Heifer Mastitis: Prevalence, Assessments of Risk Factors and Antimicrobial Suseptablity Test on Major Bacterial Isolate in Centeral Ethiopia

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Abstract: Heifer mastitis causes detrimental mammary gland development affecting the subsequent lactating stages, udder health and related culling hazard resulting in significant economic losses to the dairy development sector. Across sectional study was conducted from June 2011 to March 2012 on cross breed heifers in Debre-zeit and Sebeta towns to estimate the prevalence of heifer mastitis, isolate bacteria causing mastitis and test their antimicrobial susceptibility. One hundred fifty eight (158) heifers were able to include from one hundred forty nine (149) cooperative smallholder dairy farms during the study period. From the total of 158 heifers sampled, 46(29.1%) were positive for mastitis (9.5% clinical and 19.6% subclinical cases). Identification of the bacteria on primary culture was made on the basis of colony morphology, hemolytic characteristics, gram stain reaction including shape and arrangements of the bacteria, catalase and oxidation and fermentation (o-f) test and further differentiation within the species level were made by selective media. The most frequently isolated bacteria from quarter milk samples in for clinical and subclinical mastitis were 7(24.1%) and 22 (75.9%) CNS, 7(26.91%) and 19(73.1%) *Staphylococcus aureus* and 4(22.2%) and 14(77.8%) *E. coli*, respectively. Other bacterial isolates were *Streptococcus agalactiae* (1(11.1%) and 8(88.9%), *Kelebsella pneumonia* (3(37.5%) and 5(62.5%)), *Bacillus cerus* (1(16.7%) and 5(8.3.3%)), actinomycet pyogens (1(25%) and 3(75%)), *Streptococcus dysagalactiae* 0 and 3(100%), *Entroccoccus feacalise* (0 and 3(100%)) and *Streptococcus uberis* (0 and 3(100%)) for clinical and subclinical mastitis, respectively. The univariate logistic regression showed that among the risk factors considered, age, heifer status, mastitic milk fed to calves, body condition scoring, usage of waste disposal and udder hygiene had significant effect on the prevalence of sub-clinical mastitis. However, after multivariate analysis,

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only age (OR = 2.1; CI, 1.5-2.9), mastitic milk fed to calves (OR = 2.3; CI, 1.5-3.4), udder hygiene (OR = 1.9; CI, 1.4-2.5) and usage of waste disposal (OR = 2.7; CI, 1.6-4.4), had significant effect. The antimicrobial susceptibility test showed for the majority of bacterial isolates including the major pathogens had 75-100% susceptibility pattern. CNS and *Streptococcus dysagalactiae* were the species which showed 100% susceptibility for all of the antimicrobials tested while the remaining species had varying levels of susceptibility (50-100%). Among isolates *Staphylococcus aureus* show relatively lower susceptibility for almost all antimicrobials used. Streptomycin and Erythromycin was the most effective antibiotic followed by Sulfisoxazole and Ampicillin. The presence of mastitis in heifer in early age indicates important economic losses. Therefore, awareness creation at the smallholder dairy farm on the economic significance of heifer mastitis, risk factors that plays vital role in establishment and flourishment of potential pathogen and use of dry cow therapy before calving will help in reducing mastitis in heifer. Moreover, further studies on what extent the causative pathogen and the host itself affect the persistence of intramammary infection during calving and early lactating heifers and evaluation of other risk factors in depth will merits the dairy farms.

INTRODUCTION

Ethiopia holds large potential for dairy development due to its large livestock population and the favorable climate for improved high yielding animal breeds. Thus, the contributions of the dairy sector especially Market oriented smallholder dairy development is one of the promising avenues to improve food security and livelihood of rural households in Ethiopia[1]. Replacement heifers are critical to herd productivity as they represent the future milking and breeding stock in all dairy operations. Hence, in the long run the goal of dairy farm should be to provide an environment for heifers to develop full lactation potential at the desired age with minimal expense. Animal health and well-being play vital roles in achieving this potential and mastitis was found to be one of the major diseases that can influence such future productivity in dairy farms[2]. Mastitis is defined as any inflammatory process affecting the mammary gland[3]. Though heifers have been thought to be free of mastitis by most producers compared to multiparous cattle, nevertheless heifer can suffer from mastitis the presence of mastitis is not observed until time of calving or until the first signs of clinical mastitis in early lactation[2]. Moreover an animal may carry an Intramammary Infection (IMI) for a year or more before it is diagnosed with mastitis[4]. Research made clear that heifers are at risk for developing both subclinical and clinical mastitis more often than previously assumed and at even early age before attaining breeding age. Louisiana researchers documented mastitis in heifers as young as 6 months of age and subsequent investigations inbreeding age and pregnant heifers have shown that infection rates can be as high as 97%[4, 5]. Examination of mammary secretions collected from prepartum heifers have shown that the mammary glands of many heifers harbor organisms that frequently cause mastitis[6-8]. *Staphylococcus aureus*, *Streptococcus dysagalactiae*, *Arcanobacterium pyogenes*, *Escherichia coli* and coagulase-negative staphylococci seem to be the most important organisms that cause clinical mastitis in heifers[9]. Heifer mastitis was reported to affect the economy of farmers through reduced milk production, high culling rate, additional costs for veterinarians and drug, discarding milk during treatment period and waiting days and extra labour[10]. *Staphylococcus aureus* mastitis in heifers has shown to cause significant production losses during the first lactation and if left untreated, they produced 10% less milk in early lactation than those receiving intramammary non lactating cow therapies during gestation[11]. The greatest development of milk-producing tissue in the udder occurs during the first pregnancy, so it is important to protect the mammary gland from pathogenic microorganisms to ensure maximum milk production during the first lactation[12]. Though, heifer mastitis was found to be prevalent and economical significant in different parts of the world, there is no information available on heifer mastitis and associated factors in Ethiopia. Therefore, this study was undertaken with the following objectives:

- To determine the prevalence of heifer mastitis
- To assess the major risk factors associated with the occurrence of heifer mastitis
- To isolate the major bacterial pathogens and test their antimicrobial susceptibility

MATERIALS AND METHODS

Study areas and population: The study was conducted from June 2011 to March 2012 in smallholder dairy farms found in Debre Zeit and Sebeta. Debre Zeit town is located at 45 km South East of Addis Ababa and situated at a latitude and longitude of 8°44’N and 38°38’E, respectively. The area has an altitude of 1100 m above sea level and experiences a bimodal rainfall pattern with a long rainy season from June to September and a short rainy season from March to May. The area receives an average annual rainfall of 1100 mm with respective average maximum and minimum temperatures of 28.3 and 8.9°C[13].

Sebeta is located 25 km Southwest of Addis Ababa and situated at a latitude and longitude of 8°55'N and 38°37'E, respectively. It has an elevation of 2356 m above sea level. The area is classified as temperate Highland with an annual rainfall of about 1650 mm. The mean annual minimum and maximum temperature is 8 and 19°C, respectively. Sebeta is the administrative center of Alem Gena Woreda. Based on the report of Central Statistical Agency[14] Sebeta town has an estimated total human population of 56,131 of which 27,862 were males and 28,269 were females.

Debre-zeit and Sebeta has the potential for both crop and livestock production which is mainly undertaken by smallholder farmers. There are also a relatively growing number of commercial farms and agro-processing industries operating in the area. The district agricultural potential and the infrastructure and institutional arrangements have encouraged the emergence of private service providers such as animal feed factory, private animal health institutions, agro processors and private livestock farms. There were >900 and 700 Market Oriented Smallholder dairy farms (MOSH) which were milk suppliers for Ada'a cooperatives and Sebeta agro industry (MAMA) with an average herd size of about three animals per farm. The majority of such dairy farm holders were organized under dairy cooperatives. The majority of the smallholder kept their animal in door. The types of antibiotics used in the study areas were Alamycin (Oxytetracycline), pen-strep (Penicillin and Streptomycin combination), intramammary infusions, procaine penicillin and intertrium (Trimethoprim and Sulfonamide combination), Pen-strep (Penicillin and Streptomycin combination) and oxytetracycline were the most widely used drugs to treat mastitis and other infectious diseases.

Study design: A cross sectional study type was carried out from June 2011 to March 2012 to investigate the prevalence of mastitis, assess the risk factors associated with the prevalence of mastitis, isolate bacterial pathogens and estimate their antimicrobial susceptibility patterns to the commonly used antimicrobial agents.

Sampling technique and sample size: Non probability sampling method was used to determine the number of heifers to be sampled. The study sites were selected purposively due to the availability of large number of smallholder and commercial dairy farms in the areas also due to the fact that they are the main suppliers for the high demand of dairy products in Addis Ababa. List of households were obtained from milk collectors in the study sites (Ada'a cooperatives from Debre zeit and Sebeta agro industry from Sebeta) and through the help of veterinary experts. Unfortunately, heifers were only taken from households which were willing to cooperate. Therefore, a total of 158 heifers 85 from Debre zeit and 73 from Sebeta were included in the study.

Study methodology

Detection of clinical mastitis: The udder of selected heifers was first examined by visual inspection and then by palpation to detect the presence of visible injuries, atrophy, swelling of the supra-mammary lymph nodes, fibrosis and cardinal signs of inflammation and appearance of milk secretion from each quarter was examined for the presence of abnormalities such as clots, flakes and blood[15].

Detection of sub-clinical mastitis: Subclinical mastitis was diagnosed based on CMT results and the nature of coagulation and viscosity of the mixture (milk and CMT reagent) which show the presence and severity of the infection, respectively[16]. Before sample collection for bacteriological examination, milk sample was examined for visible abnormalities and screened by the CMT according to Quinn *et al*.[17]. From each quarter of the udder, a squirt of milk samples were placed in each of the cups on the CMT paddle and an equal amount of CMT reagents were added to each cup and mixed well. Reactions were graded as 0 and Trace for negative, 1, 2 and 3 for positive results according to Radostits. The interpretations for each result is shown in Annex 2. The CMT and milk electrical conductivity are not good predictors of IMI for Holstein heifers in the last 2 weeks precalving. Therefore, precalving subclinical mastitis were diagnosed through direct culturing method[18].

Bacteriological examination of milk samples

Preparation of udder and teats: The udder, especially the teats was cleaned or washed with tap water and dried before milk sample collection. Dust, particles of bedding and other filth were also removed by brushing the surface of the teats and udder with a dry towel. Then the teats were swabbed with cotton, soaked in 70% alcohol[19]. To prevent recontamination of teats during scrubbing with alcohol, teats on the far side of the udder was scrubbed with alcohol first, then those on the near side. Studies have demonstrated that, as long as teat ends are sanitized, samples taken aseptically and teats dipped by disinfectant and with strict follow up after sample collection, there was no effect on the development of new intramammary infection in prepartum heifers[7].

Milk sample collection, handling and storages: Udder secretion from heifers was collected by a standard milk sampling techniques[19]. Udder quarter secretions were collected aseptically to reduce contamination of the teat ends during sample collection. The near teats were sampled first followed by the far once. Then, samples were placed in racks for ease of handling and transported in an ice box to the microbiology laboratory of Addis Ababa University, school of veterinary medicine. Samples were then either stored at 4°C for a maximum of 24 h until inoculated on a standard bacteriological media or frozen at -20°C for further delay[19].

Bacteriological isolation and characterization: Bacteriological culture was performed on all quarter udder secretion samples. Out of the 632 quarters examined, 11 were found blocked and hence, udder secretion samples were collected and cultured from the remaining 621 functional quarters. Identification of mastitis pathogens was carried out following microbiological procedures for diagnosis of bovine udder infection described by Anonymous[19]. For Milk samples that had been refrigerated, dispersion of bacteria and fat were accomplished by warming the samples at room temperature (25°C) for about an hour and then mixed by shaking. The samples were allowed to stand for a while for the foam to disperse and just before inoculation the tube was inverted gently. One standard loop (0.01 mL) of milk sample was streaked on 7% blood agar. The inoculated plate was incubated aerobically at 37°C. The plates were checked for growth after 24, 48 and 72 h to rule out slow growing microorganisms such as *Corynebacterium* species. For primary identification, colony size, shape, color, hemolytic characteristics, Grams reaction and catalase production were used. The procedures followed for the identified pathogens are presented in Annex 3. Interpretation was made according to Anonymous[19].

Antibiotic susceptibility test: Antibiotic susceptibility test was undertaken to determine the resistance pattern of heifer mastitis causal bacteria to commonly used antimicrobials in the study area to provide information to concerned stakeholders. Agar disc diffusion (Kirby-Bauer method) was used as described by Quinn *et al*.[17]. The procedures for the preparation of inoculum, inoculation to the Mueller-Hinton agar and disc application are presented in Annex 4. For Streptococcus species blood was added to Mueller-Hinton agar. After measuring the zone of inhibition, isolates were classified into sensitive and resistant. National Committee for Clinical Laboratory Standard (NCCLS) breakpoints was used to interpret the inhibition zone adapted from by Quinn *et al*.[17]. The following antimicrobial discs with their corresponding concentration (Oxoid, Basing Stoke, UK) were used: Sulfisoxazole (300 μg), Tetracycline (TE) (30 μg), Erythromycin (ERY) (15 μg), Ampicillin (AMP) (10 μg), Chloramphenicol (C30) (30 μg), Polymixin B(PB) (300 μg) and Streptomycin (S) (10 μg).

The selection of the types of antimicrobial agents was made based on clinical considerations including frequent use of the drug in the study area and availability. Representative was taken for those antibiotics for which prediction is possible by the result of a representative (that is individual members within the group are related closely enough to assume cross-resistance). Tetracycline, Sulfisoxazole, Erythromycin and were used as a representative to predict the result against all other Tetracycline's, Sulfonamides and Macrolids, respectively while Streptomycin, Chloramphenicol and Polymixin B because these individual members within each group are not related closely enough to assume cross-resistance thus they were tested separately.

Data collection

Clinical examination and subclinical examination: The selected smallholder dairy farms were visited once or twice in few cases. Crossbred heifers (precalving and post calving) were clinically examined for of mastitis. Clinical mastitis was diagnosed and data were recorded on the basis of visible signs of inflammation on udder secretion and on the udder (present/absent). A quarter which was warm, swollen and had pain and upon palpation, misshaped, atrophied, hard and fibrotic quarter was considered to have clinical mastitis. Clinical mastitis were also detected in quarters that have water secretions with clots or flakes compared to those with thick, honey-like secretions in pre-fresh normal heifers[20] and appearance of milk sample from each quarter was examined for the presence of abnormalities such as clots, flakes and blood in post calving heifer[17].

The California Mastitis Test (CMT) was carried out only in post calving heifers as procedure described by Quinn *et al*.[17] for screening sub-clinical mastitis. Heifers were considered positive for clinical and subclinical, when at least one quarter turned out to be positive for clinical examination and CMT. A herd was considered positive for CM and SCM when at least one cow in a herd was tested positive with clinical examination and CMT.

Questionnaire survey: Questionnaire was compiled to collect data of potential risk factors for mastitis. Data on each sampled heifer was collected in a properly designed format (Annex 2). The factors were categorized into heifer factors (age , heifer status (before calving and after calving), body condition scoring (Category: 1-5[21] and presence of udder or teat injury (Yes versus No) herd factors (udder hygiene (1-4)[22], floor type (concrete versus soil), milking practices after calving (Yes versus No), close contact among calves (Yes versus No), contact between heifer and adult cow (Yes versus No), separate calving (Yes versus No), frequency of heifer body washing (frequent, moderate and not at all), mastitic milk fed to calves (Yes versus No) and usage of waste disposal method (Biogas versus 'fig' (a dried dung used as fire wood and fertilizer)).

Statistical analysis: All data collected were stored and prepared in Microsoft office Excel. Prevalence was calculated for clinical and subclinical mastitis at herd, heifer and quarter level as defined by clinical manifestation the CMT score and bacteriological result. The prevalence of sub-clinical mastitis was the dependent variable while age, heifer status, body condition scoring and presence of teat or udder injury were independent variables considered at heifer level. The independent variables at herd level included udder hygiene (1-4), Floor type (earth type versus concert), Milking practices after calving (use towel dry versus not used), Close contact among calves (yes versus no) contact between heifer and adult cow (yes versus no), separate calving (yes versus no), Frequency of heifer body washing (frequent, moderate and not at all) Mastitic milk fed to calve (yes versus no), Usage of Waste disposal method (biogas versus 'fig'). The association between dependent and independent variables were tested initially by using univariate logistic regression ($p<0.05$) then those factors which were significant at $p<0.15$ were fitted to multivariate logistic regression model and tested statistically by using SPSS statistical package version 16.0.

RESULTS AND DISCUSSION

Socio-demographic characteristics of small holders: Majority of respondents in these study were male (61.9%) and 70% of the respondents were illiterate whereas the remaining 30% were literate with educational level ranging from elementary to diploma (Table 1). The findings regarding the number of households headed by females (20% of the total) compared to the male owned were similar with what was reported by Mekonnen *et al*.[23] for the peri-urban area who stated that in Addis Ababa area women owned 38% of smallholder farms. Another report concerning sex and educational status were reported by Amsalu [24] with 93.6 male owners and 52.5% of the households were illiterate in Fogera Woreda of North Gonder zone whereas Tefera[25] reported 52.7% of the respondent was male in central Ethiopia. The high levels of illiterate might provide challenge for an informative interface between farmers, extensionists, researchers and development agents. The majority (77.2%) of livestock keepers depend solely on livestock herding while the rest (22.8%) were retired (10.7%) or civil servant (12.1%) involved in livestock keeping. The animals were kept as subsistence herding for the family to provide in their daily needs.

Table 1: Demographic structure of the smallholders in the study area

Observation	Group	Number	Percentage
Sex	Female	58	38.9
	Male	91	61.9
Level of education	Illiterate	104	70.0
	Literate	45	30.0
Occupation	Livestock keeper	115	77.2
	Civil servant	18	12.1
	Retired	16	10.7

Prevalence: Out of 158 heifers examined the overall prevalence of mastitis recorded was 29.1% from which 9.5% was clinical mastitis 19.6% subclinical. Even though, finding of scientific papers regarding the prevalence of heifer mastitis were scares in Ethiopia there are some studies with the objective of bovine mastitis mentioning the prevalence of mastitis at different level of parties; prevalence of mastitis at first parities were reported by Bitew *et al*.[26] and Gethaun *et al.*[27] with 23.7 and 19.8% prevalence, respectively which were comparable with the present study. Another similar study was indicated by Bart, etc. who stated the percentage of heifers with one or more subclinical mastitis infections was on average 27.2% per farm and clinical mastitis was recorded in 8.1% of the heifers with an average of 0.191 cases per 365 heifer days at risk. Nickerson *et al*.[5] also indicated 15% of mammary quarters exhibited clinical mastitis at heifer as evidenced by clots or flakes in mammary secretions. Oliver and Sordillo[28] and Pankey *et al*.[29] also reported that approximately 46% of heifers and 19% of quarters were infected at calving and during early lactation.

The prevalence of subclinical and clinical mastitis and the distribution of the causative bacteria vary among studies and the magnitude of their effect is most likely related to the virulence of the causative pathogen, the persistence of the infection when milk production has started, the time of onset of infection, the ability of the animals to cope with the disease and the response of the dairy manager to control the disease through management changes[30]. The occurrence of heifer mastitis both clinical and subclinical mastitis in the present study might be because of farmers thought towards their heifer as free of mastitis infection. And the relative increased proportion of subclinical mastitis might also be due to similar fact as in bovine mastitis were farmers specially smallholders was not well informed about the existence of subclinical mastitis[31]. Minimizing Subclinical and clinical mastitis during development of the mammary gland and in early lactation through awareness creation about heifer mastitis, subclinical mastitis and their importance might ensure future milk production, udder health and longevity and saves additional costs for veterinarians and drug.

Among 632 quarters CMT screening was only done on 403 from which 93(23.6%) quarters were CMT positive. Hundred (5.6%), 100 (5.4%), 101 (5.8%) and 102 (5.8%) of the CMT positive quarters were found in the left front, left rear, right rear and right front quarters, respectively and they were statistically not significant p>0.05 (Table 2). From 93 of quarter tested positive by CMT 22(23.7%) were culture negative these results were higher than most the observation by Aregawi[33] who reported proportions of 13.8 and 15%, respectively in bovine mastitis. The failure to isolate the bacteria from the CMT-positive samples could be attributed to bactericidal properties of the inflammatory udder secretions which have been known to destroy the infecting bacteria leaving milk with higher leukocyte counts[33]. It might also be due to some cases of delayed healing of infections from which organisms may have disappeared or been reduced, while the infiltration of leukocytes continued until complete healing[34].

In the current study out of 632 quarters examined 11 quarters (1.7%) belonging to 10 heifers were blind of which 9 (90%) heifers had only one blind quarter, 1 (10%) heifer had two blind quarters. The blind quarters were at the left front 4 (36.4%), left rear 3 (27.3%) and right rear 4 (36.4%) whereas, there was no blind quarter observed on right front positions. The occurrence of blind mammary quarters has a direct influence on milk production with a subsequent impact on food security, signifies the importance of the problem. Lack of screening and treatment of subclinical mastitis and inadequate follow-up of clinical and chronic cases coupled with persistent challenges of the mammary glands by microbial pathogens could be the main predisposing factors to quarter blindness. This hidden and gradual destruction of the mammary tissues would end with non-functional quarters.

Because mastitis is a complex disease involving interactions of several factors, mainly of management, environment and factors relating to animal and causative organisms, its prevalence is expected to vary from place to place. This study also showed difference in prevalence of mastitis between the study sites were 5.7% clinical and 10.8% subclinical in Debre zeit. Whereas the clinical and subclinical mastitis for Sebeta were 3.8 and 8.9% respectively. Despite the fact it did not show statistical significance (p>0.05) (Table 3).

The prevalence of subclinical mastitis at heifer and herd level are also shown in Table 4 and 5 were heifer status and age was significant (p<0.05) at heifer level whereas udder hygiene, mastitic milk fed to calves and usage of waste disposal were significant at herd level (p<0.05).

Risk factors associated with sub-clinical mastitis: Fifteen risk factors were considered as potential risks for the occurrence of subclinical mastitis in this study. By using univariate logistic regression analysis, heifer status, age, udder hygiene, mastitic milk fed to calve, waste disposal method and body condition score were found to be significant (p<0.05). Herd attributes (such as; floor type, milking practices after calving, close contact among calves, contact between heifer and adult cow, separate calving and frequency of heifer body washing) and host factor (udder or teat injury) considered had no significant effect (p>0.05) on the prevalence of subclinical mastitis.

Table 2: Prevalence of subclinical mastitis by using CMT test at quarter levels

Quarter	Number examined	Prevalence (%)	OR (95% CI)	p-value
LF	100	5.6	1.0(0.6-1.7)	0.998
LR	100	5.4	1.04(0.6-1.8)	
RR	101	5.8	1.04(0.6-1..8)	
RF	102	5.8		

Table 3: Heifer level Prevalence of clinical and subclinical mastitis at study site

Types of mastitis	Study sites	Number examined	Prevalence (%)	OR(95%CI)	p-value
Clinical	Debre zeit	85	9(10.6)	1.1(0.6-1.9)	0.659
	Sebeta	73	6(8.2)		
Subclinical	Debre zeit	85	17(20)	1.2(0.7-1.6)	0.658
	Sebeta	73	14(19.2)		

Table 4: Prevalence of subclinical mastitis at heifer level

				Univariate analysis	
Risk factors	Group	N	Prevalence (%)	OR(95%CI)	p-value
Heifer status	Before calving	54	12.9	1.7(1.1-2.8)	0.012
	After calving	104	23.1		
Age	<3	88	14.8	1.9(1.2-2.9)	0.000
	3-4	56	23.2	3.2(1.7-6.1)	0.030
	>4	14	35.7	1	0.000
Udder/teat injury	Present	15	0	4.5(0)	0.997
	Absent	143	21.8		
Body condition score	Poor	11	9.1	2.6(0.9-7.4)	0.072
	Moderate	147	19		

Table 5: Prevalence of subclinical mastitis at herd level in the study area

Risk factors	Group	N	Prevalence (%)	Univariate analysis	
				OR (95%CI)	p-value
Floor type	Earth type	38	21.1	1.3(0.58-1.9)	0.275
	Concert type	111	18.9		
Udder hygiene	Slightly dirty	44	13.6	2.7(1.6-4.6)	0.010
	Moderately dirty	71	16.9	1.2(0.7-2.0)	0.000
	Dirty	34	32.4	1	0.000
Heifer washing	Frequent	12	16.6	1.1(0.6-1.9)	0.795
	Moderate	137	19.7		
Mastitis milk fed to calves	Yes	59	23.7	1.9(1.2-2.8)	0.001
	No	90	16.6		
Separate calving house	Yes	20	15	1.01(0.6-1.6)	0.985
	No	129	20.15		
Milking practices after calving	Use towel to dry	10	10	1.1(0.5-2.1)	0.802
	Not use	139	20.1		
Contact among calves					
	Yes	136	18.9	1.1(0.5-1.9)	0.827
	No	13	19.7		
Contact between heifer and adult cow	Yes	137	19.7	1.01(0.5-1.8)	0.988
	No	12	16.6		
Usage of Waste disposal	Biogas	22	18.2	1.8(1.1-2.9)	0.027
	Fig	127	27.3		

Table 6: Multivariate logistic regression analysis for the potential risk factors

Risk factors	OR	95.0% C.I. for OR		p-value
		Lower	Upper	
Age	2.1	1.5	2.8	0.00
Udder hygiene	1.9	1.4	2.5	0.00
Mastitic milk fed to calve	2.3	1.5	3.5	0.00
Waste disposal practice	2.7	1.6	4.4	0.00

All risk factors that had significant effect in univariate analysis with p<0.15 were fitted in to a multivariate logistic regression model and only age, udder hygiene, mastitic milk fed to calves and usage of waste disposal show a significant effect (p<0.05) (Table 6).

Heifers that are older at calving have an increased risk of mastitis, particularly from environmental sites. In some herds, it appears that the level of infection tends to increase with age as the heifers approach calving[35, 36]. Biffa, etc., indicated an increase in the prevalence as lactation number and age increase. It has been demonstrated that yield from the subsequent lactation increased as age at first calving increased[37] and yield is evidently related to the degree of development of the udder at calving. A relationship between the susceptibility of heifers to mastitis and the degree of udder development is comprehensible.

Hence, in this study also indicated that heifer mastitis was more likely to occur in heifer that are above four years with 35.7% prevalence (OR = 2.1; 95% CI, 1.5-2.9). The increasing prevalence of mastitis with increasing age is in agreement with the findings by Busato *et al*.[38] who found that, the risk of clinical and subclinical mastitis increase significantly with the advancing age of the cow. The effect of age at first calving on subsequent risk of mastitis or IMI is not clear.

Heifers become exposed to mastitis pathogens through several routes and consumption of mastitic milk is considered as one means[5]. In the present study the occurrence of mastitis in farms where they fed mastitic milk were 2.3 times higher in farm were they fed than those did not (OR = 2.3; CI, 1.5-3.5). Until recent knowledge, this risk factor has never been reported for other pathogens only for *Streptococcus agalactiae*[5] and from an udder health point of view there is little risk of feeding mastitic or high SCC milk to calves when they are maintained in individual pens[39]. In addition, heifers fed mastitic milk as calves suffered no more udder problems than did their mates that received other liquid feed[41, 41].

Nevertheless, other concerns have been raised associated with feeding mastitic milk, including potential violative antibiotic residues in calf tissue[42] or transfer or induction of antibiotic resistance in the intestinal flora of calves[43]. Additionally, transfer of other pathogens such as *Mycobacterium avium* subspecies *paratuberculosis* may occur[44]. For these reasons feeding mastitic milk to calves appears to be contra-indicated. The transfer of mastitis-causing bacteria through cross-suckling of calves fed mastitis milk can be prevented by housing calves in individual hutches.

The finding of a high prevalence of subclinical mastitis in dirty udder when compared with slightly dirty and moderately dirty udder were ($p<0.05$; OR = 1.9; CI, 1.4-2.5). Compton *et al*.[45] also reported that heifers with poor udder hygiene have a higher risk of IMI. For the herd attribute the livelihood of subclinical mastitis were higher in heifers were they use the farm waste to produce 'fig' (2.7 times) than those whom uses to produce biogas. Poor udder hygiene and absence of immediate removal of waste in case of herds that use the dung to produce 'fig' might indicate that the potential pathogen to cause mastitis were given the immediate environment to flourish inevitably.

Most of the herd attributes considered in the current study (floor type, milking practices after calving, separate calving house, contact among calves, contact between heifers and adult cows and frequency of heifer body cleaning) and heifer factors (such as heifer status (precalving/post calving), udder/teat injury and body condition score) did not have significant effect on the prevalence of sub-clinical mastitis. However, the importance of these farm attributes in determining the prevalence of mastitis was indicated by Waage *et al*.[46], Bassel *et al*.[47] and Oliver *et al*.[48]. The homogeneity of the production environment under smallholder's condition and the little difference in farm hygienic practices could have contributed for the lack of significant effect of the farm attributes.

4 bacteriological isolate: A total of 109 bacteria were isolated from which 24 isolates were from clinical cases whereas, 85 isolates were from subclinical cases. Out of 85 subclinical cases isolates 71 were from CMT positive quarter while 14 isolates were obtained direct through culturing from pre partum heifer.

The result of various bacterial species isolated from the clinical and subclinical cases are shown in Table 7. The most frequently isolated bacteria from quarters milk sample were CNS 29 (26.6%), *Staphylococcus aureus* 26 (23.9%) and *E. coli* 18(16.5%). Other bacterial isolates were *Streptococcus agalactiae* 9 (8.3%), *Kelebsella pnumonia* 8(7.3%), *Bacillus cerus* 6(5.5%), *Actinomycet pyogens* 4(4.2%,) *Streptococcus dysagalactiae* 3(2.75%), *Entroccoccus feacalise* 3(2.75%) and *Streptococcus uberis* 3(2.75%) with decreasing order of frequency. Most *CNS* species were isolated from subclinical cases.

The prevalence of subclinical and clinical mastitis and the distribution of the causative bacteria vary among studies but a common denominator is the high proportion of subclinical and clinical mastitis cases caused by Coagulase Negative Staphylococci (CNS)[9, 49]. In the current study the quarter level prevalence for CNS was in agreement with[7, 8, 50] who stated Coagulase-Negative Staphylococci (CNS) to be the most frequently isolated pathogens from dairy heifers suffering from subclinical mastitis. In the practice area of the Faculty of Veterinary Medicine, University of Helsinki, Finland, >20% of bacterial isolates from milk samples from clinical mastitis were CNS[51]. CNS has been considered as normal skin flora which as opportunistic bacteria can cause mastitis. Some CNS isolated from mastitis may be opportunists from the environment but it is very likely that at least the main species infecting bovine mammary gland are specialized for udder environment Oliver *et al*.[52]. The increased prevalence of clinical cases caused by CNS could indicate either an increased virulence of some species or strains or an increased susceptibility of the animal to these infections.

However, because most routine laboratories do not differentiate between species and only report presence of CNS as a group, it is not clear yet how to proceed in practice[53]. Opinions are divided on CNS importance for udder health. Recent studies even found higher milk yield in CNS-infected cows than in culture-negative cows[54]. Taponen *et al*.[55] on the contrary claimed that CNS infections might be more harmful than assumed and that certain species can persist for a long time causing severe damage to the infected quarter. In solving CNS mastitis problems, focus should therefore be on the heifers, environment, feeding and management before calving. Welfare and comfort of heifers may be significant factors for good udder health.

The second leading bacteria were *Staphylococcus aureus* 26 (23.9%) these report were comparable with Trinidad *et al*.[56] and Myllys *et al*.[9] who reported 23.1 and 20.1%, respectively. Fox[57] indicate the quarter prevalence of *Staphylococcus aureus* between 1 and 4 DIM was slightly higher than the average prevalence at calving of 2.3% across different studies. Although, prevalence of *Staphylococcus aureus* IMI in heifers is generally lower compared with CNS, its importance should not be underestimated as this bacterium is one of the most difficult mastitis pathogens to control[58]. The higher incidence of the bacteria can most likely be attributed to the wide distribution of the organism is the infected udder of lactating heifers and cows which is the major reservoir site but this bacterium also colonizes teat skin, vagina, muzzle and other body sites, as well as bedding, feedstuffs, air and equipment[4, 41, 59]. The bacteria usually establish chronic, subclinical infections and are shed in the milk which serves as a source of infection for other healthy cows and heifers during the milking process. Transmission among cows increase whenever there is lack of effective udder washing and drying, post-milking teat dip and drying, inter-cow hand-washing and disinfection, washing clothes and milking machine cups[15].

Coliforms are considered to be environmental pathogens. Two of the most important members are *Escherichia coli* and *Klebsiella pneumoniae*[60]. In this study, the prevalence of mastitis caused by *E. coli* and *Kelebsella pneumoniae* were 16.5 and 7.3%, respectively. Waage *et al*.[49] reported in a study performed in Norwegian 6.7% of milk samples collected from quarters with clinical signs from heifers was positive for Coliforms. Environmental mastitis pathogens will likely be the predominant pathogens isolated in heifer mammary glands when herds are with an environmental mastitis problem. The number of hours dairy cows kept indoor is also a factor that will increase the possibility of contact of teats with the environmental pathogens according to Saloniemi. Poor hygiene of the calving area is, not surprisingly, associated with an increased prevalence and higher odds of being infected with environmental mastitis pathogens shortly after parturition. In early lactation the susceptibility of dairy cows to mastitis is increased, probably due to slow leukocyte recruitment to the mammary gland during the periparturient period and because of a negative energy balance and stress during early lactation[61]. Also severity of mastitis is a result of interaction between immune defense of the host and bacterial characteristics. Blum *et al*.[62] concluded in their review that cow factors rather than specific features of the bacterial strain mainly determine the severity of *E. coli* mastitis.

In one study 8-10% of heifer mammary glands were infected by environmental mastitis pathogens, primarily *Streptococcus* species which was consistent with the pattern of IMI in lactating cows in the herds[48]. In the present study also *Streptococcus* species such as *Streptococcus agalactiae* (8.3%), *Entroccoccus feacalise* (4.2%), *Streptococcus dysagalactiae* (2.75%) and *Streptococcus uberis* (2.75%) were reported. Reasonable hypothesis is that heifers from herds with a high prevalence of contagious mastitis will likely be infected predominantly by contagious mastitis pathogens. And whenever there is lack of effective udder washing and drying, post-milking teat dip and drying, inter-cow hand-washing and disinfection, washing clothes and milking machine cups[15]. The current study also identified a low prevalence *Bacillus cerus* 5.5%, *Actinomycet pyogens* 4.2%, *Streptococcus dysagalactiae* 2.75%, *Entroccoccus feacalise* 32.75% and *Streptococcus uberis* 32.75%.

Antimicrobial sensitivity test: As described in Table 8, only 46 of the isolates were exposed to antimicrobial susceptibility these was based on the available antimicrobial disc and the isolates were *Staphylococcus aureus* 8(17.4%), CNS 10(21.7%), *Streptococcus agalactiae* 4(8.7%), *Streptococcus dysagalactiae* 3(6.5%), *E. coli* 7(15.2%), *Kelebsella Pneumoniae* 5(10.9%), *Entroccoccus feacalise* 2(4.3%), *Actinomycet pyogen* 2(4.3%), *Streptococcus uberis*, 2(4.3%) and *Bacillus cerus* 3(6.5%) were tested for susceptibility to seven antibiotics. The antibiotics were Sulfisoxazole, Tetracycline, Erythromycin, Ampicillin, Chloramphenicol, Polymixin B and Streptomycin. Susceptibility rates for all antibacterial product indicated that all were effective (Range: 70-100%) against every isolate. When comparing the overall efficacy on all isolates Streptomycin and Erythromycin (95.6%) was the most effective antibiotic followed by Sulfisoxazole (93.5%) and Ampicillin (93.5%). In contrast Tetracycline, Polymixin B and Chloramphenicol show relatively weak efficacy with 89.1, 89.1 and 84.7%, respectively.

Table 7: Bacterial isolates from clinical and subclinical mastitic milk samples in smallholder crossbred heifer, central Ethiopia

Bacteria isolated	Clinical (%)	Subclinical (%)	Total (%)
Actinomycet pyogen	1(25)	3(75)	4(4.2)
Bacillus cerus	1(16.7)	5(83.3)	6(5.5)
CNS	7(24.1)	22(75.9)	29(26.6)
E.coli	4(22.2)	14(77.8)	18(16.5)
Entroccoccus feacalise	0(0.0)	3(100.0)	3(2.75)
Kelebsella pnumonia	3(37.5)	5(62.5)	8(7.34)
Staphylococcus aureus	7(26.9)	19(73.1)	26(23.9)
Streptococcus uberis	0(0.0)	3(100.0)	3(2.75)
Streptococcus agalactiae	1(11.1)	8(88.9)	9(8.3)
Streptococcus dysagalactiae	0(0.0)	3(100)	3(2.75)
Total	24(22)	85(78)	109(100)

CNS isolates were more susceptible to all sorts of antibiotics with 100% efficacy. *Staphylococcus aureus* isolates shows 100% susceptibility to Sulfisoxazole. *Staphylococcus aureus* also 87.5% susceptibility to Erythromycin, Streptomycin and Tetracycline. It also had relatively less effectiveness to Chloramphenicol, Ampicillin and Polymixin B with 75% susceptibility. *Staphylococcus hyicus* were 100 and 50% resistance for Chloramphenicol and Tetracycline respectively.

From Streptococcus species *Streptococcus agalactiae* were the most frequently isolated pathogens that showed (100%) susceptibility to all drugs where as *Streptococcus dysagalactiae* with the exception of Chloramphenicol and Streptomycin with 75% susceptibility the remaining antibiotics shows 100% susceptibility. *Entroccoccus feacalise* shows 100% susceptibility for all except for tetracycline (50%). For the gram negative bacteria's *E. coli* and *Kelebsella pnumonia* the drug susceptibility test result shows 80-100% susceptibility reaction to all of the selected antibiotics.

The antimicrobial sensitivity test showed in this particular study is almost all milk bacterial isolates including the major pathogens had shown 75-100% susceptibility pattern. This was in agreement with Watts *et al*.[63] showing the testing of various staphylococcal isolates obtained from heifers for susceptibility to antibiotics commonly incorporated into

Table 8: *In vitro* antimicrobial susceptibility test result of bacterial isolates

		Response to application of antimicrobial discs (susceptibility in No. and %)						
Isolates	N	C	STR	AMP	PB	E	SXT	TE
Staphylococcus aureus	8	6(75)	7(87.5)	6(75)	6(75)	7(87.5)	8(100)	7(87.5)
Streptococcus agalactia.	4	3(75)	3(75)	4(100)	4(100)	4(100)	4(100)	4(100)
Streptococcus dysagalactiae	3	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)
E.coli	7	6(85.7)	7(100)	7(100)	6(85.7)	6(85.7)	7(100)	7(100)
kelebsella pnumonia	5	5(100)	5(100)	5(100)	4(80)	5(100)	4(80)	4(80)
CNS	10	10(100)	10(100)	10(100)	10(100)	10(100)	10(100)	10(100)
Entroccoccus feacalise	2	2(100)	2(100)	2(100)	2 (100)	2(100)	2(100)	1(50)
Actinomycet pyogen	2	2(100)	2(100)	2(100)	1(50)	2(100)	1(50)	1(50)
Bacillus cerus	3	2(66.6)	3(100)	2(66.6)	3(100)	3(100)	2(66.6)	3(100)
Streptococcus uberis	2	0	2(100)	2(100)	2(100)	2(100)	2(100)	1(50)
Total	46	39(84.7)	44(95.6)	43(93.5)	41(89.1)	44(95.6)	43(93.5)	41(89.1)

N = Number of observations; AMP = Ampicillin; E = Erythromycin; PB = Polymixin B; STR = Streptomycin; TE = Tetracycline; SXT = Sulfisoxazole

mastitis infusion tubes has shown that antibiotic resistance is usually low. Greater than 90% of mastitis-causing *Staphylococci* species are generally killed by the drug preparations used based on in vitro sensitivity testing using zone diffusion analysis. From a practical standpoint, neither subcutaneous nor intramuscular injections of drugs have been found to cure IMI in heifers because sufficient antibiotic does not pass into the mammary gland to be bactericidal. Thus, intramammary infusion is the route of choice. Therefore the treatment of heifers known to be at risk for developing IMI is an option and is advantageous because the cure rate is much higher than that obtained when treating infections during lactation. Reasons for this high cure rate are unclear but the relatively small secretory tissue area of heifer mammary glands compared with mature cows might allow for greater drug concentrations in the udder of the heifer. Similarly, histological studies have demonstrated less scar tissue and abscess formation in the mammary glands of heifers compared with older cows[56], a condition which would allow for better drug distribution and greater contact with colonized bacteria.

CONCLUSION

Considerable evidence suggests that Mastitis in dairy heifers in late gestation or early lactation occurs more frequently than previously assumed and some infections may be detrimental to mammary gland development, influence subsequent lactation performance, udder health and related culling hazard. The overall prevalence of mastitis in heifer at the current study was 29.1% with 9.5% clinical and 19.6% subclinical mastitis. Although mastitis in this study seems to be less prevalent than mastitis in older cows during lactation, it is still a significant prepartum and postpartum heifer disease. The most frequently isolated bacteria from quarters sample were CNS 29 (26.6%), *Staphylococcus aureus* 26 (23.9%) and *E. coli* 18 (16.5%). Other bacterial isolates were *Streptococcus agalactiae* 9(8.3%), *Kelebsella pneumoniae* 8(7.3%), *Bacillus cerus* 6(5.5%), *Actinomycet pyogens* 4(4.2%), *Actinomycet pyogens* 4(3.2%), *Streptococcus dysagalactiae* 3(2.75%), *Entroccoccus feacalise* 3(2.75%) and *Streptococcus uberis* 3(2.75%) with decreasing order of frequency. Coagulase-negative staphylococci cause the majority of heifer mastitis in this study. These organisms seem to have a minor impact on the future milk production and udder health, although there is a difference in virulence and persistence among CNS species. The longer infection exist and the longer they persist into lactation and as in this study with the involvement of contagious pathogen such as *Staphylococcus aureus* the larger the impact on heifers' future udder health and milk production will be. The potential risk factors which influenced the prevalence of subclinical mastitis in the study were age, udder hygiene, mastitic milk fed to calves and usage of waste disposal.

Thus, it is essential for the smallholder dairy owners in the study area to monitor the udder health, to practice adequate hygienic condition of dairy environment, good milking procedure, good animal health service and giving proper attention to health of the mammary gland status regularly and implement control strategies as required. Awareness should also be created among smallholder farmers about the economic impacts and benefits of controlling mastitis.

The antimicrobial sensitivity test showed for the majority of bacterial isolates including the major pathogens had 75-100% susceptibility pattern. CNS and *Streptococcus dysgalactiae* were the species which showed 100% susceptibility for all of the antimicrobials tested while the remaining species had varying levels of susceptibility (50-100%). Among isolates *Staphylococcus aureus* show relatively lower susceptibility for almost all antimicrobials used. Streptomycin and Erythromycin was the most effective antibiotic followed by Sulfisoxazole and Ampicillin. The antimicrobial sensitivity test pattern indicates that treatment of pre-fresh heifer for mastitis is an option.

RECOMMENDATIONS

Therefore based on the above conclusive remarks the following recommendations are forwarded:

- Treatment with Both dry cow and lactating cow products have to been evaluated frequently in smallholder farms in different part of the country before practical use of antibiotics as prophylactic and control of heifer mastitis
- In order to preserve overall herd health and productivity, smallholder farm owners should be advised in evaluating udders health and improving udder and farm hygiene long before heifer's calves so that it will not be too late to effectively treat the infection
- To what extent the causative pathogen and the host itself affect the persistence of intramammary infection in around calving and early lactating heifers, merits further research
- Risk factors related to the feeding and other management factors should be evaluated more in depth as they could be valuable in optimizing the immunity around calving and in enhancing the natural resistance and the bacterial clearance during this period of immune suppression

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