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Investigation of Bovine Mastitis Pathogens in Jimma Area of South Western Ethiopia: Their Epidemiology and Antimicrobial Susceptibility Profile

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Abstract: Mastitis is one of the most common economically important diseases that require antibiotic treatment in dairy cows in the world. Antimicrobial resistance of bacterial pathogens is of concern for the antimicrobial therapy of both humans and animals. Harmonized and continuous monitoring of antimicrobial susceptibility trends over time is an important component of stewardship to ensure long-term antimicrobial efficacy. It is of particular concern in developing countries like Ethiopia, where milk and milk products are scarce. A cross-sectional study was conducted in Jimma Zone, Tiro Afeta district between January, 2017 and April, 2019 on 460 lactating cows to identify bacterial pathogens causing mastitis and determine their resistance to selected antimicrobial agents, under current farming system. A total of 441 (333 local and 108 cross breed) quarter milk samples from clinical and subclinical mastitic cows were microbiologically examined. A total of 347 isolates were obtained from 371 quarter milk collected. Staphylococcus, Streptococcus and coliform species were identified with isolation frequencies of 186 (53.6), 101 (29.1) and 60 (17.23%) respectively. The most common isolates from clinical mastitis were *Staphylococcus aureus* (19.3%), *Escherichia coli* (18.2%) and *Streptococcus agalactiae* (15.9%) while from subclinical mastitis were Coagulase Negative Staphylococcus (28.95%), *S. aureus* (21.62%), *S. agalactiae* (13.12%) and *S. dsygalactiae* (6.18%). The study showed that the contagious pathogens had predominated over the environmental pathogens and remains the significant cause of mastitis and economic loss in the area. Logistic regression analysis revealed that lactation stages, parity, drying of teat with the same towel, hygiene and presence of teat lesions are a significant contributor to this pathogens ($P<0.05$). In addition 89 (25.6%) isolates were subjected to antibacterial susceptibility tests and 91.0% (81/89) isolates showed antimicrobial resistance to at least one of the six tested antibacterials (Tetracycline, Streptomycin, Penicillin G, Gentamicin, SXT and Kanamycin). Multi-drug resistance was also observed. Therefore hygienic housing and milking practices, minimizing irrational use of antibiotics and molecular studies on the pathogens of mastitis for resistance gene isolation are very important.

Key words: Antimicrobial Pattern · Dairy Cows · Mastitis · Pathogenic Bacteria · Risk Factors

INTRODUCTION

Ethiopia holds a substantial potential for dairy development mainly due to its large livestock population coupled with the relatively suitable environment for livestock production [1]. Ethiopia constitutes the largest livestock population in Africa (59, 486, 667 cattle; 30, 697, 942 sheep; 30, 200, 226 goats; 2, 158, 176 horses; 409, 877 mules; 8, 439, 220 donkeys; 1, 209, 321 camels, 59, 495, 026 poultry and 6, 189, 329 beehives) with different distribution and quantities depending on animal husbandry system and agro-ecological zone. From these cattle population, dairy-cows are estimated to be around 7.16 million and about 11.83 million are milking cows for the private holdings [2]. Dairy cows are increasingly becoming an important in poverty reduction efforts by the improvement of households' income from sales of milk and milk products as well as generation of employment in addition to improved nutritional status of families [3]. Despite the huge potential, dairy production has not been fully exploited, mainly due to several constraints including malnutrition, traditional management and disease including mastitis [4, 5]. A number of previous reports from different parts of Ethiopia indicated

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that mastitis is a serious problem in the dairy cattle. Prevalence of mastitis was reported as 69.8 in Sebeta, 56 and 71, 36.69 and 62%, in Selalle, Asella, Holeta town and in smallholder dairy farms in Jimma, respectively [6-9].

It has been estimated that up to 150 microbial species are potential causative agents for mastitis [10]. The pathogens responsible for causing bovine mastitis in Ethiopia range from a number of gram positive bacterial species, including members of the genera *Staphylococcus* and *Streptococcus*, as well as gram negative bacteria, such as the *Coliform* species [9, 11]. These pathogens are normally classified as either contagious or environmental based on their method of infection and spread through the herd [12]. Contagious pathogens are those that are transmitted from an infected cow to a susceptible cow, which often occurs during milking. *Staphylococcus aureus, Streptococcus agalactiae* and *Mycoplasma bovis*, *Corynebacterium and* coagulase negative staphylococci (CNS) are contagious pathogens mainly cause clinical and subclinical mastitis [12, 13].

The most important change in the epidemiology of bovine mastitis over the past decade has been the rise in the importance of environmental pathogens causing clinical mastitis, relative to contagious pathogens. Environmental mastitis is caused by bacteria that are transferred from the environment to the cow, rather than from other infected quarters [13]. Despite significant progress in the control of contagious pathogens, environmental mastitis continues to be a major cause of financial loss throughout the world. Dairy herds, in which contagious mastitis has been controlled and which have low somatic cell counts, often have a higher incidence of clinical mastitis caused by environmental pathogens [14].

Environmental mastitis refers to infections caused by two groups of pathogens, the coliform bacteria and non-agalactiae *Streptococcus* species. The usual source of these organisms is the environment of the cow. Examples of conditions and situations that will favour the presence of these micro-organisms are over-crowding with zero-grazing systems, poorly designed housing, wet, unhygienic bedding, dirty lots, milking of wet udders, poor udder preparation prior to milking, housing systems that lead to teat injuries [12, 15].

Most of the previous studies in Ethiopia were concentrated on the investigation of the prevalence and risk factors for mastitis at cow level and little effort has been made on identification of pathogenic bacteria causing the disease. There is study on identification of contagious and environmental pathogens causing clinical and subclinical mastitis in Ethiopia. In Ethiopia, the isolation rates of *Staphylococcus* species from mastitic cows has been conducted in different areas: in Assela 58.6% [16], Addis Abeba 28.7% [17], in Borena 29.2% [18], in Adama 35.8% [19] and in Holota 77.1% [20]. However, correct identification of these pathogens to the species level is important to ensure proper treatment due to the variability in each pathogen's susceptibility to antibiotic treatment.

In dairy cattle operations, antimicrobials are administered for both therapeutic and prophylactic purposes for treatment of mastitis for more than fifty years, but consensus about the most efficient, safe and economical treatment is still lacking. Among main reasons of low efficacy of antibacterial treatment of mastitis cases is the resistance of the bacteria to antibacterials in many parts of Ethiopia [21, 22]. The zoonotic bacteria (*S. aureus, S. dsygalactiae, E. coli* and *K. pneumonia*) that are resistant to antimicrobials are of particular concern, as they might compromise the effective treatment of infections in humans [23, 24]. According to the UK's Review on antimicrobial resistance, a continued rise in drug resistance would, by 2050; lead to 10 million deaths every year and a reduction of 2 - 3.5% in Gross Domestic Product (GDP)" [25]. Several studies in different parts of Ethiopia indicate most of *Staphylococcus* species are resistant to penicillin and ampicillin and susceptible to ciprofloxacin, Sulphamethoxazole-trimethoprim [22, 26]. Kanamycin, Streptomycin and Gentamicin have relatively a good efficacy for treatment of mastitis [21, 27].

Tiro Afeta is located within the Jimma Zone having a high potential for dairy production. The production system in the area is categorized under "Mixed crop livestock production system". Peoples in Tiro Afeta are livestock based society where livestock and its products are more important sources of food and income and dairy production is a critical issue, but dairying has not been fully exploited and promoted mainly due to mastitis and several constraints including malnutrition and traditional management. The local communities also witnessed that mastitis was among their main problems. They also complained that milk yield is continuously decreasing per cow from year to year (personal communication). The control of mastitis has been successfully achieved through the establishment of effective herd health control programs. Comprehensive information on major bacterial pathogens causing mastitis, factors contributing to the occurrence and distribution of pathogens and economic consequences of the disease is lacking in the area. Hence, it is important that isolation and identification of those pathogens causing mastitis and susceptibility of

the pathogens to common antibacterials used in the area very are important for designing appropriate preventive and control strategies which are important for restoring economic loss and food security in the area. Therefore, this study was initiated to isolate the major bacterial pathogens causing mastitis; assess the major risk factors associated with these pathogens distribution and to evaluate the in a vitro antimicrobial sensitivity patterns of the selected isolates (disc diffusion test) under current farming system in the area.

MATERIALS AND METHODS

Description of the Study Area: The present study was conducted between January 2017 and April, 2019 in Tiro Afeta district, which is found in the eastern central part of Jimma Zone, at 64 Kmfrom Jimma town in Oromia regional state at 316 km south west of Addis Ababa at longitude of 35°52'-37°37'E and latitude of 7°36'-8°56'N. It has an area of 1001.9 km^2 and five centre of rural community (CRC) namely: Akko, Dimtu, Gebbera, Busa and Raga-siba centers with 26 peasant associations. It has common boundaries with Botor Tolayi, Sekoru, Limu Kossa, Kersa, Omo Nada districts and Southern Ethiopian Peoples Regional State. Altitudinally, the district lies between 1640 and 2800 metres above sea level. The district is classified into woinadega (85%) and dega (15%) agro climatic zones. The average minimum and maximum annual temperatures were 7°C and 30°C, respectively. Agriculture is the livelihood for more than 90% of the population in rural farming community. The main agricultural system in the area is mixed crop livestock production and animals are mainly reared in an extensive system.

The area has livestock population of 414, 297 (188, 835 cattle; 56, 338 sheep; 37, 053 goats; 8, 829 donkeys; 7, 243 horses; 4, 581 mules and 111, 418 poultry) among which 39, 379 were cows [28]. Accordingly, the study was conducted in seven kebeles of the district (Akko town, Akko, Kejelo, Busa, Dimtu, Raga Siba and Dacha Gibe) (Figure 1).

Study Population: The study population was indiginous local zebu and cross breeds of lactating cows of different age, parity, sex and body condition score all from Tiro Afeta District.The animals were categorized under small scale dairy and livestock keeping system which are kept under semi intensive and extensive husbandry system.

Study Design: Cross-sectional study design was conducted between January 2017 and April 2019. The district was purposively selected for this study, due to its high potential for dairy production and mastitis problem. Types of samples included were quarter milk samples and questioner survey.

Sample Size Determination: The sample size for lactating cow were determined according to Thrusfield [29] using 95% confidence interval, 5% absolute level of precision and expected prevalence of 85% which was reported by Tolosa, *et al.* [3], from Jimma, which has similar features with th e current study area.

$$n = \frac{1.96^2 P\exp^{11}(1-P\exp)}{d^2} = \frac{1.96^2(0.85)(1-0.85)}{(0.05)^2} = 0.\frac{5762}{(0.0025)} = 230,$$

where n = sample size; P_{exp} = expected prevalence and d = desired absolute precision.

Accordingly, the sample size was determined to be 460 (230 local and 230 cross breed) lactating cow. However, due to the fact that few cross breeds are present in the area, 368 local and 92 cross breed) lactating cow or 1840 quarters were included in the study (Table 1).

Sampling Method: In consultation with development agent (s) (DA) and Kebele experts, all households in selected kebeles were stratified as those who have 1-4, 4-7 and >8 heads of dairy cow. Then, 460 lactating cows were randomly selected from 166 households (owners of lactating cows) and included in this study. The reason for stratification was: (l) it ensures that all strata are represented in the sample; (2) the precision of overall estimates might be greater than those derived from a simple random sample. The gain in precision results from the fact that the between-stratum variation is explicitly removed from the overall estimate of variance and (3) it produces estimates of stratum-specific outcomes, although the precision of these estimates will be lower than the precision of the overall estimate.

Study Methodology

Questionnaire Survey: Data on each sampled cow were collected from the owners using a structured questionnaire to assess the associated risk factors contributing to distribution of contagious and environmental bacteria. This includes breed, age, husbandry system, stage of lactation, parity, washing of udder and hands before milking, how towel were used to dry hand and udder, previous history of mastitis occurrences and presence of teat lesion (tick infestation).

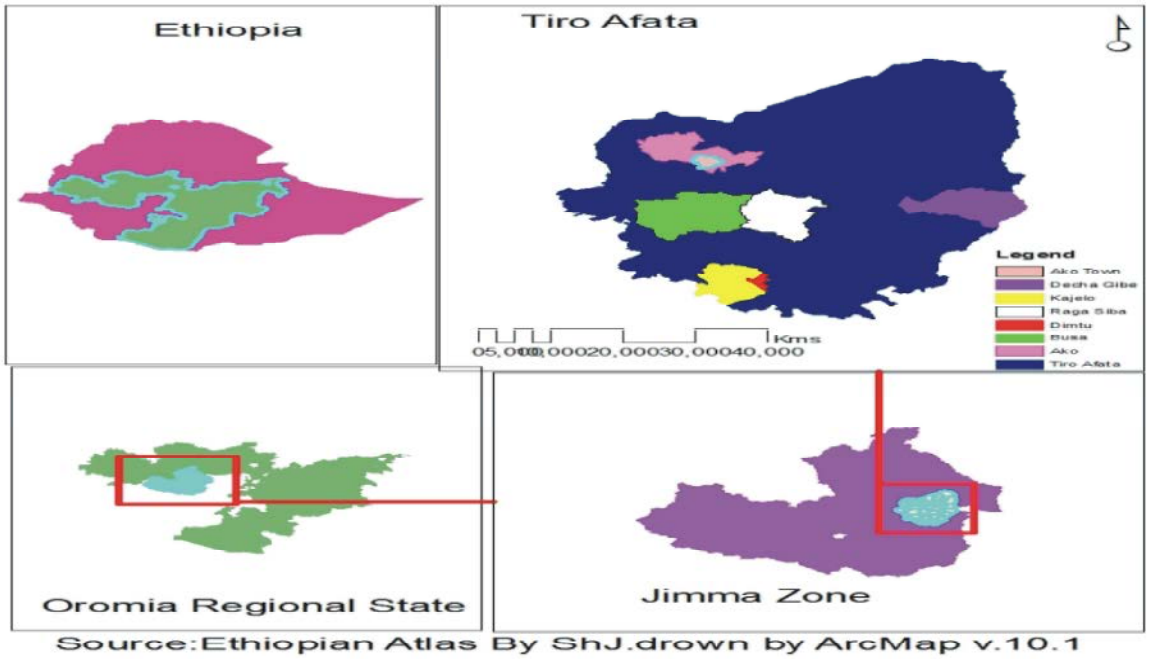


Fig. 1: The map of study area

Table 1: Towns and Kebeles selected for sample collection

		Number of selected cows in for study		
Kebeles	No. lactating cows in the area	Cross	Local	Total
Akko town,	308	12	49	61
Akko	330	13	52	65
Kejelo	241	10	38	48
Busa	385	15	61	76
Dimtu	200	8	32	39
Raga Siba	370	15	58	73
Dacha Gibe	496	20	78	98
Total	2330	92	368	460

Sample Collection and Transporting Method: Before milk sample collection the udders were carefully inspected followed by thorough palpation to detect possible fibrosis, inflammatory swellings, visible injury and appearance of milk secretion from each mammary quarter were examined to categorize cows as infected (clinical mastitis) and healthy. Clinically infected cows were restrained; udders were then washed with tap water, dried and swabbed with cotton soaked in 70% alcohol and the first 3 milking stream were discarded to make free from contamination and then approximately 10 ml the samples were taken from each quarter. Visually healthy udders were washed with tap water, dried and swabbed with cotton soaked in 70% alcohol and the first 3 milking stream were discarded to make free from contamination. The milks were subjected to California Mastitis Test (CMT). Approximately 10ml quarter milk samples were then collected only from quarters with CMT score of 1, 2, 3 and taken to JUCAVM Microbiology laboratory for isolation and identification of the pathogenic bacteria. Because, the non-pathogenic pathogens are identified more frequently in quarters with low mastitis test reactions (negative, trace and 1) and pathogenic bacteria are identified from quarters with high mastitis test reactions (CMT 2 and 3) [30].

The standard procedures of National Mastitis Council [15] were followed for sample collection and transportation. The collected milk samples were transported in an ice cooled box at 4°C and analysed within two to three days for identification of the bacterial pathogens. The samples cultured within two days were stored at refrigerator temperature and those not cultured within two days and kept for secondary culture were stored at freezer temperature. Freezing milk sample enhance the detection of coliform species in particular [14].

Microbiological Examination

Sample Processing, Culturing and Isolation of the Major Bacterial Pathogens: For primary culture, 10 µlof each quarter milk sample werestreaked onto 7% Sheep Blood Agar (SBA) plate and incubated at 37°C for up to 48hr under aerobic condition and examined for bacterial growth, morphology and hemolytic features at 24 hr and 48hr after inoculation: (1) the samples yield single (one colony) and two colonies were further identified to species level; (2) the samples yielded three or more colonies were recorded as contamination; (3) the samples with no growth (negative culture result) at primary culture were enriched within Buffered Peptone Water and recultured. The samples with one and two culture result after secondary culture were further tested. Finally the samples with three/ or more than three colonies and negative culture after secondary culture were discarded.

Isolation and Identification of *Staphylococcus* Species: Each pure colony that were grown on 7% sheep Blood Agar (SBA) was, streaked on nutrient agar and incubated at 37°C for up to 48hr under aerobic condition. Then catalase test was performed to distinguish *Staphylococcus* species (catalase-positive) from *Streptococcus* species (catalase-negative) (Figure 2).

Catalase-positive colonies were then cultured on Mannitol Salt Agar (MSA) plates and incubated at 37°C for 24-48 hours. Growth on MSA agar plates was taken as confirmative identification of the salt tolerant *Staphylococcus* species.

MSA has Mannitol (alcohol of the carbohydrate mannose) and Phenol red pH indicator. Mannitol fermentation produces acid end products which turn the medium yellow. Yellow indicates mannitol positive and no colour change indicates mannitol negative. The change in the pH of this medium was used for *Staphylococcus* classification as highly fermentative (*S. aureus*), weakly fermentative (*S. intermedius*) and non-fermentative (*S. hyicus* and CNS) (Figure 3a). Colonies from Mannitol salt medium were cultured overnight on Brain heart infusion broth medium and tested using tube coagulase test (using rabbit plasma) to differentiate coagulase positive (*S. aureus, S. Intermedius* and *S. hyicus*) from coagulase negatives *Staphylococcus* (CNS) species (Figure 3b).

Coagulase positive *staphylococcus* isolates were further streaked on purple agar base (PAB) media plate with 1% of maltose and incubate at 37°C for 24-48 hours to further differentiate species of CPS (*S. aureus, S. Intermedius* and *S. hyicus*) (Figure 4a). The identification was based on the fact that *S. aureus* rapidly ferment maltose to change the medium and colonies to yellow (Figure 4b). *S. intermedius* gives a weak or delayed reaction (Figure 4c) and *S. Hyicus* did not ferment maltose (Figure 4d) [31, 32]. Coagulase negative staphylococci were not identified to the species level, due to scarcity of reagents.

Isolation and Identification of *Streptococcus* Species: Catalase-negative colonies were sub-cultured on Edward's agar and incubated at 37°C for 24-48 hours. Edward's agar was used as a selective medium for the isolation of streptococci. *S. agalactiae, S. dsygalactiae* and *S. uberis* were identified according to their ability to split aesculine and sodium hippurate. *S. agalactiae* was differed from other by its positive result of CAMP test and negative result of both aesculine and sodium hippurate hydrolysis (Figure 5a). *S. dsygalactiae* was differed from other by negative result of CAMP test, aesculine and sodium hippurate hydrolysis (Figure 5b). *S. uberis* was identified by its negative result of CAMP test and positive result of aesculine and sodium hippurate hydrolysis (Figure 5c). The enterococci (*Enterococcus faecalis*) and group D Streptococci (*S. bovis* and *S. equinus*) were distinguished by their growth at 45°C on MacConkey agar and toleration of 40% bile.

Isolation and Identification of Coliform Bacteria: Each pure colony that were grown on 7% sheep Blood Agar (SBA) was, streaked on MacConkey (MaC) agar and incubated at 37°C for up to 48hr under aerobic condition and examined for bacterial growth, morphology and lactose fermentation. Colonies that were grown as pink on MaC Agar were Lactose fermenters and recorded as *coliform*s species (*Escherichia, Klebsiella, Enterobacter*) and *Citrobacter* species (Figure 6a).The colony which was lactose fermenters, flat dry, pink colonies with surrounding darker pink area of precipitated bile salts was recorded as *E. coli.* Colonies that those were mucoid lactose fermenters were recorded as *Klebsiella* species. Colonies which were grown as translucent and colourless are non-lactose fermenter and recorded as other gram negative bacteria.

The colonies from MacConkey agar were then tested with oxidase test to differentiate *Enterobacteriaceae* (oxidase negative) from other gram negative (oxidase positive) bacteria (Figure 6b). Oxidase negative colonies were subcultured on Edwards Medium Agar to confirm gram-negative bacteria and to differentiate between two major coliforms, *E. coli* (smaller, green-metallic sheen) and *E. aerogenes* (larger, rose colour) on other side. Only gram-negative bacteria grow on this special media [33].

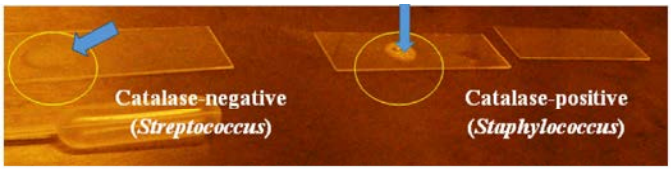


Fig. 2: Catalase-positive (*Staphylococcus*) and catalase-negative (*Streptococcus*) species

Fig. 3: (a) Salt tolerant *Staphylococcus* species grow on Mannitol Salt medium; (b) Coagulase positive *Staphylococcus* species

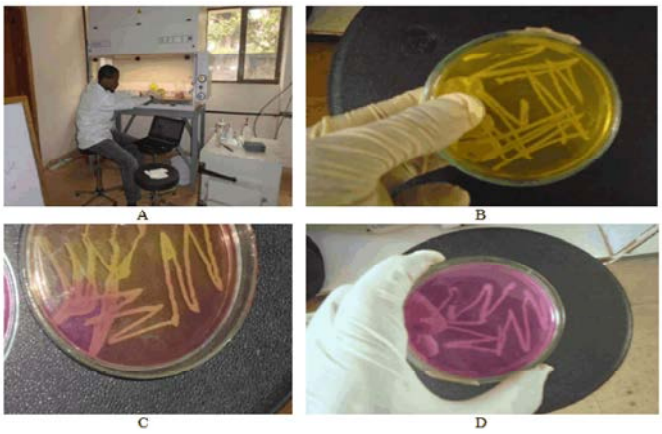


Fig. 4: (a) Identification of Pure colonies of Coagulase Positive Staphylococcus (CPS) species on PAB agar in laboratory; (b) *S. aureus* is highly fermentative after 18hr (c) *S. intermedius* start to fermentative after 28hr and (d) *S. hyicus* is non-fermentative

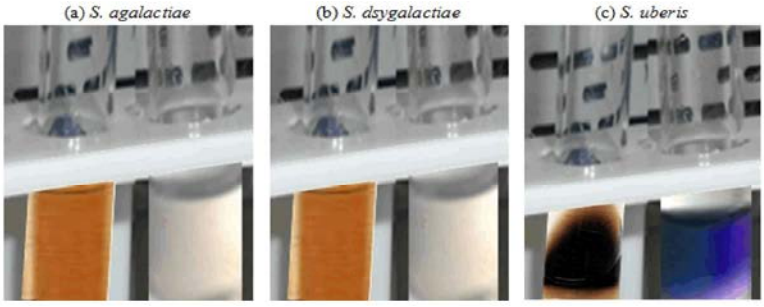


Fig. 5: Aesculine and Sodium hippurate hydrolysis tests

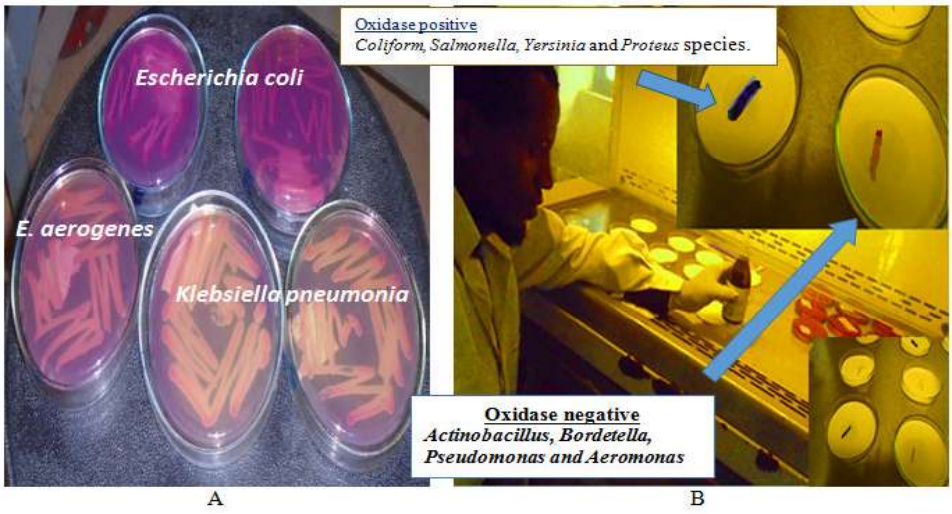
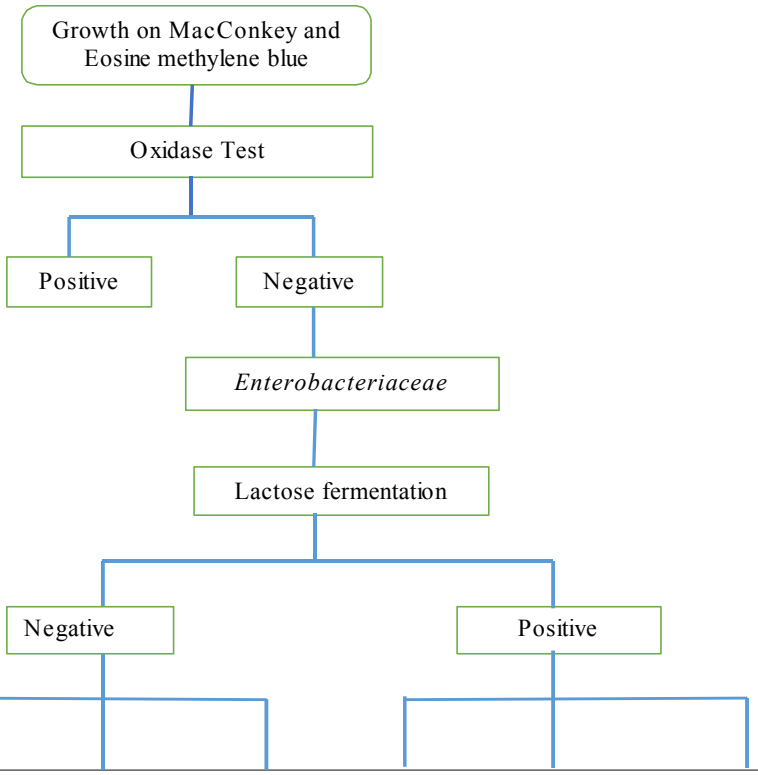


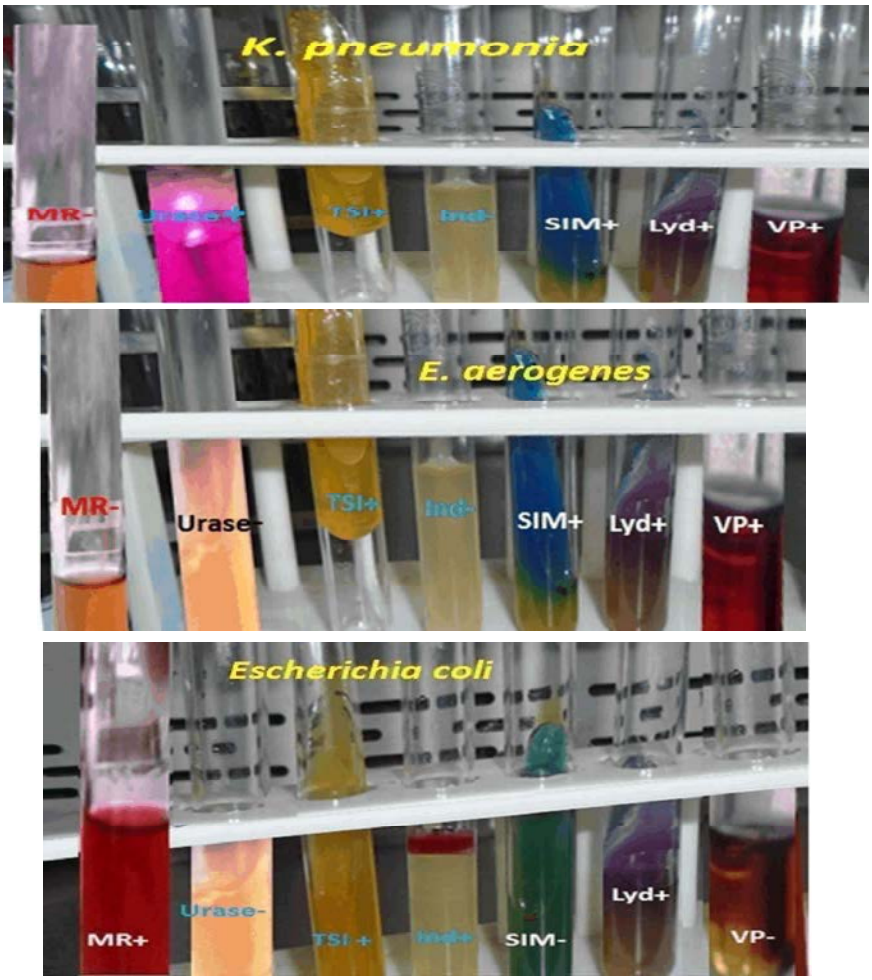
Fig. 6: Colony morphology (a) and oxidation characteristics of coliform species (b)

Table 2: Follow chart for identification of *Coliform* species. Adopted from Quine, *et al.* [33]



Types of tests	Yersinia	*Salmonella* species	Proteus species	*Escherichia coli*	*Enterobacter aerogenes*	*Klebsiella pneumonia*
Motility at 30°C	+*	+	+	+	+	-
Lactose fermentation	-	-	-	+	+	+
IMVic test						
•1 Indole production	V	-	±**	+	-	-
•2 Methyl red	+	+	+	+	-	-
•3 VP test	-	-	V	-	+	+
•4 Citrate utilization	-	+	V	-	+	+
H2S pron. in TSI	-	+	+	-	-	-
Urease	+*	-	+	-	-	+
Lysine decarboxylase	-	+	-	+	+	+

*= Except *Y. Pestis,* **=*P. vulgaris* (+): *P. mirabilis* (-); v =reaction varies with individual species



MR = Methyl red, TSI = Triple Sugar Iron (for H_2S pron), Ind = Indole Test, SIM = Simon Citrate test, Lyd = Lysine decarboxylase, VP = vogues Proskaur

Fig. 7: Biochemical characterization of coliform species

Then Coliform species were further identified to species level using primary (Motility and growth in triple sugar iron agar) and secondary biochemical tests (Lysine decarboxylase, Cimon citrate, MViC test and Urease test) (Table 2, Figure 7).

Antibacterial Susceptibility Test: The antibacterial profiles of all isolated *Staphylococcus*, *Streptococcus* and *Coliform* species were determined using the Kirby-Bauer test. Each isolates were prepared in a bacterial suspension of sterile saline with turbidity equal to 0.5 McFarland standards. The discs of antibiotic agents used in the area were chosen and placed 4 cm apart on each Mueller-Hinton agar. Of the many media available, Mueller-Hinton agar was selected as the best for routine susceptibility testing of non-fastidious bacteria because of: (a) it shows acceptable batch-to-batch reproducibility for susceptibility testing; (b) it is low in sulphonamide, trimethoprim and tetracycline inhibitors and (c) it supports satisfactory growth of most no-fastidious pathogens. Amphotericin B was added to the medium to prevent fungal competition. Plates were inverted and incubated for 18-24 hours, after which zones of inhibition for each agent were recorded in millimeters (Figure 8) according to Quine, Markey and Maguire [33] (Table 3).

Data Management and Statistical Analysis: All data collected were entered into Microsoft excel spread sheet, transferred to software SPSS version 23 and processed for analysis. The culture positive was the dependent variables while parity, stage of lactation and presence of teat lesions, milking hygiene were independent variables.

Fig. 8: Measuring Zones of inhibition for antibiotic susceptibility

Table 3: Zone diameter interpretive standards for isolates [34]

	Staphylococcus			*Enterobacteriaceae*			*Streptococcus*		
	R	I	S	R	I	S	R	I	S
Gentamycin (10 µg)	≤12	(13-14)	≥15	≤12	(12-15)	≥15	≤12	(13-14)	≥15
Erythromycin (15 µg)	≤13	(14-22)	≥23	≤13	(13-18)	≥18	≤13	(14-22)	≥23
Streptomycin (10 µg)	≤11	(12-14)	≥15	≤11	(11-15)	≥15	≤11	(12-14)	≥15
Penicillin-G (10 unit)	≤20	(20-28)	≥29	≤20	(20-28)	≥29	≤20	(20-28)	≥29
SXT (25 µg)	≤10	(11-15)	≥16	≤10	(10-16)	≥16	≤10	(11-15)	≥16
Tetracycline (30 µg)	≤14	(15-18)	≥19	≤14	(14-19)	≥19	≤14	(15-18)	≥19
Ampicillin (10 µg)	≤13	(13-17)	≥17	≤13	(13-17)	≥17	≤13	(13-17)	≥17
Kanamycin (K)	≤13	(14-17)	≥18	≤13	(14-17)	≥18	≤13	(14-17)	≥18

SXT = Sulphamethazole-trimethoprim, R = Resistance, I = Intermediate, S = Susceptible

Descriptive statistics were done to summarize the raw data. Logistic regression statistical test was used to check the presence of association between risk factors and the mastitis. Factors with $p < 0.25$ in univariable analysis were initially considered for inclusion in the multivariable analysis. Multivariable logistic regression analysis was run and only variables with $p < 0.05$ judged significant. Confounding was checked by removing and replacing variables one by one. Model results are presented as odds ratios (OR) along with their 95% confidence interval (CI).

RESULTS

Prevalence of Mastitis in the Tito Afeta District: A total of 460 (368 local and 92 cross) lactating cows were examined by visual inspection (for identification of clinical mastitis) and California Mastitis Test (for identification of sub clinically infected cows). A total of fifty two cows have blind quarters (32, 17 and 3 cows have 1, 2 and 3 blind quarters respectively). This means 35 local lactating cows have 53 blind quarters (20, 12 and 3 cows have 1, 2 and 3 blind quarters respectively) while 17 cross breed have 22 blind quarters (12 and 5 cows have 1 and 2 blind quarters respectively). Seventy cows (24 cross and 46 local breed) cows were clinically infected and 390 (68 cross and 322 local breed) cows were subjected to California mastitis test (CMT). A total of 222 (36 cross and 186 local breed) were positive with California mastitis test (CMT).

From the total positive for mastitis (clinical and subclinical), twenty eight (28), twenty nine (29), fifty seven (57) and one hundred seventy eight (178) cows have 4, 3, 2 and 1 affected quarters respectively. This means nineteen (19), seventeen (17), thirty eight (38) and one hundred fifty eight (158) local cows have 4, 3, 2 and 1 affected quarters respectively while 9, 12, 19 and 20 cross breed cows have 4, 3, 2 and 1 affected quarters respectively excluding 236 (148 local and 88 cross) Trace CMT scores.

The overall prevalence of mastitis at cow level was 63.5% (292/460), among which 15.21% (70/460) were clinical mastitis cases and 48.3% (222/460) were subclinical mastitis cases. From70 clinical mastitis cases, 26.1% (24/92) and 12.5% (46/368) were cross and local zebu cows respectively (Table 4).

Concerning prevalence of mastitis at quarter level, a total of 1840 quarters were examined and 1, 765 (95.9%) were functional quarters. The overall prevalence of mastitis at quarter level is 26.7% (491/1840). On the other hand the prevalence of mastitis at quarter level were 24.52% (361/491) and 35.33% (130/491) in local and cross breed respectively. The result showed that higher infection proportion in back quarters as compared to the

Table 4: Overall prevalence of mastitis (n = 460)

Breed (No. examined)	Clinical mastitis (%)	Subclinical mastitis (%)	Prevalence (%)	95% CI
Local (368)	46 (12.5)	186 (50.5)	232 (63.0)	[58.1- 68.0]
Cross (92)	24 (26.1)	36 (39.1)	60 (65.2)	[55.5 - 74.9]
Total (460)	70 (15.2)	222 (48.3)	292 (63.5)	[59.1 - 67.9]

CI = Confidence Interval

Table 5: Prevalence of bovine mastitis at quarter level

Breed (No. quarters)	Right front (%)	Right rear (%)	Left front (%)	Left rear (%)	Prevalence (%)	95% CI
Local (1, 472)	85 (5.8)	82 (5.6)	90 (6.1)	104 (7.1)	361 (24.52)	22.35-26.81
Cross (368)	32 (8.7)	31 (8.4)	33 (9.0)	34 (9.2)	130 (35.33)	30.44 - 40.45
Total (1840)	117(6.4)	113 (6.1)	123 (6.7)	138 (7.5)	491 (26.68)	24.68 - 28.77

Table 6: Prevalence of blind quarter (n=75)

No. of quarters examined)	Right front (%)	Left front (%)	Right hind (%)	Left hind (%)	Overall Prevalence (%)	95% CI
Local (1, 472)	13 (0.9)	12 (0.8)	17 (1.2)	11 (0.7)	53 (3.6)	2.76 - 4.68
Cross (368)	6 (1.6)	5 (1.4)	7 (1.9)	4 (1.0)	22 (6.0)	3.78 - 8.91
Total (1840)	19 (22.6)	17 (0.9)	24 (1.3)	15 (1.0)	75 (4.1)	4.03 - 6.35

front quarters in both breeds (Table 5). In addition, from a total of 1840 quarters examined 75 (4.1%) quarters were blind. On the other hand the prevalence of blind quarters are 3.6% (53/1472) and 6.0% (22/368) in local and cross breed respectively (Table 6).

Microbiological Cultures and Isolates: In this study, 441 quarter milk samples positive to clinical mastitis and CMT (score 1, 2 and 3) were collected from 292 lactating cows. The proportion of milk samples with growth (culture positive) was 87.99% (388/441) after primary and enrichment culture. The proportion of samples yielded single and double colonies were 84.13% (371/441). The proportion of milk samples with no growth culture result were 12.02% (53/441) after primary and enrichment culture. Sample yielding 2 different colonies (mixed culture) accounted for 3.63% (16/441). The samples yielding three or more different colonies and classified as contaminated were accounted to 12.9% (57/441). According to Ling, *et al.*, (2015), milk samples yielding to ≥ 3 colonies should be taken as contamination. Accordingly, total of 347 pathogens were identified from 331 quarter milk samples (Table 7).

Isolation Frequencies of Predominant Bacterial Pathogens (N= 347) from Positive Culture: The most frequently isolated bacterial pathogens in this study were *Staphylococcus* 53.60% (186/347), *Streptococcus* 29.1% (101/347) and *coliform* species 17.29 % (60/347). *S. aureus* (22.45%), *E. coli* (16.33%) and *S. agalactiae* (14.29%) occupied prime position with 28.24% (47/98) of total isolates from samples of clinical mastitis teats. While coagulase negative *staphylococcus* (CNS) (27.31%), *S. aureus* (19.9%), *S. agalactiae* (13.65%), *S. dsygalactiae* (6.18%) and *S. uberis* (5.79 %) were the predominant pathogens accounted for 75.67% (196/259) of the total isolates from the subclinical mastitis milk samples. Among the major contagious pathogens, *S. aureus* (21.29%), *S. agalactiae* (13.83%) and *S. dsygalactiae* (6.43%) were the predominant agents identified. While*coliform* species (*E. coli, K. pneumonia* and *E. aerogenes*) were the majority of the environmental pathogens accounted for 17.29% (60/347) of total isolates (Table 8).

The isolation frequency of contagious (*S. aureus, S. agalactiae* and *Coagulase negative staphylococcus*) and environmental pathogens (*E. coli*, *S. dsygalactiae*, *S. uberis*, Other *Streptococcus* species*, S. intermedius*, *K. pneumonia*, *S. hyicus*, *Enterobacter aerogenes* and other gram negative pathogens) in the area were 59.7% (207/347) and 40.3% (140/347) respectively. Among the major contagious pathogens, *S. aureus* (21.6%), *S. agalactiae* (13.83%) and *Coagulase negative staphylococcus* (25.10%) were the predominant agents identified. While the major environmental pathogens isolated were *E. coli* (8.64%), *S. dsygalactiae* (6.63%), *S. uberis* (5.76%), *S. intermedius* (4.32%), *K. pneumonia* (3.74%), *S. hyicus* (3.17%), *Enterobacter aerogenes* (2.59%) and other environmental pathogens (5.5%) of the total isolates (Table 8).

Table 7: Prevalence of Culture result and isolates after primary and enrichment culture at quarter level

Quarters	Separated culture (Single colony)	Mixed culture (two colonies)	Contamination/ Discarded	Culture-negative	Total
P°CR (No. samples)	242 (181LB+61CB)	9 (6LB+3CB)	47 (36LB+11CB)**	143 (110CB +33LB	441 (333LB+108CB)
Right front	56 (41LB+15CB)	2(1LB+1CB)x2	6 (4LB+2CB)	32 (27LB +5CB)	96 (73LB+23CB)
Right Rear	53 (39LB+14CB)	2(2LB+0CB)	13 (11LB+2CB)	35 (26LB +9CB)	103 (78LB+25CB)
Left hind	59 (44LB+15CB)	2(0LB+2CB)	16 (11LB+5CB)	37 (29LB + 8CB)	114 (84LB+30CB)
Left rear	74 (57LB+17CB)	3(3LB+0CB)	12 (10LB+2CB)	39(28LB +11CB)	128 (98LB+30CB)
ECR (No samples)	73 (65LB + 8CB)	7 (5LB + 2CB)	10 (7LB + 3CB)	53 (33LB+20CB)	143 (110CB +33LB
Right front	18 (17LB +1CB)	------	3 (3LB + 0CB)	11(7LB+4CB)	32 (27LB +5CB)
Right rear	18 (17LB + 1CB)	3 (2LB+ 1CB)	2 (0LB + 2CB)	12(7LB+5CB)	35 (26LB +9CB)
Left hind	16 (14LB +2CB)	2 (2LB + 0CB)	3 (3LB + 0CB)	16(10LB+6CB)	37 (29LB + 8CB)
Left rear	21 (17LB + 4CB)	2 (1LB +1CB)	2 (1LB + 1CB)	14(9LB+5CB)	39(28LB +11CB)
No. Isolates	315 (246LB+69CB)	16 (11LB+5CB)	57 (43LB+14CB)**	53 (33LB+20CB)	347 (268LB+79CB)
Right front	74 (58L+16CB)	2(1LB+1CB)x2	------	0	78 (60LB+18CB)
Right rear	71 (56L+15CB)	5(4LB+1CB)	------	0	81 (64LB+17CB)
Left hind	75 (58L+17CB)	4(2LB+2CB)	------	0	83 (62LB+21CB)
Left rear	95 (74L+21CB)	5(4LB+1CB)	------	0	105 (82LB+23CB)

P°CR=Primary culture result, ECR=Enrichment culture result, CB=cross breed, LB=local breed **=Not isolated to species level, contaminated=3 colonies

Table 8: Frequency of predominant bacterial pathogens in clinical and subclinical mastitis

Pathogens	% of total CM	% of total SCM	% of total isolates	95% CI (Total)
Staph. Species	47 (47.96)	139 (55.82)	186(53.60)	(48.4 - 58.5)
S. aureus	22 (22.45)	53 (21.29)	75(21.6)	(16.7 - 25.4)
S. intermedius	4 (4.08)	11 (4.42)	15 (4.32)	(2.3 - 6.6)
S. hyicus	4 (4.08)	7 (2.81)	11(3.17)	(1.4 - 5.2)
CNS	17 (17.35)	68 (27.31)	85(24.5)	(20.5 - 30.0)
Coliform species	24 (24.49)	36 (14.46)	60(17.29)	(13.5 - 21.3)
E. coli	16 (16.33)	14 (5.62)	30 (8.64)	(5.8 - 11.8)
K. pneumonia	3 (3.74)	10 (4.02)	13(3.74)	(2.0 - 6.0)
E. aerogenes	2 (2.59)	7 (2.81)	9(2.59)	(1.2 - 4.3)
Others	3 (3.06)	5 (2.01)	8(2.3)	(0.9 - 3.7)
Strep. Species	27 (27.55)	74 (29.72)	101(29.1)	(24.2 - 33.7)
S. agalactiae	14 (14.29)	34 (13.65)	48(13.83)	(10.1 - 17.3)
S. dsygalactiae	7 (7.14)	16 (6.43)	23 (6.63)	(4.0 - 9.2)
S. uberis	5 (5.1)	15 (6.02)	20(5.76)	(3.7 - 8.6)
Other *Strep.*	2 (2.04)	9 (3.61)	11(2.88)	(1.4 - 4.6)
Total isolates	98 (28.24)	249 (71.76)	347 (100)	

CM: Clinical mastitis, SCM: subclinical mastitis, CNS: Coagulase-Negative *Staphylococcus*

Risk Factors Associated with Mastitis: Different factor from management point of view, animal related and environmental were assessed for their potential contribution to the distribution of mastitis/ or bacterial pathogens causing mastitis in the study area. Risk factors with a trend toward significance ($p < 0.25$) under univariable logistic regression analysed in the multivariable logistic analysis. Table 9 illustrates multivariable logistic regression result of frequencies of bacterial pathogens as influenced by different risk factors. Lactating cows with greater than five calves are 3.17 times more high frequency of bacteria than cows those gives one to three calves and 3.01 times more likely affected than those gives three to five calves. Lactating cows at last lactation stages are 2.29 times more high frequency of bacteria than cows at early lactation stages and 2.1 more likely affected than those at medium lactation stages. Lactating cows in which there is a practice of drying udder with common (single) towel after washing are 3.32 times more high frequency of bacteria than cows in which no practice of drying udder with common towel. Lactating cows with teat lesion are 0.19 times more affected those cows without teat lesion. Those cows kept in house with poor hygiene are 5.47 and 3.04 times more high frequency of bacteria than cows kept in house with good and medium hygiene respectively (Table 9).

Antimicrobial Susceptibility Profiles of Pathogens: In this study, out of 347 bacterial pathogens identified, 89 (39, 25 and 25 *Staphylococcus*, *Streptococcus* and coliform species respectively) isolates were subjected to antimicrobial susceptibility tests against 6 antimicrobials of different classes. *Staphylococcus* species were resistant to Tetracycline 54.0% (21/39),

Table 9: Multivariable model output for frequencies of bacterial pathogens influenced with different risk factors

Risk factors	No. Exa.	Frequencies of isolates *Staph.* (%)	*Strep.* (%)	*Coliform* (%)	Total (%)	OR(95%CI)	P- Value
Parity							
1-2	164	44 (26.8)	31 (18.9)	15 (9.1)	90 (54.9)	Reference	
3-4	145	67 (46.2)	32 (22.1)	21 (14.5)	120 (82.8)	3.01 (1.03 - 4.11)	0.001*
>5	151	75 (49.7)	38 (25.2)	24 (15.9)	137 (90.7)	3.17 (1.66 - 5.34)	0.001*
Lactation stages							
Early (<2 months)	171	75 (43.8)	36 (21.1)	32 (18.7)	143 (83.6)	Reference	
Medium (3-6 months),	151	43 (28.4)	32 (21.2)	10 (6.6)	85 (56.3)	2.10 (1.14 - 3.71)	0.017*
Late (> 6 months)	138	68 (49.3)	33 (23.9)	18 (13>0)	119 (86.2)	2.29 (1.23 - 4.26)	0.009*
Washing and drying of teat*[2]							
Yes	173	50 (28.9)	21 (12.1)	12 (6.9)	83 (48.0)	Reference	
No	287	136 (47.4)	80 (27.9)	48 (16.7)	264 (92.0)	3.32 (1.95 -5.64)	0.001*
Hygiene							
Good	136	27 (19.8)	21 (15.4)	7 (5.1)	55 (40.4)	Reference	
Fair	160	63 (39.4)	38 (23.8)	24 (15.0)	125 (78.1)	3.04 (1.59 - 5.78)	0.001*
Poor	164	96 (58.5)	42 (25.6)	29 (17.7)	167 (101.8)	5.47 (2.68 -1.16)	0.001*
PHM							
No	108	45 (41.6)	32 (29.6)	25 (23.1)	102 (94.4)	Reference	
Yes	352	141 (40.0)	69 (19.6)	35 (9.9)	245 (69.6)	0.19 (0.05 - 0.67)	0.010*
Animal treatment							
Veterinarian	234	61 (26.07)	42 (17.95)	23 (9.83)	126 (53.85)	Reference	
Farmers	226	125 (55.31)	59 (26.11)	37 (16.37)	221 (97.79)	8.98 (2.74 -9.49)	0.001*
Total	460	186 (40.43)	101 (21.96)	60 (13.04)	347 (75.43)		

PHM: Previous history of mastitis, [2a]: Drying of teat with the same towel, OR: Odds ratio, CI: Confidence Interval, * there was significant association

Table 10: Antimicrobial susceptibility patterns of selected pathogenic Staphylococcus species (n=39)

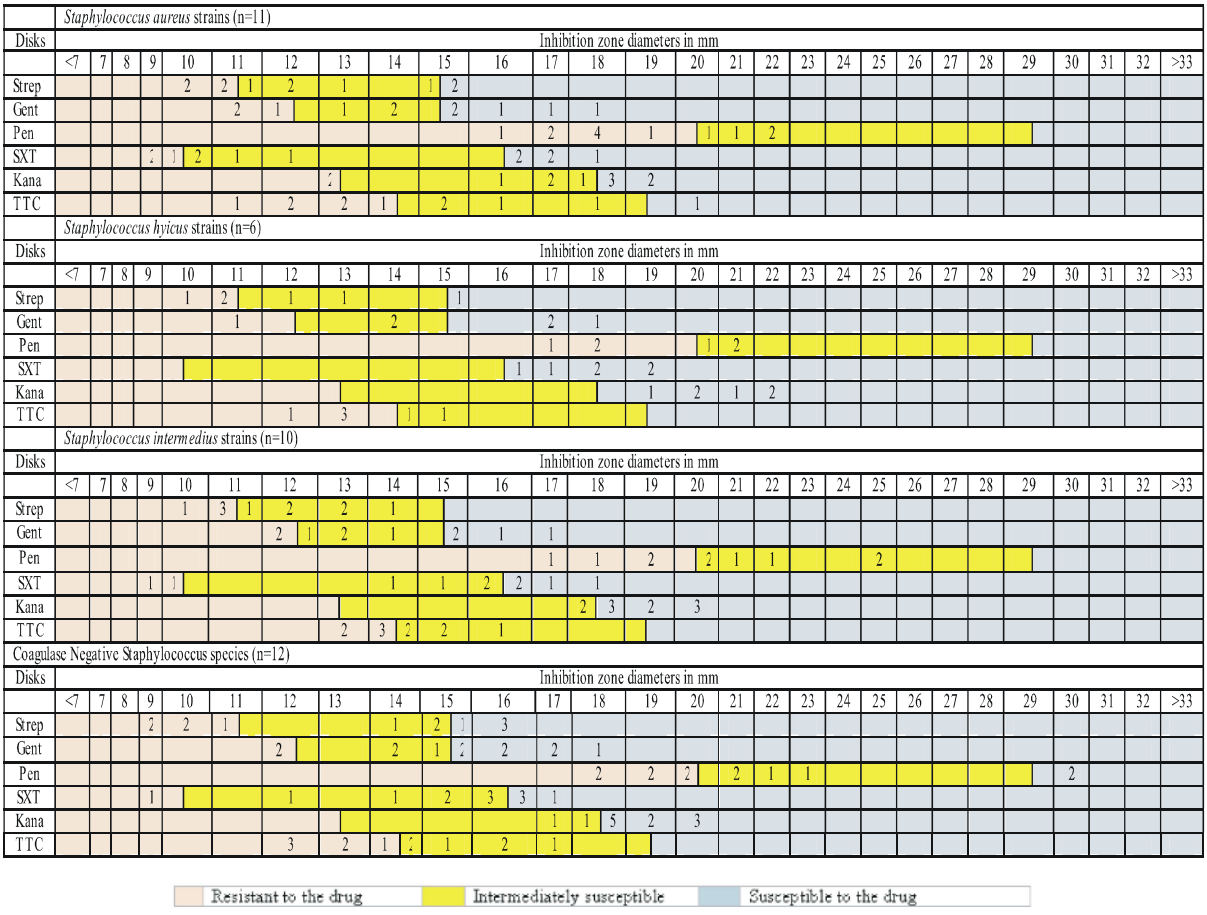


Table 11: Antimicrobial susceptibility patterns of selected pathogenic Streptococcus species (n=25)

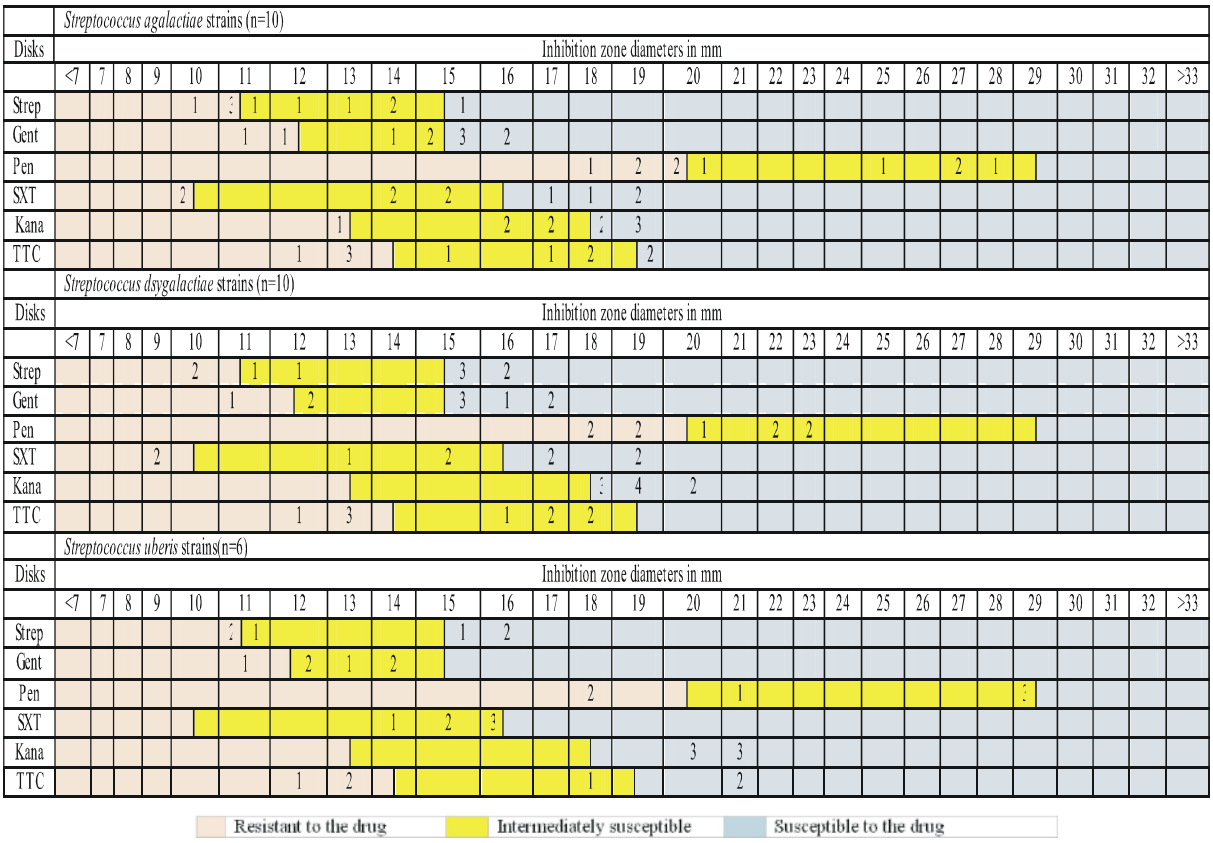


Table 12: Antimicrobial susceptibility patterns of selected pathogenic Enterobacteriaceae species (n=25)

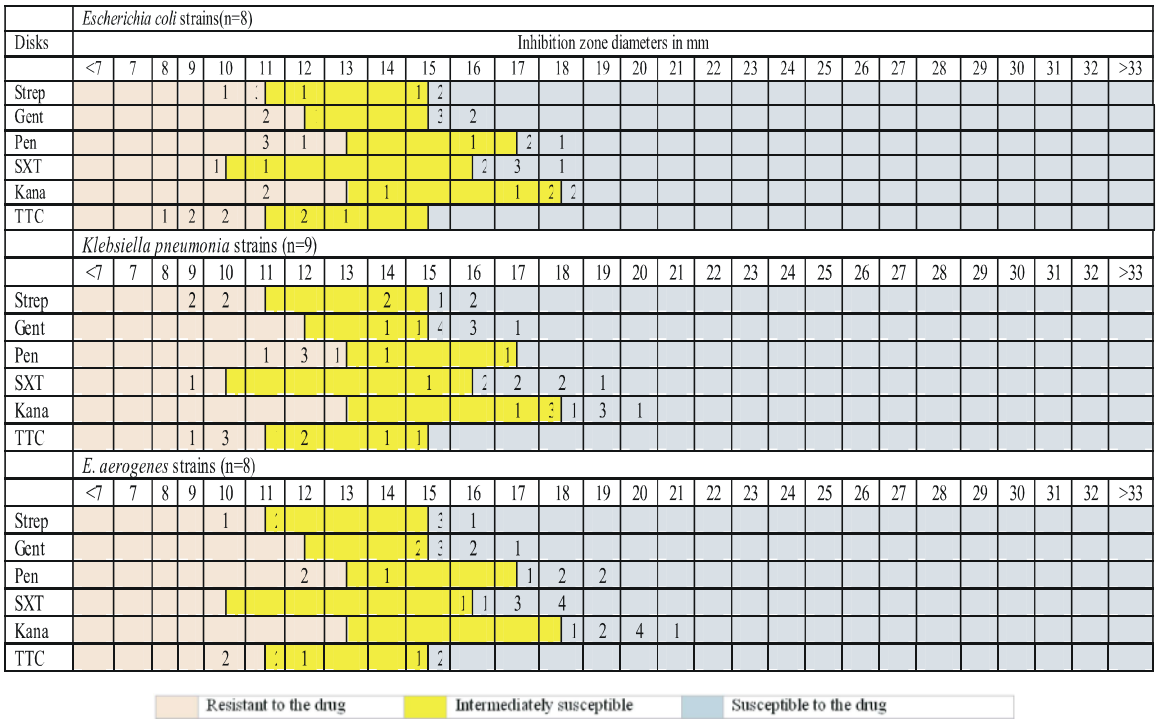


Table 13: Multi-drug resistance profiles of selected pathogenic bacteria

Antibiotics	Total (n=81)	Selected Bacterial Species (%)								
		S. aureus	*S. intermedius*	*CNS*	*S. agalactiae*	*S. dsygalactiae*	*S. uberis*	*E. coli*	*K. pneumonia*	*E. aerogenes*
P/S	26 (32.5)	4 (36.4)	4 (40)	5 (41.6)	3(30.0)	2 (22.2)	2 (33.3)	3(30.0)	2 (22.2)	1 (12.5)
P /S/TE	25 (30.8)	3 (27.3)	3 (30.0)	5 (41.6)	3 (30.0)	2 (22.2)	2 (33.3)	4 (50.0)	2 (22.2)	1 (12.5)
SXT/S/TE	11 (13.6)	2 (18.0)	2 (20.0)	1 (8.0)	2 (20.0)	1 (11.1)	1 (16.7)	1 (12.5)	1 (11.1)	0
G/S/TE	11 (13.6)	3 (27.3)	2 (20.0)	2 (16.7)	1(10.3)	1(11.1)	0	2 (25.0)	0	0
K/S/TE	4 (5.0)	2 (18.0)	0	0	1 (10.0)	0	0	1 (10.0)	0	0
G/S/K/TE	4 (5.0)	2 (18.0)	0	0	1 (10.0)	0	0	1(10.0)	0	0

TE= Tetracycline; S= Streptomycin; GN= Gentamicin; P= Penicillin G; SXT= Sulphamethoxazole trimethoprim; K= Kanamycin

Penicillin 53.8% (21/39), Streptomycin 41.1 % (16/39), Gentamicin 20.5% (8/39), Sulphamethoxazole-trimethoprim (SXT) 15.4% (6/39) and Kanamycin 5.1% (2/39) (Table 10). *Streptococcus* species were resistant to Tetracycline 68% (11/25), Streptomycin 44% (8/25), Penicillin 44% (11/25), Gentamycin 16% (4/25), SXT 16% (4/25) and Kanamycin 4% (1/25) (Table 11). Coliform species were also resistant to Tetracycline 44% (11/25), Streptomycin 36% (9/25), Penicillin 44% (11/25), Gentamycin 8% (2/25), SXT 8% (2/25) and Kanamycin 8% (2/25) (Table 12).

Multiple Drug Resistance of Isolates: Multi-antibiotic resistance (MDR) (defined as lack of susceptibility to at least two antibiotics from different classes) was also recorded in several bacteria isolates with 91.0% (81/89) of the bacterial isolates showing multi-antibiotic resistance patterns, whereby 32.1% (26/81) isolates were resistant to two antibiotics and 67.9% (54/80) were resistant to more than two antibiotic drugs (Table 13).

DISCUSIONS

Prevalence of Mastitis: Numerous diseases are responsible for reduction in milk production, among these mastitis is the most important and various contributory factors have been reported, but such reports are scanty in Jimma Zone Tiro Afeta District. Therefore, it was necessary and crucial to know the prevalence of mastitis and different extrinsic and intrinsic risk factors which favour the entry of different pathogens in the udder. Therefore the current study revealed that the overall prevalence mastitis at cow level was 63.5%. The result of the present finding is in line with the previous findings different researchers who reports 62.5% [35], 65.6% [7] and 61.11% [36] in different parts of Ethiopia. In contrast, higher prevalence were also reported by different authors who reports 88% [4], 71% [8] and 69.9% [6] in North Showa Zone of Ethiopia, dairy farms of Holeta town and Addis Ababa, respectively. This difference might be due to different management system and milking practice.

The overall prevalence of mastitis at quarter level in the present study was 26.68%. About twenty eight [37], 37% [38] and 20.4% [72] were also reported in different parts of Ethiopia. The higher mastitis infection was observed in hindquarters than front quarters. This is similar with previous report in different parts of Ethiopia [35, 39]. This might be due to increased milk production performance followed with relaxed teat sphincters and contaminated hind legs as a result, the pressure on teat canal forces the canals to be opened widely which allows entrance of microbes. In the present study, the proportion of blind quarters was 4.1%. This result is in line with the previous findings different researchers who reports 4.5% [7], 6.1% [3] and 3.8 % [38] in different parts of Ethiopia. This is an indication of serious mastitis problem in dairy farms and absence of culling that should have served to remove a source of mammary pathogens/or infections.

In the present study, the overall prevalence of clinical mastitis at cow level in this study was 15.2%. The result of this finding is in line with the previous findings different researchers who reports 10% [41], 16.11% [42] and 11% [3] in southern, in central Ethiopia and Jimma town respectively. About 4.8% [43], 7.75% [44] and 3.9% [45] which are lower than this report were also reported in Bahir Dar, in and around Jimma town and Adama, Ethiopia respectively. Overall 48.3% prevalence of subclinical mastitis at cow level was recorded. The prevalence of sub clinical mastitis in present study is comparable with previous reports such as 62% [3], 34.8% [46] in Jimma and Adama town, respectively and other country 56.3% [71] in Egypt. About 89.5% [47] and 80.6% [4], which is higher than this finding was also reported in North Showa Zone of Ethiopia and Selale, North Shewa Zone (Central Ethiopia) respectively. The difference in prevalence of subclinical mastitis may be due to the fact that subclinical form of mastitis received little attention and efforts have been concentrated on the treatment of clinical cases Ethiopia. In summary, the variations in reports of mastitis prevalence between authors at cow and quarter level as well as clinical and subclinical mastitis could be due to

types and burden of pathogens that can transmit between lactating cows as results of improper milking hygiene, lack of post milking teat dipping and poor housing facilities.

Isolation Frequencies of Predominant Bacterial Pathogens (n= 347): The commonly isolated pathogens in current study were *Coagulase negative staphylococci* (*CNS*) (25.10%) followed by *S. aureus* (21.04%), *S. agalactiae* (13.83%), *E. coli* (8.64%), *S. dsygalactiae* (6.63), *S. uberis* (5.76%) and *S. intermedius* (4.32%), *K. pneumoniae* (3.74%), *E. aerogenes* (2.59%) and other streptococcus and coliform species(5.2%). This result was in agreement with most of previous studies [38-48]. Fufa *et al.* [48] reported *S. aureus* (21.13%), *S. agalactiae* (18.31%) and *K. pneumonia,* (4.23%), *S. uberis* (4.23%), *S. dsygalactiae* (5.63%), *E. coli* (7.04%) and CNS (51.9%) in Addis Ababa city. About 19.6%, 9.4%, 5.8% and 4.3% isolation frequency of *S. aureus, E. coli, K. pneumoniae* and *E. aerogenes* respectively was reported in Algae state dairy farm [38]. Bitew *et al.* [43] reported isolation rates of *S. aureus* (20.3%), *S. agalactiae* (8.8%) and *S. dsygalactiae* (5.1%), *E. coli* (20.3%) and CNS (51.9%), in Bahir Dar and its Environs. Rafik *et al.*[71] reported *E. coli* (25.5%), *S. aureus (14.8%), Coagulase negative Staphylococci (CNS)* (12.7%), *S. agalactiae* (12.7%), *Klebsiella pneumonia (*4 8.5%), *S. pyogens* (10.6%), *Pseudomonas aeruginosa* (4.2%) and *Salmonella species* (4.2%) as well as other mixed environmental and contagious species in Egypt.The milk samples positive for CMT and negative on culture could be due to difference of causative agents of the disease i.e. it could be caused by pathogens which need special media such as *Mycoplasma*, *Nocardia* or *virus* [10].

The commonly isolated pathogens from clinical mastitis cases were *S. aureus* (19.32%), *E. coli* (18.18%) and *S. agalactiae* (15.9%) in descending order; while from subclinical mastitis cases coagulase negative *staphylococcus* (*CNS)* (29.34%), *S. aureus* (21.23%), *S. agalactiae* 33(12.74%) and *S. dsygalactiae* (6.17%) were the predominant pathogens. Sub-clinical mastitis on the other hand often goes unnoticed and can only be detected if specific tests are performed on a milk sample [69]. Generally, the findings in the current study showed that the contagious pathogens such as *S. aureus* and *S. agalactiae* were the most frequently isolated pathogens which could be described as predominant mastitis causing agents and most of lactating cows have similar mastitis pathogen profile. According to Hussein *et al.* [70] *S. aureus* was affirmed as the most transcendent among contagious mastitis causing pathogens in Egypt utilizing bacteriological and molecular systems. On the other hand, those bacteria spread from an infected animal to herd mates on hands, inflations, common towels and other items used during the milking process [49]. The high occurrence of contagious mastitis is usually because of improper hygienic and poor farm management of small-scale farms [70].

Risk Factors Associated with Mastitis: The present study showed that lactation stages, parity, drying of teat with common towel after washing, hygiene of the housing and presence of teat lesion (tick infestation) are statistically significantly associated with burden of pathogens ($p < 0.05$). The isolation frequencies of isolates were 67.3, 49.7 and 73.9 in early (<2 month), medium (3-6 month) and late (> 6 month) respectively. The occurrence of mastitis in this study was 2.08 times higher in late (> 6 month) lactation stage than early lactation stages. ie. [OR=2.08 (1.14 - 3.83)]. The increased prevalence of mastitis with advancing lactation stages agrees with previous investigations [21, 50]. Isolation frequencies of pathogens increase with lactation stage. The linear increasing of pathogens with lactation stages in this study indicates the lack of proper milking procedure before milking, during the time of milking and post milking which can contribute to the spread of these pathogens from infected teats to healthy ones and remaining persistence. Most owners of the lactating cows did not use towel and a few of them used a single towel for all cows commonly to dry the udders as well as their hands. The reuse of towel for cleaning and sanitizing may result in recontamination of the udder. Pre milking udder preparations play an important part in the contamination of udder during milking [51]. Furthermore, milkers wash their hands at the beginning of milking but did not dry their hands and not repeat washings between milking and some of the milkers used milk to moisten the teats when they became dry in between milking, which could be additional sources of contamination for udder.

The isolation frequency of the pathogens was 3.17 times higher in cows having >5 calves i.e. [OR=3.17 (1.66 - 5.34)]. A significant association of increased parity with isolation frequency has been reported [20, 52]. This might be due to the increased opportunity of infection with time and the prolonged duration of infection, especially in a herd without mastitis control program. In addition, the prolonged duration of infection and the physiological defence mechanism of the udder reduced with advancing age to overcome bacterial pathogens, so that pathogenic organisms get access to

the glandular tissue and cause inflammation of mammary glands [53]. On the other hand this might be caused by contagious pathogens. Contagious pathogens (*S. aureus* and *S. agalactiae*) survive in the udder of the cow and difficult to eliminate from mammary gland due to very low rate of self-cure and treatment result.

In this study, hygiene was also highly significantly related with isolation frequencies of pathogens ($p<0.001$). The burdens of pathogens were 6.13 times higher in poor hygiene. i.e. [OR=6.13 (3.05-12.35)] than those kept in good hygiene. Cows kept in poor hygiene were severely affected with mastitis than those with good hygiene practices. The high frequencies of bacteria in dairy cows kept under poor hygiene indicate that the surrounding environment is not sufficiently clean and provides a risk for spreading of environmental udder pathogens. As observed throughout the study period, poor hygiene practices contributed to the presence of environmental pathogens at a considerable higher percentage. The high frequencies of pathogens were reported by different authors in different areas of Ethiopia [54-56]

Lactating cows in which there is a practice of drying udder with common (single) towel after washing are 3.28 times more like affected with pathogens than cows in which no practice of drying udder with common towel. Similar finding was reported [57-59]. This might be due to using of common udder cloths, which could be vectors of spread especially for contagious mastitis.

Antibacterial Susceptibility Patterns of Isolated Bacterial Pathogens

Drug susceptibility patterns of *Staphylococcus* Species: The result of present study indicates that *S. aureus* isolates were resistant to Tetracycline 54.6% (6/11), Streptomycin 36.36% (4/11) and Gentamycin 27.3%, Sulphamethoxazole-trimethoprim (27.3%), Penicillin-G (72.7%) and Kanamycin (18%). This is similar with the findings of Lencho [26] who reported (78.9%) resistant level of *S. aureus* to Tetracycline; Alemayehu [35]; who reported the resistant level of *S. aureus* to Streptomycin (73.1%), Tetracycline (72.2%) in Bishoftu as well as Fitsum [60] who reported *S. aureus* isolates were highly resistant to Streptomycin (53.3%), Tetracycline (40%) and Sulphamethoxazole-trimethoprim (26.7%) in Wolayta Sodo, Southern Ethiopia, The probable explanation for this could be; those drugs are commonly used antimicrobials for the treatment of other infections as well as mastitis in the area and *S. aureus* strains have the capacity to change their resistance behaviour to the exposed antimicrobials [61].

Both *S. hyicus and S. intermedius* isolates were found to be resistant to most antimicrobials. *S. hyicus* isolates were resistant to Tetracycline 50.0% (3/6), Streptomycin (50.0%), Penicillin-G (50%), Gentamicin (16.7%) and 100% susceptible to Kanamycin and SXT. *S. intermedius* isolates were resistant to Tetracycline 50% (5/10), Streptomycin (40%), Penicillin-G (40%), Gentamicin (20%), SXT (20%) and 100% susceptible to Kanamycin. While CNS species are resistant to Tetracycline 50% (6/12), Streptomycin (41.7%), Penicillin-G (50%), Gentamicin (16.7%), SXT (8.3%) and 100% susceptible to Kanamycin. There was similar report that CNS isolates were susceptible to Sulphamethoxazole-Trimethoprim and Gentamicin, with efficacy rate of 95% and 100%, respectively [38]. The reason might be due to their frequent and long-term use of antibiotics and *staphylococcus* species have the capacity to change their resistance behaviour to the exposed antimicrobials. Antibiotic resistant genes carried on plasmid and transposons can pass from one *Staphylococcus* species to another [62]. The most common strategy used by *S. aureus* to circumvent the action of the penicillins is by the production of enzyme ß-lactamase, which hydrolyses the β-lactam ring, rendering the entire compound inactive [63].

Drug Susceptibility Patterns of *Streptococcus* Species: *Streptococcus agalactiae* were resistant to Tetracycline 40% (4/10), Streptomycin (40%), to Penicillin-G (50%), Sulphamethoxazole-trimethoprim (20%), Gentamycin (20%) and to Kanamycin (10%). *S. dsygalactiae* were resistant to Tetracycline 44.4% (4/9), Streptomycin (22.22%), Penicillin-G (40%), Gentamicin (11.1%) and Sulphamethoxazole-trimethoprim (22.2%) and susceptible to and Kanamycin (100%). While *S. uberis* isolates were resistant to Tetracycline 66.7% (4/6) Streptomycin (33.33%), to Penicillin-G (33.33%), Gentamycin (16.6%), (100%) susceptible to Kanamycin and Sulphamethoxazole-trimethoprim. Similar finding was also reported [64, 65].

Drug Susceptibility Patterns of *Coliform* Species: *Escherichia coli* were resistant to Tetracycline 62.5% (5/8), Streptomycin (50%), Penicillin-G (50%) and Gentamycin (25%), Sulphamethoxazole-trimethoprim (12.5%), Gentamycin (25%) and Kanamycin (25%). There was similar report that, *Escherichia coli* was resistant to Tetracycline (40.5%), Streptomycin (34.2%), Gentamycin (3.8%) and Sulphamethoxazole-trimethoprim (38%), in Addis Ababa abattoirs enterprise and Alema

farm slaughter slab [66]. *Klebsiella pneumoniae* were found to be resistant to Tetracycline 44% (4/9), Streptomycin (44%), Penicillin-G (55.6%), Sulphamethoxazole-trimethoprim (11.11%) and (100%) susceptible to Kanamycin and Gentamicin. *E. aerogenes* were 100% susceptible to Kanamycin, Gentamycin and Sulphamethoxazole-trimethoprim, but resistant to Tetracycline (25%) and Streptomycin (12.5%) and Penicillin-G (25%).

The difference in susceptibility patters of bacteria to different anti-biotic might be attributed to difference in utilization of anti-microbial agents for treatment regimen and development of resistance due to repeated use of similar antibiotics for longer period. The other reason might be their ability to grow in biofilm (self-produced matrix). When growing in this mode of life, microorganisms become more tolerant to opsonophagocytosis and conventional antibiotics, being 100-1000 times less susceptible to antibiotics than their planktonic counterparts [67]. Other reasons for development of resistant bacteria to antibiotic could be inappropriate use of the antibiotics in cattle, wrong dosage and routes of administration, arbitrary drug combinations and the acquisition of mobile genetic characteristics of the pathogens [68].

Multiple Drug Resistance of Isolates: From the results of this study it was found that many bacteria isolates were resistant to all or most of the commonly used antibiotics in the area. Multi-antibiotic resistance (MDR) was also recorded in several bacteria isolates with 91.0% (81/89) of the bacterial isolates showing multi-antibiotic resistance patterns, whereby 32.1% (26/81) isolates were resistant to two antibiotics and 67.9% (54/80) were resistant to more than two antibiotic drugs. Multi drug resistance was also reported by different authors in different areas of Ethiopia [35, 38, 60]. There are several factors which might account for the observed multi-antibiotic resistance, this include long-term exposure, organism type and antibiotic type. Other factors relate to under dosing, incomplete treatment of animals and/or the long period of inappropriate use of antibiotics, since in Tiro Afeta these are dispensed without a prescription. Therefore, based on the findings that majority of farmers in the area has tendency of treating animals themselves and rarely seek advices from veterinary or extension officers; the inappropriate use of veterinary drugs increases the risk of resistant bacteria in herds, which do not respond well to the antimicrobial agents in use and this will lead to chronic diseases. Finally, when comparing the overall efficacy of antimicrobials on isolates, Kanamycin, SXT and Gentamicin were the most effective antibiotics where more than 50% of the total isolates were found to be susceptible respectively.

CONCLUSION

The current study revealed that the overall prevalence mastitis in the area was 63.5% (15.2% clinical and 48.3% subclinical mastitis). Sub-clinical infections constituted the major component of prevalence indicating the fact that the farmers are only concerned with clinical mastitis and often are unaware about sub-clinical infection in their herds. The present study showed that *Staphylococcus, streptococcus* and *coliform* species are the major pathogens causing mastitis in the area. Contagious pathogens (*S. aureus* 21.04%, *S. agalactiae* 13.83% and *Coagulase negative staphylococcus* (25.10%) are the most causative agents of the mastitis in the area. Several risk factors such as parity and lactation stage, hygiene and previous history of mastitis were found to be significantly associated with isolates. From the results of this study it was found that many bacteria isolates were resistant to all or most of the commonly used antimicrobials in the area. Antimicrobials are used unconsciously to treat mastitis in the area and it is observed that the level of antibacterial resistance has been raising more and more compared to other area. As a result, bacterial pathogens are the most important factor that contributes for reduced milk production and increased losses in dairy farm in different expenses, which are treatment cost, veterinary and other costs that could affect the profitability of the farmers business and has public health importance. Based on this conclusion the following points are recommended:

- This study revealed that subclinical mastitis was the main performance of the disease and attention should be given to subclinical mastitis diagnosis, treatment and control.
- Awareness creation by implementing short-term training on the importance of applying high hygienic standards of housing and milking practices such as separate drying of udder with single towel for each cow before every milking is recommended to effectively stop the spread of both contagious and environmental pathogens in the area.
- It is important if antibiotic susceptibility test is carried out to choose effective drug for treatment of mastitis.

- It is also important if further research is carried on both contagious and environmental pathogens by using molecular methods on the genes favour multidrug resistance of the pathogens so as to block the economic impact of mastitis in the study area.
- The milk samples positive for CMT and negative on culture could be due to difference of causative agents of the disease: it could be caused by Mycotic, Mycoplasmal, Nocardial or viral mastitis. Therefore further study should be conducted on Mycoplasmal mastitis.

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