

Prevalence of Bacteria Associated with Subclinical Mastitis in Haramaya University Dairy Cattle, Goat and Sheep Farms

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Abstract: A cross-sectional study was conducted from November 2011 to May, 2012 to estimate the prevalence of major bacteria associated with subclinical mastitis in lactating animals. Lactating animals were screened for subclinical mastitis using California mastitis test (CMT). The CMT positive milk samples were cultured on media to isolate major bacterial pathogens. An overall prevalence of 31.6, 26.3, 21.1, 15.8 and 5.3% were recorded for *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CNS), *Acinetobacter* species, *Micrococcus* species and coliforms, respectively. The result revealed a subclinical mastitis of 48, 30.8 and 25% in cows, goats and ewes, respectively. The dominant bacteria isolated in cow milk was *S. aureus* (33.3%) followed by CNS species (25%), *Micrococcus* species (16.7%), *Acinetobacter* species (16.7%) and coliforms (8.3%). Similar frequency of *S. aureus*, CNS and *Acinetobacter* species were obtained for doe (25%) and ewe (33.3%). The prevalence of *Micrococcus* species in doe was 25%, while it was not present in ewes. Among the isolated bacteria, *S. aureus* and coliform species are important in clinical as well as subclinical mastitis, and they are human pathogens that cause septicemia and gastrointestinal illness. Therefore, attention should be given to the management of the dairy animals in the farm in order to reduce potential losses of productivity as well as risk of human illness due to infection and intoxication.

Keywords: Bacteria isolates, CMT, Lactating animals, Subclinical mastitis

Introduction

Among the health constraints of dairy animals, mastitis (inflammation of the mammary glands) is becoming important disease of lactating animals that leads to reduced milk production and economic losses in the dairy industry across the world, particularly in developing nations, where there is no adequate facility to control the spread of the disease. Besides, people that consume mastitic milk are at risk of acquiring pathogenic microbes and their toxic metabolites that leads to different forms of disease syndromes (Radostits *et al.*, 2006).

Mastitis is usually caused by bacterial pathogens that gains access to the mammary gland. In Ethiopia, studies show that bacterial agents such as *Staphylococcus aureus*, CNS, *Streptococcus* species, *Staphylococcus intermedius*, *E. coli*, *Pasteurella haemolytica*, *Pseudomonas aeruginosa*, *Bacillus* species, *Micrococcus* species, *Actinomyces* species, *Arcanobacterium* species, *Klebsella pneumoniae* and *Enterobacter* species were frequently associated with clinical and subclinical cases of bovine mastitis (Kerro & Tareke, 2003; Hunderra *et al.*, 2005; Kifle & Tadele, 2007; Berhanu *et al.*, 2010; Molalegne *et al.*, 2010; Nibret *et al.*, 2011; Lidet *et al.*, 2013) and sheep and goat mastitis (Assefa *et al.*, 2006; Tsegay *et al.*, 2012). Factors such as cold or wet environments, muddy areas, and nutritional stress may predispose to mastitis by reducing the blood flow to the udder and/or suppressing local immune function (secondary to increased stress-related cortisol output). Grazing improved pastures and implementing feeding practices

that favor high milk production may also predispose to mastitis (Smith and Sherman, 1994) since injury and leakage of milk between milking are increased.

Haramaya University has different livestock farms which include dairy, sheep and goat farms. Farm records and close inspection indicates that mastitis was among the most commonly encountered disease in the dairy farm resulting in decreased milk yield. This problem has required an in-depth investigation of sub clinical mastitis and isolation of major bacterial pathogens from udder of lactating dairy cows, sheep and goats. Therefore, the present study was undertaken to assess the magnitude of subclinical mastitis in lactating cow, doe and ewe as well as to isolate and identify the predominant bacterial agents of mastitis in Haramaya University farms.

Materials and Methods

Study Area Description

The study was conducted in Haramaya University dairy cattle, sheep and goat farms. The University is located at a geographical coordinate of 41°51' 58" N latitude and 90°24'10" S longitude at an altitude of 2000 m above sea level. The area receives 492 mm average annual rain fall ranging from 118-866 mm with a bimodal occurrence having short and long rain season that cover February to May and June to September, respectively. The maximum and minimum temperatures are 24°C and 9°C, respectively. The relative humidity of the area is 65% (HADB, 2007).

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Study Animals and Management

The study animals were lactating cow, ewe and doe kept in the respective farms. The cows were all cross bred (Holstein Friesian x Zebu), while ewe and doe were local breeds. The cows were managed under intensive husbandry practice in stall barn made of concrete floor. They were mainly fed hay, brans and silage. The cows were milked twice a day (in the morning and afternoon) in a separate milking parlor either by hand or milking machine depending upon the presence of facilities such as electric power. Regular washing of milker's hand before and after milking of the cows is an established practice at the farm. The ewes and does were frequently released to graze and browse in the farms vicinities and sheltered under loose housing system. They are not regularly milked and milking was done only to collect milk samples.

Study Design and Sampling

A cross-sectional study was conducted from November 2011 to May 2012 to isolate and identify bacteria species associated with sub clinical mastitis. During the study, a total of 53, 24 and 26 lactating animals were present at the dairy, sheep and goat farms, respectively. From these populations 50 cows and all of the ewes and does, showing no visible signs of clinical mastitis and in the mid-lactation stage were taken. Three of the lactating cows were not taken due to the development of clinical signs and subsequent antimicrobial therapy, as it is not recommended for bacterial isolation. Relevant information on general health conditions, any active antimicrobial therapy, regular udder health management, handling of chronically affected animals, and awareness level of farm attendants about mastitis management were gathered and recorded.

Milk Sample Collection and Transportation

Milk sampling was done according to the recommendations given by NMC (1990). Milk sample was collected before milking early in the morning to minimize/avoid contamination. Milk samples were taken from animals that were not treated with either intramammary or systemic antimicrobial agents. Udder and teats with paste of dirt materials were washed with potable water and disinfected with cotton soaked in 70% Ethanol before milking. In cases where there was no dung on the udder and teats, 70% Ethanol was applied directly on the teats without washing. The first two to three streams of milk were removed in to a beaker and representative milk samples of about 10ml were collected aseptically into screw capped sterile universal bottles for laboratory analysis. Three ml of the sample milk was used for CMT and the remaining was transported using a box containing an ice to Haramaya University, College of Veterinary Medicine Microbiology Laboratory and processed for bacterial culture up on arrival.

Detection of Mastitis

Before sample collection, the udder was examined visually and by palpation for evidence of gross pathology. Milk from each quarter was visually examined for evidence of physical changes. When the udder is confirmed as clinically normal, milk samples were subjected to CMT to test for subclinical infection of udder. Equal amount of milk and commercial reagent (California mastitis reagent) which contains 3% alkyl aryl sulfonate and bromocresol purple as pH level indicator were mixed in the cup on a paddle and gentle swirling was applied to the mixture in a circular motion. The result of the test was recorded based on the intensity of the gel formation and score were graded according to Smith and Sherman (2009). Accordingly, grades 0 was given for negative reaction, trace (T) for slight reaction and strong (S) for enhanced reactions.

Bacteriological Analysis of Samples

Culture media preparation and sample inoculation:

The media used to cultivate bacteria associated with sub clinical mastitis were selected and prepared based on the recommendation given by Quinn *et al.* (2011). Accordingly, blood agar was used for initial isolation of microorganisms from milk samples. In addition, MacConkey agar and mannitol salt agar were used for further identification of target microorganisms. Dehydrated media containing agar were dissolved in a hot plate which incorporate magnetic stirrer until it boils. Then the media were sterilized by autoclave at 121°C for 15min holding time, and dispensed with a volume of about 15 ml in to sterilized petri-dishes. To prepare blood agar, non-coagulated blood was collected from sheep that did not receive antibiotic therapy and gently added to molten agar base and cooled to 50°C on water bath. All CMT positive milk sample were subjected to culture on blood agar. A loop full of milk was streaked on 7% sheep blood agar plate and incubated aerobically at 37°C. The primary culture was examined for growth after 24, 48 and 72 hours of incubation to rule out slow growing microorganisms. For further identification, top 3 isolated and similar colonies were picked with sterile wire loop and transferred to other blood and MacConkey agar plates.

Identification of bacterial colony: The isolated bacterial colonies were identified based on colony characteristics such as size, shape, color, consistency, growth on MacConkey agar and hemolytic characteristics. A loop full of colony from each representative colony types were subjected to Gram's staining in order to observe Gram's reaction, cellular morphology and arrangements, catalase test, oxidase test and O-F test. After primary characterization, suspected colonies were further cultured on mannitol salt agar and subjected to coagulase test to identify pathogenic *Staphylococcus* species. All test protocols and means of bacterial identification were performed according to Quinn *et al.* (2011).

Data Management and Analysis

Laboratory results were entered into and managed using Microsoft Excel (2007) and descriptive statistics (frequency and percentage) were used to describe the prevalence of sub clinical mastitis and to show the prevalence of bacterial species associated with subclinical mastitis.

Results and Discussion

Prevalence of Sub Clinical Mastitis

The prevalence of sub clinical mastitis was higher in cow (48%) followed by doe (30.8%) and ewe (25%). All CMT positive samples showed strong reaction and harbor at least one type of bacterial isolate (Table 1). The higher percentage of subclinical mastitis and bacterial isolates in cow could be due to the hand milking practices and relatively high milk production, as they promote bacterial invasion and multiplication (Radostits *et al.*, 2006). Moreover, the cows were managed under confined environment, which can result in increased concentrations of bacteria inside the shed and subsequent risk of intramammary infections (Bergonier *et al.*, 2003).

In Ethiopia, similar findings were reported from Sodo district (Kerro & Tareke, 2003) and Bahir Dar (Molalegne *et al.*, 2010) in which bacteria were isolated from 100% and 96% of cow milk samples from sub clinical mastitis, respectively. Berhanu *et al.* (2010) and Kifle & Tadele (2007) also reported prevalence of 90% and 89% in cow from farms located in Selale and Holeta areas, respectively. In accordance with the finding of the present study, Bourabah *et al.* (2013) recorded a prevalence rate of 99% in milk samples from goats with subclinical mastitis that hold either single or combination of bacteria isolates. Lower prevalence of bacterial isolates than the present study were also reported for Kenyan (28.7%) lactating goats

(Ndegwa *et al.*, 2001) and Ethiopian goats kept under small scale farms in Adami Tulu (89.9%) (Assefa *et al.*, 2006) and in Kafta Humera district (52.3%) (Tsegay *et al.*, 2012). In sheep, Tsegay *et al.* (2012) reported a prevalence rate of 37.5% bacterial isolates from subclinical mastitis. The higher number of isolates in subclinical mastitis in the present study could be related to factors such as cold temperature that predispose animal to bacterial pathogens by suppressing the local immune function (Smith and Sherman, 1994) and confinement due to cold environment, which increases the chance of udder infection by environmental pathogens (Radostits *et al.*, 2006). This can be explained by the observation that the lower (9°C) and higher (24°C) temperature of Haramaya area is relatively more hostile and in fact a predisposing factor to lactating animals than the Rift Valley temperature of Adami Tulu (12°C and 27°C, respectively) as well as Kafta Humera district (17.5°C and 41.7°C, respectively).

The relatively high prevalence of bacteria associated with subclinical mastitis might lead to drop in milk production potential of the farm animals, which subsequently influences the food security, revenue generation as well as weaning rate of young animals. The high percentage of bacterial associated mastitis has a negative impact on the farms, particularly the dairy cattle farm, due to high cost of treatment (Radostits *et al.*, 2006) and public health implication as a result of medicine residues and zoonotic pathogens (Blowey & Edmondson, 2010). Subclinical mastitis is a hidden threat which harbors human pathogens such as *Staphylococcus aureus* and *Streptococcus uberis*, which causes urinary tract, blood stream and deep wound infections (Valentiny *et al.*, 2015) as well as gastrointestinal intoxications (Zhang & Stewart, 2002).

Table 1. Prevalence of subclinical mastitis and bacterial isolates in cows, does and ewes at Haramaya University farms

Animal species	Total animals examined	Total animals positive to CMT (%)	CMT reaction		Number of CMT positive animals	
			T	S	With isolates	Without isolates
Cow	50	24 (48)	0	24	24	0
Doe	26	8 (30.8)	0	8	8	0
Ewe	24	6 (25)	0	6	6	0

CMT= *California mastitis test*; T= *Trace*; S= *Strong*.

Phenotypic Profiles of Bacterial Isolates

Based on the phenotypic characteristics, the frequent and dominant bacterial isolates were grouped in to five categories. All the isolates grow well on blood agar, whereas there were variability on growth ability on MacConkey agar plate (Table 2), indicating that the bile salt in the MacConkey agar is inhibitory to some organisms.

The isolated colonies constitute *Staphylococcus aureus*, coagulase negative *Staphylococcus* species, *Micrococcus* spp., *Acinetobacter* species and coliforms. Previous reports

also revealed that these bacterial isolates are among the common microbial agents of mastitis in farm animals (Radostits *et al.*, 2006; Smith & Sherman, 2009; Quinn *et al.*, 2011). It has been reported that significant percentage (up to 88%) of bovine and ovine mastitis isolates of *Staphylococcus aureus* produce one or more enterotoxins responsible for human intoxication characterized by symptoms of vomiting, diarrhea, and abdominal cramping, even with pasteurized milk because of the thermo stable enterotoxins (Zhang & Stewart, 2002).

Table 2. Profiles of bacteria isolated from subclinical mastitis cases

Tests	Character	Group of isolates				
		1	2	3	4	5
Growth on blood agar	Pigmentation	+	(-)	(-)	(-)	(-)
	Hemolysis	+	(-)	(-)	(-)	(-)
Growth on MacConkey agar	Lactose fermenter	NG	NG	NG	+	+
	Lactose non-fermenter	NG	NG	NG	(-)	(-)
Growth on mannitol salt agar	Growth only	(-)	+	NP	NP	NP
	Growth and fermentation	+	(-)	NP	NP	NP
Gram's reaction		+	+	+	(-)	(-)
Cell morphology and arrangement		Cocci in cluster	Cocci in cluster	Dispersed cocci	Short rods	Short rods
Catalase test		+	+	+	+	+
Coagulase test		+	(-)	(-)	NP	NP
O-F test		F	F	O	O	FA
Oxidase test		(-)	(-)	+	(-)	(-)

1= *Staphylococcus aureus*; 2= *Coagulase negative Staphylococcus* species; 3= *Micrococcus* species; 4= *Acinetobacter* species; 5= *Coliforms*; += Positive; (-)= Negative; NG= No-growth; NP= Test not performed; F= Fermentative; O= Oxidative; FA= Facultative anaerobe.

Prevalence of Bacteria Associated with Subclinical Mastitis

Staphylococcus aureus, CNS, *Micrococcus* species, *Acinetobacter* species and coliforms were associated with subclinical mastitis among dairy animals with varied frequencies (Table 3).

In lactating cows, *Staphylococcus aureus* constitutes higher percentage (33.3%) among the isolates followed by CNS (25%), *Micrococcus* species (16.7%), *Acinetobacter* (16.7%) and coliforms (8.3%) species. This is in agreement with the findings of Berhanu *et al.* (2010) and Birhanu *et al.* (2013) who reported that *S. aureus* to be dominant isolate (43.1% and 35.7%) followed by CNS (28.8% and 15.7%) and *Micrococcus* (3.26% and 7.14%, respectively) in different parts of the country. The present finding is not in the same order with the findings of Molalegne *et al.* (2010) who reported the

dominant isolate to hold CNS (56.2%) followed by *S. aureus* (16.4%). In the present study, the prevalence of coliforms is far lower than reported by Kerro & Tareke (2003) in dairy cow (14.1%) in southern Ethiopia. In goats *Acinetobacter* species were reported to be responsible for 5% of subclinical mastitis in Kenya (Ndegwa *et al.*, 2001). This indicated that the animals are kept under poor hygienic status, because *Acinetobacter* species are environmental pathogens. The organism is particularly important due to multi-drug resistant pattern and their ability to produce heat labile toxins, which causes food poisoning in human and triggering infections in severely ill patients in intensive care units or immunocompromised individuals (Bergogne-Berezin & Towner, 1996; Villegas & Hartstein, 2003).

Table 3. The prevalence of bacterial pathogens among lactating animals with subclinical mastitis

Organism	Frequency (%)			
	Cow (n=24)	Doe (n=8)	Ewe (n=6)	All animals (n=38)
<i>Staphylococcus aureus</i>	8 (33.3)	2 (25)	2 (33.3)	12 (31.6)
CNS	6 (25)	2 (25)	2 (33.3)	10 (26.3)
<i>Micrococcus</i> species	4 (16.7)	2 (25)	-	6 (15.8)
<i>Acinetobacter</i> species	4 (16.7)	2 (25)	2 (33.3)	8 (21.1)
Coliform species	2 (8.3)	-	-	2 (5.3)

CNS= *Coagulase negative Staphylococcus* species.

Except *Micrococcus* species (not isolated in ewe) the prevalence of each bacterial isolates in the present study was equal in lactating doe (25%) and ewe (33.3%). Assefa *et al.* (2006) indicated that the dominant isolates were *S. aureus* (17.6%) followed by CNS (15.6%), *Acinetobacter* species (8.8%) and *Micrococcus* species (7.6%) in goat's with subclinical mastitis. Similarly, Tsegay *et al.* (2012) reported that CNS (21.7%) was dominant isolate followed by *S. aureus* (6.5%), in goats as well as sheep with prevalence rate of 36.7% for CNS and 26.3% for *S. aureus* in Tigray

region. Another study (Ndegwa *et al.*, 2001) from Kenya indicated that CNS was dominant isolate (37.5%) followed by *S. aureus* (22.7%), *Micrococcus* species (17.7%) and *Acinetobacter* species (5%) in goats. Moreover, Bourabah *et al.* (2013) from Algeria reported prevalence's of CNS (15.5%), *Micrococcus* (10.1%) and *S. aureus* (2.8%), in dairy goats with subclinical mastitis. The similar occurrences of bacterial isolates among sheep and goats could be due to the greater chance of environmental microbes accessing the udder of animals as a result of poor hygienic status and the limited

emphasis given to the farms. However, it is clear that various bacterial agents are responsible for the udder infection and pose risk for human that consumes milk, since most of the isolated bacteria are thought to cause illness due to infection or intoxication.

Overall, the present study revealed that among animals with subclinical mastitis, *S. aureus* was isolated at higher percentage (31.6%) followed by CNS (26.3%), *Acinetobacter* species (21.1%), *Micrococcus* species (15.8%) and coliforms (5.3%). *Staphylococcus aureus* is the main pathogenic species causing sub clinical form of mastitis and economically the greatest concern wherever dairy farming is practiced (Radostits *et al.*, 2006). The relatively high prevalence of pathogenic *S. aureus* could be associated with the total absence of dry cow, ewe and doe therapy and post milking teat dipping, the invariable hand milking practice, low culling rate of chronically infected animal and limited knowledge of farm workers on segregation as control options.

Conclusion

The present study display that pathogenic *S. aureus* is the major bacteria along with other environmental bacteria to be associated with sub clinical mastitis. This could be an indicator of poor hygienic practices and absence of regular health monitoring of animals. Therefore, careful hygienic milking practice, regular health monitoring and culling of chronically infected animals that could harbor potentially harmful and multi-drug resistance pathogens should be practiced to reduce reservoir of infection and contamination of the rest of the herd.

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Conflict of Interests

The authors declare that they have no competing interests.

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