kg/D/C	Brugemann *et al.* (2012)

Effects on milk composition

In another study, decreased milk protein, lactose, and fat values were recorded during the summer (Gaafar *et al*., 2011). Elevated temperature and humidity can reduce the ability of cattle to dissipate excess heat, which can ultimately lead to TS and associated physiological changes such as reduced milk fat and protein (Chebel *et al*., 2004; Novak, *et al*., 2007; Hossein-Zadeh *et al*., 2013). TS significantly reduced contents from 3.79% milk solids, 3.20% fat, 4.78% protein, 8.69% lactose, 12.48% SNF, and 0.71% ash during the winter season to 3.49%, 3.07%, 4.59%, 8.34%, 11.83%, and 0.67% during the summer season, respectively (Gaafar *et al*., 2011). In addition, milk solid, fat, and protein concentrations in Holstein-Friesian (HF), New Zealand Jersey (NZJ), and HF×NZJ cows tend to decline for THI values of 60, 64.3, 66.7, and 73.3, respectively (Bryant *et al*., 2007). Zedeh *et al.* (2013) reported that Iranian Holstein cows that calved in montTS in THI1 (30-40), and THI2 (41-50) group had a greater amount of milk and milk fats ($p<0.05$) than the cows that calved in months belonging to a month in THI group 4 (81-90).

Economic losses from thermal stress

Thermal stress causes severe economic loss in approximately 45%-60% of dairy farms' incomes around the world (Bernabucci *et al.,* 2010; Pragna, *et al*., 2017). Researchers (St-Pierre *et al.,* 2003; Bernabucci *et al.,* 2010) have documented the adverse impact of warm environments on cattle's productive performance, which can have devastating economic consequences for the global dairy industry. They have estimated a total economic loss incurred by the US livestock sector due to Thermal stress at between 1.69 and 2.36 billion US$. About 45%-60% of this loss was observed in the dairy industry [$897 million] (Bernabucci *et al.,* 2010; Pragna, *et al*., 2017). Upadhyay *et al.* (2009) reported that the annual total milk loss due to thermal stress at the all-India level was 1.8 million tons or approximately 2% of the total milk production of the country amounting to a whopping 2661.62 rupees per year. The negative impact of global warming on total milk production in India is also estimated to be about 3.2 million tons by 2020 and more than 15 million tons by 2050. The study showed that without heat abatement (minimum intensity), total losses across animal classes averaged $2.4 billion annually. However, by 2050, an estimated nine billion people will live on the planet and to feed the planet, we will need to increase food production dramatically.

Thermal stress reduction measures

Researchers (Armstrong, 1994; Silanikove, 2000; Chandra *et al.,* 2015; Amaral-Phillips, 2016) have identified and documented measures for mitigating thermal stress in animals. Commonly used Thermal stress reduction strategies included modification of the physical environment, breeding for heat-tolerant dairy cattle, nutrition management, and timed artificial insemination (TAI) protocol (Armstrong, 1994; Silanikove, 2000). Herd's physical environment management (introduction of shaded areas under which cows can graze via the retention of trees in paddocks and the planting of shelter belts and housing in pens in which they are exposed to evaporative cooling and spray and/or fan cooling (Ghosh *et al*., 2017*).* Breeding heat-tolerant animals, such as indigenous (Sahiwal), Senepol, and Carona, is a long-term strategy aimed at developing heat-resistant breeds for breeding purposes. Nutrition management is supplying high-energy feeds along with bypass protein and ration with >18% protein, low fiber, vitamins C, E, and A, and minerals such as zinc (Amaral-Phillips, 2016). Scientific literature provides several clear examples where the availability of shade for cattle can significantly reduce their heat load largely by reducing the solar radiation they receive (Armstrong, 1994; Kendall *et al*., 2006; Fisher *et al*., 2008). The scientific literature that monitors animal behavior also reports that cows with access to shade begin to preferentially use it when air temperatures rise above 25°C or when THI is above 73 and that uptake increases exponentially as air temperature increases (Kendall *et al*., 2006). Ghosh *et al*. (2017) did a study on the efficacy of shading and spraying to alleviate Thermal stress and found that the shade of the structure proved to be more successful at reducing moderate and severe Thermal stress occurrence than spray treatment. There were 53% fewer moderate and 86% fewer severe Thermal stress events with the shade treatment. The spray treatment proved effective at reducing only the number of severe Thermal stress events. During the 182 days, 46% fewer severe Thermal stress events were measured for the spray treatment. For low-susceptibility herds, simulated milk loss was 67% lower for the shade treatment and only 20% lower for the spray treatment.

Thermoregulation by the animal itself

In 2009, Verkek identified several processes utilized by dairy cattle to regulate their body temperature. These included seeking areas of pasture with the greatest air movement, standing to increase the body's surface area exposed

to air and/or changing orientation concerning the sun, increasing respiratory rate, panting and/or sweating to transfer heat away, reducing or stopping feed intake to minimize heat-generating metabolic processes, searching for water and shade, and seeking minimal shade from the herd itself if no other source of shade is available.

Conclusions

Thermal stress resulted in a decrease in reproductive efficiency, growth, milk production, and composition. The THI is the most commonly used index to measure the level of thermal stress in dairy cattle. The productive and reproductive traits of cattle are susceptible to the negative impacts of Thermal stress with an increase in THI above 68. The effects of thermal stress were higher in the summer seasons from April to September where the average THI level was above 75 than spring and winter seasons. The high-producing dairy cows seem to be more affected by thermal stress than the low-producing ones. The shade structure proved to be successful at ameliorating calculated milk production loss across herds with different susceptibilities to Thermal stress**.** Mitigation of stress due to meteorological factors should be considered an integral component of the cattle management system. Consideration of heat tolerance potential along with performance traits during selection can help to mitigate the impact of Thermal stress. In general, integrated heat mitigation measures need to be adopted to reduce thermal stress to maintain the welfare of animals and prevent economic losses.

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Bovine mastitis: Prevalence, causes and associated risk factors in Silte Zone, Ethiopia

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Abstract

Mastitis is an important disease of dairy cows, and it causes huge economic losses to dairy farm owners due to a decrease in milk production, and reduction in milk quality, and an increase in the cost of cow treatment. A cross-sectional study was conducted in Southern Ethiopia's Silte zone from October 2020 to June 2021 aimed to estimate mastitis prevalence, assess related risk factors, and identify prevalent bacterial causes. Three hundred eighty-four lactating cows were examined for abnormalities in udder quarters and teats. Milk samples were tested for subclinical mastitis via the California mastitis test (CMT) and cultured for causative agents from clinical mastitic and CMT-positive cows. The overall prevalence of mastitis was 54.9% (95% CI=49.9-59.9), of which 52.1% (95% CI=47.1-57.1) was subclinical and 2.9% (95% CI=1.6 -5.1) clinical mastitis. From a total of 1536 quarters examined 41 (2.7%) quarters were found blind and 427 (27.7%) quarters were affected by mastitis. Overall, more hind quarters, 236 (55.5%) were affected than the front quarters, 189 (44.5%) of udder. The prevalence of mastitis was significantly higher during early lactation, ≤4 months ($p< 0.05$); and it was increased with increasing parity ($p< 0.05$) and age ($p< 0.05$). Cows with no bedding were more affected than those with bedding ($p< 0.05$). Multivariable logistic regression showed that cows with round and flat teat ends were 2.84 and 11.85 times more likely to contract mastitis. Also, cows with pendulous udder, producing more than 10 liters per day milk and during the early 4 months of lactation were 1.87, 6.81, and 2.14 more likely affected by mastitis than normal udder, producing less than 10 liters of milk per day and lactation after five months, respectively. Milk samples collected from 211 mastitis-positive cows were cultured using standard bacteriological technique, and the isolated bacteria were *Staphylococcus aureus* (29.5%), *Staphylococcus epidermidis* (14.2%), *Staphylococcus intermedius* (11.6%), *Staphylococcus hyicus* (11.1%), *Streptococcus agalactiae*

(8.9%), *Streptococcus disgalactiae* (6.3%), *E. coli* (5.8%), *Streptococcus uberis* (5.3%), *Klebsiella* spp. (1.6%) and *Enterococcus* spp. (1.1%). Owners should give proper udder care and are advised to apply dry cow therapy. Extension workers should raise awareness.

Keywords: Mastitis; Prevalence; risk factor; Silte zone; Ethiopia.

Introduction

Mastitis is inflammation of the parenchyma of the mammary gland regardless of the cause. It is characterized by a range of physical and chemical changes in the milk and pathologic changes in the glandular tissue (Constable *et al*., 2017). Bovine mastitis is a multifactorial inflammatory disease that depends on a combination of animal, environmental, and pathogen-related factors (Hillerton and Berry, 2005).

Mastitis is one of the most prevalent and costly diseases in dairy production with losses attributable to milk production losses, milk quality, discarded milk, early culling, veterinary services, and labor costs (Seegers *et al*., 2003; Hogeveen, and *Østerås*, 2005). Mastitis has two forms: clinical and subclinical. The major causes of bovine mastitis are classified into contagious pathogens, for example, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *M. bovis*) and environmental pathogens, for example, Streptococcus uberis, Streptococcus dysgalactiae, E. coli, *Klebsiella* spp., and *Enterobacter* spp.) (Constable *et al*., 2017).

In both clinical and subclinical mastitis, there is a loss in milk production. Moreover, the loss in milk production does not only occur during the case itself, even after the mastitis case is cured, the milk production level of the cow stays lower. Milk production loss is not obvious to the producer, because this is milk never produced, and therefore never seen. It is a hidden cost or lost income opportunity (Hogeveen, and *Østerås*, 2005). Moreover, milk from mastitic cows is potentially a risk to public health, because it may transmit zoonoses and sicknesses associated with food toxins (Zouharva and Rysanek, 2008; FAO, 2014). *Staphylococcus aureus* produces staphylococcal enterotoxins that may cause food poisoning, which is characterized by acute onset of nausea, vomiting, abdominal cramps, and diarrhea (Le Loir *et al*., 2003; Zouharva and Rysanek, 2008).

In Ethiopia, the prevalence of mastitis ranged from 25.1% to 73.7% (Fesseha *et al*., 2021; Girma and Tamir, 2022). The higher prevalence of mastitis in lactating cows is greatly influencing the productivity of the animals. The potential predisposing factors so far studied include breed, parity, age of cow, lactation stage, bedding, udder position, teat end shape and morphology, and other management factors like feeding and hygiene (Getaneh and Gebremedhin, 2017; Kitila *et al*., 2021; Girma and Tamir, 2022).

There is no information about bovine mastitis from the Silte zone; thus, the study gives some information about mastitis in selected districts of the Silte zone. So, this study is useful to add to the existing knowledge of mastitis status in the country. Therefore, the purpose of this study is to estimate the prevalence of bovine mastitis, to identify the major bacterial causes circulating in the area, and to assess the potential predisposing risk factors in the study area.

Materials and methods

Study areas

The study was conducted from October 2020 to June 2021 in selected districts of the Silte zone, Southern Ethiopia. The zone is located at 7.43^0 N to 8.1^0 N latitudes and 37.86^0 E to 38.53^0 E longitudes. The area is generally characterized by diverse agro-climatic conditions with altitudes ranging from 1500 meters to 2,100 masl. The mean annual temperature and annual rainfall range from 15.1°C to 27.5°C and 800 to 2,000 mm, respectively (SZLFRD, 2020).

Study animals and study design

During the informed consent for cow udder examination and milk sample collection as well as the willingness to participate in the study was obtained from the animal owners. The study animal includes lactating cows (local zebu, and Holstein-cross breeds), that are found in and around Worabe. They were kept under small-scale and semi-intensive management systems. All the study cows were hand milked and milked twice a day, and the study included small-holder (i.e. having 1-10 lactating cows) and large-scale (i.e. having ≥10 lactating cows) dairy farms.

A cross-sectional study design was applied to estimate the prevalence of mastitis in lactating dairy cows and to investigate the major bacterial causes of mastitis

in the study areas. Also, the risk factors contributing to the development of mastitis were assessed. The study was conducted from October 2020 to June 2021. The potential risk factors considered for this study were teat end shape, udder position, milk yield per day, parity of cow, lactation days, breed, age, parity, bedding material, and district. Teat end shape and udder position were assessed as described by Bakken (1981) and the age of cows was determined by using dentition (Parish and Karisch, 2013). But information about milk yield per day, parity, and lactation days was collected by direct interviews of the owners during farm visits for the selection of the study cows and milk sampling.

Sample size and sampling methods

The study areas: Worabe town and two surrounding districts (Dalocha and Hulbarag) were selected because of their dairy production potential. In this study, lactating cows were selected by using a systematic random sampling technique. The sample size was determined by considering 50% expected prevalence (i.e. to have the maximum sample size), 95% confidence level, and 5% desired level of precision (Thrusfield, 2018). Hence, the total sample size required for the study was 384 lactating cows. The computed sample was proportionally allocated to the three study areas based on the number of dairy cows present in each area (Table 1).

Table 1. Proportional allocation of the number of study animals

District	Number of Lactating Cows	Sample size
Worabe	4202	138
Dalocha	3957	130
Hulbarag	3531	116
Total	11690	384

Source: Dairy cows data (SZLFRD, 2020).

Study methodology

Clinical examination of cows and milk

All quarters of the udders and teats of selected lactating cows were examined visually and by palpation for the presence of the manifestation of general clinical signs like a gross abnormality, inflammatory swelling and pain, hardness,

atrophy of tissue, and teat blindness. The milk sample was checked for the presence of clots, flakes, and blood by using a strip cup (Andrews *et al*., 2004; Quinn *et al.,* 2004).

Screening test (CMT)

All udder quarters irrespective of the physical, clinical, and examination results were tested by using California Mastitis Test (CMT) to investigate the affected quarter. During this time teat area was cleaned by using 70% denatured alcohol. After the first few squirts of milk were discarded, about 2ml of milk sample (i.e. approximately ½ teaspoon) were collected into a CMT paddle with four shallow cups. An equal amount of CMT reagent was added to each milk sample. Then after, the paddle was rotated to mix the contents. The CMT result was interpreted as negative=0, weak positive=+1, positive=+2, and strongly positive=+3 (Quinn *et al*., 2004; Constable *et al*., 2017). A cow was considered positive when at least one-quarter was positive for CMT.

Milk sample collection and culture

For identification of the causative agent/s milk samples were collected from all cows with clinical mastitis and CMT-positive cows. Udder and teat were cleaned thoroughly and dried with a clean towel, then, each teat end was cleaned with 70% ethyl alcohol. The first few streams of milk were discarded and approximately 10 ml of milk was aseptically collected from each quarter of lactating cows into a sterile test tube. Each test tube was labeled with the necessary information for identification. The samples were immediately transported by the ice box (4°C) to Sodo Regional Veterinary Laboratory, Wolaita Sodo town. Collected milk samples were subjected to bacteriological examination by following the standard procedure described in Quinn *et al.* (2004), and then it was immediately cultured. A loop full of milk samples was streaked on blood agar base enriched with 7% sheep blood and MacConkey agar, and incubated aerobically at 37ºC for 24 to 48 hours. Then, the plates were examined for growth, morphology, and hemolytic characteristics of colonies. Colonies were identified based on their morphology, color and size, presence or absence of hemolysis, and gram stain (Quinn *et al.*, 2004). Gram stain reaction including shape and arrangements of the bacteria, catalase, and O-F tests. And then, subcultures were done to obtain pure isolate for further identification. For further identification, biochemical tests were conducted by following the standard methods

described by Quinn *et al*. (2004) and Carter and Cole (1990). Gram-positive bacteria were further identified into genera and species based on catalase and oxidase test, tube coagulase test; and ability to produce hemolysis and ferment maltose and growth on mannitol salt agar. Gram-negative bacteria were identified based on the growth characteristics on MacConkey agar, as well as Oxidase reaction, catalase test, triple sugar iron agar, and "IMViC" test.

Data analysis

All collected data were entered into a Microsoft Excel spreadsheet, edited, coded, and then summarized by descriptive statistics like percentages and mean. For the data analysis, STATA 14.2 software (Stata Corp 4905 Lakeway Drive, College Station, Texas 77845 USA) was used. The prevalence was computed by dividing the number of mastitis-positive cows by the total number of examined cows and multiplying by 100. The association of the potential risk factors and the prevalence of mastitis was analyzed by univariable logistic regression analyses followed by multivariable logistic regression analysis. Then, after multicollinearity testing, all non-collinear variables with $p< 0.25$ were subjected to multivariable logistic regression analysis. Then, the model fitness was assessed by the Hosmer–Lemeshow goodness-of-fit test (Dohoo *et al*., 2009).

Results

Animal-level prevalence of mastitis

From a total of 384 cows examined both clinically and by CMT for subclinical 211 (54.9%, 95% CI=49.9-59.9) of them were found affected by clinical and subclinical mastitis (Table 2). The prevalence of subclinical mastitis was significantly (χ^2=12.31, $p< 0.05$) higher than that of clinical mastitis.

Table 2. The overall prevalence of mastitis in lactating cows in selected districts of the Silte zone

Study areas	No. Examined	Subclinical Mastitis		Clinical Mastitis		Overall	
		No Pos.	Prevalence (95% CI)	No Pos.	Prevalence (95% CI)	No Pos.	Prevalence (95% CI)
Worabe	138	72	52.2 (43.7-60.5)	3	2.2 (0.7-6.6)	75	54.3 (45.9-62.6)
Dalocha	130	66	50.8 (42.1-59.4)	6	4.6 (2.1-10.0)	72	56.2 (47.4-64.5)
Hulbarag	116	62	53.4 (44.2-62.4)	2	1.7 (0.4-6.8)	64	54.3 (45.1-63.3)
Total	384	200	52.1 (47.1-57.1)	11	2.9 (1.6 -5.1)	211	54.9 (49.9-59.9)

No. Pos. = number positive

Quarter-level prevalence of mastitis

From a total of 1536 quarters examined 41 quarters were found blind (i.e. 39 cows were with a blind quarter of which thirty-seven and two cows with one and two-quarters blind, respectively). So, the remaining 1495 quarters were examined and 425 (28.4%) of them were found positive for mastitis. Of the total positive quarters the subclinical mastitis accounted for 56.7% (n=241) and clinical mastitis for 46.3% (n=184). Relatively the right hind quarters were more frequently affected (55.5%) than the left hind quarters (44.5%) (Table 3).

Table 3. Prevalence of mastitis at the quarter level

Quarter sampled	Total examined	Positive for mastitis		Blind No. (%)
		Number positive (%)	95% CI	
Right front	384	95 (24.7)	20.7-29.3	3 (0.8)
Right hind	384	128 (33.3)	28.8-38.2	2 (2.1)
Left front	384	94 (24.5)	20.4-29.1	6 (4.2)
Left hind	384	108 (28.1)	23.8-32.9	14 (3.6)
Total	1536	425 (27.7)		41 (2.7)

Bacterial isolation

For causative bacterial agents isolation milk samples were collected from 211 mastitic cows (i.e. clinical and subclinical mastitis) and cultured. From these samples (n=211) about 21(9.95%) showed no growth and in 190 (90.05%) of them there was bacterial growth. In culture-positive samples, both contagious and environmental bacteria were isolated, and a total of eleven bacterial spe-

cies were identified. The most commonly identified bacteria were *Staphylococcus* spp. (n=126, 66.3%), followed by *Streptococcus* spp. (n=39, 20.5%).

The most commonly isolated bacterial spp. was coagulase-positive staphylococci [*Staphylococcus aureus* (29.5%), *Staphylococcus intermedius* (11.6%), and *Staphylococcus hyic- us* (11.1%)] which is followed by coagulase-negative staphylococci [*Staphylococcus epidermidis* (14.2%)]. The proportion of causative bacterial agents isolated during this study is shown in Figure 1.

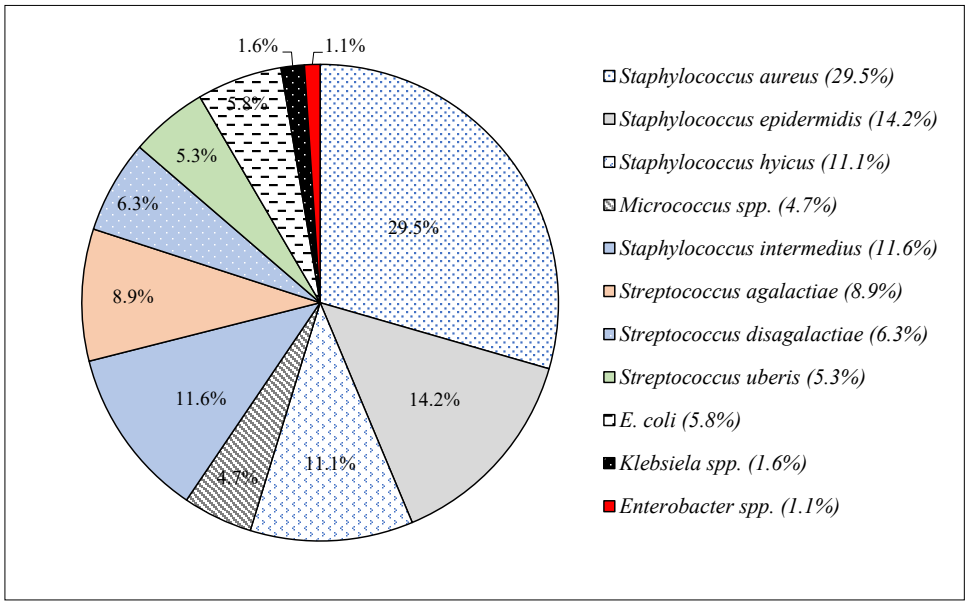


Figure 1. Proportion of bacterial species isolated from mastitic lactating cows

Risk factors analysis

A univariable logistic regression analysis revealed that the occurrence of mastitis was significantly ($p<0.05$) associated with parity, lactation days, age, the shape of the teat end, udder position, amount of milk yield, and bedding materials (Table 4).

The multicollinearity matrix revealed that the age of the cow and parity are collinear to each other (**Γ**= 0.97). Similarly, udder position and parity are col-

linear to each other (Γ = 0.83), and udder position and age are collinear to each other (Γ = 0.79). Biologically it is plausible to drop age and parity from the multivariable logistic regression analysis and consider the udder position that is influenced by age and calving numbers of the cow. So, the stage of lactation, teat end shape, milk yield per day, udder position, and bedding was selected for the final multivariable regression model (Table 4). The multivariate logistic regression model has Hosmer-Lemeshow $\chi^2(7) = 13.26$, p=0.066, ROC = 0.839, and this suggests that there is no significant difference between the observed and predicted values. The sensitivity, specificity, and positive and negative predictive values of the models are 77.25%, 74.57%, 78.74%, and 72.88%, respectively.

Table 4. Univariable logistic regression analysis of the association of mastitis risk factors considered for the study area

Risk factors	Total	Infected (%)	95% CI	Univariable			Multivariable		
				OR	95% CI	P-value	OR	95% CI	P-value
Teat end shape									
Pointed	146	50 (34.25)	27.0-42.4	Ref	-	-	Ref	-	-
Round	133	70 (52.63)	44.1-61.0	2.1	1.31-3.46	0.002	2.84	1.60-5.04	0.000
Flat	105	91 (86.67)	78.7-92.0	12.5	6.46-24.11	0.000	11.85	5.48-25.62	0.000
Udder position									
Normal	237	107 (45.15)	38.9-51.6	Ref			Ref	-	-
Pendulous	147	104 (70.75)	62.8-77.6	2.9	1.90-4.55	0.000	1.87	1.07-3.29	0.029
Milk yield per day									
<10 liter	155	47 (30.3)	23.6-38.1	Ref					
≥10 liter	229	164 (71.6)	65.4-77.1	5.8	3.71-9.07	0.000	6.81	3.95-11.73	0.000
Age (Years)									
3–5	145	67 (46.21)	38.2-54.4	Ref					

Risk factors	Total	Infected (%)	95% CI	Univariable			Multivariable		
				OR	95% CI	P-value	OR	95% CI	P-value
6–9	148	91 (61.49)	53.4-69.0	1.86	1.17-2.96	0.009			
≥10	91	53 (58.24)	47.8-68.0	1.62	0.96-2.76	0.073			
Lactation days									
≤ 120 days	250	162 (64.8)	58.6-70.5	3.20	2.06-4.94	0.000	2.14	1.25-3.67	0.006
≥ 121 days	134	49 (36.6)	28.8-45.1	Ref			Ref	-	-
Parity									
1-2	145	66 (45.5)	37.5-53,7	Ref					
3-4	155	92 (59.4)	51.4-66.8	1.75	1.11-2.76	0.017			
≥5	84	53 (63.1)	52.2-72.8	2.05	1.18-3.55	0.012			
Bedding material									
Yes	254	150 (59.1)	52.9-65.0	1.63	1.07-2.50	0.024	1.38	0.80-2.39	0.245
No	130	61 (46.9)	38.5-55.6	Ref					
District									
Worabe town	138	75 (54.3)	45.9-62.6	Ref					
Dalocha	130	72 (56.2)	47.4-64.5	1.08	0.66-1.74	0.77			
Hulbarag	116	64 (54.3)	45.1-63.3	1.0	0.61-1.64	1.00			

Discussion

The current study showed that the overall prevalence of mastitis in lactating cows was 54.9% of which the clinical and subclinical mastitis accounted for 2.9% and 52.1%, respectively. This study finding is relatively comparable with various reports from different parts of the country (Sori *et al*., 2005; Lakew *et al*., 2009; Abunna *et al*., 2013; Teklesilasie *et al*., 2014; Pal *et al*., 2017; Abebe *et al*., 2020). Subclinical mastitis was more prevalent (54.9% and 95% CI= 49.9-

59.9) than clinical mastitis (2.9% and 95% CI= 1.6 -5.1) in the study area, which is in agreement with various studies reported from various parts of the country (Getaneh and Gebremedhin, 2017; Abebe *et al*., 2020; Fesseha *et al*., 2021; Belay *et al*., 2022; Demil *et al*., 2022; Girma and Tamir, 2022). Subclinical mastitis causes huge economic losses (Azooz *et al*., 2020; Singh *et al*., 2021), which is mostly not recognized timely by owners. According to Sinha *et al*. (2014), the economic loss due to a reduction in milk yield was estimated to be 49%.

A relatively higher proportion of mastitis was observed in the hind quarter of cows, which is similar to previous study reports (Abera *et al*., 2012; Zenebe *et al*., 2014; Abebe *et al*., 2016; Zeryehun and Abera, 2017). The hind quarters are characterized by higher milk yield and longer time of milking (Tacin *et al*., 2006), and these factors are potential indicators for the development of mastitis and high somatic cell count (Kokca, 2006).

The multivariable logistic regression analysis showed that among the potential risk factors considered for this study lactation days, milk yield per day, udder position, and teat end shape were significantly influencing the prevalence of mastitis. This finding is in general agreement with Sori *et al*. (2005), Zenebe *et al*. (2014), and Abebe *et al*. (2016). The occurrence of mastitis is significantly ($p< 0.05$) higher during the early lactation period (Zenebe *et al*., 2014; Getaneh and Gebremedhin, 2017; Zeryehun and Abera, 2017), especially with environmental pathogens (Constable *et al*., 2017). This might be due to the absence of a dry cow therapy regime could be the major contributing factor to a high prevalence of mastitis at early lactation. Higher milk yielding and large teat canal diameter were associated with an increased risk of intramammary infection (Sori *et al*., 2005; Constable *et al*., 2017). There is a genetic correlation between milk yield and mastitis, and this correlation suggests that genes that increase milk yield tend to increase susceptibility to mastitis (Constable *et al*., 2017).

The prevalence of mastitis was significantly higher in round and flat shape teat ends ($p< 0.05$), which is in accord with the reports of Bakken (1981) and Hickman (1964). The teat apex is the first barrier against invading pathogens (Bakken, 1981; Seykora and McDaniel, 1985; Constable *et al*., 2017). Generally, the occurrence of mastitis is higher in cylindrical-shaped teats than the funnel-shaped teats (Hickmann, 1964; Rathore, 1976; Bakken, 1981). This might be due to the most frequent teat orifice prolapse in cylindrical-shaped

teats (Rathore, 1977; Bakken, 1981). According to Constable *et al*. (2017), the efficiency of the teat defense mechanism depends on the integrity of teat tissue; and its impairment leads to an increase in the risk of intramammary infection.

The multicollinearity test showed that the age of the cow and parity, udder position and age, and udder position and parity were found collinear to each other. This might indicate that these variables are confounding one another. So the multivariable logistic regression analysis revealed that the stage of lactation, teat end morphology, milk yield per day, and udder position were significantly ($p< 0.05$) influencing the occurrence of mastitis. These independent variables are the best predictor of mastitis in lactating cows in the Silte zone, Ethiopia.

Among the isolated bacteria, especially *Staphylococcus* spp. and *Streptococcus* spp. are commonly reported from various parts of the country (Abera *et al*., 2012; Abunna *et al*., 2013; Zenebe *et al*., 2014; Belay *et al*., 2022; Demil *et al*., 2022; Girma and Tamir, 2022; Tora *et al*., 2022).

Conclusions

This study revealed that more than half (50%) of examined lactating cows were affected by mastitis, clinical and subclinical mastitis. Subclinical mastitis was the dominant type of mastitis in the study area. Relatively, more proportion of the hind quarter of the udder is affected by mastitis. The prevalence of mastitis increased with the increasing of parity number and age of cows. Mastitis is significantly higher during the early period of lactation, in cows with round and flat teat ends, and pendulous udder, and in cows producing more than ten liters of milk per day. *Staphylococcus aureus* (29.5%) was the major bacterial agent isolated from affected cows.

Based on this study finding the following recommendations are forwarded: Proper attention should be given to mammary gland health by the owners to reduce the burden of mastitis. Strengthening animal health extension work to aware dairy owners about regular testing for early detection of mastitis, and improving the management of dairy cows will enable to reduce of the problem of mastitis. Dry cow therapy should be advocated and introduced into the areas, which will reduce the prevalence of mastitis during the lactation period. Further study for antimicrobial susceptibility of the common isolates is very

important that is for designing an appropriate drug selection and treatment regime.

Limitations of the study

Antimicrobial susceptibility of isolates is not done that is due to budget constraints.

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Ethical consideration

Informed consent was obtained from the animals' owners

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Knowledge, attitude, and practice of goat farmers towards contagious caprine pleuropneumonia in Amhara region, Ethiopia

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Abstract

Contagious caprine pleuropneumonia (CCPP) is known for its high mortality, morbidity, and economic losses. A cross-sectional study using a multistage cluster sampling technique was conducted in Amhara Regional State from January to June 2019 to assess the knowledge, attitude, and practices of goat farmers towards CCPP in the region. A semi-structured questionnaire was used to collect information from the goat farmers found in 12 districts. A total of 386 goat producer respondents participated in the questionnaire survey. Out of all the households of goat farmers interviewed, 370 (95.8%) were headed by males, while 16 (4.2%) were headed by females. Of all the respondents, 73.58% did not know the disease. The remaining 26.42% were familiar with the CCPP and had seen the disease in their goats or nearby goat flocks, and from this 4%, they experienced CCPP with their goats. Half of the participants who experienced the disease in their flock reported that mixing with neighboring flocks was the major source of CCPP infection. The goat farmers who are familiar with the disease had a high-risk perception of CCPP with a mean score of 4 out of 5 for the seriousness of the disease and 4.12 out of 5 for the risk of infection. These farmers showed a good perception of the effectiveness of prevention practices with a mean score of 4 out of 5 for the usefulness of vaccinations and reporting disease outbreaks to veterinary authorities. This collective understanding demonstrates their awareness of the disease and the proactive measures they are willing to take to mitigate its impact on their flocks. They have a good practice of vaccinating and treating their goats. Most of the farmers use veterinary clinic services to control CCPP occurrence in their herds. Although the farmers familiar with the disease have a good understanding of the risk of

the disease and a positive attitude towards control measures, most farmers are not aware of the disease and hence better animal extension about the disease is needed in the study area.

Keywords: Amhara region; Attitude; Contagious caprine pleuropneumonia; Goats; Knowledge; Practice.

Introduction

Goats are important assets for ensuring the livelihood of farmers. They are an important source of income for millions of people in Ethiopia (Farm Africa, 2017). They play a significant role in the livelihood of farming communities by providing milk, meat, and income to cover various household expenditures. However, the productivity and well-being of goats are constrained by several infectious diseases, among which contagious caprine pleuropneumonia is a major one (Gelagay *et al*., 2007; Tesfaye *et al*., 2011; MoA, 2012; MoA and ILRI, 2013; Asmare *et al*., 2016).

Contagious caprine pleuropneumonia (CCPP) is one of the most severe diseases in goats. It is caused by *Mycoplasma capricolum* subspecies *capripneumoniae* (Mccp) (OIE, 2014; Spickler, 2015). *Mycoplasma capricolum* subspecies *capripneumoniae*, formerly known as Mycoplasma biotype F-38, is classified within the genus *Mycoplasma* and the family *Mycoplasmataceae* (Spickler, 2015). The disease is transmitted through the inhalation of respiratory droplets during close contact, and it can be brought into a flock by a carrier or infected animal. CCPP primarily affects the respiratory tract, leading to symptoms such as anorexia, fever, and respiratory signs including dyspnea, polypnea, productive coughing, grunting, inability to move, standing with front legs wide apart, stiff and extended neck, as well as continuous salivation and nasal discharges (Ahaduzzaman, 2021).

CCPP hampers goat production due to its high morbidity and mortality (Gelagay *et al*., 2007; Tesfaye *et al*., 2011). The economic impact of the disease is linked to several factors, including high mortality, decreased meat and milk production, expenses related to diagnosis, treatment, and control, as well as disruptions of trade of goats and the associated products (Rushton, 2009). The main preventive and control measures for this disease include vaccination,

movement restriction, medical treatment, quarantine, cleaning, and disinfection of premises (OIE, 2009).

In a system characterized by a lack of guidelines for both farmers and local veterinarians, as well as the absence of national strategies to prevent and control the introduction or spread of diseases, farmers' knowledge and practices have an important impact on disease occurrence. A knowledge, attitude, and practice survey is a representative study of a specific population aimed at collecting valuable information on what is known, believed, and acted upon concerning a particular topic (WHO, 2008). Such surveys help generate data on farmers' knowledge, attitudes, and practices regarding animal diseases in a resource-scarce country (Tiongco *et al*., 2012) like Ethiopia. So far, little is known about goat farmers' knowledge, practices, and attitudes towards CCPP in Ethiopia in general and in the Amhara region in particular. This study was undertaken to assess the knowledge, attitude, and practices of farmers towards CCPP in goats in the Amhara region.

Materials and methods

Description of the study area and population

The study was conducted in Amhara regional state. The region is located in the northern and western parts of Ethiopia between 9°20' and 14°20' North and 36° 20' and 40° 20' East. The region comprises 11 administrative zones: North Gondar, South Gondar, West Gojam, Bahirdar special zone, Awi, East Gojam, Wag Hemra, North Wollo, South Wollo, Oromia special zone, and North Shoa (ANRS BoFED, 2012). The region covers approximately 161,828.4 km^2 area and encompasses three major agroecological zones: highlands (above 2,300 m.a.s.l), mid-highlands (1,500 to 2,300 m.a.s.l) and lowland (below 1,500 m.a.s.l) accounting 20, 44, and 28%, of the land area respectively. In the region, the daily average temperature ranges from 16 to 27°C. The mean annual rainfall over the whole region varies from 300 mm in the East to well over 2,000 mm in the West (ANRS BoFED, 2011).

The region has a total of 16,148,390 head of cattle, 11,086,083 sheep, 7,766,661 goats, 488,626 f horses, 220,940 mules, 3,279,179 asses, 151,143 camels, 19,809,915 poultry and 1,154,094 beehives (CSA, 2018). The goat population

in the region is kept under traditional smallholder farming in an extensive management system (CSA, 2017).
For this study, one or two districts were selected from all administrative zones except Bahirdar special zones. The districts included in the study were Aberegelle, Kobo, Thehulederie, Bati, Kewet, Ankober, Hulet Ejju Enese, Wemberma, Jawi, Ebenat, Wegera, and Dembia (Figure 1).

Sampling methods

Selection of districts, kebeles, and villages

The study was carried out from January to June 2019. District, kebele, and village were primary, secondary, and tertiary sampling units. The study districts were selected purposively considering the absence of previous studies, goat population, accessibility, and representativeness of the district to the zone. After excluding the kebeles that were remote, unsafe, or unlikely to cooperate, simple random sampling was employed to select two kebeles from each district of the remaining kebeles. For each selected kebele, simple random sampling was used to select two to four villages in proportion to the number of villages available. The lists of kebeles were obtained from each district agricultural office, while the lists of villages were obtained from the respective selected kebele representatives.

Selection of farmers

The multistage cluster sampling technique was employed to select sampling units for the questionnaire survey. Before commencing the study, general information about goat production in the area was obtained from animal health experts during a preliminary visit to each selected village. The inclusion criteria for farmers were being a goat producer, having at least 2 years of experience in goat production, and being willing to participate in the interview. For each selected village, a sampling frame was constructed by obtaining a list of goat farmers from a village representative. The 'RAND' function in Microsoft Excel 2010 (Microsoft Corporation, 2018) was employed to generate random entries for the selection of households in the selected village. If the selected farmer was unwilling to participate, another randomly selected farmer who met the criteria was substituted. Farmers were informed of their selection on the day before the village visit. Verbal consent from farmers was obtained before the interviews.

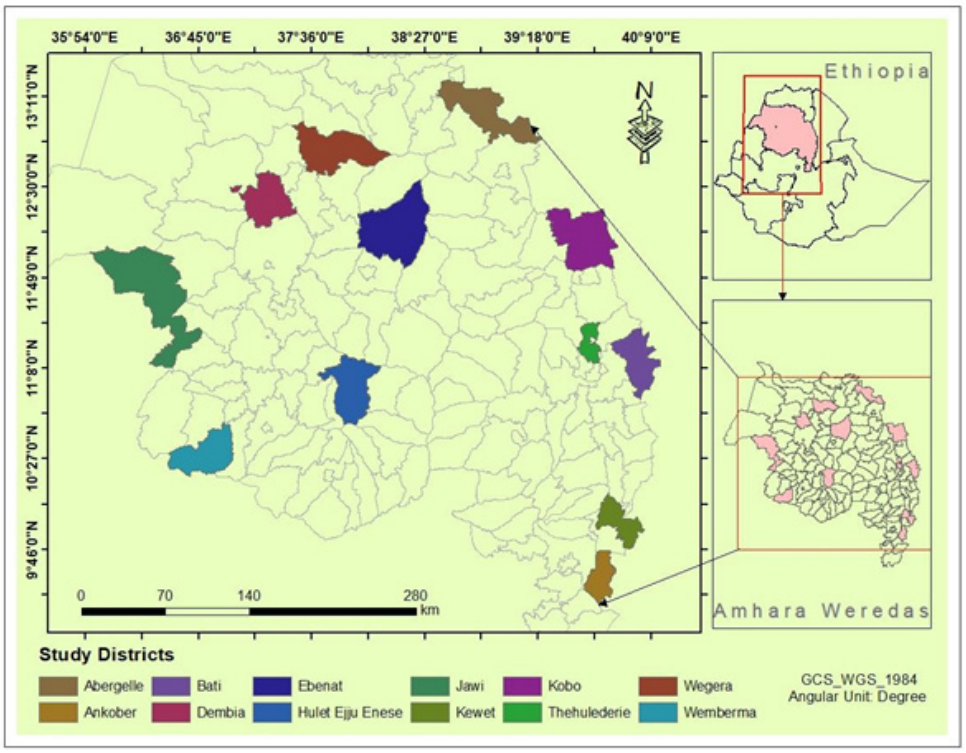


Figure 1. Map of the study areas

Sample size determination

For the questionnaire survey, the sample size was determined using Cochran's sample size formula for categorical data (Bartlett *et al.*, 2001).

$n=(Z)^2 * p(q))/d^2$

Where Z = the value for a selected alpha level of 0.05 = 1.96. (p)(q) = estimate of variance across the population with 0.5 value for each. P = proportion of the population that knew about the disease, **q** = proportion of the household who did not know about the disease, **d =** acceptable margin of error = 0.05. A total of 384-sample size was determined and 386 households were interviewed from twelve districts.

Study design and data collection procedure

A cross-sectional study design was employed for data collection. The questionnaire survey on management factors, the occurrence of CCPP, and attitude towards the disease were developed according to the guidelines of Frary (1998). The questionnaire had four components for collecting the data: socio-demographic characteristics of goat farmers, previous occurrence of goat diseases, farmers' practices for goat disease management, and their attitude, practice, and knowledge of CCPP. Both open and closed-ended questions were incorporated into the list of questions. Before starting an actual data collection, a pretest was conducted on goat farmers to see the gap in the questionnaire.

The socio-demographic data were related to location, educational status, household size, and goat flock size. Knowledge of the disease was assessed by asking questions on manifested goat disease, clinical signs of CCPP, morbidity, mortality, and case fatality rate. For this purpose, a case definition of CCPP developed previously was used as a guide (FAO, 2010). Accordingly, if a respondent mentioned a combination of the following clinical signs (labored breathing, nasal discharge, inability to move or stand with front legs wide apart, stiff and extended neck, and continuous drooling of saliva); postmortem lesions (fibrous pleuropneumonia, massive lung hepatization, straw-colored fluid in the pleura, enlargement of lung lymph nodes); and epidemiological situations (high morbidity, high mortality, affecting all age and both sex), he/she was considered knowing the disease. This was followed by further questions about his/her knowledge, attitude, and practices towards CCPP. If the respondent did not know CCPP based on the case definition above, the interview was stopped there.

The attitude of farmers towards disease occurrence was collected by asking questions related to the nature of the disease, risk of illness, and handling of the disease by clinicians. Data associated with controlling and preventing goat diseases were collected by asking questions about the prevention and treatment approaches employed when diseases occur.

For the ranking of goat diseases, the index value of each respondent's disease prioritization was calculated based on the guidelines provided by Kosgey (2004), using the following formula: -

Index = Sum of (n x number of households ranked first) + (n-1) x number of households ranked second + (n-2) x number of households ranked third +…+ 1 x number of households ranked last) for one factor divided by the sum of (n x number of households ranked first + (n-1) x number of households ranked second +…. +1 x number of households ranked last) for all factors, where n = the number of factors under consideration. The variable with the highest index value was the most economically important disease occurring commonly.

Knowledge, attitude, and practice were considered latent variables and were assessed using item questions and statements. The attitude was measured on an ordinal scale, including options such as strongly agreed, agreed, neither agreed nor disagreed, disagreed, and strongly disagreed. The mean score of farmers' perceived attitudes regarding the risk of infection and prevention of CCPP was calculated by dividing the total sum score by the number of respondents.

Data management and analysis

Data were recorded, cleaned, coded, and edited in Microsoft Excel. The analysis was conducted using STATA software version 14.1 (Stata Corp, 2014). Tables, frequencies, percentages, proportions, and pie charts were utilized to describe the characteristics of the study population, as well as the attitudes of farmers towards prioritization of economically important diseases and disease control practices.

Ethical consideration

Farmers were made aware of the purpose of data collection and how the researchers planned to use the collected information. Verbal consent was obtained from each participant and data were collected based on their willingness.

Results

Socio-demographic description of the participants

The general characteristics of interviewed households were presented in Table 1. Among the total goat farmer households interviewed, 370 (95.8%) were

male-headed, and 16 (4.2%) were female-headed households. Regarding the respondent's literacy, 41.7% were illiterate, 16.3% were adult education level, 31.3% were primary school completed, 8.8% were secondary school completed and the remaining 1.8% were college diploma level. The household size of respondents ranges from 4.5-6.8.

Table 1. Background characteristics of 386 goat producer respondents in selected districts of Amhara region, 2019

Zone	District	Number of kebeles Sampled	No. of villages sampled	Number of partici pants (%)	Average house hold size	Sex of partici pant						
						Female	Male	Prim ary sch ool	Secon dary school	Illite rate	Adult educ ation	College diplo ma
Wag Humira	Abergele	2	6	37 (9.6)	6.8	0	37	10	6	12	7	2
North Shoa	Kewet	2	4	33 (8.5)	6.1	0	33	1	1	11	20	0
	Ankober	2	5	41 (10.6)	6.3	0	41	16	10	5	10	0
Oromia Liyu zone	Bati	2	4	31 (8.0)	6.0	3	28	9	4	16	1	1
North Gondar	Dembia	2	4	30 (7.8)	5.7	1	29	6	1	18	4	1
	Wegera	2	2	18 (4.7)	6.2	1	17	0	0	18	0	0
South Gondar	Ebenat	2	5	36 (9.3)	6.1	1	35	15	0	16	5	0
East Gojam	Hulet Eju Enese	2	4	36 (9.3)	5.9	0	36	10	0	21	5	0
Awi	Jawi	2	5	34 (8.8)	4.7	2	32	8	2	21	3	0
South Wollo	Thehule dere	2	4	26 (6.7)	4.5	2	24	14	3	5	4	0
West Gojam	Wembe rma	2	3	31 (8.2)	6.6	6	25	13	2	11	2	3
North Wollo	Kobo	2	3	33 (8.5)	6.2	0	33	19	5	7	2	0
Total		24	49	386 (100%		16 (4.2%)	370 (95.8%)	121 (31.3%)	34 (8.8%)	161 (41.7%)	63 (16.3%)	7 (1.8%)

Goat management, flock size, and production goals

The management practices and purpose of goat rearing are shown in Table 2. Majorities (93.52%) of the respondents practiced a sedentary husbandry system and larger portions (79.27%) of the households used communal grazing (Table 2). Of the total respondents, 26.94% (95% CI: 22.7-31.6%) used housed barn type while 68.65 (95% CI: 63.8-73.1) used fenced type of housing. Concerning treatment practice, 91.97% (95% CI: 88.8-94.3%) of the goat farmers used modern treatment. Regarding the purpose of goat keeping, 68.8% of respondents rear goats for meat and cash income, 27.2% for cash income only, and 5% for meat, milk, and cash income.

Table 2. Description of management systems employed by goat farmers in selected districts of Amhara region, 2019

Management practice	Frequency	Percent	95% CI
Type of housing			
Fenced stable barn	265	68.65	63.8-73.1
Housed barn	104	26.94	22.7-31.6
Both fenced stablers and housed barn	17	4.4	2.7-6.9
Type of treatment used			
Modern	355	91.97	88.8-94.3
Traditional	11	2.85	1.6-5.1
Both modern and traditional	20	5.18	3.4-7.9
Who care goats			
Husband	98	25.39	21.3-29.9
Children	218	56.48	51.5-61.4
Other family members	54	13.99	10.9-17.8
Wife	1	0.26	0.04-1.80
Keeper	15	3.89	2.3-6.3
Type of husbandry system			
Sedentary	361	93.52	90.6-95.6
Trans-human	25	6.48	4.4-9.4
Type of grazing			
Fencing/paddock	30	7.77	2.31-13.56
Both paddock and communal	50	12.95	6.50-23.34
Communal	306	79.27	64.32-85.21

The average number of goats at the household level was 15.67 (95% CI: 13.2-18.56 %), whereas the minimum and maximum number of goats were 2 and 150, respectively. From a physiological category perspective, the average number of bucks, does, kids, and doelings were 11.79, 10.9, 8.97, and 4.58, respectively.

Priority goat diseases reported by farmers

According to 386 respondents' responses, goat diseases in the study districts of the Amhara region were prioritized (Figure 2). Peste des Petitis Ruminitis (PPR), Pox, CCPP, Mange, Anthrax, and Orf were the most common disease problems in the twelve districts of the Amhara region.

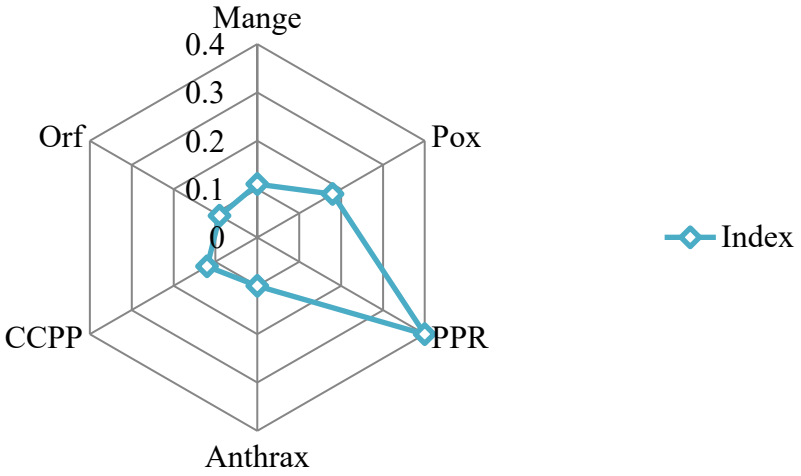


Figure 2. Radar plot of prioritized goat disease problems in the study district of Amhara region, 2019

Knowledge and attitude of farmers towards contagious caprine pleuropneumonia

Regarding CCPP knowledge, 73.58% (284) of the respondents had not heard of CCPP before participating in this survey. The remaining 26. 42% (102) of the goat farmers were familiar with CCPP and had seen the disease in their goats or nearby goat flocks and out of these 4.14% % (n = 16) of the respondents experienced CCPP with their goats. Fever, weakness, lethargy, coughing, difficulty

breathing, frothy nasal discharge, stringy saliva, and poor appetite were the reported clinical signs of the disease by these farmers.

According to 16 respondents who had experienced the disease in their goats and owned a total of 202 goats, the morbidity, mortality, and case fatality rate of goats due to CCPP was 40.60%, 17.3%, and 42.7%, respectively. They also reported that mixing with neighboring flocks (50%), mixing with traded animals (18.75%) and purchasing animals from the market (31.25%) were the major sources of goats' infection.

The mean score of farmers' perception was higher on the seriousness of CCPP (4.00), the risk of CCPP infection for goats (4.12), the dependability of CCPP vaccine (4.0), and the importance of reporting CCPP occurrence (4.00) (Table 3).

Table 3. Perception of farmers about the risk of infection and prevention of CCPP in goats of Amhara region, 2019 (N=16)

Attitude statements	Responses					Sum (Total score)	Mean
	SA	A	ND	D	SD		
1. CCPP is a serious or important disease	8	3	3	1	1	64	4.00
2. Goats are at risk of CCPP infection	7	5	3	1	0	66	4.12
3. Spread of CCPP from goat to goat can be prevented	1	1	3	5	6	34	2.12
4. Vaccination of goats against CCPP is trustful (if available)	7	4	3	2	0	64	4.00
5. Healthcare providers can handle CCPP outbreaks very well	6	4	3	3	0	61	3.81
6. Reporting of sick or dead animals by CCPP to the local authorities/ veterinary officers	7	3	5	1	0	64	4.00

Remarks: SA= Strongly agreed, A = Agreed, ND = Neither agreed nor disagreed, D = Disagreed,

SD = Strongly Disagreed, SA = 5, A = 4, ND = 3, D = 2, SD = 1

The practice of prevention and control of contagious caprine pleuropneumonia

The practice of farmers for controlling CCPP, when it occurs, is presented in Figure 3. The majority of the farmers (56%) control CCPP by using conventional veterinary treatment (Figure 3).

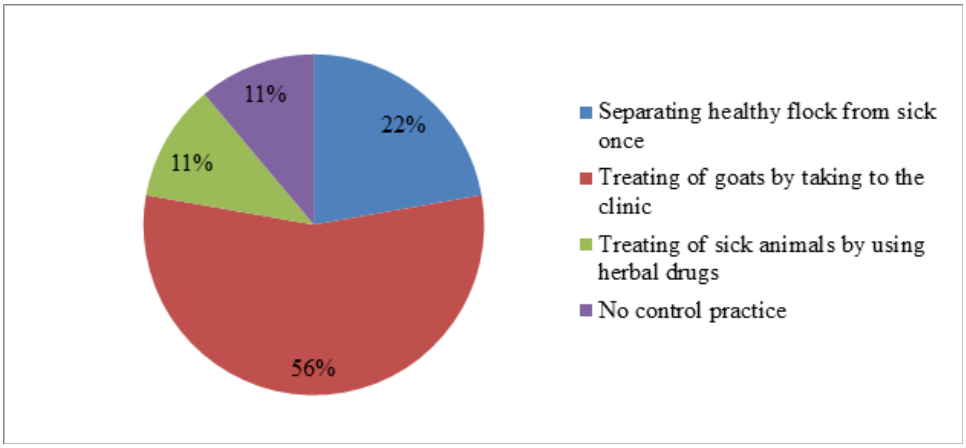


Figure 3. Control practice of farmers on the occurrence of CCPP in goats of Amhara region, 2019 (N=16)

Discussion

Assessment of goat disease information from goat farmers is crucially important for developing intervention strategies to control and prevent animal diseases. The study population in this study was in a mixed crop-livestock production setting, where transhumant and sedentary husbandry systems were practiced by 6.48% and 93.52% of the goat farmers, respectively. Both reported production practices could favor the transmission of CCPP. Traditional livestock production is commonly practiced in developing countries, especially in Africa, which increases the likelihood of the spread of CCPP among goats gathered at watering and grazing areas. Effective transmission occurs through close contact with infected animals and the inhalation of infective aerosols from infected goats (Lefevre *et al.*, 1987).

Most of the respondents (68.65%) used a fenced stable barn, which exposes the animal to wind, rain, and hunters, with poor hygiene floors and narrow spaces. This type of housing induces stress on goats so that it decreases their ability to fight against disease and makes them more susceptible to respiratory infection. Around 79.27% of households used communal grazing, an important predisposing factor for CCPP transmission. This condition allows animals to congregate in the grazing area, and there could be close contact with other animals. In the study area, when goats get sick, the farmers take them to a veterinary clinic for treatment. The treatment is primarily based on modern veterinary drugs (91.97%) rather than using local herbal medicines. This observation differed from a pastoral community practice highly dependent on local medicinal plant materials to treat and prevent animal diseases (Githiori *et al*., 2005; Giday and Teklehaymanot, 2013).

The respondents ranked CCPP as the third most important disease, following PPR and Pox. This ranking was based on factors such as economic losses associated with morbidity, frequency of occurrence, mortality rate, contagious nature of the disease, and the probability of recovery. However, this finding contradicts the results of a participatory investigation of CCPP in Southern Ethiopia, where farmers ranked the disease as the primary health problem in goat production (Mekuria *et al*., 2008). This variation may be attributed to the differences in socio-economic activities between the study areas. The previous studies were conducted in areas inhabited by pastoral communities whose livelihoods primarily depend on goat production. These communities undergo seasonal movement during the dry season towards the Omo River in search of feed and water for their animals, leading to a mixing up and aggregation of flocks that favor CCPP infection. Nevertheless, our study aligns with the report by Bett *et al*. (2009) in the Turkana Southern district of Kenya and Atim's (2010) in the Agagao districts of Uganda. Philemon *et al*. (2014) reported in the Manyara region of Tanzania that 61.5% of household respondents ranked CCPP as a more severe disease than other goat diseases. According to Senait (2012), CCPP is the second most significant goat disease, following foot and mouth disease, in the Ghindae sub-region of Eritrea. Kipronoh *et al*. (2016) in Kenya also reportedly stated that the occurrence of CCPP causes high morbidity and mortality in most goat flocks in the Rift Valley region of Kenya. Generally, CCPP is a common problem in pastoral production systems, which heavily rely on strategic mobility to access grazing land and water (Mekuria and Asmare, 2010).

The present study has shown that some goat farmers (26.7%) have experienced the occurrence of CCPP disease within their own and neighboring goat populations. The observed clinical signs were consistent with the documented case definition of CCPP (Radostits *et al*., 1994; Thiaucourt *et al*., 1996; Wesonga *et al*., 2004; FAO, 2010; OIE, 2014). However, the majority of respondents (73.6%) did not know CCPP, indicating an awareness gap among goat farmers regarding this disease. This knowledge gap could be partly attributed to the complexity of the disease, as it is often confused with other respiratory diseases such as PPR and pasteurellosis. It might also be influenced by recall bias due to the time elapsed between the occurrence of the disease and the study period. Furthermore, it could be associated with a lack of animal health extension services addressing each specific disease. These findings are consistent with studies conducted in Southern Ethiopia (Mekuria *et al*., 2008), pastoral areas of the Afar region (Gezahegn, 2006; Amare, 2012), in the regions of Somali, Oromia, Afar, and Southern Nation Nationalities of Ethiopia (Berhanu *et al*., 2017), Agago district of Uganda (Atim, 2010), and Manyara region of Tanzania (Philemon *et al*., 2014). Understanding the timing of disease occurrence is critically important for the development of disease prevention and control strategies.

In this study, the morbidity, mortality, and case fatality rate of goats due to CCPP were 40.60%, 17.3%, and 42.7%, respectively. This is in contrast to what is commonly quoted in the literature, the morbidity of CCPP can reach 100% and mortality can reach 70% in susceptible goat populations. However, the current findings indicate lower rates, which might be related to the endemicity of the disease in the study area. Nevertheless, our findings are comparable to a study conducted by Philemon *et al.* (2014) in the Manyara region of Tanzania, where the estimated mortality and morbidity rates were 11.5% and 38.5%, respectively. The morbidity rate is higher than the rate reported by Amare (2012), which was 30%, while the mortality rate is lower than the estimated rate of 31.7% in the Gulina district of the Afar region. Swai and Eselle (2010) reported an estimated morbidity and mortality rate of 31.6% and 61.4%, respectively, in the Maasai flocks of Northern Tanzania. The Maasai ethnic group, mainly relying on goat production, is found in both Kenya and Tanzania regions and is prone to drought and mobility with their livestock during the dry season in search of grazing areas and watering points, which increases exposure to infectious diseases. Mixing with neighboring flocks, trading animals, and purchasing animals from the market were identified as major sources of infection. The results indicate that members of the interviewed households

believe that the mixing of infected and uninfected stock, whether through communal grazing, watering, or markets, significantly contributes to the spread of CCPP from one flock to another. This finding is consistent with the report by Philemon (2014) in Tanzania.

Those farmers who had exposure to the occurrence of CCPP were asked to express their perception by using six-item statements. Most of the farmers showed high perception scores about the seriousness of CCPP, the risk of CCPP to goats, the capability of animal health providers to handle CCPP outbreaks, the reporting of CCPP cases, and the importance of vaccines in the prevention of CCPP (if they had access). These perceptions reflect the right attitude for easy control and implementation of intervention strategies. However, a higher number of farmers disagreed (strongly disagreed and disagreed) on the preventability of CCPP spread from goat to goat (mean score = 2.12), which has adverse implications for the prevention of CCPP. This discrepancy in perception could be associated with differences in farmers' knowledge levels. The level of knowledge can influence farmers' behavior regarding disease risk management, and each specific practice is also influenced by their attitudes (Jansen *et al*., 2009; Ellis-Iversen *et al*., 2010; Garforth *et al*., 2013).

Goat farmers control the occurrence of CCPP by separating healthy flocks from sick ones (22%), treating goats by taking them to the clinic (56%) and using herbal medicines (11%) to treat sick animals. Treating sick animals is one of the control measures for CCPP, as indicated by Thiacourt *et al*. (1996). However, approximately 11% of respondents did not implement any control measures when CCPP occurs. These practices indicate a low awareness among farmers regarding disease transmission and its impact. This lack of action poses a significant obstacle to the implementation of intervention strategies. In various questions, respondents were asked to recall information from the last 24 months, and study participants might have provided inaccurate information due to poor reminiscences from the past, this might be taken as a weakness of the study.

Conclusions

Most goat farmers in the study areas are not familiar with CCPP. Farmers who knew the CCPP perceived that it is a serious disease and goats in the study areas are at high risk of CCPP infection. These farmers who are familiar

with the disease had also a positive attitude towards vaccination and CCPP case reporting to a veterinarian, and good practice in vaccinating and treating their goats. Generally, although the farmers familiar with the disease have a good understanding of the risk of the disease and a positive attitude towards control measures, most farmers are not aware of the disease and hence better animal extension about the disease is needed in the study areas.

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Conflicts of Interest

No conflict of interest.

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Seroprevalence of *Brucella* infection in cattle and small ruminants in South Omo zone, southern Ethiopia

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Abstract

A cross-sectional study was conducted in selected districts of the South Omo Zone to estimate the seroprevalence of brucellosis and its associated risk factors. Additionally, the knowledge, attitude, and practices (KAP) of livestock keepers about the disease were also assessed. A total of 1349 sera samples were collected from 450 cattle and 899 small ruminants (450 goats and 449 sheep) kept under an extensive production system. Rose Bengal Plate Test was used for screening and ELISA as a confirmatory test for the detection of antibodies against *Brucella* species. Based on confirmatory tests, the overall seroprevalence of brucellosis was 2.2 % (95% CI: 1.1 – 4.1%) in cattle, 2.0% (95% CI: 0.9, 3.8%) in goats and 1.3% (95% CI: 0.5, 2.9%) in sheep with higher seropositivity in cattle compared to small ruminants. Seropositivity did not vary significantly (p> 0.05) with agroecology, age, and sex groups in cattle. However, a higher seroprevalence of 2.7% was detected in male cattle compared to 1.6% for females. Seroprevalence was higher in small ruminants from the lowland agroecology (3.0%) than those from the midlands (0.8%). Study respondents had a low level of knowledge on brucellosis with only 43% of them having some information about the disease. Most of the respondents have routinely engaged in practices that can expose them to infections such as assisted delivery (65%), contact with after-birth materials (50%), and handling aborted materials (15%) without any protection. High consumption of unpasteurized dairy products (93%) such as raw whole milk and traditionally fermented dairy product is also common. This study provides information on the occurrence

of brucellosis in major livestock species kept at cross-border marginal areas with limited disease information. The existence of various exposure practices implies the need for creating awareness among livestock keepers on general disease transmission and its zoonotic role.

Keywords: Brucellosis; Exposure practices; Risk factors; Ruminants; Seroprevalence; Southern Ethiopia.

Introduction

Brucellosis is an infectious disease of humans, livestock, and wildlife with public health and economic importance globally (Ducrotoyetal *et al*., 2015). It is one of the re-emerging zoonotic diseases that pose public concerns, particularly for livestock keepers. The disease has complex epidemiology involving multi-species with the sources of infection being fetal membranes, aborted fetuses, discharge from aborted or delivered animals, and consumption of raw milk and meat from infected animals (Adugna *et al*., 2013; Mangen *et al*., 2015). The means of transmission in the animal is through contact with contaminated materials, inhalation of aerosols, ingestion, and mucous membranes (Radostits *et al*., 2007).

Ruminant brucellosis is mainly caused by *B. abortus* and *B. melitensis;* the susceptibility of animals to brucella infection depends on various factors like natural immunity, environment, sex, and age (Kebede *et al*., 2008). Predominant impacts on livestock include reproductive problems such as abortion, decrease milk production, culling of infected animals; weak offspring, infertility, and weight loss (Mangen *et al.*, 2015). In livestock and most wild ungulates, death is rare, except in fetuses and neonates, and the mortality rate ranges from 12% – 14% in sheep and 11% - 13% in goats (Behnke, 2010). The three species; *B. abortus, B. melitensis,* and *B. suis* have an essential zoonotic role with no pathognomonic symptoms in human which make them difficult to differentiate from febrile conditions including malaria and typhoid fever (Ducrotoy *et al*., 2017). Thus, human brucellosis may be missed and undiagnosed by health professionals in developing countries and cases remain infected with consequent chronic disorders.

In developed countries, the disease has been controlled in animals and humans, while the re-emergence of the disease in humans is associated with travelers or immigrants coming from developing countries. On the other hand, a high

prevalence of *B. melitensis* in small ruminants has been reported from Middle Eastern countries with more than 100 cases per 100,000 herds. In Africa and South/Southeast Asia, the prevalence of brucellosis in livestock populations range from 0 to 68.8% in cattle, 0 to 88.8% in small ruminants, 0.4 to 20% in camels, and 0 to12.9% in other species (McDermott *et al*., 2013).

Brucellosis is an endemic zoonotic disease in most developing countries including Ethiopia. Seroprevalence results have been documented in cattle and small ruminants across different production systems and regions of Ethiopia (Megersa *et al*., 2011; Tadess, 2016, Tesfaye *et al*., 2017; Edao *et al*., 2018; Gutema and Tesfaye, 2020, Wossene and Teshager, 2021). However, there are few seroprevalence reports of brucellosis in cattle (Megersa *et al*., 2011; Mekonnen, 2021) and in small ruminants (Feyera *et al*., 2020; Sintayehu *et al*., 2015) in the South Omo zone.

Nevertheless, the complexity of the disease occurrence, dynamic production system with high herd mobility, multi-species herding, and intermixing of the herds at communal grazing and water point, proximity interaction of people and animals, together with limited knowledge of zoonoses and consumption of raw animal products make brucellosis an important disease in the country. Therefore, understanding the magnitude of the disease and the level of awareness and exposure risks among herders is vitally important in any intervention measures. Hence, this study investigated the seroprevalence of the disease and associated risk factors and livestock keepers' knowledge and risky practices related to brucellosis in the South Omo Zone of Southern Ethiopia.

Materials and methods

Study area

The present study was conducted in three districts (Dasenech, BenaTsemay, and Debub Ari) of the South Omo Zone. The Zone is located in the Southwestern part of Ethiopia bordering Northwestern Kenya. The Zone has a total area of 243,780 hectares, which is subdivided into agroecological features of 93.4% lowland arid and semi-arid areas and 5.6% midland areas (SOZFED, 2017). South Omo Zone is characterized by pastoral, agro-pastoral, and mixed farming production systems. Livestock species like cattle, goats, sheep, and equine are kept in the area. The Zone is found between latitude ranging from 4° 43' N to 6° 46' N and longitude ranging from 35° 75' E to 37° 07' E (Figure 1). It

receives 400-1600 mm of rainfall and has an average daily maximum temperature of 29.5^{0}C and a minimum of 12.3C^0 (NMA, 2018).

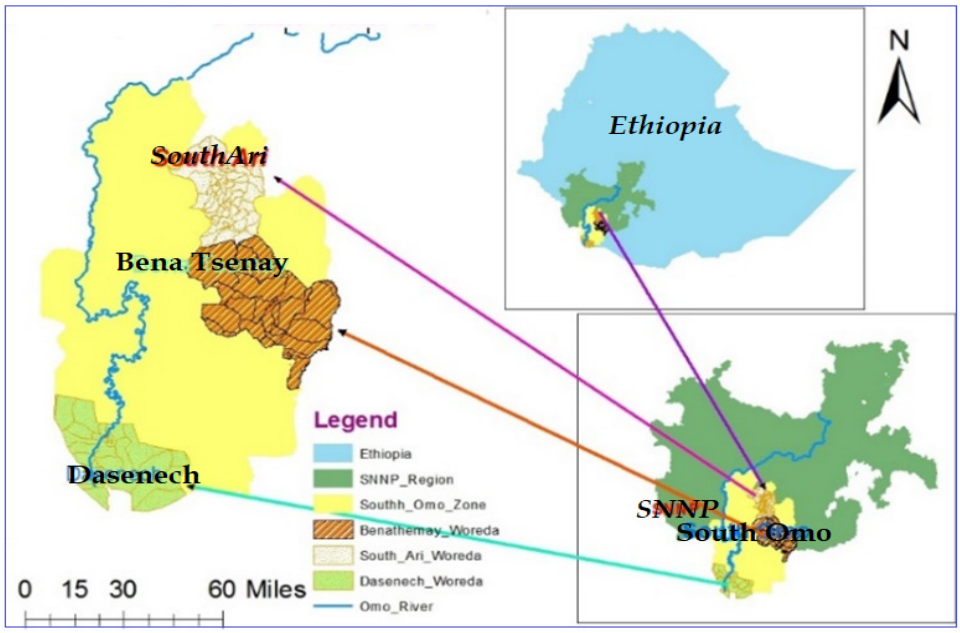


Figure 1. Map of Ethiopia showing the study area

Study design and study population

A cross-sectional study was carried out to assess the seroprevalence of brucellosis in cattle and small ruminants in three districts of the South Omo Zone. The study animals are indigenous cattle and small ruminants (both sex and above six months of age), which are kept under an extensive production system in Bena Tsemay, Debub Ari (south Ari), and Dasenech districts. The criteria for the selection of districts were based on complaints of ruminant abortion as a major problem in the areas during the animal disease surveillance by the Jinka Regional Veterinary Laboratory, in addition, to the large livestock population, and the diverse agro-ecology of the area.

Sample size determination and sampling technique

A mix of purposive and random sampling methods was applied to select the study units. Hence, zone and districts were purposively selected while *kebeles*, the lowest administrative unit, were sampled randomly, taking 3 *kebeles* from

each district, making a total of nine *kebeles*. South Omo Zone was purposively selected based on the abortion complaints reports by the Jinka Regional Veterinary Laboratory while the three districts were sampled considering agro-ecology, livestock populations, and production systems.

The respondents for the questionnaire survey were selected randomly or subsampled. The required number of respondents (herders) for the questionnaire survey was estimated using the formula described by Arsham (2007) as $N= 0.25/(SE)^2$; Where 0.25 is a constant value, SE refers to Standard Error (SE=0.05), N refers to the total number of respondents. Thus, 100 respondents were selected randomly for the questionnaire survey.

In selecting livestock for blood collection, as random sampling of herds was not feasible for the pastoral and agro-pastoral system, herds were selected through transect travel in the selected villages, and the encountered households were contacted for their willingness. If livestock keepers were willing, their animals were sampled until the required number of animals from a *kebele* was collected. If a contacted livestock keeper refused to cooperate, then the next household on the transect route was contacted to sample their animals. The livestock sample size was determined using the WinEpi formula (Universidad de Zaragoza©2010) with a 95% Confidence Interval (Z) and 5% marginal error (e), a design effect (deff) of 1.5%, and an expected prevalence (p) of 50% considering each species as an independent population.

An estimated designed effect of 1.5% was considered in the sample size estimation to cope with the expected sampling error resulting from the application of cluster sampling of animals from voluntary herders since the random sampling of herds and animals was not feasible. Thus, a total of 450 cattle and 900 small ruminants (450 goats and 450 sheep) were selected making a total sample size of 1350 animals. A total of 1,349 blood samples were processed and results were analyzed as a sheep sample was lost).

Questionnaire survey

The semi-structured questionnaire was administrated to livestock keepers to assess the knowledge and practices of herders toward brucellosis. The questionnaires focused on demographic variables including age, sex, education, knowledge of herders regarding the zoonotic nature of brucellosis, and expo-

sure practices of herders. The questionnaire covered also husbandry practices, livestock species composition, herd mobility, and consumption of animal products including dairy products. Local animal health workers were used to contact the owners of selected herds to explain the purpose of the study in the local language. Then, the interview was conducted in the local language of the herders, by translating the questionnaire originally developed in English to Dasenechigna, Benigna, Arigna, and Amharic languages.

Blood sample collection and testing procedure

After disinfecting the area of the jugular vein with alcohol, blood samples were collected from cattle (10 ml) and small ruminants (5 ml) using plain vacutainer tubes and disposable needles. The identity of each animal was marked on the corresponding vacutainer tubes. Then, tubes were kept tilted overnight at room temperature to separate the serum. The serum samples were harvested in cryo-tubes and transported on ice to Animal Health Institute at Sebeta, where they were stored at - 20°C until processed.

The Rose Bengal Plate Test antigen Kits (CACOGENICS Corporation, USA) were used for screening the presence of *Brucella* antibodies from sera. The test was carried out by adding an equal volume (25µl) of antigen and sera into each well in the direction from left to right as indicated in the laboratory protocol. Then the plate was agitated gently for 4 minutes. After 4 minutes of rocking the plate, the mixtures were observed for the formation of agglutination, and results were recorded.

Enzyme-linked Immunosorbent Assay (ELISA)

Positive reactor samples were tested by indirect ELISA (IDvet, 310, rue Louis Pasteur-Grabels-France) for antibodies to the *Brucella* species at the Animal Health Institute in Sebeta. In the 96 microplate wells, 190 µl of dilution buffer was added; then 10 µl of the positive, negative controls, and serum samples were added to their respective wells. Then the plates were incubated for 45 minutes at 21°C (+/- 5). Following incubation the contents were removed by flicking the plate over a sink, and the plate was washed three times with washing solution. Then, 100 µl of Conjugate 1x was added to each well; incubated for 30 minutes at 21°C (+/- 5) after which the plates were emptied and washed three times with washing solution. Then, 100 µl substrate solution was added to each well and incubated for 15 minutes at 21°C (+/- 5). Finally, 100 µl of the

Stop solution was added to each well to stop the reaction, and the plates were read by the microreader and their optical density (OD) values were recorded at 450 nm. The serum samples were considered positive if SP% > 120%.

Data management and analysis

Data were entered in the Microsoft Excel spreadsheet and analyzed using Stata version 14.2 (Stata Corp, College Station, Texas, USA). The data were carefully checked, cleaned, and imported from a Microsoft Excel Spreadsheet to Stata for further analysis. The seroprevalence was calculated by dividing the number of test-positive samples by the total number of samples multiplied by 100. The chi-square test was used to assess the association between seroprevalence and environmental and animal factors (species, sex, and age) and the statistical test was regarded as significant at $p < 0.05$.

Ethics statement

The ethical considerations (approval) for using study animals for the collection of blood samples was reviewed and approved by The College of Veterinary Medicine and Agriculture of Addis Ababa University and approval was given through a letter with Reference No VM/ERC/ 01/12/.11/2019. South Omo Zone and the selected district livestock offices were also consulted to carry out the study in selected areas. Informed consent was obtained from the participants after informing them about the purpose of the study and assuring them their identity would not be disclosed.

Results

Demographic and production system characteristics

Of the 100 respondents interviewed, 83% were male participants. Their age distribution showed 35% were from 18 to 35 years, 42% were from 36 to 45 years, and 23% were older than 46 years. Generally, most of the respondents (60%) were illiterate and 40% of them had some informal education, mostly elementary. A large proportion of the households (60%) had average family size below 7 persons and 40% of them had more than seven individuals per household. The respondents were engaged in agro-pastoral (22%), mixed farming (36%), and pastoralism (42%) production systems. Most of the respondents owned diverse species of livestock with 97% of them keeping cattle and small

ruminants, and few of them (3%) possessed other species such as donkeys and dogs.

Hygienic practices, attitudes, and awareness of respondents

Most of the respondents (65%) routinely assisted birth delivery and half of them (50%) had contact with after-birth materials, and some of them (15%) handled aborted fetus materials (Table 1). The majority of participants (58%) did not use any protection while assisting at birth. Knowledge about brucellosis is generally low and only 43% of them have some information about the disease, and only 27% of the respondents mentioned the correct route of transmission for the disease. They mentioned the main symptoms of the diseases to be abortion in cattle and small ruminants. Stillbirths, vaginal excretion, reduced milk production, and weakness were also mentioned as signs of the disease. Most of the respondents (93%) reported applying some disease prevention measures like isolation from other animals and culling. The respondents indicated that they believe that the disease is treatable by traditional medicine (15%) and modern medicine (66%), while a few of them (19%) did not know about the treatments. Study participants also reported that they do not prefer going to health institutions due to their dependence on traditional medicine (14%), remoteness (19%) from health centers, lack of awareness (28%), and cost of treatment (39%). The majority of respondents (93%) consume unpasteurized dairy products such as whole milk and traditionally fermented milk "Ergo" (yogurt) whereas very few individuals (7%) had no habit of consuming any raw dairy product (Table 1).

Table 1. Socio-demographic and exposure practices of respondents (n=100) in the South Omo zone

Variables	Levels	Proportion (%)
Sex	Male	83
	Female	17
Marital status	Married	92
	Single	4
	Divorce /widow	4
Age (years)	18-35	35
	36-45	42
	>46	23
Education level	Illiterate	60

Variables	Levels	Proportion (%)
	Literate	40
Production system	Agro-pastoral	22
	Mixed farming	36
	Pastoral	42
Heard about brucellosis	Yes	43
	No	57
Assist at birth	Yes	65
	No	35
Contact with birth material	Yes	50
	No	15
	Do not know	35
Use of protective materials	Yes	7
	No	58
	Do not know	35
Route of transmission	Contact	14
	Ingestion	9
	Inhalation	4
	Do not know	73
Raw milk intake	Yes	93
	No	7

Seroprevalence of brucellosis and the effect of risk factors

The results of the RBPT tests were 6.4% for cattle, 5.6% for goats, and 4.6% for sheep. Following confirmatory test by indirect ELISA, the overall seroprevalence of brucellosis was found to be 2.2 %; (95% CI: 1.1 – 4.1%) in cattle, and 1.7% (95% CI: 0.8 – 3.5%) in small ruminants. Seroprevalence of brucellosis was significantly higher in cattle than in small ruminants ($p<0.001$). Table 2 shows the summary of the seroprevalence of bovine brucellosis with potential risk factors. The results showed that the seroprevalence was not affected significantly by district, agroecology, sex, and age. However, the seroprevalence of bovine brucellosis was marginally higher in Bena Tsemay (3.3%) and lowest in Debub Ari (0.7%) district. The seropositivity was slightly higher in the lowland (2.7%) compared to the midland agroecology (2.0%). The seroprevalence also

did not differ significantly between female (2.7%) and male (1.6%) cattle young (0.9 %) and adult (2.7 %) cattle (p>0.05).

Table 2. Seroprevalence of brucellosis in cattle

Variables	Samples	Positive	Seroprevalence (%)	X^2	P-value
District					
Dasenech	150	4	2.7	2.7	0.265
BenaTsemay	150	5	3.3		
Debub Ari	150	1	0.7		
Agroecology					
lowland	150	4	2.7	0.2	0.651
midland	300	6	2.0		
Sex					
female	257	7	2.7	0.7	0.405
male	193	3	1.6		
Age					
young	115	1	0.9	1.3	0.254
adult	335	9	2.7		
Overall	450	10	2.2 (95% CI: 1.1, 4.1%)		

In small ruminants, brucellosis was prevalent in 2.0% (95% CI: 0.9, 3.8%) of the goats and 1.3% (95% CI: 0.5, 2.9%) of sheep with marginally higher seropositivity in Dasenech (3.0%) than other districts (χ^2= 5.3, p=0.072) based on ELISA results. There was also a significant difference in *Brucella* seropositivity with higher seroprevalence in lowland (3%) than midland (0.8%) agroecology (χ^2= 4.9, p=0.027). However, there was no significant effect on species (2.0% vs 1.3%, sex (1.8% vs 1.4%), and age (1.8% vs 1.6% of small ruminants (Table 3).

Table 3. Seroprevalence of Brucellosis in small ruminants

Variables	Samples	Positive	Prevalence (%)	X^2	P-value
District					
Dasenech	300	9	3.0	5.3	0.072
Bena Tsemay	299	2	0.7		
Debub Ari	300	4	1.3		
Agro-ecology					
lowland	300	9	3.0	4.9	0.027
midland	599	5	0.8		
Specie					
goats	450	9	2.0	0.6	0.437
sheep	449	6	1.3		
Sex					
female	611	11	1.8	0.2	0.653
male	288	4	1.4		
Age					
young	167	3	1.8		
adult	732	12	1.6	0.01	0.886
Overall	899	15	1.7(95% CI: 0.7, 3.5)		

Discussion

In the present study, we assessed the knowledge and risky practices of livestock keepers about brucellosis using a semi-structured questionnaire. In this result, a higher proportion of herders (65%) had exposure to *Brucella* infections through unprotected assistance of animals giving birth or aborting. This exposed the respondents to contaminated fetuses, afterbirth, and aborted fetus. They also mostly consume raw dairy products, suggesting high exposure risks among livestock keepers. A previous research finding demonstrated that the major means of brucellosis transmission is via exposure to aborted animals and assisting animals at birth (Kozukeev *et al*., 2006), which is in line with our findings.

The knowledge of herders on brucellosis and its means of transmission is vitally important in minimizing the risk of exposure to infection. A significant proportion of the livestock keepers (43%) had good knowledge about brucellosis whereas most of the respondents (57%) had no information about the

disease, which increases the likelihood of exposure of these respondents to infections. Similar survey findings were also reported from the Bench Maji zone in which most of the livestock keepers (72.7%) did not have information and basic knowledge about brucellosis (Kenea and Megersa, 2021). Additionally, others also reported low awareness among respondents of semi-intensive farms in and around the Alage district (Asgedom *et al*., 2016) and Addis Ababa dairy cattle workers (Edao *et al*., 2018). In the present study, the majority of respondents (93%) consumed unpasteurized milk and dairy products, which is one way of the transmission of brucellosis from animals to humans (Omore *et al*., 1999).

In the present study, through RBPT 6.4%, and ELISA test the overall seroprevalence in cattle was found to be 2.2%. Such high variation between the tests might be related to the high sensitivity of RBPT particularly in chronic cases, and relatively low specificity in endemic areas (Díaz *et al*., 2011). The observed prevalence is in agreement with the previous finding carried out in other parts of Ethiopia such as 2.2% (Gutema and Tesfaye, 2021) from Afar Region pastoral system where there is high livestock mobility, multi-species herding and livestock-dependent livelihood similar to the present study. Similar seroprevalence figures (2.1 to 2.4%) were reported by Tesfaye *et al*., (2017) from Kombolcha, Asgedom, *et al.* (2016) from Alage district, and Yohannes *et al.* (2016) from Arsi Zone. Our seroprevalence result of cattle brucellosis is lower than the reports of other authors, which range from 5.2%-42.31% (Yohannes *et al.,* 2016; Mekonnen, 2021; Wossene and Teshager, 2021) which could be due to difference in the production system and agroecology.

In this study a higher seroprevalence of brucellosis was observed in cattle (2.2%) than in small ruminants (1.8%), which is similar to previous reports in Ethiopia (Megersa *et al*., 2011) and other African countries; Tanzania (Assenga *et a*l., 2015), and Sudan (Mokhtar *et al*., 2007). Seroprevalence variation might be due to prevailing *Brucella* species, as *Brucella abortus* infection is more prevalent in cattle than in small ruminants; also among different domestic ruminant cattle were the most infected with *Brucella* spp than other animals (James, 2012; Temba, 2012).

In the present study, seroprevalence was slightly higher (2.7%) in lowland than midland (2.0%) areas, though not statistically significant. Findings from several authors also revealed a higher seroprevalence in lowland pastoral production systems than in mid-altitude areas and crop-livestock mixed framing

practice (Megersa *et al*., 2011; Dinka and Chala, 2009; Omer *et al*., 2000). A possible reason might be the production system, composite of livestock species kept, and frequent migration of pastoral herds in search of pasture and water points due to conflicts.

In this study, seroprevalence did not vary with animal factors such as age and sex of animals unlike most of previous reports which showed adults were more affected than young animals (Godfroid *et al.,* 2010). A similar finding has been also documented in Tanzania by Shafee *et al.* (2012) in which there was no variation of the brucella seroprevalence between sex groups. Nevertheless, findings of other studies reported from Tanzania, Nigeria, Pakistan, and Ethiopia (Megersa *et al*., 2011; Mai *et al*., 2012; Shafee *et al*., 2012; Assenga *et al.,* 2015) showed a significant difference between male and female animals. A possible reason could be due to sample size, the number of positive reactors in which few male animals are often tested, in addition to the effect of the age difference between male and female animals in which females are more aged than their male counterparts, (Godfroid *et al*., 2010). Likewise, a study by Akinseye *et al.* (2016) also indicated age of cattle did not have a significant effect on the seropositivity of brucellosis. Most of the previous studies in Ethiopia and other African countries (Megersa *et al.,* 2011; Assenga *et al*., 2015; Mekonnen, 2021) reported a significant association of age with Brucella seropositivity with higher seroprevalence in adults than in young animals. Age-related seroprevalence is ascribed in literature to the availability of erythritol hormones in mature animals that stimulated the multiplication of Brucella organisms and concentration in the reproductive organs (Radostits *et al.,* 2007). Additionally, increasing the age of the animals also increases the risk of exposure associated with longer contact with infected animals or with the environment.

The finding of 1.8% seroprevalence of brucellosis in small ruminants is similar to the findings of Mohammed *et al.* (2017) who reported 1.72% prevalence from the Somali Region, Ethiopia; Sintayehu *et al.* (2015) recorded seroprevalence of 1.9% in pastoral and agro-pastoral lowlands of Ethiopia, and Teshale *et al.* (2006) reported 1.9% seroprevalence from Afar and Jijiga areas in Ethiopia. The current study area has many similarities with the study areas of the aforementioned findings in terms of animal husbandry practices, population density, use of communal grazing lands and water points, and high mobility of herds that facilitate the transmission of the disease within the livestock population. But a significantly higher seroprevalence (21%) was reported from 124 female small ruminants with an abortion history in the same study area (Feyera *et al*.,

2020). The variation could be due to sample size, sampling techniques i.e. considering of flock with abortion history, and types of tests used. Another study in the Borana area also showed a higher seroprevalence (9.11%) of the disease compared to our findings (Yohannes *et al.,* 2013). The study area shared the same production system but, showed different seroprevalence results possibly due to differences in sample size, the test used, and *Brucella* species circulating in the area.

In this study, seropositivity to *Brucella* infection in small ruminants is significantly higher in the lowland than in the midland area, which is in accordance with the findings of Aloto *et al*. (2022) in Southern Ethiopia. The higher prevalence in the lowland areas could be related to the livestock husbandry practice in lowland areas, where different livestock species are kept together and large flocks can come into contact at communal grazing and water points which could facilitate *Brucella* infection in small ruminants.

We did not find a significant difference in the seropositivity of *Brucella* infection between sheep and goats unlike most of the previous study findings in which higher seroprevalence was reported in goats than sheep (Teshale *et al*., 2006; Ashenafi *et al.,* 2007; Tesfaye *et al.,* 2012; Kelkay *et al.,* 2017; Aloto *et al*., 2022). This result is in line with the report of Deddefo *et al.* (2015) in Arsi and East Shewa which reported seroprevalence of brucellosis in goats (4.9%) and sheep (4.4%) was not statistically different. The variation in seroprevalence between the species of animals could be due to the different sample proportions between species and the size of the flock and their management. In general, a wide range of seroprevalence of *Brucella* infection; ranging from 1.9% to 15.4% was previously reported (Teshale *et al.,* 2006; Ashenafi *et al.,* 2007; Tesfaye *et al*., 2012; Wedajo *et al*., 2015; Teshome *et al*., 2018; Aloto *et al.,* 2022; Dosa *et al*., 2022) in goats and sheep in Ethiopia.

Conclusions

The present study showed that antibodies against *Brucella* species are prevalent in cattle and small ruminants in southern Ethiopia where flock mobility and cross-border movement of animals are also high. The results provide baseline information on brucellosis occurrence in livestock in cross-border marginal areas with limited disease information. Agroecology was an important risk factor for brucellosis occurrence in small ruminants in that higher seroprevalence

was recorded in the lowland pastoral production system than midland area. The existence of various practices which expose people and animals to brucella infection implies the need to create awareness among livestock keepers about the general nature of the disease and the zoonotic role it plays.

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Contributing factors to repeat breeding and postpartum anestrus and pregnancy rate subsequent to hormonal intervention in crossbred dairy cows

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Abstract

A cross-sectional study was conducted from January to July 2021 to determine the prevalence and contributing factors to repeat breeding (RB) and postpartum anestrus (PPA) in crossbred dairy cattle in the central highland of Ethiopia. Furthermore; a retrospective study was used to collect data on the occurrence of RB and PPA and the associated risk factors. The pregnancy rate was determined by rectal palpation on day 60 after hormonal therapy using double $PGF_{2\alpha}$ and GnRH in combination with $PGF_{2\alpha.}$ (Ovsynch). The prevalence of RB and PPA was 33.85% and 30.73%, respectively. Body condition score, parity, milk yield, herd size, abortion, and mastitis all revealed a statistically significant association ($p < 0.05$) with the prevalence of RB and PPA. Cows with a previous history of abortion were 2.58 times at risk of repeat breeding than those without. Similarly, cows with a previous history of mastitis were 2.63 times more at risk of RB than cows that didn't encounter the disease. Previously aborted and older cows with greater parity numbers were 2.89 and 1.23 times more affected by PPA, respectively. Moreover, endometritis and retained fetal membrane showed significant association ($p<0.05$) with postpartum anestrus. The pregnancy rates in cows treated with double $PGF_{2\alpha}$ and GnRH + $PGF_{2\alpha}$ (Ovsynch) were 41.46% and 29.17%, respectively. It is concluded that the occurrence of RB and PPA in the research area was influenced by parity, body condition, and daily milk yield of the cow. Reproductive health problems including abortion retained fetal membranes, endometritis, and mastitis affected the prevalence of RB and PPA. Although the use of double $PGF_{2\alpha}$ or in combination with GnRH has resumed cyclicity in RB and PPA dairy cows, the pregnancy rates are yet low. Hence, a study that utilizes detailed hormonal assay profiles and reproductive-related blood metabolites should be done.

Keywords: Anestrus; dairy cow; GnRH; PGF_{2a}; postpartum; repeat breeding.

Introduction

Reproductive inefficiency is one of the most costly and production-limiting factors facing the dairy industry. Dairy cattle reproductive and production efficiency in Ethiopia is lower than the average African levels (CSA, 2021). Limited genetic potential of dairy cows, inadequate nutrition, and prevailing animal diseases (Belay *et al.*, 2012) as well as reproductive problems (Hadush *et al.*, 2013) that are related to an ovarian malfunction (Cenariu and Jospe, 2017), fertilization failure, and embryonic mortality (Levine, 1999), are among the major limitations resulting in poor reproductive performance (Bartlett *et al.*, 1986; Ibrahim *et al.*, 2011). Repeat breeding (RB) and postpartum anestrus (PPA) are among the major causes of infertility in dairy cows (Dinka, 2013) resulting in economic losses, replacement costs as well as loss of genomic improvement.

Repeat breeding is a breeding problem that results in substantial financial losses for dairy producers due to lower conception rates and inadequate milk yield (Lafi *et al.,* 1992). The causes of RB are multifactorial (Sarder *et al.*, 2010; Nath *et al.*, 2014) that include managerial Asaduzzaman *et al.*(2016) and Malik *et al.*(2019), dietary and reproductive diseases and disorders including endometritis, ovarian cysts, and anovulation (Wodaje and Mekuria, 2016; Cenariu and Jospe, 2017). Poor estrus detection and untimely insemination also contribute to repeat breeding (Keskin *et al.*, 2010). Similarly, PPA is considered a reflection of faulty management, poor nutrition (Rhodes *et al.*, 2003; Montiel and Ahuja, 2005), chronic illnesses, or uterine and ovarian disorders (Opsomer *et al.*, 1996; Gundling *et al.*, 2012). In addition, the incidence of PPA is thought to be influenced by season, breed, age (McDougall, 1994; Mwaanga and Janowski, 2000), parity number, dystocia, and by extended effects from the previous pregnancy (Opsomer *et al.*, 2000).

Various hormonal interventions have been suggested to treat repeat breeding and postpartum anestrus, the potential causes of infertility (reduced or absent capacity to produce viable offspring) in female cattle (Tiwari *et al.,* 2019; Ambrose, 2021). The most commonly applied treatment option is to stimulate or induce estrus and ovulations using GnRH (Fricke *et al.*, 2003) or prostaglan-

dins ($PGF_{2\alpha}$) or in combination with both hormones (Karki *et al*., 2018; Tiwari *et al*., 2019).

In Ethiopia, studies that have been conducted on reproductive problems in dairy cattle are fragmented and focused on identifying the major reproductive disorders (Mitku *et al*., 2012; Haile *et al*., 2014). However, the extent of occurrences of RB and PPA in crossed dairy cows is not yet well documented. Moreover, there were no reports related to the potential risk factors and hormonal intervention on PPA and RB in the study area. Though the study area belongs to the central highland of Ethiopia with a relatively large number of crossbred dairy cows and is one of the main milk sheds of the country that supply milk and milk products to Addis Ababa, the reproductive and productive efficiency of dairy cows remained low. Therefore, the current study was conducted to assess the prevalence, contributing risk factors, and pregnancy rate subsequent to hormonal intervention in RB and PPA dairy cows.

Material and methods

Study area

The research was conducted in Debre Berhan town and its surrounding from January to July 2021. The town is found in Amhara Regional State of North Shoa Zone, some 120 kilometers north of Addis Ababa, at 9^0 30 and 9^0 50 latitudes and 39^0 20 and 39^0 44 longitudes with an Altitude of 2,840 meters above sea level. The average annual temperature is 20 °C, 7°C, and 8.2°C during the day and at night, respectively with 964 milliliters of precipitation. The mean monthly temperature of the study area ranges from 2.8 to 21.9°C with a mean of 13.18°C, and the mean annual rainfall ranges from 698.5 to 1083.5mm with a mean annual of 920 mm (EMA, 2017).

According to the classification by Nish *et al.* (2018), extensive, semi-intensive, and intensive farms are prevalent in the study area. Cows in extensive management conditions were grazing and given straw as a supplement feeding and they were housed on a conventional floor without any additional facilities. Cows that were held in a semi-intensive system were provided with hay, straw, and some concentrate feeding; they were kept in farms with concrete floors. Cows in intensive farms were housed in a standard barn with a concrete floor and were provided with feed consisting of hay, and concentrate with vitamin

and mineral mixture before and after calving, they were also fed brewery by-product (spent grain) and straw. Water in most cases was given ad libitum.

Study population

Dairy cows belonging to small-scale (extensive and semi-intensive) farmers and commercial (intensive) dairy farms keeping Zebu*HF crosses located in and around Debre Berhan were considered as the study population. The farms were selected based on the owners' willingness and accessibility to records. Cows that were in estrus for at least three cycles and have been inseminated but failed to conceive were classified as repeat breeders. Cows that have not experienced estrus for more than 60 days succeeding parturition were designated as postpartum anestrus.

Study design and method of data collection

In dairy farms with organized records, retrospective data were collected. In farms without a recorded history of cows, data was collected with a structured questionnaire. The collected information includes herd management-related (herd size, housing, and animal-related (previous calving history, age, parity, milk yield, retained fetal membrane, endometritis, mastitis, and abortion). To determine the nutritional status of the dairy cows, body condition was scored on a one-to-five scale according to Wildman *et al.* (1982). Accordingly, cows were categorized into body condition scores (BCS) of < 3 and ≥ 3.

The sample size required for this study was calculated using Thrusfield's (2005) method. The confidence interval was calculated to be 95 percent, the absolute precision was 5%, and the projected prevalence was estimated to be 50% because there were no comparable reports in the study area. The sample size was calculated as follows:

$\mathbf{n= 1.96^2 Pexp\ (1\text{-}Pexp)/\ d^2}$, where Pexp = expected prevalence; d= absolute precision; n =sample size.

Accordingly, the estimated sample size was 384 dairy cows. Animals were obtained by inquiring about 3 commercial farms and 164 smallholder farmers to collect data using a questionnaire survey.

Selection of repeat breeding and postpartum anestrous cows for hormone treatment

Cows identified as repeat breeders failed to conceive after three or more inseminations but were regularly cycling and were subjected to hormonal therapy. Similarly, cows that were identified as having postpartum anestrous were also subjected to hormonal therapy. Additionally, all cows in the treatment were properly evaluated to be free from any detectable malfunctions of reproductive organs, and free of reproductive diseases or disorders that are associated with dystocia, retained fetal membrane, or endometritis.

After selecting repeat breeder cows and those with postpartum anestrus, cows were categorized into two groups based on the presence or absence of CL. Further cows were evaluated for BCS (<3 or ≥3) and parity (≤3, 4-6 or ≥7). All dairy cows were also subjected to rectal palpation and trans-rectal ultrasonography to detect apparent reproductive abnormalities and the presence of the corpus luteum.

Treatment group I (double $PGF_{2\alpha}$**)**: The first group consists of dairy cows with CL (n=50) on either of the ovaries that received 2 ml of $PGF_{2\alpha}$ (Synchromate ®, Bremer, GMBH, Warburg, Germany) intramuscular on day zero (Figure 1). Estrus detection was started 24 h after the first $PGF_{2\alpha}$ administration and cows in estrus were inseminated 12-24 hrs after standing heat. If the cows failed to show estrus signs within five days of the first $PGF_{2\alpha}$ injection, a second $PGF_{2\alpha}$ was administered on day eleven, and insemination followed after 18 hrs of standing heat.



Figure1. Diagrammatic representation of double $PGF_{2\alpha}$ **protocol**

Treatment Group II (Ovsynch): All cows without detectable CL (n=24) at rectal palpation and ultrasonography were injected with 100 µg Gonadotropin Releasing Hormone (GnRH) (Vetoquinol, Canada) on day 0 (Figure 2) followed

by $PGF_{2\alpha}$ (Synchromate ®, Bremer, GMBH, Warburg, Germany) administration on day 7. The cows were then monitored for signs of estrus three times per day for two days. When cows were seen in estrus, two inseminations were performed; the second insemination was conducted 12 hrs later. This was done to improve pregnancy rates by minimizing mistakes in estrus detection and the disparity between insemination time and ovulation.

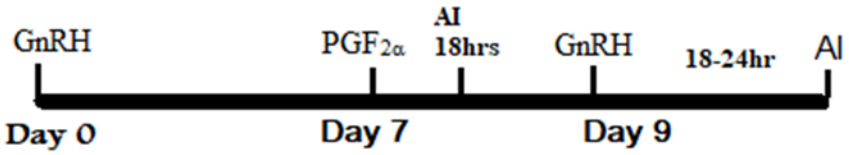


Figure 2. Diagrammatic representation of Ovsynch protocol

Dairy cows that did not come to heat/estrus within 48 hours of administration of $PGF_{2\alpha}$ were given the second shot of 100 µg GnRH on day nine and were inseminated 19 hrs later without estrus detection (fixed time AI). In both groups, when cows were seen in estrus, two inseminations were performed; the second insemination was conducted 12 hrs later. This was done to improve pregnancy rates by minimizing mistakes in estrus detection and the disparity between insemination time and ovulation. To minimize individual variance due to inseminator skill, every cattle was inseminated by the same inseminator and with semen from the same batch of production from the Kality Livestock Development Institute (LDI).

Pregnancy diagnosis

To eliminate individual variance, pregnancy diagnosis was performed by the same professional after 60 days of insemination using rectal palpation, where the asymmetry of the uterine horns and CL on the ovary ipsilateral to the pregnancy and palpation of the allantoin-chorion (membrane slip) are very good indicators of pregnancy (ultrasonography was not used due to organizational limitations).

Data analysis

Statistical Package for Social Science (SPSS) software version 25 was used to sort, code, compute, and analyze the data that had been collected. Descriptive statistical analysis was used to calculate the prevalence of repeat breading

and postpartum anestrous in percentages. The chi-square (X^2) test was used to determine the associations between risk factors with the prevalence of repeat breading and postpartum anestrous. Logistic regression was also employed to check the influence of the risk factors on the occurrences of RB and PPA, $p < 0.05$ was considered to declare a significant effect.

Results

Repeat Breeding (RB)

Out of 384 dairy cows that were included in the study, 130 (33.85%) were repeat breeders (RB). In cows with a previous history of repeat beading, the associated potential risk factors such as body condition score, parity number, and milk yield were statistically significant ($p<0.05$) as shown in Table 1.

Table 1. Association of cow-related risk factors with repeat breeding

Risk Factors	Categories	N (% Prevalence of RB)	X^2	P-value
BCS	≥3	61 (22.93)	14.9336	0.001
	<3	69 (25.94)		
Parity (number)	≤3	105 (39.47)	20.4143	0.016
	4-6	22 (8.27)		
	≥7	3 (1.14)		
Daily milk yield (litters)	<10 L	33 (12.41)	49.2897	0.000
	≥10 L	97 (36.47)		

Among management-related variables; herd size revealed a significant association ($p<0.05$) with the prevalence of repeat breading (Table 2), whereas the production system has no statistically significant relationship ($p>0.05$) with repeat breading.

Table 2. Association of management-related factors with repeat breeding

Risk Factors	Categories	N (% Prevalence of RB)	X^2	P-value
Production system	Extensive	40 (15.04)	3.6968	0.157
	Intensive	24 (9.02)		
	Semi-intensive	66 (24.81)		
Herd Size	≤10	106 (39.85)	17.2584	0.000
	>10	24 (9.02)		

Among reproductive disorders and diseases; abortion and mastitis showed a strong association ($p<0.05$) with repeat breeding. Cows with an abortion history had 2.58 times more chance of becoming repeat breeder cattle than those that were not affected cows. Similarly, cows with mastitis exhibited 2.63 times more risk of becoming repeat breeder cattle (Table 3) than those that were not affected by mastitis. Dairy cows with lower BCS (<3), higher milk production, and smaller herd size had a statistically significant ($p<0.05$) association with the prevalence of repeat breeding (Table 3).

Table 3. Strength of association (logistic regression) between risk factors and repeat breeding

Risk Factors	Odds Ratio	p-value	[95% Conf. Interval]
BCS	0.36	0.000	0.22 - 0.61
Milk yield	0.16	0.000	0.09 - 0.27
Herd Size	0.85	0.000	0.80 - 0.91
Abortion	2.59	0.044	1.03 - 6.51
Mastitis	2.63	0.005	1.35 - 5.14

Postpartum anestrus (PPA)

Of 384 dairy cows, 118 (30.73%) were post-partum anestrous (PPA), cows that didn't return to estrus within 60 days of parturition. Body condition score and milk yield were among the risk factors that had statistically significant ($p<0.05$) associations with postpartum anestrus. The management-related factors such as production system and herd size had no eminent relationship ($p>0.05$) with postpartum anestrus (Table 4).

Table 4. Association of management-related factors with postpartum anestrus

Risk Factors	Categories	N (% Prevalence of PPA)	X^2	P-value
Production system	Extensive	35 (13.78)	2.9683	0.227
	Intensive	31 (12.2)		
	Semi-intensive	52 (20.47)		
Herd size	≤10	77 (30.33)	27.9611	0.112
	>10	41 (16.14)		

From the reproductive health problems (risk factors), when an equal number of cows without reproductive health problems were compared with those

with reproductive problems, abortion, endometritis, retained fetal membrane (RFM), and mastitis all showed significant association ($p<0.05$) with postpartum anestrus.

Among the risk factors, RFM has shown a strong association with PPA, and cows that had previous RFM were 9.904 times more at risk of facing postpartum anestrus than those cows free of RFM. Moreover, cows with older age and thus with greater parity were 1.23 times more at risk of suffering from postpartum anestrus than those cows that were younger.

Table 5. Strength of association (Logistic regression) between risk factors and PPA

Risk Factors	Odds Ratio	p-value	[95% Conf. Interval]
Parity	1.24	0.002	1.08-1.42
BCS	0.24	0.000	0.14-0.42
Daily Milk Yield	0.29	0.000	0.17-0.48
RFM	9.90	0.006	1.95-7.29
Endometritis	3.58	0.013	1.31-9.75
Mastitis	2.56	0.041	2.32-5.89
Abortion	2.89	0.025	1.15-7.29

Response to hormonal treatment and associated pregnancy rate

The overall pregnancy rate of repeat breeder and postpartum anestrus female cattle that were treated with double $PGF_{2\alpha}$ and Ovsynch protocols is given in Table 7.

Table 6. Pregnancy rate of repeat breeding and postpartum anestrus cows by hormonal therapy type

Treatment protocol	No. of treated animal	No. of animals in estrus	No. of pregnant animals (%)
Double $PGF_{2\alpha}$	50	41	17 (41.46)
GnRH+$PGF_{2\alpha}$ (Ovsynch)	24	24	7 (29.17)
Total	74	65	24 (36.92)

Among the total of 50 cows that were diagnosed as having corpus luteum (CL) on either of the ovaries, 33 cows exhibited overt signs of estrus after the first

injection of $PGF_{2\alpha}$. Consequently, 12 (36.36%) cows were conceived after artificial inseminations. A second shot of $PGF_{2\alpha}$ was given to the rest of the cows (n=17) of which 8 cows exhibited estrus signs and were inseminated, from which 5 (62.5%) were diagnosed pregnant on rectal palpation 60 days after insemination.

Twenty-four (24) cows that did not possess CL upon ultrasonography examination received GnRH injection on the day of start, among which six cows showed estrus signs and inseminated, where only 1 (16.67%) cow confirmed pregnant. Consequently, from 18 cows that failed to show estrus and further received $PGF_{2\alpha}$ injection after 7 days of GnRH administration, three cows showed estrus and inseminated, from which 1 (33.3%) cow was able to conceive. The remaining 15 cows were given the second GnRH injection on day 9 of the first GnRH injection and inseminated 19 hrs later (fixed time AI) regardless of manifestation of estrus signs, where 5 (33.3%) cows were found pregnant on rectal palpation 60 days of insemination.

Discussion

The prevalence of repeat breeding (33.85%) in the cross-sectional study is consistent with the findings of Bartlett *et al.* (1986), who reported the prevalence of repeat breeding (RB) ranging from 14.6 and 36.8 %. In disparity to studies that indicated a prevalence of 7.03%, 13.08%, and 8.72% (Mitku *et al.,* 2012), (Haile *et al.*, 2014) and (Haftu and Gashaw, 2009) in various regions of Ethiopia, respectively and 10.1%, 11.3%, 27.6% and 20% (Gustafsson and Emanuelson, 2002), (Nath *et al*., 2014) (Malik *et al*., 2019) and Sarder *et al*., 2010), respectively from abroad, the prevalence of RB in the present study was quite high. The high rate of occurrence of RB may be the consequence of insufficient estrus detection and errors in timing insemination (Keskin *et al.*, 2010). Moreover, the variances across these studies may be a result of study design, individual variation, topographical variances, and discrepancies in the interpretation of repeat breeding.

The findings of the present study revealed a significantly higher prevalence of RB ($p < 0.05$) in dairy cows with low BCS (<3) than in cows with BCS of ≥3. This result was consistent with previous works of Asaduzzaman *et al*. (2016) and Malik *et al.* (2019), who also found a substantial link between decreasing body condition consequent to inadequate supply of nutrition and reproductive

problems including RB. Dairy cows that have insufficient nutrition tend to have low BCS, thus putting them at a higher chance of repeat breeding. More specifically, poorly fed dairy cows are more likely to develop a negative energy balance, which in turn affects the release of reproductive hormones and hence, increases the risk of fertilization failure and/or early embryonic mortality that leads to a higher prevalence of RB (Malik *et al.*, 2019).

The current investigation also revealed that milk yield had a statistically significant ($p < 0.05$) impact on the prevalence of RB. The findings show that the higher a dairy cow's milk output is, the greater the chances of being a repeat breeder. In agreement with the current study, Gustafsson and Emanuelson (2002), Nath *et al.* (2014), and Malik *et al.* (2019) observed that the high-producing cattle were more susceptible to RB. The reason could be changes in the reproductive physiology of higher milk-supplying cows experiencing RB and hence subjected to lower fertility (Gustafsson and Emanuelson, 2002). In addition, Yusuf *et al.* (2010) reported that shorter days in milk at first AI were among the risk factors for repeat breeding.

The present study indicated that dairy cows in smaller herds ($n \leq 10$) were more likely to be repeat breeders. The result was consistent with Gustafsson and Emanuelson (2002), who also reported a direct relationship between the decrease in herd size and the rise in the number of repeat breeder cows. However, Asaduzzaman *et al.* (2016) found that RB was considerably less common on farms with fewer animals. The differences may be explained by management differences in those herds; with the assumption that herds with fewer animals were of part-time livestock production and larger herds tend to be commercial farms and hence more attention was given to fertility/reproductive and productive performance of the cows (Asaduzzaman *et al.*, 2016). In the present study, dairy cows with a history of reproductive problems of abortion and mastitis during the previous calving had a considerably greater prevalence of RB ($p < 0.05$): dairy cattle that had abortion and mastitis were 2.58 and 2.63 times more likely to turn out to be a repeat breeder, respectively. This finding agreed with the reports of Levine (1999) and Gustafsson and Emanuelson (2002).

The 30.73% prevalence of postpartum anestrus in the present study fairly agrees with the findings of Ducrot *et al.* (1994) who reported a 32.5% prevalence of PPA. In contrast, Hadush *et al.* (2013) and Haile *et al.* (2014) reported considerably lower prevalence of 12.9% and 10.26%, respectively in various regions of Ethiopia; and McDougall (1994), Walsh *et al.* (2007) and Kamal *et al.*

(2012) of 19.5%, 17.2% and 18.5%, respectively from abroad. The difference in prevalence between these studies might be topographical variations, differences in breed studied, and individual variations (Mwaanga and Janowski, 2000). Cows with a body condition score (BCS) of less than 3 and poorly fed animals had a substantial association with the occurrence of postpartum anestrus in the present investigation. The findings were in agreement with reports of Ducrot *et al*. (1994), Rhodes *et al*. (2003), and Montiel and Ahuja (2005), who found a strong interrelation between PPA, body condition scoring, and poor nutrition. The findings of the present study also revealed a substantial association between the higher milk yield of dairy cows and postpartum anestrus. Comparable results were also reported in previous studies of Mwaanga and Janowski (2000) who found that higher-yielding cattle were more at risk of PPA.

Among the reproductive problem-related risk factors considered in this study, endometritis, RFM, mastitis, and abortion was found to have a strong association ($p < 0.05$) with PPA. These results correlate with the findings of Opsomer *et al*. (2000) who were able to show that the risk of postpartum anestrus increases with multiple reproductive disorders and diseases.

The pregnancy rate obtained in the present study when using double $PGF_{2\alpha}$ protocol was 41.46%, which is lower than the findings of Mansour *et al.* (1999) (65.2%) and El-Shahat and Badr (2011) (71.42%) who also used the same treatment protocol. The variations may lay on differences in the breed of cattle used for the study or inseminator skill. In contrast, the hormonal intervention using the Ovsynch protocol resulted in a pregnancy rate of 29.17%, which is fairly close to the findings of Caraba and Velicevici (2013) who reported a rate of 25% for the same hormonal therapy. On the other hand, Zobel *et al*. (2011) found a higher pregnancy rate of 79.88%, in 131 out of 164 treated cows. The difference can be justified by the breed of cattle studied and or the skill of the inseminator as well as the late pregnancy diagnosis time of 60 days post insemination in the present study.

Conclusions

This study confirmed that repeat breeding and postpartum anestrus are among the frequent causes of infertility in dairy cows in the study area. The occurrence of RB and PPA was influenced by parity, reproductive health problems

including abortion, retained fetal membranes, endometritis and mastitis, body condition, and daily milk yield of the dairy cows. Treatment of cows with RB and PPA with double $PGF_{2\alpha}$ and/or Ovsynch protocol brought them back to cyclicity thereby increasing the chance of becoming pregnant up on insemination. It is thus, recommended that smallholder and commercial dairy farms need to improve the dairy management and reproductive health monitoring strategy since most risk factors of RB and PPA arise from poor care for dairy cattle. Moreover, hormonal therapy can be used to reduce the wastage of dairy cows that are prone to culling due to RB and PPA.

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Conflict of interest

The authors declare that they have no competing interests.

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Comparison of quantitative real-time PCR targeting *nuc* gene and culture-based plate count methods for quantification of *Staphylococcus aureus* in raw cow milk

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Abstract

Staphylococcal food poisoning (SFP) is caused by ingestion of enterotoxins produced by enterotoxigenic *Staphylococcus aureus* when the cell population exceeds 5 Log CFU per gram/ml of food. The Objectives of this study were to evaluate the performance of SYBR Green 1-based quantitative real-time PCR (qPCR) targeting the *nuc* gene for the quantification of *S. aureus* in milk and to compare the assay with the plate count method. The qPCR and the plate count were applied for the quantification of *S. aureus* in 92 naturally contaminated and artificially spiked bulk milk samples. Optimized standard curves were generated as the qPCR employed the absolute quantification method. The qPCR assay discriminates *S. aureus* from other *Staphylococcus* species with a large difference in quantification cycle (Cq) (Mean *S. aureus* Cq = 13.83± 0.93; other staphylococci Cq= 30.34 ± 2.65). The standard curve showed 91 % amplification efficiency and 0.98 coefficients of correlation (R^2). The detection and quantification limit of the assay was 18 copies of the *nuc* gene. The precision of the assay as expressed by standard deviation was 0.12 – 0.3 for intra-assay and 0.29 – 0.5 for inter-assay variability. In artificially contaminated milk, the R^2 between CFU ml^{-1} and *S. aureus* cell equivalent (SCE) ml^{-1} was 0.95, which implies, the estimation of CFU ml^{-1} in raw milk by qPCR is possible. A statistically significant ($p< 0.05$) difference in *S. aureus* count was documented between qPCR and plate count. The average SCE (5.59 ±1.55 Log SCE ml^{-1}) estimated by qPCR was one Log higher than CFU (4.46 ± 1.06 Log CFU ml^{-1}) estimated by plate count. Furthermore, 28% of the samples with < 5 Log ml^{-1} *S. aureus* by plate count had > 5 Log ml^{-1} by qPCR. Hence, the qPCR is recommended for routine and research work for its advantage of rapid, sensitivity, and reliability. Further study on validation of the qPCR protocol in different food matrixes for quantification of foodborne pathogens and cost-benefit analysis of the assay is required.

Keywords: *S. aureus*; qPCR; nuc gene; bulk milk.

Introduction

Staphylococcal food poisoning (SFP) is an important foodborne disease worldwide. *S. aureus* has many potential virulence factors; staphylococcus enterotoxin (SE) is one among others which is responsible for food poisoning. Ingestion of less than 1.0 µg of SE causes SFP (Cretenet, 2011). Milk and milk products are commonly associated with SFP. Human and mastitis cows are the major sources of *S. aureus* to contaminate foods including milk and milk products (Hennekinne *et al.,* 2011; Loir *et al.*, 2003). *S. aureus* has been reported at a prevalence of 38.7% from cow bulk milk and milk products in the Tigray region (Tarekgne *et al.*, 2015).

Quantitative data of *S. aureus,* if any*,* in raw milk and dairy products during production, processing, and storage is of food safety concern. A high *S. aureus* counts in the product is an indicator of poor hygiene and the possibility of SE production. Enterotoxigenic *S. aureus* can produce SE at levels that can cause illness when the population exceeds 10^5 CFU per gram of contaminated food (Food and Drug Administration, 2012). However, a low count of *S. aureus* in food does not necessarily indicate the absence of SE. Although large bacterial populations, which are the source of SE, may die during the process, the SE can still survive in products, as it is thermostable and resistant to different enzymatic actions (Hennekinne *et al.*, 2011; Loir *et al.*, 2003).

Several countries set a microbiological criterion for the presence of *S. aureus* in milk and dairy products to be taken as microbiological standards. For example, the European Commission, directive 92/46/EEC, stated that in raw milk intended for processing, < 500 CFU/ml of *S. aureus* is considered an acceptable level. The enumeration of *S. aureus* is not only important as a microbiological criterion but also is a prerequisite for the microbial risk assessment study of SFP (Postollec *et al.*, 2011). In the diagnosis of SFP outbreak enumeration, 10^5 CFU/g of *S. aureus* in the food remnants is confirmatory (Hennekinne *et al.*, 2011).

There are two methods for the detection and enumeration of *S. aureus* in milk and milk products; the culture-dependent (conventional) and the culture-in-

dependent (molecular) methods. The conventional culture-dependent methods are laborious and time-consuming (Alarco'n *et al.,* 2006).

Real-time quantitative PCR (qPCR) is one of the culture-independent methods that detects and quantifies *S. aureus* and other pathogens from different foodstuffs. Compared to the culture-based methods, qPCR is fast, sensitive, and more specific (Postollec *et al.*, 2011). It enables quantifying specific nucleic acids in a complex mixture even if the initial amount is very low. The quantification of the DNA is performed by monitoring the amplification of the target sequence in *real-time* using fluorescent technology. Although many detection chemistries are available, in food microbiology, SYBR Green I and the Taqman or Molecular Beacons are common (Fraga *et al*., 2014).

For the detection and enumeration of *S. aureus* many molecular markers have been developed and tested such as the enterotoxin genes (Johnson *et al.*, 1991), 23S rDNA (Straub *et al.,* 1999), and *femA, fmhA* genes (Riyaz-Ul-Hassan *et al*., 2008). The *nuc* gene was initially developed by (Brakstad *et al.*, 1992) and was widely used as a molecular marker for the detection and enumeration of *S. aureus* from clinical and food samples. However, it has been reported to form a primer dimer and non-specific products when used with SYBER Green I in qPCR assay (Hein *et al*., 2001b). To solve this problem, a new SYBER Green-based primer that targets the *nuc* gene has been developed, tested, and reported as efficient (Alarco'n *et al.*, 2006). As SYBER Green I is cheaper than the TaqMan probe and the designed primers are appropriate, it is important to evaluate and assess qPCR based on these items for local use. If one intended to introduce the qPCR for local use as an alternative approach to the conventional culture-based method, it is imperative to assess and evaluate the qPCR assay in the local food matrixes and local pathogen strains.

Therefore, the objectives of this study were to evaluate the qPCR that targets the *nuc* gene and uses SYBER Green I as a dye for quantification of *S. aureus* in raw cow's milk and to compare the assay with the conventional plate count method.

Materials and methods

Microbiological analysis

A total of 310 samples (168 raw bulk milk and 142 milk products) were collected from the Tigray region, Northern part of Ethiopia with the objective of detection and enumeration of *S. aureus*.

The isolation and enumeration of *S. aureus* from the samples were performed as detailed by (Ahmed, 2003a). Briefly, from the raw milk samples tenfold serial dilutions were prepared, and from each dilution step 100 µ*l* were inoculated onto duplicate plates of Baird-Parker agar (Oxoid, England) supplemented with egg yolk tellurite (Merck, Germany). The plates were incubated aerobically at 37 °C for 48 hours. Typical colonies, which were circular, black to gray-black, and surrounded by an opaque halo clear zone, were counted. Gram staining was made for each colony and tested for catalase activity. Five typical colonies were subcultured onto brain heart infusion (BHI) (Oxoid, England) and incubated overnight at 37 °C. From the BHI broth cultures, a coagulase test on rabbit plasma with EDTA (Remel, USA), DNase test (Sigma-Aldrech, USA), and anaerobic fermentation of mannitol (Sigma-Aldrech, USA) was performed.

Final identification of the isolates to species level was done by sequencing the 16S r RNA gene.

DNA Extraction: DNA was extracted from the overnight BHI growth by GenElute ™ bacterial genomic DNA kit (Sigma- Aldrich, USA) as per the manufacturer's guideline. In brief, one ml of the broth was pelleted by 16, 363 x g for 2 min and washed twice with 0.9% aqueous NaCl solution at the same speed. The content was homogenized with 200 ul of lysosome solution and incubated at 37 °C for 90 minutes. The suspension was treated with 20 ul of RNAase and Proteinase K followed by 200 ul of lysis solution C and incubated at 55 °C for 60 min. The lysate was homogenized with 200 ul of ethanol to precipitate the DNA and transferred into a new binding column where it was centrifuged at 3968 x g for 1 min. The content was washed twice with washing solution I. Then, the concentrated wash solution and the elute were discarded. Finally, the column was put into a new 2 ml tube and the DNA was harvested with 100 ul (twice 50 ul each) elution buffer solution. The concentration (ng/ul) and quality (260/280 and 260/230 ratio) of the DNA was determined by NanoDrop

ND-2000 spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA, USA). The genomic DNA was stored at – 20 °C until use for the next 16srRNA gene sequencing and later for qPCR assay.

The forward and reverse universal primers described by Vebø *et al.* (2011) were used to amplify the 16 S rRNA gene with the expected 1200 bp product. The amplification was conducted with a final PCR reaction volume of 50 µl. It contained, 5 µl of 10x ThermopolTm reaction buffer (BioLabs, New England, USA), 0.25 µl Taq Polymerase of 5,000 µ/ml (BioLabs), 1 µl of each primer of 10 pMol con, 1 µl of 10 Mm deoxynucleotide triphosphate mixture (Sigma-Aldrich, USA) 2 µl of genomic DNA and finally adjusted to a final volume of 50 µl with Milli-Q water. The amplification was carried out in a C1000 TMThermal cycler (BIO-RAD laboratories) programmed to initial denaturation of 95°C for 1 min, 30 cycles of 95°C for 30 sec., 55°C for 30 sec. 68°C for 80 s and a final extension period at 68°C for 5 min. After amplification, the products were verified by gel-electrophoresis, purified, and sent to GATC Biotech AG (European Genome and Diagnostic Centre, Germany) for sequencing as per the company's instruction. The sequence result was bio-edited and compared to GenBank using the nucleotide BLAST algorithm (http://www.ncbi.nlm.gov/blast).

Among the total 120 milk and milk product samples positive for *S. aureus,* 92 were randomly selected for qPCR assay.

The qPCR assay

Bacterial isolates and strains used in the study: The *S. aureus* strain MSSA 476 (accession no. BX571857) purchased from National Veterinary Institute, Debre-Zeit, Ethiopia, was used as a reference strain for the study. It was used for specificity study, artificial contamination of milk, preparation of standard curve, and in all cases as a positive control.

Specificity study

For the specificity study, the following confirmed twelve *staphylococcus* species, namely *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus warneri, Staphylococcus cohonii, Staphylococcus haemolyticus, Staphylococcus carnosis, Staphylococcus scineri, Staphylococcus hominis, Staphylococcus divresia, Staphylococcus chromogenes, Staphylococcus saprphyticus,* and *Staphylococcus caprie,* which were previously isolated from dairy products of

the Tigray region were used (Tarekgne *et al.*, 2015). DNA was extracted from overnight cultures of reference *S. aureus* and other 12 species of *Staphylococcus* in BHI using the GenEluteTM Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instruction, as described previously. To assess the specificity of the primers, the DNA of the isolates involved in the specificity study (n=12), were standardized to 5 ng/µl concentration and subjected to qPCR assay using the same PCR equipment and reaction conditions in duplex. MillQwater was used as a negative control. Then mean Cq and TM values with standard deviation were calculated.

Artificial contamination of raw milk

Raw milk from a healthy cow was collected and confirmed to be free from *S. aureus* by conventional microbiological as well as molecular methods to check for any *S. aureus* DNA. The milk was artificially contaminated by the reference *S. aureus* strain and subjected to molecular analysis as per the method described by (Hein *et al.*, 2005). Briefly, the reference *S. aureus* strain was cultured in BHI broth (Oxoid, England) at 37 °C for 15 hours. From this broth, five tenfold dilutions, from 10^{-1} to 10^{-5},(1 ml of broth to 9 ml peptone water) were prepared with peptone water (Sigma-Aldrich, Switzerland). From each dilution, 100 µ*l* was inoculated in duplex onto Baird-Parker agar (Oxoid, England) supplement with egg yolk tellurite (Merck, Germany) incubated at 37 °C for 48 hours, and CFU/ ml of each dilution was calculated as per Ahmed (2003b). Furthermore, from each of the above dilutions, (10^{-1} to 10^{-5}), 100 µ*l* was added, to five, 900 µ*l* of raw milk to obtain a tenfold dilution of 10^{-1} to 10^{-5} bacteria spiked raw milk. The artificially contaminated raw milk samples were subjected to DNA extraction, as described below, in triplicate and the *nuc* gene copy number was measured twice by qPCR.

DNA extraction from Milk samples

Ninety-two milk samples (n=92) and the five tenfold dilution (10^{-1} to 10^{-5}) of the artificially contaminated milk in triplicate (n=15), were subjected to DNA extraction as follows. Four ml of the content was added to 6 ml of sterile 2% w/v sodium citrate in 15 ml Eppendorf tube and centrifuged for 5 minutes at 16 x g rpm in 4 °C. As much as possible the fat layer was removed with sterile swab tips and the supernatant was transferred into a new 10 ml Eppendorf tube and pelleted at 4500 x g for 15 minutes in 4 °C and transferred into 1.5 ml Eppendorf tube. The content was washed twice with 1 ml of 2% w/v sodium

citrate solution by centrifuging at 16,363 x g for 2 min. in 4 °C and subjected to DNA extraction with GenElute ™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) as described previously.

Quantification Standard for qPCR

The reference *S. aureus* was cultured in BHI broth (Oxoid, England) at 37 °C overnight. One ml of the broth (pure culture) was subjected to DNA extraction as per the aforementioned procedure. After quantification of DNA concentration by Qubit® 2.0 Fluorometer assay (Invitrogen, USA), tenfold dilutions containing 1.8 x 10^1 to 1.8 x 10^5 copies of *nuc* gene were prepared assuming that1 *ng* of DNA equals 6 x 10^5 times the entire genome and that the *nuc* gene is a single-copy gene (Hein *et al.*, 2001a), hence 0.003 ng/µg equals to 1.8 x 10^2 *nuc* gene. Each dilution was subjected to qPCR assay in triplicate. For optimization purposes, many runs have been conducted till the best standard curve is acquired.

The slope(s) of the standard curve was used for the calculation of the PCR efficiency using the equation $E = 10^{-1/s} - 1$ Where E=efficiency, and s=slope. The qPCR assay employed the absolute quantification method to qualify the copy gene in each sample using the standard curve as a reference.

Detection limit

Three tenfold dilutions containing 1.8 copies of the *nuc* gene (0.000003 ng/µl), 1.8 x 10^1 copies of the *nuc* gene (0.00003 ng/µl), and 1.8 x 10^2 copies of the *nuc* gene (0.0003 ng/µl) were prepared from the reference *S. aureus* genomic DNA extracted from pure culture as well as from artificially inoculated milk. The genomic DNA was quantified by Qubit® 2.0 Fluorometer assay (Invitrogen, USA). Each dilution, both from the two sources was subjected to qPCR assay in ten replicates. The number of positive signals exhibited in each dilution as well as the mean and standard deviation of the Cq were documented. For the dilution that was considered as the limit of detection (positive signals exhibited), the relative standard deviation (RSD) of the DNA measurement of the ten replicates was calculated.

Repeatability (Intra-assay) and reproducibility (Inter-assay)

To assess the precision of qPCR, three dilutions containing 1.8 x 10^4 copies of the *nuc* gene (0.03 ng/µl), 1.8 x 10^5 copies of the *nuc* gene (0.3 ng / µl), and 1.8

x 10^6 copies of *nuc* gene (3 ng / µl) were prepared by Qubit® 2.0 Fluorometer assay (Invitrogen, USA). Ten replicates of the three dilutions were subjected to qPCR to determine repeatability whereas three independent assays (by different personnel and time) were performed to document the reproducibility of the qPCR. For all runs, the mean Cq and standard deviations (SD) were calculated.

Primers for the quantitative qPCR assay

Primers (F372/R465) that targeted the *nuc* gene of *S. aureus* recommended by (Alarco'n *et al.,* 2006) for qPCR, were used for the detection and quantification of *S. aureus* from the bulk milk. The oligonucleotide sequence of the primers was verified by the BLAST algorithm in Genbank.

F 5`TGTAGTTTCAAGTCTAAGTAGCTCAGCAA 3`, (F372)

R 5`TGCACTATATACTGTTGGTCTTCAGAA 3 ` (R465). They have expected an amplified product of 94 bp. The amplicon (94 bp) was verified by electrophoresis in 2% agarose gel. They were synthesized in Invitrogen by life technologies (Trondheim, Norway).

Quantitative qPCR amplification condition

All samples (n=92) and the artificially contaminated milk were quantified by qPCR in a duplex. The qPCR was run with the final volume of 20 *ul*. The LightCycler ® 480 SYBR Green I Master mix (Roche, Mannheim, Germany) that contains FastStart Taq DNA polymerase, reaction buffer, d NTP mix, SYBR Green I dye, and $MgCl_2$ was utilized. Besides, 300 nM of each primer and 2 µ*l* of genomic DNA were added. The H_2O PCR grade (Roche, Mannheim, Germany) was used to adjust the reaction component to the final reaction volume. In all, qPCR runs the reference *S. aureus* as a positive control, and the PCR-grade H_2O as a negative control was included. All amplification was conducted in LightCycler ® 480 Instrument (Roche, Mannheim, Germany) in 96 well plates and sealed with sealing foil (Roche, Mannheim, Germany).

The LightCycler ® 480 Instrument was programmed as follows. Pre-incubation of 1 cycle at 95 °C for 5 min. Amplification of 35 cycles with denaturation at 95 ° C for 15 sec., annealing at 60 °C for 1 min. and elongation at 72 °C for 5

sec. *Melting curve* of 1 cycle at 95 °C for 5 sec. 65 °C for 1 min. and continuous at 97 °C and cooling at 40 °C for 10 sec.

Quantitative qPCR results were given as an increase in fluorescence signals of the reporter dye, SYBR Green I, detected and visualized by the LCS480 1.5.1.62 software (Roche, Mannheim, Germany).

At the end of each run, an analysis of melting curves, generated by the LCS480 1.5.1.62 software, was performed to determine the melting point of the amplicons.

The absolute quantification method was applied in this assay. This method depends on the comparison of the Cq value of the samples with the standard curve generated from the amplification of the known/reference *S. aureus* gene. In every qPCR, a new standard curve was included that has relatively similar amplification efficiency. Quantification of the *S. aureus* load in each sample was registered by extrapolating the Cq values of the standard curve which was generated by the light cycler software.

Calculation of the *S. aureus* cell equivalent (SCE) per ml was performed by multiplying the number of copies of the *nuc* gene per qPCR with the ratio of the volume of the DNA containing supernatant (Vs) which is two µl. The initial 4 ml of the milk employed for the extraction of DNA was taken into consideration during the calculation of SCE/ml of milk (Hein *et al.*, 2005).

Data analysis: Microsoft Excel for Windows (MS Office version 8.1 version) was used as a database. Descriptive analysis of the data was conducted with the Microsoft Excel program. The independent sample t-test from The Unscrambler X version X 10.3 software was used to compare the plate count with the qPCR quantification data. Before analysis, all the data were checked for possible outliers. P-value < 0.05 was considered statistically significant.

Results

Standard Curve

In the absolute quantification method of qPCR, the generation of the standard curve is mandatory, as the Cq value of the samples will compare with the standard curve which is generated from the known /reference *S. aureus* gene as previously described.

One of the standard curves generated from a tenfold dilution of 1.8 X 10^1 to 1.8^5 copies of the *nuc* gene is shown in Fig 1. It has a slope of -3. 362, which gives an amplification efficiency of 98.3%. The coefficient of correlation between the Cq value and copy number is calculated as 0.99, which shows the linearity of the quantification.

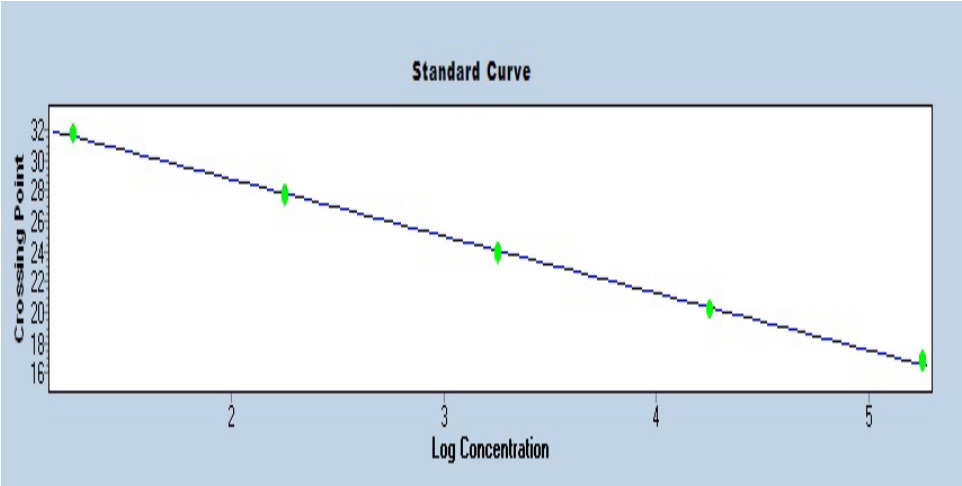
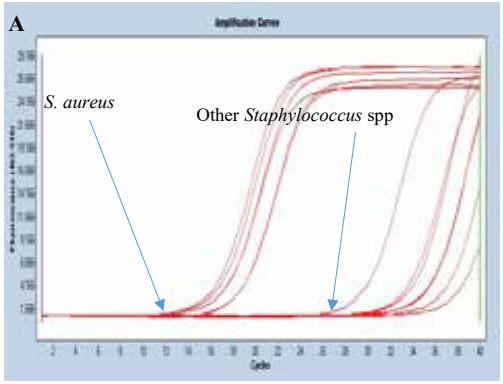


Figure 1. The standard curve generated (Slope-3.36, Y/intercept 38.32, error 0.22) from serial dilution of 1.8 x10^1, 1.8x10^2, 1.8x10^3, 1.8x10^4, 1.8 x10^5 copies of *nuc* gene.

Specificity study

The specificity of the primers that targeted the *nuc* gene was studied, with optimized qPCR reaction, on *S. aureus* and other *Staphylococcus* species of dairy isolates. The result showed that the mean Cq value of *S. aureus* was 13.83 ± 0.93 (13.08 ± 0.18 for ref. *S. aureus*) whereas the mean Cq value for the other *Staphylococcus* species was 30.34± 2.65 (Fig.2). The mean melting temperatures of the amplicon for *S. aureus* was 77.49 ± 0.04 ^{0}C while for other staphylococcus species was 77.71 ± 0.33 ^{0}C.



B. Staphylococci species	Mean Cq
Staphylococcus aureus	13.8
Staphylococcus epidermidis	29.6
Staphylococcus cohnii	29.4
Staphylococcus hemolyticus	27
Staphylococcus carnosus	30.9
Staphylococcus sciuri	34
Staphylococcus hominis	31.9
Staphylococcus devriesei	32.6
Staphylococcus chromogenes	27.9
Staphylococcus saprophyticus	29.1
Staphylococcus warneri	26.4
Staphylococcus caprae	34.6

Figure 2. One of the qPCRs runs for specificity study. A. The marked difference in the Cq value of *S. aureus* from the other *Staphylococci* species in the amplification curve of the qPCR assay B. The summarized mean Cq value of *S. aureus* and other *Staphylococcus* spp.

The limit of detection (LOD)

There was no difference in the LOD between the pure culture and milk-extracted DNA. Out of the ten replicates (in both sources) of 1.8 x 10^2 copies of the *nuc* gene, ten showed positive signal with mean Cq value of 29.09 $\pm$, 0.22 1. Of the ten replicates containing 1.8 x 10^1 copies, ten revealed a positive signal with a mean Cq value of 31.98 $\pm$ 0.47. However, in the dilution containing 1.8 copies, only six showed positive signals from pure culture and four from the milk with a mean Cq value of 33.23+ 0.14. Therefore, the detection limit of this qPCR assay was 18 copies of the *nuc* gene per PCR reaction with and without the matrix effect. It was also possible to quantify the ten replicates of the 18 copies/qPCR with the assay. The measurement showed a variation of 31. 1 % as expressed by RSD or the coefficient of variation (CV). Hence, the 18 copies of *nuc* gene /qPCR were not only the detection limit but also a quantification limit of the assay.

Repeatability (Intra-assay) and Reproducibility (inter-assay)

The repeatability (intra-assay) and reproducibility (inter-assay) of the qPCR assay as expressed by the standard deviation (SD) of Cq are presented in Table 1. The SD values range from 0.12 to 0.3 for intra-assay and 0.29-0.59 for inter-assay (all< 1.0 SD) variation, which were in an acceptable range. This showed that the precision of the qPCR assay was good.

Table 1. The mean and SD of three tenfold dilutions of copies of the *nuc* gene subjected to qPCR to determine the intra-assay and inter-assay variation of the assay

	Number of replicates N	1.8 X 10 ^{4} copies		1.8 X 10 ^{5} copies		1.8 X 10 ^{6} copies	
		Mean Cq	SD	Mean Cq	SD	Mean Cq	SD
Intra-assay	10	21.8	0.30	18	0.17	14.8	0.12
Inter-assay	3	22.3	0.59	18.22	0.32	14.8	0.29

CFU and R^2 of artificially contaminated milk

In log-transformed data, the mean SCE count (5.9 log SCE/ml) obtained by qPCR was higher than the mean CFU count (5.3 log CFU/ml) obtained by the plate count. The coefficient of correlation (R^2) between the log counts of SCE / ml and CFU/ml was 0.9578, which showed that it is possible to calculate the number of colonies forming the unit of *S. aureus* in raw milk by running the qPCR.

Comparison of plate count data (CFU/ml) and qPCR (SCE/ml) data of naturally contaminated raw milk

There was no sample below the quantification limit of the assay. The mean quantification value was 5.85 +/- 1.55 log SCE/ ml and 4.46 +/- 1.06 log CFU/ml in the qPCR and the plate count, respectively. The median of the counts was 5.59 log SCE/ml and 4.8 log CFU/ml for qPCR and plate count, respectively. There was a statistically significant difference ($p < 0.05$) in counts of *S. aureus* between the two methods.

Classification of the two methods by S. aureus count level

Classification of the two methods based on the two categories of counts thus: ≤ 5 log /ml *S. aureus* counts and > 5 log/ml of *S. aureus* count has been ana-

lyzed. The results showed that, out of the total 92 samples, 59 (64%) samples, S. *aureus* > 5 log SCE/ml were counted by qPCR assay whereas > 5 log CFU/ml of *S. aureus* were counted only from 33 (36%) of the samples by plate count method. Twenty-six (28 %) samples, which were considered as having < 5 log/ml of *S. aureus* count with the plate count method, were found to have > 5 log /ml count with qPCR assay.

Discussion

The present study evaluated the performance of qPCR, targeting the *nuc* gene and using Syber Green 1 as a dye, for quantification of *S. aureus* in raw milk and compare the results with the conventional plate count method. The overall result showed that the qPCR assay is more sensitive and specific than the plate count method in the qualification of *S. aureus* in raw milk.

In the present study, the primers showed good specificity that can differentiate *S. aureus* from other *Staphylococcus* species due to low Cq value. This finding is in agreement with *Alarco'n et al*. (2006) and Brakstad *et al*. (1992).

The sensitivity of the qPCR both from pure culture and from milk was 18 copies /PCR reaction. Although the food matrixes have a direct effect on the sensitivity of an assay (Hein *et al.*, 2001a), however, it was not evident in the present study. The reason may be associated with efficient DNA extraction procedures. A good DNA extraction procedure could significantly improve the sensitivity of PCR assay as reported by Kim *et al*. (2001) in the detection of *S. aureus* from mastitis milk. Alarco'n *et al*. (2006) demonstrated a detection limit of 10-20 cells of *S. aureus* from food by qPCR using aSYBR-Green I as detection chemistry, which is comparable with our study.

The repeatability and reproducibility of the qPCR assay were in the acceptable range, which showed the good precision of the qPCR assay. Variation in the qPCR results could be occurred due to temperature variation that could affect the annealing and/or denaturation of the DNA, or due to template concentration variation introduced by pipetting error and stochastic variation (Bustin *et al*., 2009).

There was a statistically significant difference ($p < 0.05$) in the qualification of *S. aureus* in the milk between the two assays. The qPCR assay showed > one

log higher count than the plate count method in the naturally contaminated method. In consistence with this observation, other research also reported higher *S. aureus* count in naturally contaminated raw milk (Hein *et al.*, 2005; Studer *et al.*, 2008) by qPCR assay than the plate count method. Postollec *et al.* (2011) reviewed the following possible reasons for the higher bacterial count in the culture-independent molecular method than in the culture-dependent method. The presence of intact DNA from dead cells as well as the presence of viable but non-cultivable bacteria, which can only be detected by molecular method, could increase the counts. Moreover, one CFU on the plate may be originated from more than one cell, and, in addition, some primers involved in the molecular assay may target several multi-copy genes (e.g. 16S rRNA) that could increase the count.

The detection of dead *S .aureus* in food is a public health concern. It helps for retrospective analysis of the level of contamination starting from the production up to that point in time, which is important for the microbial risk assessment study (Hein *et al.*, 2005). In the present study 28 % of the samples, which were considered as having < 5 log/ml *S. aureus* count with the plate count method, were found to have > 5 log /ml count with qPCR, which indicated the high sensitivity of the molecular method. Samples with > 5 log /ml count of *S. aureus* may perform SE if the *S. aureus* population has enterotoxigenic potential and yet the SE could stay biologically active for a long period as SE is thermostable and resistant to enzymatic activities, although the bacteria it produced is dead (Hennekinne *et al.*, 2011). A recent study in the same area reported that 51 % of dairy isolates of *S. aureus* have enterotoxigenic potential (Tarekgne *et al.*, 2016). Taking these facts, it is relevant to suggest that there is a high risk of an SFP outbreak in the study area.

In comparison with the plate count method, the level of contamination in qPCR is minimum even better than the conventional PCR (use of gel electrophoresis) because the data are generated in real-time immediately after amplification. People working in the qPCR do not afraid of contamination by *S. aureus* and other pathogens as they are mostly working with dead DNA. Furthermore, in Ethiopia where clinical and subclinical mastitis is a major dairy cow problem (Duguma *et al.*, 2014; Tolosa *et al.*, 2015) monitoring, of the etiological agents with the qPCR, is preferable as the conventional plate count methods are costly and time-consuming. The qPCR has been reported as an important and efficient alternative to the plate count method in monitoring inframammary infections elsewhere (Graber *et al.*, 2007; Studer *et al.*, 2008). Although it did

not perform in the current study, it is also possible to multiplex the assay for simultaneous detection and quantification of more than one pathogen in food.

One limitation of the primers, observed in the present study is that when the level of *S. aureus* in the samples is low, the Cq value becomes high which could overlap with the Cq value obtained from high levels of other staphylococcus species (data not shown). To minimize this problem, during the optimization of the current qPCR assay, we reduced the qPCR amplification cycle from 40 to 35 cycles.

The current study showed us the following advantages and prospects of qPCR over the conventional plate count method. Due to its automated nature, the qPCR generates quantified results within a short period, which is practically important during the diagnosis of food poisoning outbreaks such as SFP. Not only the speed but gives also quantified data of viable and dead *S. aureus* cells in the samples, which shows the high sensitivity of the assay. From a microbial risk assessment perspective such quantified and sensitive data are so important to characterize and quantify the microbial risks that influence food safety (Postollec *et al*., 2011).

Conclusions

The present study showed good comparative performance between qPCR and culture-based plate count methods for the quantification of *S. aureus* in raw milk. In light of these findings, the author strongly recommended the use and application of qPCR assay for routine and research work for its advantage of rapid, sensitivity, and reliability. Further study, on validation of the qPCR protocol in different food types for quantification of the common foodborne pathogens and assessing the cost-benefit analysis of the assay are required.

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Case Reports

Cllinical features and postmorteum findings of sheep-associated malignant catarrhal fever in a 2-years old bull

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Abstract

Malignant Catarrhal Fever (MCF) is a fatal lymphoproliferative disease of cattle and other ungulates caused by alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2), the main causative agents of wildebeest-associated MCF (WA-MCF) and sheep-associated MCF (SA-MCF), respectively. The virus is mainly spread by aerosols from pregnant or newborn sheep, goats, and wildebeest to susceptible animals. This case report presents the clinical features and post-mortem findings of an unusual case of malignant catarrhal fever (MCF) in a two-year-old bull brought to the Professor Feseha Gebreab Memorial Veterinary Teaching Hospital in Bishoftu, Ethiopia. The bull was semi-intensively managed, co-housed, and fed with sheep and other domestic animals. The animal was shivering upon arrival, with naso-ocular discharge and clouding of the eyes. The bull was febrile, with a rectal body temperature of 41.4 ^{o}C and a respiratory and heart rate of 40 and 48 beats per minute, respectively. On physical examination, the bull was emaciated, with bilateral yellowish mucopurulent naso-ocular discharge, frequent blinking, bilateral corneal opacity, salivation, a foamy mouth, head pressing, and enlargement of superficial lymph nodes. Malignant catarrhal fever was suspected based on the history and clinical signs, and empiric therapy with 10% oxytetracycline, diclofenac, and IV fluid was initiated. The bull died after receiving the third day of treatment. At necropsy, hemorrhages were found in the esophagus, trachea, and small and large intestines. In the kidney, white foci, enlargement, and fatty degeneration were observed. An ulcerated lesion was seen on the abomasum. In the gall bladder, enlargement and vascularization were also noted. The current case report confirms the rare case of clinical SA-MCF based

on the history, exhibited clinical pictures, post-mortem findings, and PCR results. Separation of cattle and sheep is strongly advised to prevent SA-MCF, as no vaccine has yet been developed.
Keywords: Bull; Clinical signs; Malignant catarrhal fever; PCR; Postmortem findings.

Introduction

Malignant catarrhal fever (MCF) is a fatal lymphoproliferative disease of cattle and other ungulates caused by the ruminant gamma-herpesviruses alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2). The other recently identified viruses known to cause MCF include caprine herpesvirus 2 (CpHV-2), which is enzootic in domestic goats. These viruses infect their reservoir hosts efficiently and without apparent disease (Dettwiler *et al.,* 2011). Alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2) are the major causative agents responsible for wildebeest-associated MCF (WA-MCF) and sheep-associated MCF (SA-MCF), respectively, in cattle and other ruminant species. Wildebeest-associated MCF (WA-MCF) is an economically important disease of cattle in Africa, where wildebeest are present, and SA-MCF is prevalent worldwide where sheep husbandry is practiced (Gelaye *et al.,* 2013).

MCF is a fatal lymphoproliferative disease upon infecting susceptible hosts, including cattle, deer, bison, water buffalo, and pigs. However, there are outbreaks in which several animals are affected, with evidence of recovery and mild or inapparent infections in some cases (Baxter *et al.,* 1993; O'Toole *et al.,* 1997; OIE, 2020). The disease is transmitted to the reservoir host both vertically *in utero* and by close contact. While, from the reservoir to the susceptible host (such as cattle), the transmission is by close contact through aerosols and contamination of pasture by the discharge of the reservoir animal. However, cattle are a dead-end host for MCF because once they are infected; they do not transmit the disease to other animals (Horner, 2003).

In diseased animals, the clinical signs of MCF are highly variable and range from peracute to chronic forms involving multiple organs, including the head and eye, alimentary tract, nervous system, and skin (Sood *et al.,* 2013; 0IE, 2020). In a peracute case, either clinical signs are not evident, or depression followed by diarrhea and dysentery may develop 12–24 hours before death. In general, the disease is characterized by high fever, inappetence, ocular and

nasal discharge, keratoconjunctivitis with bilateral corneal opacity, and a decrease in milk yield (Li *et al.*, 2011).
The diagnosis of MCF has multiple challenges, including clinical resemblance to many viral diseases and highly complex pathogenesis and epidemiology. The diagnostic method is different in different clinically affected animals and reservoir hosts due to the biology of the MCF viruses and the hosts' responses to the viruses. Clinical signs and different assays such as serological tests, PCR, histopathology, isolation, and identification of viruses are used for the detection of MCFVs or the diagnosis of the disease caused by the viruses (Wallman and Thompson, 1982). The severity of clinical symptoms is reflected in gross pathological alterations, which are often broad and may encompass multiple organs (OIE, 2020). Salivation and oral hyperaemia may be early signs and may progress to erosions of the tongue, hard palate, gums, and particularly the tips of the buccal papillae. Sometimes skin ulceration and necrosis may develop which may extend or be restricted to the udder and teats. Erosions and hemorrhages in the gastrointestinal tract may be evident (Moore *et al.,* 2010). The urinary bladder often has characteristic ecchymoses and hemorrhages of the epithelial lining, especially in bison. In the kidney, extensive multiple raised white foci, each 1–5 mm in diameter, may appear (Sood *et al.,* 2013; OIE, 2020). The brain may also show signs and symptoms of non-suppurative meningoencephalitis (OIE, 2020).

The use of PCR allows sensitive confirmation of the presence of MCF viruses in infected animals and is also useful for phylogenetic and epidemiological studies in both natural and MCF-susceptible hosts. Both conventional and quantitative real-time PCR assays have been developed for the detection of OvHV-2 and AlHV-1 viral DNA (Toole and Crawford, 2000). The OIE-approved nested PCR was found to be 10-fold more sensitive than quantitative PCR. However, real-time PCR assays have the potential to define viral loads in a range of tissues from both natural and MCF-susceptible hosts (Swai *et al.,* 2013). This case report presents a PCR-confirmed case of sheep-associated malignant catarrhal (SA-MCF) fever in a two-year-old bull in Bishoftu, Ethiopia.

Case description and management

A two-year-old bull was brought to Addis Ababa University College of Veterinary Medicine and Agriculture Professor Feseha Gebreab Memorial Veterinary Teaching Hospital with a history of reduced feed intake, shivering, nasal discharge, and lacrimation. The bull was semi-intensively managed, cohoused,

and fed with sheep, horses, and donkeys. Body vital parameters revealed that the bull was febrile with 41.4 °C, 40 breaths per minute, and 48 beats per minute of temperature, respiratory rate, and heart rate, respectively. The bull was highly emaciated, with bilateral yellowish mucopurulent naso-ocular discharge, frequent blinking of the eyes, bilateral corneal opacity, salivation, foamy mouth, and prescapular and prefemoral lymph node enlargement (Figure 1). The team of veterinarians went to the village where the animals lived and discovered that the bull and other animals (a donkey, a horse, and sheep) shared the same house and communal grazing land. Based on the clinical presentations, viral diseases like infectious bovine rhinotracheitis (IBR), malignant catarrhal fever (MCF), rickettsial infections like ehrlichiosis, and other diseases with related signs were suspected.



Figure 1. Clinical evidence of the case: (A); Yellowish mucoid nasal discharge (B); Foamy salivation (C); Corneal opacity (D); Emaciation with prominent ribs and pelvic bone

To prevent secondary bacterial complications and relieve pain, 10% oxytetracycline (Shanghai Thongren Pharmaceutical Co., Ltd., China) at a dose of 10 mg/kg body weight daily was prescribed for five days in conjunction with 2.5 mg/kg diclofenac sodium (Jiangsu Pengyao Pharmaceutical Co., Ltd., China).

The bull was in good condition on the first and second days of follow-up. However, it became lethargic and unable to move. The following day, after the third shot, the bull died.

Postmortem findings and PCR result

Immediately after the bull died, the cadaver was opened for post-mortem investigation. The trachea, esophagus, lymph nodes, lung, liver, kidneys, gall bladder, and gastrointestinal tract were thoroughly investigated and sampled. The gross postmortem findings were hemorrhages in various organs, including the esophagus, trachea, and small and large intestines. In the kidney, white foci, enlargement, and fatty degeneration were noted. In addition, ulcerated lesions on the abomasum, enlargement, and vascularization of the gall bladder were seen, as indicated in Figure 2 below.

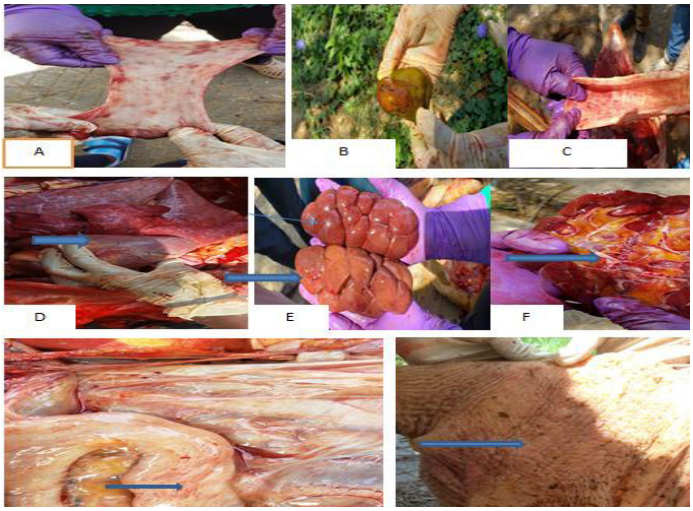


Figure 2. Post-mortem findings of the case: (A); Hyperemic esophagus (B); Eroded lesion on gall bladder (C); Hemorrhage on the trachea and diphtheritic mucus membrane (D); Engorged heart (E); Infarct and white foci on the kidney (F); Fatty degeneration (G); Hemorrhage on intestine and (H); ulcerated lesions on abomasum.

For molecular detection, MCF1TK-Fow-5 pm/ µ 5-CCCGGGAAAACTTCTACCAC-3 and MCF1TK-Rev-5pm/ µ 5-CGCTTAGGTCAATGACAACG-3 primers (NVI, Ethiopia) were used, DNA extraction was made from pathological tissue samples (kidney, liver, and lymph node) and Ovine herpesvirus type two (OHV-2) DNA was detected in the samples using the conventional PCR diag-

nostic method (Figure 3). This result has confirmed that the bull's death was caused by SA-MCF, a rare and underreported condition in Ethiopia.

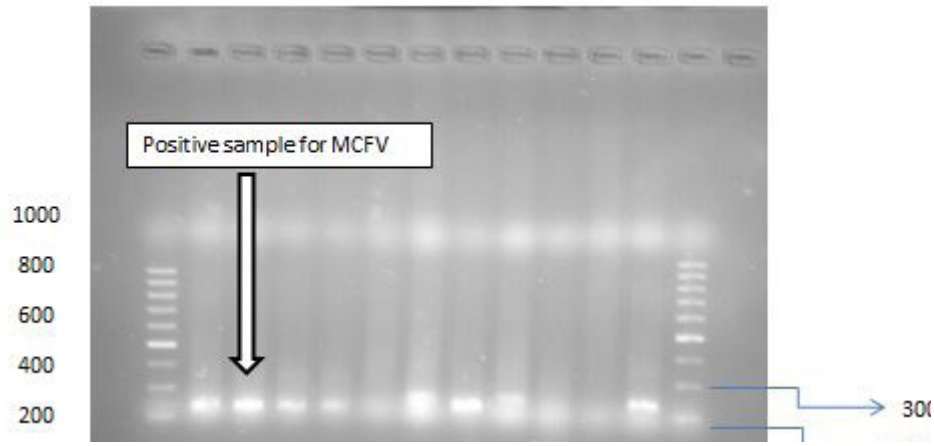


Figure 3. PCR test result

KEYS: M- Molecular ladder, S1- Sample one, D1-D4- Doubilacated samples of the original sample, NC- Negative control, PC- Positive control, and Left side and right side molecular base pairs put by numbers

Discussion

The present case of MCF was diagnosed based on clinical findings, post-mortem, and a PCR result, as described by Horner (2003). Similar to the current case, MCF is characterized by clinical signs of appetite loss, self-isolation from the stock, lethargy, emaciation, fever, lymph node enlargement, bilateral corneal opacity, nasal and ocular discharge, and hyperesthesia (Zamila *et al*., 2011; Gelaye *et al.,* 2013; OIE, 2020). The gross pathological findings obtained in the present case report are similar to those described in previous reports by Horner (2003) and Pesca *et al*. (2019), which revealed erotic lesions in the mouth and nasal cavities, diphtheritic mucus membranes, hemorrhage on the serosal surface, an infarct, white foci in the renal cortex, and enlarged superficial lymph nodes. The conventional PCR test has confirmed the presence of Ovine herpesvirus type two (OHV-2) DNA in the pathological tissue samples (kidney, liver, and lymph node) collected during the post-mortem examination of the present case. However, PCR detection was not performed on sheep, which are potential reservoir hosts. The lack of clarity of the gel photo used in this report and the failure to include histological investigation due to a lack of facilities are limitations of this work.

Conclusion

The current report confirms a rare clinical case of SA-MCF based on history, clinical signs, post-mortem findings, and PCR results. Therefore, separating sheep from cattle could be a possible control measure because there is no effective vaccine against SA-MCF.

Funding

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Institutional Review Board Statement

All the procedures performed on the bull included in this study were done in the framework of routine clinic veterinary care and post-mortem procedure. Thus, ethical review and approval were not needed for this case report.

Informed Consent Statement

Informed consent was obtained from the owner.

Acknowledgments

We are grateful to CVMA, Professor Feseha Gebreab Memorial Veterinary Teaching Hospital in Bishoftu, NVI (National Veterinary Institute) of Ethiopia for their cooperation to do PCR detection and animal owner for his consent for post-mortem examination.

Conflicts of Interest

The authors declare no conflict of interest.

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Caseous lymphadenitis: A case of sheep and its management in Ethiopia

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Abstract

Caseous lymphadenitis (CLA) is a contagious and chronic bacterial disease of animals that affects the lymphatic system with the formation of abscesses. This case report documents a sheep diagnosed with CLA that was brought to the Veterinary Teaching Hospital of Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVMA), Bishoftu on March 06/2022. The primary complaint was weakness, anorexia, and a slight, fluctuating, sickle-shaped, enlarged swelling in the neck region between the ear and jaws, which developed due to a laceration by a wire on the fence. Physical and clinical examination revealed increased body temperature (40.7°C) and respiratory rate (44 breaths/min) and mildly fluctuating swelling on lymph nodes. Anorexia, coughing, general ill thrift, exercise intolerance, and enlargement of subcutaneous tissues and lymph nodes around the neck region were observed. Aspiration of the swelling revealed thick, pale greenish-cheesy pus. Using the Ethiopian Differential Diagnosis and Information Environment App-based diagnosis and bacterial culture of the pus revealed the case as CLA. It was managed by surgical removal of the pus, topical wound spray, and systemic administration of fortified procaine penicillin for five days intramuscular and once topically on the site, respectively. The sheep recovered after a month. In conclusion, *CLA* is a challenging suppurative disease of sheep and goats that can be successfully treated by topical wound management and systemic penicillin therapy.

Keywords: Abscess; Caseous lymphadenitis; Penicillin; Sheep.

Introduction

Caseous lymphadenitis (CLA) or cheesy gland disease is caused by *Corynebacterium pseudotuberculosis*, is a significant and chronic contagious disease that potentially threatens the majority of sheep-rearing countries. This disease is characterized by the formation of widespread abscesses in lymph nodes, subcutaneous tissues, internal organs, and reproductive disorders (orchitis, abortion, and stillbirth). It occurs worldwide and is considered one of the most economically important diseases, particularly in the sheep and goat industry. That is why it's so-called the plague of small ruminant producers (Pépin and Paton, 2010; Umer *et al*., 2017).

Caseous lymphadenitis is caused by *Corynebacterium pseudotuberculosis (C. pseudotuberculosis)* which is a gram-positive, pleomorphic, rod-shaped, non-sporulating, non-capsulated, non-motile, intracellular, facultative anaerobic bacterium in the family *Corynebacteriaceae*. This micro-organism also causes other diseases; external subcutaneous abscesses, ulcerative lymphangitis, and internal infection in horses (Çetinkaya *et al*., 2002). Transmission occurs mainly through contamination of superficial wounds by contact, during common procedures (shearing, castration, and ear tagging), through contaminated environment (*C. pseudotuberculosis* is able to penetrate skin lesions and oral mucosal injuries), through injuries by other traumatic events and aerosols from animals with lymphadenitis in the lung (Osman *et al*., 2018; Moura *et al*., 2020).

In small ruminants, the clinical signs of CLA are primarily characterized by weight loss, poor growth, purulent nasal discharge, fever, general ill health, and the development of abscesses in peripheral and/or internal lymph nodes and organs such as the lungs, kidneys, liver, and spleen. The abscesses are caused by bacteria-induced caseation necrosis of the lymph glands. Additionally, affected animals may exhibit signs of respiratory disease, including cough, dyspnea, and tachypnea (Ribeiro *et al*., 2013; Constable *et al*., 2017). The diagnosis of this disease can be mainly based on the history of a wound, clinical signs, isolation of the agent from discharging abscesses, serology, PCR, and ultrasonography or radiography for internal organs. The differential diagnosis of CLA are localized tumor, abscess, hernia, oedema, and haematoma (Dercksen *et al*., 2010; Nassar *et al*., 2016).

CLA due to *C. pseudotuberculosis* can be treated with penicillin, tetracyclines, and cephalosporins. However, treatment with these drugs alone is generally not effective because of several factors including the protective nature of the capsule, the formation of the encapsulated abscess as well as the intercellularity of the organism. Therefore, surgical lancing or removing superficially located abscesses along with the above antibiotics is the effective way of treatment (Constable *et al*., 2017; Alves *et al*., 2020).

Due to the rapid spread of the disease, once introduced into a flock and those infected individuals serve, as a reservoir of infection, the control and eradication of CLA is difficult. Generally, it is agreed that the finest strategic way to control the disease is the vaccination of healthy animals, along with identification and isolation/quarantine of infected ones. The disease is highly prevalent and economically important in most sheep-producing countries like Ethiopia. It produces a negative influence on the growth, wool, meat, and milk production, skin and carcass condemnation, and reproductive efficiencies of flocks of small ruminants in which the most efficient strategy for the control and prevention remains in a matter of debate (Fikre and Abraha, 2014; Osman *et al*., 2018; Yitagesu *et al*., 2020). Therefore, the current case report describes the case of CLA and its treatment in a young sheep.

Description of the case

A young sheep with a body weight of 20 kg was presented to the Veterinary Teaching Hospital (VTH) of Addis Ababa University, College of Veterinary Medicine and Agriculture (AAU-CVMA), Bishoftu on March 06/2021 with the primary complaint of anorexia, weakness, weight loss and hot, slightly fluctuating, sickle-shaped and enlarged swelling between the ear and jaws around the neck region. The owner has also stated that the swollen mas has developed due to a laceration by a wire on the fence when the sheep was managed under a semi-intensive system with other animals. Physical examination revealed that the sheep has a body temperature of 40.7 °C, respiratory rate of 44 breaths/ minute, heart rate of 88 beats/ minute, and hot and thick (mildly fluctuating) swelling externally between parotid and mandibular lymph nodes while during palpation involvement of retropharyngeal or other associated lymph nodes were felt.

During clinical examination, the sheep displayed symptoms such as loss of appetite, coughing, general ill health, purulent nasal discharge, fever, and increased respiratory rate. Additionally, there was enlargement of subcutaneous tissues and lymph nodes around the neck region, and a hot, fluctuating, sickle-shaped, and enlarged swelling (Figure 1) was observed. Aspiration of this swollen mass with a sterile 20-gauge needle and syringe revealed thick, pale green, watery to cheesy pus, which confirmed the presence of a pus-filled cyst around the neck region. The EDDIE App-based diagnosis revealed the case as CLA and its treatment options. Therefore, based on the history of a wound, characteristic clinical signs, and EDDIE App-based diagnosis result, the case was tentatively diagnosed as CLA, which is differentially diagnosed with localized tumor (lymphoma), abscess oedema, and haematoma.



Figure 1. Picture of the sheep affected by caseous lymphadenitis (**A**) and after therapy (**B**).

Laboratory investigation and its findings

A fresh sample was collected from the swollen mass with a sterile needle with a syringe and placed into a screw-caped bottle containing 3ml of tryptone soya broth and immediately transported to AAU-CVMA laboratory, Bishoftu and enriched overnight by placing it inside the microbial incubator at 37 °C. The next day, some of the enriched samples were inoculated on a blood agar base supplemented with 7% sheep blood and incubated at 37 °C for 48 hours. After these hours, the sparse growth of small, white to grayish (cream-like) and waxy colonies surrounded by a weak zone of *hemolysis* (Figure 2A) characteristic of *Corynebacterium* species, the etiologic agent of CLA was observed.

From the grown colonies on blood agar, a loop full of the pure colony was taken and a smear was made for gram staining, which provides small, short, gram-positive, pleomorphic (angular, palisade arrangement), looking like rod-shaped (Figure 2B below) were observed. Generally, the history of the wound due to laceration, clinical signs, EDDIE App-based diagnosis, and laboratory results showed to conclude that the disease affecting the sheep was finally confirmed as caseous lymphadenitis.

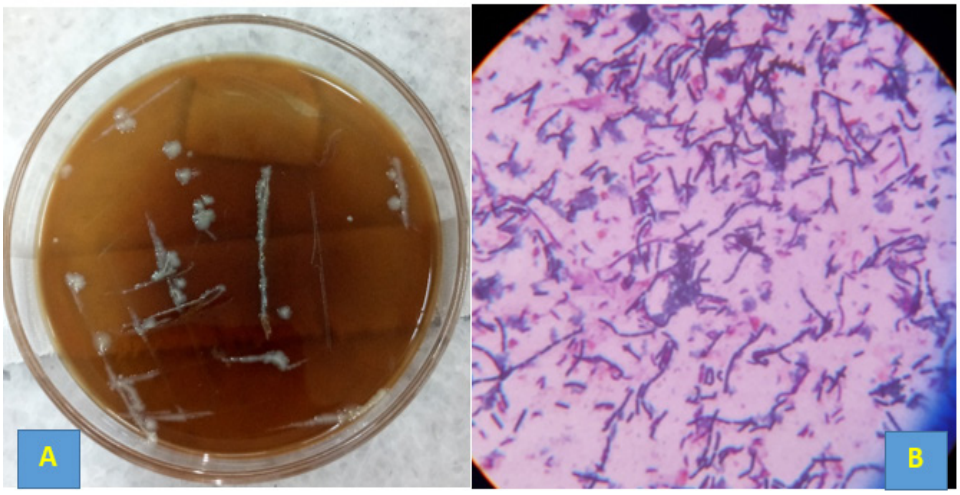


Figure 2. Laboratory results of caseous lymphadenitis suspected sheep (**A**, growth of *C. pseudotuberculosis* on blood agar and **B**, result of gram stain of colonies from blood agar).

Case management and treatment outcome

After confirmation of the disease affecting the sheep as CLA, the management and treatment approaches used were surgical draining of the abscess by the following procedures; the hair around the swollen area was clipped and shaved, followed by cleaning with clean water and savlone, then lidocaine HCL 2% was locally infiltrated around the swelling to avoid pain during manipulation. Then the abscess was surgically lanced out and flushed with diluted iodine solution followed by application of fortified procaine penicillin for five days intramuscular and into the cavity topically once stat where the pus content was removed. Subsequently, external chlortetracycline (*cyclo) spray* (Farvet Laboratories B.V., Netherlands) was applied for three days.

Finally, an intramuscular injection of 4,000,000 IU/10ml fortified procaine penicillin (North China Pharmaceutical Co. Ltd., Shijiazhuang China) at the recommended dose of 22, 000 IUs/ kg body weight for five days stat was applied. Because of the contagious nature of the disease, all materials used to treat this animal were appropriately disinfected and the drained purulent material was also carefully collected and disposed of by burning. The owner was advised to isolate this sheep from other animals by cleaning and disinfecting its bedding until the lesion stops draining and heals. Finally, the sheep were reported to be recovered from this disease after a month and sold to another owner.

Discussion

Caseous lymphadenitis (CLA) is a transmissible, chronically insidious, and serious disease of small ruminants caused by *Corynebacterium* species (Galvão *et al*., 2017; Osman *et al.*, 2018) which is in line with the cause of the present case report.

CLA can occur as an outbreak in a population of small ruminant farms involving flocks or as a single case (Colom-Cadena *et al*., 2014) an Iberian ibex (Capra pyrenaica hispanica. This is in agreement with the presented case that a single sheep was infected from four. It is not a notifiable disease in many countries, since the actual prevalence in small ruminants is usually underestimated and animal owners are not aware of its economic impacts. The morbidity rate can usually range up to 15%, and morbid animals will often eventually succumb to the disease (Osman et al., 2015). Correspondingly, studies that were carried out in different parts of Ethiopia have shown that CLA is a widespread disease, especially in sheep (Fikre and Abraha, 2014; Abebe and Sisay, 2015; Yitagesu et *al.,* 2020; Osman *et al.,* 2015).

The hallmark clinical finding in cases of CLA is the development of an external abscess in the location of marginal lymph nodes. Common sites of development include behind the ears, beneath the jaw or neck, on the shoulders, or in the rear flank region and the involved lymph nodes that include parotid, submandibular, prescapular, and prefemoral lymph nodes (Ribeiro *et al*., 2013). Caseous lymphadenitis abscesses are characteristically thick-walled. Soft pasty white to green exudate filled these abscesses when they are fresh, but the typical onion-like appearance of rings of thick dry pus is displayed in older abscesses (Constable *et al*., 2017). Other clinical signs which include

loss of appetite, coughing, general ill thrift, purulent nasal discharge, fever, increased respiratory rate, and exercise-intolerant can also be displayed in addition to enlargement of the lymph nodes and subcutaneous tissue that contain pus (Osman *et al*., 2015; Abba *et al*., 2017; Reshma *et al*., 2017). This agrees with the clinical signs of the current case report.

The diagnosis of CLA disease can be mainly based on the history of a wound, clinical signs, isolation of the agent from discharging abscesses by microbiological techniques, and other advanced laboratory techniques (Jabar *et al.,* 2016; Dercksen *et al.,* 2010). This is in line with the diagnostic approaches followed in the current case report that discharging abscess was collected in and cultured on the blood agar followed by primary and secondary bacteriological tests.

The best approach in the management and treatment of CLA is surgical lancing and draining of the pus from the enlarged lymph nodes and subcutaneous tissues and lavage with dilute iodine solution followed by the use of antibiotics topically and parentally. In addition to proper management of the case, strict biosecurity measures, elimination of diseased animals from the flock, vaccination, disinfection of shearing materials used for production procedures, and removal of hazards in the environment that injure the skin can prevent and reduce the disease (Colom-Cadena *et al*., 2014; Abba *et al*., 2017). Thus, the diagnostic, management for removal of the pus along with antibiotic therapy for inhibition of secondary complications and the methods used for the control and prevention of CLA in the current case report were in line with the approaches of the above authors.

Conclusions

This case report showed that CLA is a challenging suppurative disease of sheep that can be treated by topical wound spray, systemic therapy with fortified procaine penicillin, and surgical management by drainage. As a preventive measure, there is a vaccine available for use in sheep in other countries. Generally, because CLA is a chronic infection, efforts should be focused on preventing the spread to uninfected animals. However, if a suspected animal is presented, palpation of the affected area and laboratory bacterial examination usually work for the identification of the disease. Animals with visible CLA abscesses should be isolated until the abscesses get completely healed. Usu-

ally, it is recommended to work in close collaboration with a nearby veterinarian or animal health worker to develop an on-farm disease control and testing plan. In addition, when farmers purchase used sheep handling equipment, it is advised to disinfect appropriately to prevent the introduction of CLA from outside farms. Regular disinfection of farms that may become spoiled by abscess material is highly recommended. Close follow-up and separation of new farm additions for a considerable time is also one of the preventive measures to reduce the risk of entry of CLA infection. Drinking raw milk is a potential source of human infection, especially in immunosuppressed individuals.

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Ethiopian Veterinary Journal

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Acknowledgements

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