



INVESTIGATION OF BACTERIAL PATHOGENS AND ASSOCIATED RISK FACTORS FOR SUBCLINICAL MASTITIS IN GOATS AT CHITTAGONG IN BANGLADESH

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Roll No. 0214/02

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degree of Master of Science in Medicine**

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee will be addressed

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List of Abbreviations

Abbreviation	Elaboration
A	Alpha
%	Percentage
<	Less than
>	Greater than
°C	Degree Celsius
µl	Microlitre
BDT	Bangladesh Taka
BA	Bachelor of Arts
BBG	Black Bengal Goat
CI	Confidence interval
CVASU	Chittagong Veterinary and Animal Sciences University
CNS	Coagulase-negative staphylococci
CMT	California Mastitis Test
DNA	Deoxyribo Nucleic Acid
EMB	Eosin methylene blue
et al.	And his associates
GNB	Gram negative bacteria
GEE	Generalized Estimating Equation
HSC	Higher Secondary Certificate
IDF	International dairy federation
IMI	Intramammary infection
ml	Milli Litre
MA	Master of Arts
MS	Master of Science
MS	MS Microsoft Excel
MSA	Mannitol salt agar
MR	Methyl red
NMC	National mastitis council

N	Number
OR	Odds Ratio
p	Probability
PRTC	Poultry Research and Training Centre
SE	Standard Error
SCM	Subclinical mastitis
SCC	Somatic cell count
TSI	Triple sugar iron
VP	Voges-Proskauer
SSC	Secondary School Certificate

Abstract

Goat plays a considerable role in economy and major part of subsidiary income of the rural people in Bangladesh. Interest in dairy goats and goat milk products is increasing with the demand for healthy food, as goat milk has similar nutritional qualities to human milk and is less allergenic for human consumption than cow milk. This importance is also reflected by increase in goat's population during the last 20 years in Bangladesh. Therefore, any factor that adversely affects the quantity and quality of milk from the goat is of great financial interest. Milk quality is mainly affected by bacterial infection of the mammary gland which causes mastitis. Dairy animals including lactating goats are prone to the intramammary infection and associated with a lot of economic impact on the farmers but the scientific reports on caprine subclinical mastitis are limited in Bangladesh.

The present study was undertaken to determine the proportionate prevalence of subclinical mastitis at farm and goat level along with the potential risk factors associated with subclinical mastitis and mastitis related organisms during the period from October 2015 to December 2016 at Chittagong metropolitan city. The teat and udder of a lactating population of 106 goats from 88 goat farms were physically examined.

Most of the goats were reared under intensive as well as semi-intensive rearing system and rests were at free range system. Wooden floor was more common floor type among the studied farm and others were constructed with concrete and brick and some were found only plastic and jute bag over mud. Goat farmers had got predominantly the secondary level of education.

In this study the proportionate prevalence of subclinical mastitis at goat level was 50% whereas; the quarter level proportionate prevalence was 46.2%. The major bacterial pathogens isolated were coagulase-negative staphylococci (35.4%); (95% CI: 28.9-42.2%) followed by *Pseudomonas* spp (23.1%); (95% CI: 17.6-29.4%), *Staphylococcus aureus* (11.3%); (95% CI: 7.4-16.4%), and other organisms (8.9%).

Fisher's exact test followed by Generalized Estimating Equation was applied to identify potential risk factors associated with SCM in goat. Source of goat (OR=2.2: Own stock versus external), Floor materials (OR=3.7: Concrete and brick; Wood versus Plastic and jute

bag), Parity (OR=2.0: First and second versus Third to fifteenth), Teat shape (OR=3.6: Bottle and collapsed; Pencil and short versus Conical and cylindrical) and Lactation period (OR=5.3: Up to 60 versus 61 to 180) were identified as the risk factors for SCM in goat. Knowledge obtained from the study could help in practicing hygienic management system for goat farming to reduce SCM in goat.

Key words: Subclinical Mastitis, Goat, Risk factors, Prevalence, Chittagong, Organisms

Chapter I: Introduction

Goat milk production is a dynamic and growing dairy industry that is fundamental to the wellbeing of hundreds of millions of people worldwide and is an important part of the economy in many countries (Silanikove et al., 2010). On a global scale an annual goat milk production reaches 15,510,411 tons per year, of which 80% are produced in developing countries where goat's milk plays an important role in the livelihood of hundreds of millions of human (Silanikove et al., 2010). The current goat population in the world is around 921 million of which ~90% and 60% goats are from developing countries and Asian countries, respectively (FAO, 2012). In Bangladesh goat population is estimated to be about 34.5 million and these are kept by mostly rural women for production of meat, milk and skin and thereby contribute to the family income, nutrition and welfare (FAO, 2001). Goat milk production is of major economic importance in many countries including Bangladesh (Haenlein, 2004). According to recent FAO (2010) report, Bangladesh remained in the top position for producing goat milk globally as it reached 2,168,000 tons in 2009. Goat rearing sector is contributing about 50 million Euros (appx.4.23billion BDT) to the national economy by producing respectively 0.17 and 0.08 metric tons meat and milk (MOFL, 2002). Goats rank the second position in terms of total goat milk production in the world (FAO, 2001).

Considering the prospects, a participatory approach in goat rearing as a small scale subsistent family enterprise has been promoted by the government and various non-government organizations with the credit and technical input support since 1980's in Bangladesh (Das, 2004; Islam et al., 2012b). The importance of goat is strongly emphasized for their versatile production profile and valuable contribution like meat, milk, industrial raw product such as skin, fiber and manure. Therefore, any management and disease factor that adversely affects the quantity and quality of goat milk is of great financial interest in the goat rearing countries of the world including Bangladesh. Goat rearing is an enterprise which has been practiced by a large section of population in rural areas. Goats can efficiently survive on available bush and trees (such as Jackfruit) in adverse harsh environment in low fertility lands where no other crop can be grown. Goat rearing is emerging as an important source of livelihood particularly for landless laborer's and marginal farmers across the country. Family educational status and exposure to the communication sources are also vital in goat keeping (Chandra et al., 2005). Therefore, management and rearing system of goat farms are often

related to farmer's socioeconomic status. The present study therefore explores the socioeconomic status of goat farmers in Chittagong metropolitan area. The farm management factors are directly or indirectly related to outcome of diseases and production. Mastitis (clinical) is an inflammatory condition of the mammary gland, characterized by changes in the physical characteristics of the udder or milk, while detection of subclinical mastitis depends on various test procedures including microbial examination, somatic cell count, and California mastitis test. (Islam et al., 2012c). However, it is worth noting that the interpretation and methods for enumerating somatic cell counts in goat milk are different due to higher somatic cell count of milk of uninfected goats compared to cows or sheep (Contreras et al., 2007). Depending on the severity of the disease, mastitis could result in decreased revenues for producers (Nazifi et al., 2011). Mastitis also hamper animal welfare and holds public health risk of zoonotic pathogen transmission from drinking raw milk and antimicrobial residues (Razi et al., 2013). Multiple pathogens were previously isolated from mastitic goats. Extensive literature review by Contreras et al. (2007) has demonstrated that several pathogens have been reported to cause mastitis in goats while *Staphylococcus* spp. are the most frequently diagnosed causal microorganisms. Other pathogens such as *Streptococcus* spp., *Enterobacter* spp., *Pseudomonas aeruginosa*, *Mannheimia haemolytica*, *Corynebacterium* spp., and fungi can produce intra-mammary infection in small ruminants. Similarly, in a study by Wakwoya et al. (2006) *Staphylococcus* spp. were the major isolates in Ethiopia, while *Escherichia coli*, *P. aeruginosa*, *Citrobacter* spp., *Klebsiella* spp., *Acinetobacter* spp., *Micrococcus* spp., *Corynebacterium* spp., *Bacillus* spp., *Streptococcus* spp., and *Pasteurella* spp. were also identified and reported.

Prevalence of clinical and subclinical mastitis in goat has been estimated in different countries of the world (Contreras et al., 2007; Koop et al., 2009). The overall prevalence reported 5-30% for SCM while only <5% for clinical mastitis (Bergonier et al., 2003; Contreras et al., 2007). However, in a hospital base descriptive study the proportionate prevalence of clinical mastitis was found 15-20% (Koop et al., 2016) in Chittagong, Bangladesh. However the proportionate prevalence of different germs responsible are usually CNS (about 80% of infections), *S. aureus* (4- 40%), gram-negative bacteria (8%) and *Streptococci* (6%) (Contreras et al., 2007; Leitner et al., 2008; Bagnicka et al., 2011). Understanding the distribution of common causal agents of mastitis will facilitate the rapid and effective management. The species and management system wise distribution of causal agent is also important for mastitis prevention and control.

Many studies have been conducted to identify the putative risk factors of SCM in goats in many countries including Bangladesh (Moroni et al., 2005b; Razi et al., 2013). Goats raised under a traditional farming system had 2.1 times higher risk compared to semi-intensive farming system. Farms that had earth floor type had 2.1 times more risk of SCM compared to farms with slatted and concrete floors. Higher parity and late lactation period had also higher odds of SCM in goat (Razi et al., 2013). However, these were not complete set of risk factors. Hence, the current study was attempted to determine a full set of risk factors associated with subclinical mastitis at goat level. The risk factors associated with the proportionate prevalence of types of organism in goat isolated were not properly determined before. So identification of risk factors associated with mastitis pathogens was also crucial to manage subclinical and clinical mastitis in goats.

Somatic cell count is a useful tool for the assessment of udder health status as well as quality of the milk. They are mainly phagocytes and lymphocytes and indicate intramammary infection when elevated (Jimenez-Granado et al., 2014). Using a composite SCC to detect mastitis in goats, Koop et al. (2011) proposed a cut-off value of $1,500 \times 10^3$ SC mL⁻¹, with 0.9 and 0.95 sensitivity and specificity, respectively. California Mastitis Test (CMT) developed by Schalm and Noorlander (1957) has been routinely used to evaluate the content of leucocytes and epithelial cells, defined as the somatic cells (SC), in milk. California Mastitis Test is helpful in screening out SCM in goats (Persson and Olofsson, 2011). It was found that the sensitivity and specificity, of the CMT is 0.74 and 0.74, respectively at a cut point of greater than trace (McDougall et al., 2001). The current study was conducted with the aim of the following objectives:

Objectives of the study:

1. To assess socio-economic status of goat farmers and to find out the goat farm characteristics with its management traits in Chittagong metro city.
2. To determine potential risk factors associated with subclinical mastitis and mastitis associated organisms.
3. To estimate proportionate prevalence of subclinical mastitis at farm and goat level along with the prevalence and distribution of isolated organisms.

Chapter II: Review of Literature

Relevant literatures on goat farm demography, mastitis, classification, causal agents of mastitis, prevalence of mastitis of goats and associated risk factors, economic consequences, diagnostic tools, prevention and control of subclinical and clinical mastitis are reviewed in this chapter. The main purpose of this chapter is to provide updated information concerning this research work which is addressed here and find out the research gap in subclinical mastitis in goats in our context.

2.1. Mastitis

Mastitis is considered as one of the most serious and costly diseases in dairy goats, causing significant economic losses mainly derived from reduced milk production and discarded milk, veterinary costs, and culling or death of animals especially due to gangrenous mastitis (Bergonier et al., 2003; Aires-de-Sousa et al., 2007; Leitner et al., 2007; Leitner et al., 2008; Marogna et al., 2010). In addition to its sanitary and economic importance, mastitis has hygienic and legal implications in terms of food safety (Bergonier et al., 2003). Malher et al. (2001) reported that in goat herds voluntary culling is mostly dependent on reduced milk production, and that mastitis represents the most frequent cause of culling for sanitary reasons.

2.2. Classification of Mastitis

Mastitis is an inflammation of the mammary gland occurring with a variety of causes and bacterial infections are the predominant (Wellenberg et al., 2002). In general, mastitis is classified into clinical, sub-clinical, and chronic in the scientific literature (Bergonier et al., 2003) and according to the International dairy federation (IDF) definitions of 1999 (Smith, 1999). Clinical mastitis presents with evident pathological signs affecting the udder, with both quantitative (from reduction to absence) and qualitative (changes in the macroscopic appearance and in composition) milk alterations. Subclinical mastitis is characterized by presence of an intra-mammary infection (IMI) without clinical signs and is often accompanied by a raise in milk somatic cell count (SCC) (White and Hinckley, 1999) and reduction in milk production. It is the most common form in goats and is mainly caused by contagious bacteria (Persson and Olofsson, 2011). Zeng and Escobar (1995) suggested that

high SCC can be associated with lower milk yield. Moreover, a decrease in milk yield was also observed by (Leitner et al., 2004a) for goats after inoculation with CNS in udders. Reductions in milk yield are largely due to physical damage of the mammary gland alveolar cells, and to the consequent reduction in the synthetic and secretory functions of mammary gland. Chronic mastitis is defined as an inflammation of the udder that continues over a long period of time (Hamadani et al., 2013). Chronic mastitis can be clinical or subclinical. Other definitions are available for classification of clinical mastitis as per acute, acute, and sub acute. Per-acute mastitis is characterized by severe udder inflammation accompanied by an evident systemic reaction; acute mastitis is characterized by severe inflammation without systemic signs; sub acute mastitis is clinically less evident compared to acute mastitis (Hussain et al., 2014).

2.3. Intra-mammary Infection

The term intramammary infection is not strictly synonymous with the term mastitis, as IMI is more commonly used in the context of a defined etiology following completion of diagnostic culture procedures. Intramammary infection refers to infection of ductal and secretory glandular tissues (mammary gland parenchyma) and/or luminal spaces (i.e. alveolar and ductal lumen, gland cistern, teat cistern). Several pathogens can cause mastitis but *Staphylococcus* spp. is the most frequently diagnosed causal microorganisms of IMI in goats. Other pathogens such as *Streptococcus* spp. *Enterobacter* spp. *Pseudomonas* spp. *Mannheimia haemolytica*, *Corynebacterium* spp. and fungi can produce IMI in small ruminants, but occurrence rates are lower. In addition, severe cases of mastitis related to incorrect preventive strategies have been attributed to the pathogens *Aspergillus fumigatus*, *Serratia marcescens*, *P. aeruginosa* (Berriatua et al., 2001; Bergonier and Berthelot, 2003; Contreras et al., 2003; Gonzalo et al., 2004). Lentiviruses are also known to infect goats, but because they rarely produce clinical symptoms or elevated mean somatic cell count (MSCC). They are not usually considered as classic small ruminant intramammary pathogens (Turin et al., 2005). Nevertheless, caprine lentiviruses should still be included in the general plan for controlling mastitis (Contreras et al., 2003). Because contagious agalactia syndrome produces symptoms other than mastitis, some authors fail to consider *Mycoplasma* spp. as the etiology of goat IMI. However, the intense effects of this pathogen in reducing milk production and increasing the MSCC, means that contagious agalactia should be considered as one of the

most important causes of mastitis in endemic areas, where subclinical cases are frequent (Corrales et al., 2004).

2.4. Etiological agents of caprine mastitis

2.4.1. *Staphylococcus aureus*

Staphylococcus spp. is the most frequently isolated bacterial genus during IMI in goats, and it can account for over 90% of all bacterial species identified in this condition (Contreras et al., 1997; Contreras et al., 1999; Ndegwa et al., 2001; McDougall et al., 2002; Kyozaire et al., 2005; Hall and Rycroft, 2007; Min et al., 2007). Among staphylococci, *S. aureus* is considered the most important pathogenic agent of mastitis in dairy goats, where it has been found with frequencies ranging from 4 to 40% of all isolated microorganisms (McDougall et al., 2002). Although certain strains of *Staphylococcus aureus* are responsible for subclinical mastitis, the pathogen is also one of the main agents responsible for caprine clinical mastitis and main organism for gangrenous mastitis (Maisi and Riipinen, 1991).

2.4.2. Coagulase-negative staphylococci

The *Staphylococci* most involved in subclinical mastitis are principally coagulase-negative staphylococci (CNS). Although CNS are less pathogenic than *Staphylococcus aureus*, they can also produce persistent subclinical mastitis and even clinical mastitis (Deinhofer and Pernthaner, 1995; Contreras et al., 1997) accompanied by a significant increase in milk SCC (Moroni et al., 2005b; Corrales et al., 2007). The main CNS species causing intramammary infection reside in the skin of the udders and teat of the goats (Valle et al., 1991). The importance and pathogenicity of the different CNS species varies widely. In goat, *Staphylococcus epidermidis* is usually the most frequently isolated species (Contreras et al., 1999; Leitner et al., 2004a; Moroni et al., 2005d; Leitner et al., 2007), followed by *Staph. caprae* which, in some studies, was reported as being the most prevalent species (Contreras et al., 1995; Sánchez et al., 2004; Moroni et al., 2005d). *S. simulans*, *S. chromogenes*, and *S. xylosus* are other CNS frequently found in mastitic milk. Several other CNS has been isolated from goat milk, but inconstantly and at low frequencies, suggesting their marginal role in the onset of IMIs. Among different species of CNS *S. caprae* is responsible both for sub-clinical and clinical mastitis (Deinhofer and Pernthaner, 1995; Contreras et al., 1997). However, no differences between goat halves infected with *S. caprae* and healthy goats have been

demonstrated for SCC, milk yield and composition. Moreover *S. caprae* is capable to persist during the dry period (Poutrel, 1984).

2.4.3. *Streptococcus* spp.

Streptococcus is the second most frequently isolated genus in goat milk, with a prevalence ranging from 1% to 9% (Moroni et al., 2005b; Marogna et al., 2012). They mostly cause clinical mastitis, and a number of epidemiological studies on subclinical mastitis even report the absence of these pathogens. This situation seems clearly different in certain areas of dairy sheep production, in which major problems on farms are diagnosed due to *Streptococcus agalactiae*. Because of this absence of *Str. agalactiae* in etiology of goat's mastitis, most of the diagnoses are related to environmental streptococci. So, these occasional diagnoses of Streptococcal mastitis in dairy goats must be associated with problems of environmental contamination, particularly from poor bedding conditions (Kuusi et al., 2006).

2.4.4. Gram-negative bacilli and other intramammary pathogens

The agents known as “Gram-Negative Bacilli” (GNB) are less common in goats than cattle, but when they appear they usually cause severe acute clinical mastitis which can lead to symptoms similar to those caused by *S.aureus*, even acute mastitis of a gangrenous nature (Ribeiro et al., 2007). *Escherichia coli* and *Pseudomonas aeruginosa* are the most frequently isolated GNB agents in goats (East et al., 1987; Contreras et al., 1997). Goat milk contains high SCC, and most of them are neutrophils (Paape et al., 2001). It is well known that low SCC is a risk factor for environmental clinical mastitis, especially in cases caused by *Escherichia coli* (Barkema et al., 1998).

E. coli is a member of the family of Enterobacteriaceae. Over 700 antigenic types or serotypes of *E. coli* have been recognized based on O,H, and K antigens and 8% prevalence was recorded in case of small ruminant. These organisms represent “environmental” mastitis. Thus control involves keeping sleeping areas clean and dry, drying teats thoroughly before milking, and avoiding teat-end injuries. Using post-milking teat dipping does not aid much in controlling coliform infections, because these are initiated between milking, except when wet udders are milked (Beheshti et al., 2010).

In spite of the absence of specific studies in goats, this low rate of mastitis by GNB in goats could be related with the high SCC. Furthermore, the environmental conditions of goat husbandry (generally drier and sunnier than those in which cattle are raised) help to reduce the prevalence of disease due to this type of pathogen. Similarly, some large-scale outbreaks of mastitis due to fungi have also been associated with the latter (incorrect handling of intramammary cannulas). These iatrogenic problems may be so serious that after parturition they bring about the mass infection of treated animals and make it necessary to remove a large number of lactating goats (Jensen et al., 1996). Other pathogens are less common in goats, such as gram-positive bacilli. Among them *Arcanobacterium pyogenes* is a germ which, when it appears, causes a major alteration in milk secretion, which is why it is mainly detected in clinical mastitis of an incurable nature. Conversely, other gram-positive bacilli, such as *Corynebacterium* spp., are regarded as “minor pathogens”. Their role is particularly based on causing subclinical infections, with very little increase in cell counts and an incapacity for producing long lasting persistent infections, which is why they may be the cause of “false positive” diagnoses (Contreras et al., 1997). Other gram-positive bacilli, such as *Bacillus* spp. or *Clostridium perfringens*, are rare in small ruminants mastitis, although they have been reported on occasions (Kalogridou-Vassiliadou, 1991). The latter, which is strictly an anaerobic pathogen, might be a complicating agent in the cases of gangrenous mastitis caused primarily by *S. aureus*. Although caprine mastitis due to *Nocardia asteroides* has only been reported in tropical countries, its existence is disturbing due to the importance of this zoonotic pathogen, which can withstand pasteurization (Bassam and Hasso, 1997).

Earlier studies have been done on causal agents of sub-clinical mastitis in goats. However these studies have not been systematic. So, in the present study causal agents have also been investigated.

2.5. Diagnostic tools for mastitis in small ruminants

2.5.1. Bacteriological detection

A standard tool in the diagnosis of *mastitis* in small ruminants is provided by bacterial culture (Contreras et al., 2007). For two reasons, economic and practical, only one sample of milk is taken to diagnose IMI. It was found that a positive diagnosis in isolating the same pathogen

from the half of the udder in the following samples of milk shows high sensitivity (96.2%) and specificity (96.1%) (Contreras et al., 1997). Intramammary infection (IMI) by CNS is one of the important factors affecting IMI of the flock, and as such it reduces profits from small ruminants (McDougall et al., 2002; da Silva et al., 2004; Leitner et al., 2008). The mean prevalence of subclinical mastitis in three herds of goats in a study by Hall and Rycroft (2007) ranged from 33 to 42%. Among the pathogens isolated from infected udder halves CNS ranked first (47%), followed by *Corynebacterium* spp. (31%), *Staphylococcus aureus* (13%), and α -haemolytic streptococci (6%). Knowledge concerning the etiology of infection in a flock can be a useful tool in reducing economic losses due to decreased milk yields and the deterioration of its quality (Leitner et al., 2008).

2.5.2. California Mastitis Test (CMT)

The California Mastitis Test (CMT) is based on a reagent destroying the membranes of the somatic cells in milk and binding to the cellular DNA. This process results in an increase of the milk viscosity depending on the amount of cells. Thus, CMT allows to roughly estimating the number of cells of the immune system and epithelial cells in given milk sample (Schaeren and Maurer, 2006). California mastitis test is influenced by factors causing variations in SCC as well. Therefore CMT levels correlate well with SCC levels found in caprine milk (Stuhr and Aulrich, 2010) but the comparison between studies is hindered by the different scores used by the various authors. Furthermore, classification in CMT tests is solely based on individual assessment of visual changes of the sample. CMT can be classified from 1 to 5, recently the scores range on a scale of 0 (negative), 1, 2 or 3, but usually authors use a scale of 0 (negative), trace, 1, 2 or 3 (Schalm et al., 1971; Schaeren and Maurer, 2006; McDougall et al., 2010). Some authors decided to combine 0 and trace to one CMT score for a more informative value (Contreras et al., 1996). The relationship between SCC, CMT and infection status was reviewed within many studies (Contreras et al., 1996; Schaeren and Maurer, 2006). It was proposed that low levels of CMT indicate an absence of mammary gland infection (Maisi, 1990).

California mastitis test is influenced by factors which are also associated with variations in SCC. Therefore, the factors that influence SCC in goat is also influence the CMT scores as well.

2.6. Factors associated with somatic cell count (SCC)

2.6.1. Parity

Parity affects milk fat and protein concentrations, yield, and SCC. Milk production is lower for primiparous than for multiparous dairy goats; highest production is for parity 3 or 4 (Carnicella et al., 2008). Similarly, Zahradeen et al. (2009) found increasing partial daily milk yield as parity increased from 1 to 3. The SCC increases with increasing parity (Wilson et al., 1995; Salama et al., 2003). The rise in SCC with increasing parity relates to increasing bacterial presence or cumulative mammary gland stress. (Boscos et al., 1996; Paape et al., 2007) concluded that much of the increase in SCC is due to non-infectious factors, although increasing frequency of intra-mammary infections is also involved.

2.6.2. Stage of lactation

Somatic cell count (SCC) in goat milk increases as lactation advances (Gomes et al., 2006). Physiologically, dairy goats have SCC with an upward trend corresponding to the progression of the productive period (Poutrel et al., 1997). This trend shows an inverse relationship with milk production (Rota et al., 1993; da Silva et al., 2015). Thus, the cellular concentration of goat milk is so high that, according to Corrales et al. (2007), at the end of lactation it is impossible to distinguish between uninfected and healthy glands through SCC. Several authors have explained that the increase in SCC during the lactation due to a dilution effect and because the advancement of the lactation implies a decrease in production and there is a significant negative correlation between SCC and milk, the SCC being higher at the end of lactation (Gomes et al., 2006). In another similar studies, Paape et al. (2007) counts were lowest at first parity, averaging $\sim 200 \times 10^3 \text{ SC ml}^{-1}$ at 15 days of lactation and these reached maximums of around $500 \times 10^3 \text{ SC ml}^{-1}$ at 285 days. By the fifth parity, counts averaged $\sim 250 \times 10^3 \text{ SC ml}^{-1}$ at 15 days and increased to a maximum of $\sim 1,150 \times 10^3 \text{ SC ml}^{-1}$ at 285 days of lactation.

2.6.3. Prolificacy

Most studies found that the type of birth influences the SCC (Luengo et al., 2004). Highest counts are obtained in animals with multiple birth ($1,666.9 \times 10^3 \pm 137.1 \times 10^3 \text{ SC ml}^{-1}$, $p <$

0.05) and breeding than in those with simple birth, although the former produce more milk than the latter. This result could be attributed to a worse health status of the udder in mothers who breastfeed two kids compared to those who only nurse one. However, this explanation seems insufficient, since Luengo et al. (2004) also found that when raising kids with artificial feeding, the goats with multiple births also have higher counts than those of single birth. Despite the above, it should be pointed out that some studies found that the prolificacy does not influence the SCC.

2.6.4. Breed

The authoritative information contrasted on the SCC in different dairy goat breeds (Zeng et al., 1996), cannot categorically confirm the genetic implications of that factor. However, according to Boscos et al. (1996), possible relationship is not existed between breeds.

2.6.5. Milk yield and contents

Milk production is lower for primiparous than for multiparous dairy goats; while the highest production is for parity 3 or 4 (Goetsch et al., 2011). The information about goats indicates that in the absence of infection, less productive animals result in higher SCC (Chen et al., 2010) found that milk composition (fat, protein, lactose, casein, and total solids), did not change when milk SCC varied from 214,000 to $1,450 \times 10^3$ SC ml⁻¹.

2.6.6. Estrus

Some authors have found that when goats are on estrus, either natural or induced (Moroni et al., 2007); both at the station of estrus and of anestrus (McDougall and Voermans, 2002) there is a significant increase in SCC. This increase is unexplained by the slight decrease in milk production, suggesting that it is the estrus directly responsible for the cell growth, probably due to still unknown physiological mechanisms (McDougall and Voermans, 2002). On the other hand, it seems that common situations and events affecting stress levels on farms apparently did not affect the SCC. There is a controversy about whether the effect of estrus on the SCC is dependent on the infection status of the udder in the case of infected glands. In this sense, Bergonier et al. (2003) indicate that estrus can cause a greater increase in SCC in infected glands than in healthy glands.

2.6.7. Time between milking

In goats, it has been found that the SCC of milk obtained from the evening milking is between 17-78% higher than that obtained from the morning (Cedden et al., 2008). Some authors explained that this is due to a dilution effect, because the amount of milk obtained in the morning milking is between 35-69% higher than in the afternoon one.

2.6.8. Feed

An unbalanced ration (nitrogen, energy or minerals) can be the cause of the increase in the SCC of milk (Sánchez et al., 2007). Generally, when feeding causes metabolic disorders (acidosis, alkalosis, etc.) it causes elevation of SCC (Fedele et al., 1996). This increase is probably due at least in part, to a reduced milk production in animals suffering from such disorders, which translates into a higher cell concentration. Fedele et al. (1996) studied the effect of different types of rations on the SCC, noting that when the goat diets are only based on grazing, the SCC values are slightly lower than when these are supplemented with a concentrated energy; whereas higher counts are obtained when supplemented with a protein concentrate.

2.6.9. Stress

There are various handlings of the herd that presumably produce some kind of stress, causing spontaneous elevations of SCC in bulk tank milk. For example, at the time of goats mating, when introducing the males into the herd, there is usually an increase of SCC in the milk bulk tank (Borges et al., 2004). However, it is unclear whether this increase is due to the effect of heat or stress by the introduction of males or both factors. On the other hand, various management practices such as blood draws and tuberculin skin testing can temporally increase the level of SCC in bulk tank milk (Contreras et al., 1997). There has also been detected an increase of SCC after vaccination against enterotoxaemia (Lerondelle et al., 1992) proved that the stress level of goats in response to parasitic diseases (*i.e.* mange) produces a reduction in milk production and an increase in SCC, reaching levels of $950,000 \times 10^3$ SCC ml⁻¹.

2.6.10. Seasonality

The binomial photoperiod-temperature influences on milk production and, indirectly, the SCC (Wilson et al., 1995) associated effects of long day photoperiod on increased percent milk fat and decreased SCC ($< 1,705 \cdot 10^3 \text{ SC ml}^{-1}$). Thus, in the spring season (increasing photoperiod, mild temperatures, and sometimes better feed) the production tends to increase and therefore the SCC is reduced. In contrast, in the autumn months the situation tends to be opposite. Moreover, in the summer it is expected that the SCC will tend to increase as temperatures decrease the production (Delgado-Pertiñez et al., 2003)

2.6.11. Farming system

When comparing indoors farms with semi-intensive ones, levels of SCC are lower in the former (Razi et al., 2013). This could be due to better milking facilities and routines, as well as better hygiene in the intensive and indoors farms. Furthermore, when systems based on grazing and indoor systems are compared, the milk components (fat, protein, lactose) appear to be rather less influenced by the type of farming system than by the level of milk production. Natural pasture based farming systems produce milk rich in fat, micro components and volatile components.

Chapter III: Materials and methods

3.1. Description of the study area:

Chittagong, considered as the second financial capital city of Bangladesh located at 22°22'0"N and 91°48'0"E (Anon, 2016). The tropical monsoon climatic condition characterizes by annual average temperature of 13°C to 32°C, humidity of 70 to 85% and rainfall of 5.6 mm to 727.0 mm (Anon, 2016). Around 6.5 million people live in this metro city. Livestock rearing is common practice in Chittagong as major or subsidiary income source. The predominant livestock includes cattle, goat and sheep as well as poultry (backyard and commercial). In metropolitan city, the total population of cattle, buffalo, sheep and goat are 51290, 578, 2438, and 30320, respectively (DLS, 2017). Goats are reared in intensive, semi intensive, free range and tethering systems in Chittagong. Farmers use to keep their goats in their own houses as well as separate goat houses with low biosecurity and hygienic standard. The backyard and smallholding goat farmers rear goats as meat purpose while some people also rear goats as their hobby in metropolitan area of Chittagong.

3.2. Reference population, source population and study population

Goats of smallholder farms under Chittagong metropolitan city were considered as reference population. Goats of smallholder farms having at least one lactating goats per farm were treated as source population. A total of 88 smallholder goat farms were conveniently chosen, but ensured representation of different suburbs of the Chittagong metropolitan city. These farms were used for sampling under a cross-sectional study. All lactating goats ranging 1-5 per farm were sampled. Only clinically healthy animals without changes in udder consistency or milk appearance were included in the study. Distribution of sampled farms among different sub-sites of Chittagong Metropolitan City was as follows: Khulshi (27), Bayezid (8), Akbarshah and Firozshah (35), Haliashahar, EPZ and Bondar (6), Pahartali (7), Kalurghat and Panchlaish (2), Doublemuring and Kotowali (3).

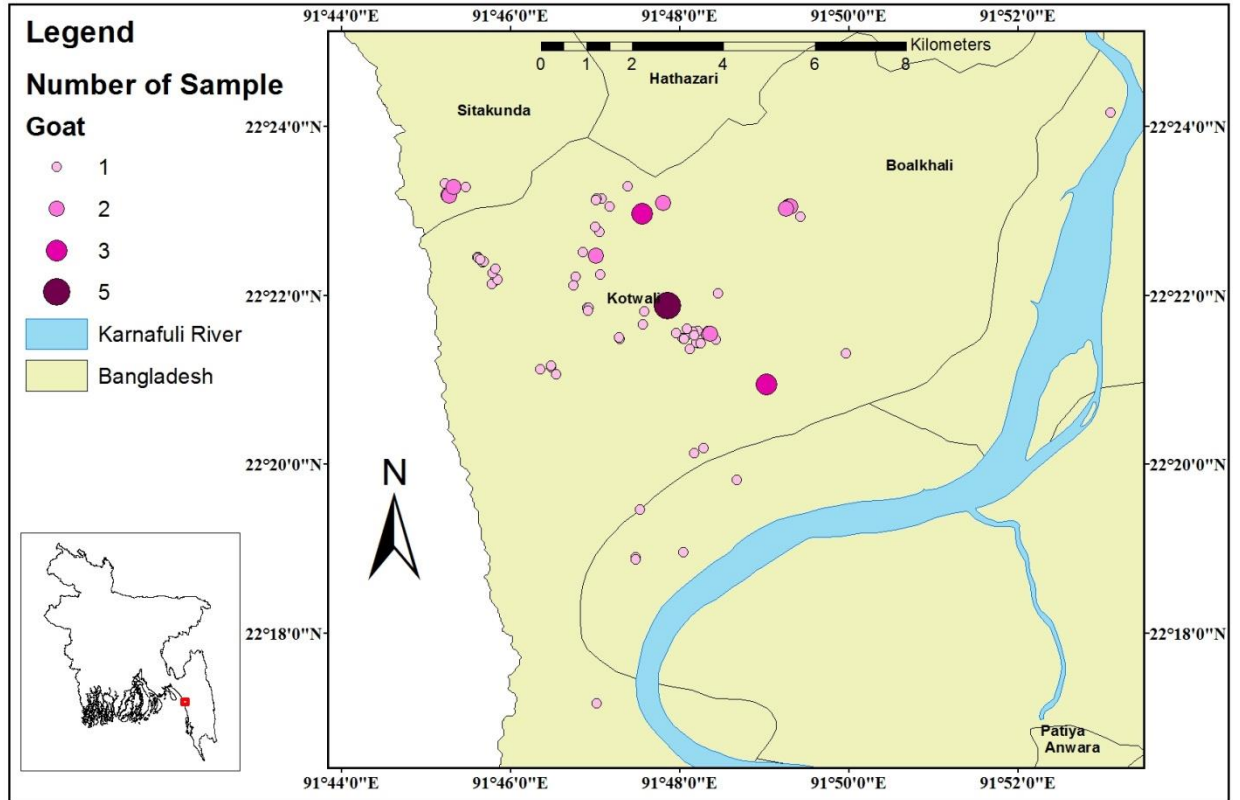


Figure 1: Location of goat farms of Chittagong metropolitan city (as coordinates of some of the farms are very closely situated so all 88 goat farms are not visualized individually)

Google map (<https://www.google.com.bd/maps>) was used to get geocoordinates of the location of the individual goats. ArcGIS-ArcMap version 10.2 (ESRI, USA) was used to produce map and locate individual studied farms.

3.3. Collection and transportation of milk samples

All udder quarters of the 106 lactating goats were sampled in this study. A total of 10 ml milk sample per quarter was collected aseptically and placed in 15 ml sterile falcon tube with an unique identity number. All samples collected were immediately transported using insulated ice box to the laboratory at CVASU. Samples were stored at -20°C before further analysis. The detailed sample collection procedure is given in Appendix-I.

3.4. Data collection

A validated structured questionnaire was made and used to collect epidemiological field information. Information related to farmers' demography, farms' demography, characteristics and management features of goat farms were recorded through face to face interview and close inspection. The detailed information in questionnaire is given in Appendix-II. 2-3 trained interviewers were administered the questionnaire. Around 45-60 minutes were required to complete each interview and observational check list. The interview was usually taken during morning time.

3.5. Laboratory evaluation

3.5.1. California Mastitis Test:

The California Mastitis Test (CMT) was applied to all samples collected using the method modified from Schalm et al. (1971). The CMT test is based on the reaction between the CMT reagent and DNA in the somatic cells and high concentration of somatic cells leads to a higher CMT score. Two ml milk samples from each quarter were taken in a cup of the CMT plate and the same amount (2 ml) of CMT reagent was added. A circular motion was made with the plate for 10 seconds to mix the reagent and milk, and after 20 seconds, changes in the milk were observed. According to the reactions obtained, the results were classified as: negative (no change in consistency), traces (slightly positive), 1 (mild positive), 2 (moderate positive) and 3 (highly positive), recorded as -, ±, +, ++ and +++, respectively. The scores are ranked according to an increase in viscosity, where the highest viscosity (CMT 5) is more or less correlated to the highest SCC.

3.5.2. Bacteriological culture

Following gentle inversion after thawing of the milk sample (if needed), one loopful of milk was spread onto a 5% sheep blood agar plate regardless of CMT positive and negative samples. Inoculated plates were incubated at 37 °C for 16-24 h. If there was no growth, the plates were reincubated, and the final assessment was made at 48 h. Any isolate present with >2 colonies/plate was speciated per National Mastitis Council (NMC) recommendations (Council, 1999). A sample was defined as contaminated when >3 distinct

colony types of bacteria were present. The organisms were identified on the basis of colony morphology on different selective media, Gram staining results, characteristic hemolytic patterns, and biochemical tests (Lennette et al., 1985). Gram staining was performed as described by Murray et al. (1994). The morphology of the bacteria were observed by microscopy (Quinn et al., 2011).

3.5.2.1. *Staphylococcus aureus*

The following characteristics were considered for identification of *S. aureus* were golden yellow colony with beta hemolysis on blood agar, yellow colonies with yellow zones on mannitol salt agar, grey white to yellow colonies on nutrient agar, gram positive cocci in cluster in grams staining, catalase positive and coagulase negative.

3.5.2.2. Coagulase-negative staphylococci (CNS)

Coagulase-negative staphylococci (CNS) were identified by observing the following characteristics: moderately-sized and white or golden color colonies and non-hemolytic on blood agar, small red colonies with no color change to the medium on mannitol salt agar, gram-positive cocci in clusters resembling bunches of grapes on gram's staining and non-motile, catalase test positive and coagulase test negative.

3.5.2.3. *Streptococcus spp.*

Streptococcus spp. was diagnosed based on alpha or beta hemolytic on blood agar, small translucent colonies (beta-hemolytic streptococci), gram-positive cocci and characteristic chains occur in broth cultures and staining (with gram's stain) and catalase negative.

3.5.2.4. *Bacillus spp.*

Characteristics considered to identify *Bacillus spp.* were colonies of dull, opaque, grayish-white with an irregular border from which long strands of cells arranged parallel, giving the typical 'medusa head' appearance and beta hemolytic on blood agar, large, gram-positive rods, cells occurred singly, in pairs or in long chains microscopically, catalase positive and oxidase-negative.

3.5.2.5. *Enterobacter* spp.

Characteristics considered to identify *Enterobacter* spp were large, mucoid colonies and surrounding medium was pink due to acid production from lactose on Mac Conkey agar and large, mucoid, blue-black on EMB agar, yellow (acidic) color in the slant and butt of the TSI agar tube was observed with gas (CO₂) and without H₂S production, gram-negative, pink color, motile rod bacterium (on grams' staining), oxidase, indole and methyl red (MR) test negative and voges-proskauer (VP) test positive.

3.5.2.6. *Pseudomonas* spp.

To identify *Pseudomonas* spp. the following criteria were considered such as lactose-negative, pale colonies on MacConkey agar, on EMB agar irregular colorless or pinkish mucoid colonies, no color change of butt and slope of TSI agar tube, gram-negative rods, actively motile, catalase and oxidase positive, indole, MR and VP tests negative.

3.5.2.7. *Klebsiella* spp.

Characteristics considered for identification of *Klebsiella* spp. were pale pink mucoid colonies on primary isolation indicative of the presence of a large capsule around individual cells on Mc Conkey agar, lactose-fermenters, oxidase-negative.

3.5.2.8. Maintenance of stock culture

Fifty percent sterile buffered glycerin was made by mixing 50 parts of pure glycerin and 50 parts of buffered saline. Pure culture of isolated bacteria from blood agar was incubated overnight in Brain heart infusion broth which was prepared according to manufacturer's instruction (Oxoid Ltd, Basingstoke, Hampshire, UK). Then the bacteria were mixed with 50% sterile buffered glycerin in 1.5 ml eppendorf tubes (700µl broth culture and 300µl gluserol) and were preserved at – 80° C for long term use. The detailed information about isolation and identification of organisms are given in Appendix III.

3.6. Statistical evaluation

Field and laboratory data were stored in the spread sheet of Microsoft Excel (MS) 2007 programme. Data were cleaned, coded, reoded and checked for integrity in MS Excel 2007 before exporting to STATA-IC-13 (*Stata Crop, 4905, Lakeway Drive, College station, Texas 77845, USA*) for performing epidemiological analysis.

3.6.1. Descriptive analysis

Descriptive analysis was performed on the data of farmers' demography (frequency distribution), farms' demography (summery statistics), characteristics of sampled goats and management features of farm as well as different organisms (frequency distribution). The result was expressed as frequency number, percentage, mean and median, minimum and maximum where applicable.

3.6. 2 Risk factor analysis

3.6.2.1 Univariate analysis

Source of goat, floor materials of farm, BCS, parity, lactation period and teat shape were initially assessed by Fisher's exact test to identify univariate association between the status of SCM and the selected factors. The level of significance of the test was set at $p \leq 0.05$.

3.6.2. 2. Multivariate analysis

Factors determined as significant ($p \leq 0.05$) by univariate Fisher's exact test were used for multivariate analysis. To know the characteristics of the dataset first of all an ordinary logistic regression model ignoring clustering of goat was fitted. Then the same model was fitted asking for robust standard error. The calculated difference between likelihood-based (model-based) standard error and robust (residual-based) standard error represented the dataset as clustered where the goat identity number was the cluster variable. Based on this context the Random Effect model was tried to be fitted for the dataset. But the Likelihood Ratio Test (LRT) did not satisfy the model due to greater quadrature points (>0.01) at the 8th number cut point. So, alternative Generalized Estimating Equation model was performed

considering the significant variables at 5% level. A backward-elimination procedure was applied by fitting the full model and reducing the model based on significance of the variables one by one. The confounding and interaction was checked by adding or removing a variable from the model. The co-linearity was evaluated using Chi-square test between the factors. Interaction was also checked. Variables that were significant ($p \leq 0.05$) based on Wald test were considered as risk factors for the SCM. The results were expressed as Odds Ratio (OR) and Standard Error (SE) with 95% confidence interval.

Chapter IV: Results

4.1. Descriptive analysis

4.1.1. Demography of goat farmers

Goat farmers had a diverse profession in this study; such as self business (34%), housewife (25%), job (12.5%), agricultural farming (10.2%) etc. The educational qualification of goat farmers' was primary (14.8%), secondary (30.7%), higher secondary (3.4%) and graduate level (10.3%). However, 32.9% farmers were illiterate (Table 4.1).

Table 4.1: Goat farmers' demographic information in Chittagong metropolitan city (N=88)

Variable	Category	Frequency number	%
Profession	Self business (Electrician, shopkeeper, rickshaw puller etc.)	30	34.1
	Service (Driver, guard, gardener etc.)	11	12.5
	Housewife	22	25.0
	Farmer	9	10.2
	Student	11	12.5
	Unemployed and others (like retired persons)	5	3.7
Education	Illiterate	29	32.9
	Class I-V (Primary)	13	14.8
	Class VI-IX (Secondary)	22	25.0
	SSC and equivalent (vocational) (Secondary)	5	5.7
	HSC and equivalent (Diploma) (Higher secondary)	3	3.4
	BA equivalent (Honors, engineering etc) and higher (MA and MS)	9	10.3
	Missed to record	7	7.9

SSC: Secondary School Certificate; HSC: Higher Secondary Certificate; BA: Bachelor of Arts; MA: Master of Arts; MS: Master of Science

4.1.2. Demography of goat farms

The median goat farm size was 5 for intensive farm, 6 for semi-intensive farm and 3 for free-ranging farm. Irrespective of farm types, each farm had median 1-2 lactating goats in the study. Other status of farm composition is given in Table 4.2.

Table 4.2: Composition of goat farm in Chittagong metropolitan city

Variable	Intensive			Semi-intensive			Free-range		
	Mean (n)	Median	Min- Max	Mean (n)	Median	Min- Max	Mean (n)	Median	Min- Max
Farm size	7.5 (29)	5	2-40	7.5 (34)	6	2-24	5.3 (21)	3	2-18
Doe	3.2 (29)	2	1-20	2.8 (34)	2	1-10	2.2 (21)	2	1-5
Buck	2.8 (11)	2	1-7	2 (13)	1	1-7	2.2 (5)	2	1-5
Kid	3.3 (29)	2	1-17	3.9 (34)	3	1-11	2.8 (21)	2	1-11
Pregnant	2.4 (16)	1	1-15	1.9 (12)	1	1-5	1.6 (10)	1	1-3
Lactating	1.8 (28)	1	1-5	2.3 (34)	2	1-10	1.7 (21)	1	5-21

4.1.3. Characteristics of sampled goats in Chittagong metropolitan city

Multiple goat breeds were recorded in the study area where cross bred was (72%) Jamnapari was (24%) and Black Bengal goat was (5%). About 23.6% goats had the first parity followed by 63.2% the second to fourth parity and 13.2 % more than the fourth parity. The BCS of goats was very thin (47.2%), thin (40.6%) and moderate (12.2%). On close observation udder of goats was found free of dirt (26.4%), slightly dirty (65.1%), mostly covered by dirt (6.6%) and completely covered by dirt (1.9%). Thigh of goats was completely free of dirt (16.9%), slightly dirty (71.7%), mostly covered by dirt (9.5 %,) and completely covered and caked on dirt (1.9%) (Table 4.3).

Table 4.3: Characteristics of sampled goats in Chittagong metropolitan city (N=106)

Variable	Category	Frequency number	%
Breed	Pure Black Bengal	5	4.7
	Pure Jamnapari	25	23.6
	Cross	76	71.7
Parity	First	25	23.6
	Second to fourth	67	63.2
	More than fourth	14	13.2
Body Condition Score	Very thin	50	47.2
	Thin	43	40.6
	Moderate	13	12.2
Udder cleanliness	Completely free of dirt	28	26.4
	Slightly dirty	69	65.1
	Mostly covered by dirt	7	6.6
	Completely covered, caked on dirt	2	1.9
Thigh cleanliness	Completely free of dirt	18	16.9
	Slightly dirty	76	71.7
	Mostly covered by dirt	10	9.5
	Completely covered, caked on dirt	2	1.9
Leg cleanliness	Completely free of dirt	2	1.9
	Slightly dirty	70	66.0
	Mostly covered by dirt	29	27.4
	Completely covered and caked on dirt	5	4.7

4.1.4. Management features of goat farms in Chittagong metropolitan city

The farm floor was made of concrete and bricks (17.0%), plastic and jute bag (22.7%) and wood (60.3%). Goats were generally fed with bran (14.8 %), grass (14.8%), grass, pea husk and/ kaora leaf together (67.0%), grass and cabbage (2.3%), coconut shell and bran together (4.6%). Farm floor condition was variable of which 86.4% were clean and dry and 13.6% were muddy and dirty. Around 63% farm floor was covered with feces in variable depth on the day of visit. Majority of farmers (75%) responded that they cleaned their farms daily (Table 4.4).

Table 4.4: Management features of goat farms in Chittagong metro city (N=88)

Variable	Category	Frequency number	%
Floor materials	Concrete and bricks	15	17.0
	Plastic and jute bag	20	22.7
	Wood	53	60.3
Feeding	Bran	13	14.8
	Grass	10	11.3
	Grass, pea husk and/ kaora leaf	59	67.0
	Grass and cabbage	2	2.3
	Coconut shell and bran	4	4.6
Muddiness	Perfect (100% floor is dry)	56	63.7
	Clean (75% floor is dry)	20	22.7
	Medium (Maximum 50% floor covered with mud)	9	10.2
	Dirty (Floor totally covered with mud)	3	3.4
Feces	Clean (no feces on floor)	33	37.5
	Medium (Most of the floor is dry)	34	38.6
	Dirty (Feces is seen everywhere on floor)	21	23.9
Cleaning frequency of floor	Once daily	66	75.0
	More than once daily	17	19.3
	More than once per week	5	5.7

4.1.5. Isolated organisms and the status of California Mastitis Test

The proportionate prevalence of subclinical mastitis at goat level and quarter level was 50% and 46.2%, respectively. The prevalence of organism in relation to CMT was calculated based on quarter level. The prevalence of coagulase-negative staphylococci was 35.4% followed by *Pseudomonas* spp. (23.1%), *Staphylococcus aureus*(11.3%) and other organisms (8.9%) (Table 4.5).

Table 4.5: Descriptive association of different bacterial organisms and the status of California Mastitis Test

Organism	CMT 1	CMT 2	CMT 3	CMT 4	N (%)	95% CI
Coagulase-negative staphylococci	47	21	7	0	75 (35.4)	28.9-42.2
<i>Pseudomonas</i> spp.	30	11	8	0	49 (23.1)	17.6-29.4
<i>Bacillus</i> spp.	21	9	6		36 (16.9)	12.2-22.7
<i>Staphylococcus aureus</i>	7	8	8	1	24 (11.3)	7.4-16.4
<i>Staphylococcus epidermidis</i>	1	3	6	0	10 (4.7)	2.3-8.5
<i>Enterobacter</i> spp.	4	0	1	0	5 (2.4)	0.8-5.4
<i>Streptococcus</i> spp.	1	1	0	0	2 (0.9)	0.1-3.4
<i>Klebsiella</i> spp.	0	1	1	0	2 (0.9)	0.1-3.4

CMT: California Mastitis Test; N: Number; CI: Confidence Interval

4.2. Risk factor analysis of subclinical mastitis in goat in relation to California Mastitis Test

4.2.1. Univariate Analysis

The proportionate prevalence at quarter level varied significantly by source of goat, floor materials, body condition score, parity, lactation period and teat shape ($p \leq 0.05$). The prevalence of SCM was significantly higher (52.5%) in goats of external source than the goats of own stock (38.0%) ($p=0.038$). Goats housed on the floor with the bedding materials of plastic and jute bag had significantly greater level of SCM (61.9%) than that of the floor with bedding materials of wood only (44.8%) or no bedding materials (33.3%) ($p=0.039$). Goats of poor and fair body condition score had significantly higher prevalence of SCM (55.4%) as compared to goats of cachectic body condition score (36.0%) ($p=0.006$). The prevalence of SCM was significantly higher in goats with 3-5 parities (52.9%) than in goats with 1-2 parities (40%) ($p=0.073$). Subclinical mastitis was commonly affected in goats during late lactation (71.4%) than in early lactation (37.2%) ($P < 0.001$). Goats having conical and cylindrical shape of teat had significantly higher prevalence of SCM (73.7%) than in goats having other teat shape (38.9-46.7%) ($p=0.001$) (Table.4.6).

Table 4.6 : Univariate association between selected factors and California Mastitis Test status (Yes/No) at quarter level of lactating goats in Chittagong metro city

Factor	Category	N	CMT status		p (Fisher's exact test)
			Positive (%)	Negative	
Source of goat	Own stock	92	35 (38.0)	57	0.038
	External	120	63 (52.5)	57	
Floor materials	Concrete and brick	36	12 (33.3)	24	0.039
	Plastic and jute bag	42	26 (61.9)	16	
	Wood	134	60 (44.8)	74	
Body condition score	Cachectic	100	36(36.0)	64	0.006
	Poor and fair	112	62(55.4)	50	
Parity	First and second	110	44(40.0)	66	0.073
	Third to fifteenth	102	54(52.9)	48	
Lactation period (days)	Upto 60	156	58(37.2)	98	0.000
	61 to 180	56	40(71.4)	16	
Teat shape	Bottle and collapsed	144	56 (38.9)	88	0.001
	Conical and cylindrical	38	28(73.7)	10	
	Pencil and short	30	14 (46.7)	16	
Teat end callosity thickness	None	140	60 (42.9)	80	0.192
	Thin and moderate	72	38 (52.8)	34	
Teat size	Normal	162	78 (48.2)	84	0.334
	Larger	50	20 (40.0)	30	
Teat end shape	Pointed	138	62 (44.9)	76	0.665
	Rounded	74	36 (48.7)	38	
Rearing system	Intensive and semi-intensive	156	70 (44.9)	86	0.535

	Free ranging and tethering	56	28 (50.0)	28	
Breed	Pure bred (BBG and Jamnapari)	60	24 (40.0)	36	0.268
	Cross bred	152	74 (48.7)	78	
Age (months)	Min to 36	146	67 (45.9)	79	0.883
	37 to maximum	66	31 (46.9)	35	

BBG: Black Bengal Goat; N: Number; CMT: California Mastitis Test; p: Probability

4.2.2. Generalized estimating equation

Factors identified as significant ($p \leq 0.05$) by univariate Fisher's exact test were forwarded to multivariate Generalized Estimating Equation (GEE) analysis to assess the adjusted population averaged effects on the prevalence of SCM in goats. After adjustment of the effect of factors each other source of goat, floor materials, parity, teat shape and lactation period were turned out as potential risk factors for the occurrence of SCM in goats.

Goats of external source was 2.2 times more likely to have odds of SCM than in goats of own stock ($p=0.07$). Goats housed on floors with bedding materials of plastic and Jute bag was 3.7 times more odds of SCM in comparison to goats housed on floor with bedding materials of wood or concrete and brick ($p=0.08$). Goats with 3-5 parities had 2 times more odds of SCM than that of goats with 1-2 parities ($p=0.09$). Does with conical and cylindrical teat shape had 3.6 times greater odds of SCM than does with other teat shape ($p=0.03$). Goats having late lactation period were 5.3 times higher odds of SCM than in goats having early lactation period ($p=0.002$).

Table 4.7: Outputs of Generalized estimating equation model on the factors for California Mastitis Test status (Yes/No) status in goats of Chittagong metropolitan city

Factors	Category	OR	95% CI	p-value
Source of goat	Own stock	1	0.9- 5.3	0.07
	External	2.2		
Teat End Callosity Thickness	None	1	0.5-3.2	0.6
	Thin and Moderate	1.3		
Body Condition Score	Cachectic	1		

	Poor and Fair	2.2	0.9-5.5	0.10
Floor Materials	Concrete and Brick	1		
	Plastic and Jute bag	3.7	0.8-16.7	0.08
	Wood	1.8	0.6-5.9	0.33
Parity	First and second	1		
	Third to fifteenth	2.0	0.9-4.5	0.09
Teat Shape	Bottle and Collapsed	1		
	Conical and Cylindrical	3.6	1.1-11.8	0.03
	Pencil and Short	1.4	0.4-5.6	0.57
Lactation period (days)	Up to 60	1		
	61 days to 180	5.3	1.9-14.9	0.002

OR: Odds Ratio; CI: Confidence Interval; p: Probability

4.3: Risk factor analysis of subclinical mastitis in goat in relation to organisms

4.3.1: Univariate association of different factors in correspondence to binary response variable of different organism at sample level.

Intramammary infection due to CNS was significantly higher in own stock goats (45.6%) in contrast to goats of external source (27.5%) ($p=0.009$). CNS was significantly higher in cachectic goats (46.0%) than that of poor and fair body condition score (25.9%) ($p=0.003$). The prevalence of CNS was significantly higher in pencil and short teat shape (50.0%) in comparison to bottle and collapsed, conical and cylindrical teat shape (37.5% and 15.8%, respectively) ($p=0.007$). CNS was more in goats reared under semi-intensive system (52.3%) than in goat under free range system (25.0%), intensive (23.5%) and tethered (12.5%) ($p<0.001$). More prevalence of CNS was noticed in goats housed on floor with no bedding materials (52.8%) in contrast to goats housed on floor with wood type (32.1%) and Jute and plastic bag type (30.9%) ($p=0.06$).

The prevalence of *S. aureus* was significantly higher in conical and cylindrical teat shape (26.3%) in contrast to pencil and short and bottle and collapsed teat shape (10.0% and 7.6%, respectively) ($p=0.008$).

The prevalence of *S. epidermidis* was significantly greater in thin and moderate teat end callosity thickness (9.7%) than moderate teat end callosity thickness of none (2.1%) ($p=0.03$).

S. epidermidis had significantly high prevalence in tethered system (25.0%) than the system of intensive (10.3%) and semi-intensive (1.1%) ($p=0.001$).

Pseudomonas spp. had significantly higher prevalence in tethered system (50.0%) compared with the system of intensive (29.4%), free-range(20.8%) and semi-intensive (17.1%) ($p=0.08$). The prevalence of *Pseudomonas* was significantly higher in floor cleaned weekly (50.0%) than that of more than once daily (23.7%) and once daily (15.2%) ($p=0.06$).

Table 4.8: Univariate association of different factors in correspondence to binary response variable of different organism at sample level

Factor	Category	N	CNS			<i>S. aureus</i>			<i>S. epidermidis</i>			<i>Pseudomonas</i>		
			+(%)	- (%)	p	+(%)	- (%)	p	+(%)	- (%)	p	+(%)	- (%)	p
Source of goat	Own stock	92	42 (45.6)	50		10 (10.9)	82		2 (2.2)	90		19 (20.7)	73	
	External	120	33 (27.5)	87	0.009	14 (11.7)	106	1.0	8 (6.7)	112	0.2	30 (25.0)	90	0.5
Body condition score	Cachectic	100	46 (46.0)	54		12 (12.0)	88		1 (1.0)	99		19 (19.0)	81	
	Poor and fair	112	29 (25.9)	83	0.003	12 (10.7)	100	0.8	9 (8.0)	103	0.02	30 (26.8)	82	0.2
Teat shape	Bottle and collapsed	144	54 (37.5)	90		11 (7.6)	133		6 (4.2)	138		35 (24.3)	109	
	Conical and cylindrical	38	6 (15.8)	32		10 (26.3)	28		3 (7.9)	35		8 (21.1)	30	
	Pencil and short	30	15 (50.0)	15	0.007	3 (10.0)	27	0.008	1 (3.3)	29	0.6	6 (20.0)	24	0.9
Teat end callosity thickness	None	140	46 (32.9)	94		17 (12.1)	123		3 (2.1)	137		34 (24.3)	106	
	Thin and moderate	72	29 (40.3)	43	0.3	7 (9.7)	65	0.7	7 (9.7)	65	0.03	15 (20.8)	57	0.6
Rearing system	Intensive	68	16 (23.5)	52		5 (7.4)	63		7 (10.3)	61		20 (29.4)	48	
	Semi-intensive	88	46 (52.3)	42		13 (14.8)	75		1 (1.1)	87		15 (17.1)	73	
	Free range	48	12 (25.0)	36		6 (12.5)	42		0	48		10 (20.8)	38	
	Tethered	8	1 (12.5)	7	0.000	0	8	0.4	2 (25.0)	6	0.001	4 (50.0)	4	0.08
Parity	First and	110	41	69		16	94		2 (1.8)	108		30 (27.3)	80	

Factor	Category	N	CNS			<i>S. aureus</i>			<i>S. epidermidis</i>			<i>Pseudomonas</i>		
			+	-	p	+	-	p	+	-	p	+	-	p
	second		(37.3)			(14.6)								
	Third to fifteen	102	34 (33.3)	68	0.6	8 (7.8)	94	0.13	8 (7.8)	94	0.05	19 (18.6)	83	0.14
Flooring	Concrete and brick	36	19 (52.8)	17		2 (5.6)	34		2 (5.6)	34		7 (19.4)	29	
	Jute and plastic bag	42	13 (30.9)	29		7 (16.7)	35		4 (9.5)	38		9 (21.4)	33	
	Wood	134	43 (32.1)	91	0.06	15 (11.2)	119	0.3	4 (2.9)	130	0.15	33 (24.6)	101	0.8
Breed	Black bengal and Cross	162	56 (34.6)	106		20 (12.4)	142		7 (4.3)	155		38 (23.5)	124	
	Jamnapari	50	19 (38.0)	31	0.7	4 (8.0)	46	0.6	3 (6.0)	47	0.7	11 (22.0)	39	1.0
Cleaning floor	Once daily	156	60 (38.5)	96		17 (10.9)	139		7 (4.5)	149		37 (23.7)	119	
	More than once daily	46	14 (30.4)	32		6 (13.0)	40		2 (4.4)	44		7 (15.2)	39	
	Weekly	10	1 (10.0)	9	0.14	1 (10.0)	9	0.9	1 (10.0)	9	0.53	5 (50.0)	5	0.06

Chapter V: Discussion

Mastitis is one of the most costly diseases in the dairy industry. The majority of studies on mastitis in ruminants are non-systematic. There are few scattered studies have been found dealing with mastitis in goats in Bangladesh and suggested that goat farms also have a potential role in epidemiology of subclinical mastitis. Therefore, the present study was performed in Chittagong metropolitan city in order to assess subclinical mastitis status and its associated risk factors and distribution of pathogens along with farmers' socioeconomic characteristics and farm characteristics and management traits. This section of the thesis has discussed important findings of the current study and their implications along with limitations, conclusion and recommendations.

5.1. Descriptive association of the status of California Mastitis Test and different bacterial organisms

The overall proportionate prevalence of SCM estimated as 50% by CMT in the goat population in Chittagong metropolitan area in the current study which is supported by number of studies conducted in northern districts of Bangladesh (56.2%) (Yeruham et al., 2004; Begum et al., 2016) and 20 to 50 % reported by Bergonier et al. (2003). However, the current prevalence of SCM was higher than the prevalence found in another study conducted at Bangladesh Agricultural University Veterinary Clinic, Mymensingh (37.2%) (Amin et al., 2013) and other countries such as in Brazil 22.5% (Schmidt et al., 2009), in Pakistan 13% (Ali et al., 2010) and in Ethiopia 18.03% (Gebrewahid et al., 2012).

The proportionate prevalence of SCM at udder half of goat in this study was 46.2% which is supported by the study conducted in Italy (49.8 %) (Moroni et al., 2005a). This finding can also be compared with the findings of previous study (33.9-35.1%) (Leitner et al., 2004b; Islam et al., 2012a).

Goat level proportionate prevalence of SCM was higher than quarter level, also agreed with another investigation (30% goats and 18% udder) (Contreras et al., 1995). The difference in prevalence of SCM in some of the cited studies and the current study could be due to

difference in management practices, breed, geographic location and study methods (Radostits et al., 2006).

In this current study, coagulase-negative staphylococci (CNS) were the most frequent bacterium encountered. This is in agreement with number of previous findings, which reported prevalence ranging from 60 to 93% (McDougall et al., 2002; Bergonier et al., 2003; Hall and Rycroft, 2007; Min et al., 2007). Coagulase-negative staphylococci was usually isolated from the respiratory tract and the teat skin or the teat-end from milk (Kiossis et al., 2007).

The proportionate prevalence of *Staph. aureus* was 11.3% in the present study which is lower than the study conducted previously in Bangladesh (29.41%) (Razi et al., 2013) and in Bulgaria (19.8%) (Bochev and Russenova, 2005). In other studies, *Staph. aureus* was detected with prevalence of 2 to 13% of all isolated microorganisms (McDougall et al., 2002; Moroni et al., 2005b; Moroni et al., 2005c; Hall and Rycroft, 2007). This pathogen can frequently reach even higher values, peaking to 40% of all isolated bacterial species (Ameh and Tari, 1999; Leitner et al., 2007), and in some cases it was reported as the most frequently occurring species among all isolates (da Silva et al., 2004).

The proportionate prevalence of *Pseudomonas* spp. was 23.1% in the present study which is well agreed with an earlier study performed in Algeria (27.6%) (Bourabah et al., 2013) but higher than in Italy (3%) (Marogna et al., 2012).

The present study identified 16.9% proportionate prevalence of *Bacillus* spp. It has also been identified from the caprine subclinical mastitis quite frequently (Kalogridou-Vassiliadou, 1991; Kostelič et al., 2009).

5.2. Discussion on risk factor analysis of subclinical mastitis in goat in relation to California Mastitis Test

The current study evidenced of that goats being purchased from other sources are 2.2 times more likely to be positive in CMT than the own farm raised stock (95% CI: 0.9- 5.3). The results herd immunity of goats in certain farm environment could be the reason for the increased risk. This result is supported by Moroni et al. (2005b). They found 4 times higher

odds of SCM in goats being collected from external sources than internal own stock. The lower odds of SCM in own stock goats could be due to develop herd immunity somehow against mastitis pathogens (Schukken et al., 2011).

A higher odds of SCM was found in goats which were managed under poor floor having plastic and jute bag (OR:3.7; 95% CI: 0.8-16.7) than those managed with floor having bedding materials of wood only or no bedding materials and this result could be attributed to the presence of environmental mastitis pathogens with reservoirs in mud (Ali et al., 2010). The floor surface is a potential hazard to the animal; mud and excessive moisture increases likelihood of organism contaminating udder (Ndegwa et al., 2000; Megersa et al., 2010). This observation could be explained by the fact that dirty mud and moisture would be easily attached with jute bag and plastic bag retain the moisture of urine, feces for long time tends to harbor a wide range of infectious organisms that may also contaminate the udder and teats. Based on these findings, we can suggest using either dry raised floor or concrete floor to rear goats in order to reduce the prevalence burden of subclinical mastitis.

Parity was also determined as a risk factor for SCM in the present study. Goats with 3-15 parities had 2.0 times more odds of SCM than that of goats with 1-2 parities (95% CI: 0.9-4.5). The increased incidence of SCM in relation to advance parity and stage of lactation has been reported by different author in case of goat (Paape and Contreras, 1997; Luengo et al., 2004; Paape et al., 2007). Subclinical mastitis in goats increases significantly with increasing days in milk which is correlated with the results of Zeng and Escobar (1995) who reported the increased somatic cell count in goat with the parity but Paape et al. (2007) reported no relationship of SCM with parity. (Wilson et al., 1995; Paape et al., 2001; Stuhr et al., 2013). The increase in SCC with increasing parity is due to increasing bacterial presence or cumulative mammary gland stress (Boscos et al., 1996; Paape et al., 2007).

The current study found that the goat in later lactation period was 5.3 times more likely to be positive in California mastitis test than the early lactation period (95% CI: 1.9-14.9). It is well known that SCM tends to increase during and towards the end of lactation period, without necessarily seeing an increased incidence of IMI during this period (Luengo et al., 2004). At the end of lactation SCC can increase to such an extent that it cannot be distinguished between infected and non-infected udders. e.g., Moroni et al. (2005a) could not find

significant differences in SCC between healthy and infected animals at the end of the lactation period.

The anatomical shape of teat was one of the important risk factors for being positive in CMT in this study. The conical and cylindrical shaped teats were 3.6 times at higher risk of being CMT positive than bottle and collapsed teat (95% CI: 1.1-11.8). The effect of teat shape on SCM in goat is not well documented but a study of Porcionato et al. (2010) on milk flow, teat morphology and subclinical mastitis prevalence in Gir cows reported similar trend.

5.3. Risk factors in correspondence to binary response variable of different organisms at sample level

In the current study, coagulase-negative staphylococci (CNS) infection was significantly higher in own stocked goat than external sources ($p=0.009$). Different host related (immunity, infection, body condition and teat shape) and environmental factors (management, grazing system etc.) might be responsible for the significant difference (El-Jakee et al., 2013). Coagulase-negative staphylococci infection was higher in poor and fair in comparison to cachectic goats in this study. The rearing system has a significant role in positivity with different organisms. The goats under tethered system of rearing had the highest risk of being positive with CNS, *S. epidermis*, *Pseudomonas* spp. The environmental exposure of pathogens in a confined grazing land in tethered area might cause the increased infection (Megersa et al., 2010).

Pseudomonas infection was also significantly higher in those goats where floor cleaning performed weakly in this study. Yeruham et al. (2004) reported as *Pseudomonas* spp. associated outbreak of mastitis cases in sheep and goat related with overcrowding, wet bedding and lack of water facility to clean the floor. Therefore, cleaning of the floor once in a week might increase the chance of infection with *Pseudomonas* spp. Thus, this finding calls for frequent cleaning and drying of goat houses in order to reduce udder infections.

5.4. Farmers' and farms' demography and management traits of goat farms

Regardless of types, most of the goat farmers had secondary level of education (30.7%) with 32.9% farmers was illiterate in the present study. These results suggest that most of them can

easily understand and read Bengali literature easily; therefore it would be easy and efficient to educate goat farmers on structured goat farming system by providing simple leaflet and manual with the aim of reducing the risk of SCM as well as clinical mastitis.

Intensive, semi-intensive and traditional (free ranging and tethered) goat rearing system are commonly practiced among the study area where the respective median farm population size was 5, 6 and 3. As there is limitation of hygienic management practiced at traditional farming therefore, it could have high risk of having SCM in goats which is also supported by the study conducted at Mymensingh (Razi et al., 2013).

The present study identified that the floor of goat house was constructed with concrete and bricks (17.0%), plastic and jute bag (22.7%) and wood (60.3%). Concrete floor is easy to clean up in comparison to wooden floor specially floor having plastic and jute materials.

This observation could be explained by the fact that dirty and wet bedding, which was a common finding on the earthen floors, tends to harbor a wide range of infectious agents, which may contaminate the udder and the teats (Razi et al., 2013).

5.5. Limitations of the study

- i. **Sample size:** The study was conducted in 88 smallholder goat farms only. The sample size was small which might have introduced lower statistical power and bias on the reliability of estimates in the study.
- ii. **Sampling techniques:** The studied farms were selected conveniently based on the location and accessibility to the farms. Therefore, the sampling techniques might induce the selection bias in the study which might not have reflected the population from which it was drawn. The convenient sampling approach may reduce the external validity of the study.
- iii. **Recall bias during information recoding:** The study used structured questionnaire to record the response on variables from the farmers/ attendance of the farmers. The response might have introduced recall bias which could affect the study results by overestimating the odds or vice versa for instance.
- iv. **Diagnosis bias:** The diagnosis was done by using California Mastitis Test for detection of subclinical mastitis in goat. So poor sensitivity and specificity of the CMT could

introduce diagnosis bias during the laboratory testing which could under estimated or overestimated the true prevalence of subclinical mastitis in the study population.

- v. Financial constrains: Due to limitation in the study funding, the larger sample size and random sampling was not possible for the study. deLaval cell counter for measuring SCC could not be used here which have more sensitivity and specificity. The molecular diagnosis of the identified organism was not performed also in the study.
- vi. Absence of goat database: In the regional livestock authority there is no reliable goat farms and population data base which might help to conduct the study in a more scientific and systematic ways

Chapter VI: Conclusions, Recommendations and Future Directions

6.1. Conclusion

The goat farming was practiced among diverse professional groups mainly with lower and marginal income group of people with no education (32.9%) or primary education (14.8%) background in the study areas of Chittagong metropolitan city. Intensive, semi-intensive, free range and tethered goat rearing practice were available where the median farm population size 5, 6 and 3, respectively in the study area.

Regardless of production systems Cross (72%) and Jamnapari goats (24%) were dominant breeds. Goat house was made of wood as prime material (60.7%). Goats were fed with easy available feeds like grass and pea husk.

The proportionate prevalence of SCM was 50% in goats and 46.2% in udder quarter. The most prevalent organism was coagulase-negative staphylococci (CNS) (35.4%) followed by *Pseudomonas* spp. (23.1%) and *Staphylococcus aureus* (11.3%).

The poorly managed goat having plastic and jute bag on the floor ($p=0.08$), goats with more than third parity ($p=0.09$), does with conical and cylindrical teat shape ($p=0.03$), goats having late lactation period ($p=0.002$) and purchased goat from external sources ($p=0.07$) were significant risk factors for being positive for subclinical mastitis at udder quarter level.

The proportionate prevalence of *Pseudomonas* was higher in floor cleaned once weekly (50.0%) than floor cleaned once daily (23.7%) and more than once daily (15.2%) ($p=0.06$).

6.2. Recommendations:

1. The current study found that goats with higher parity and late lactation period are more susceptible to subclinical mastitis. Proper feeding along with health care should be ensured to prevent subclinical mastitis in goat.
2. The management practice of farm cleaning is significantly related with higher prevalence of mastitis in goat. Clean dry raised floor or concrete floor is recommended for goat housing.

3. Cleaning the floor more frequently is also recommended for prevention of mastitis in goat.
4. In the current study most of the goat farmers have the secondary level of education. They can easily be trained by providing training of simple leaflet about good management practice for goat farming.

6.3. Future direction:

1. A longitudinal study with larger samples to understand the seasonal and environmental factors associated with the sub clinical mastitis in goats can be conducted.
2. Somatic cell count should be measured by using deLaval cell counter.
3. Advance molecular diagnosis with genetic sequencing of the causal agent could be helpful for better understanding the molecular epidemiology of SCM.
4. Antibigram of identified organisms could be conducted to choice the drugs for mastitis management and drug management.
5. A large scale cross-sectional study and repeated cross-sectional study should be conducted to explore the results that would be more generalized and to assess temporal variations of SCM.

Chapter VII: References

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Appendix- I

Collection and transportation of samples

Samples were collected and transported to the laboratory according to the National Mastitis Council (Council, 1999). The collection procedure was as follows: grossly dirty teats and udders were thoroughly washed and dried before proceeding with sample collection. Udder was washed as a last resort. First three or four streams of milk were removed from the quarter being sampled to minimize chances of sample contamination from bacteria in the teat end. Then each teat was dried using single, dry cotton per goat with particular emphasis on the teat end. The teat end and orifice was carefully and vigorously scrubbed with a cotton pad moistened with 70% ethyl alcohol. Separated swab was used for each teat being sampled, even within the same goat. The teat end was cleaned until the swab was completely clean and white. The collection vial was hold at a 45° angle to keep debris (hair, manure, dirt) from accidentally falling into the collection vial. The teat should never touch the collection vial or cap. Then the samples were transported immediately to the laboratory in a special box with ice at 4° C.

Appendix-II

A QUESTIONNAIRE FOR DETECTING SUBCLINICAL MASTITIS

Hospital information

Hospital Reg no: Date:/...../..... Diagnosis:

Farm level information

Farm ID no (FID): Goat ID no (GID):

Sample ID no (SID)..... &

Name of Owner: Occupation: Education:

Vill/ward: Upazilla:..... Dist:

Contact no:

Source of Animals: Farm borne/ bought

Types of breed reared: Local/BBG/Jamnapari/Cross/Others.....

Farm size: No. of adult does: No. of adult bucks:

No. of goat kids..... No of Pregnant animal:..... No of Lactating animal:.....

Housing system: Intensive/Semi-intensive/Tethered/Free range/ others.....

Feeding regimen: Roughage: Concentrate:..... Others:
.....

Flooring material: Dirt score for muddiness:1/2/3/4

Dirt score for faeces:1/2/3

Cleaning frequency of the floor:

Cleaning frequency of the feeding trough:

Cleanliness of the feeding trough: Clean/not Clean No. of animals per square meter:
.....

Animal information

Type of Breeding: Natural / AI Date of parturition:/...../..... No. of parity:

Days in milk:

Age:..... D/M/Y Body weight: kg BCS : 1/2/3/4/5

Color:..... Breed:..... Milk yield:ml/L

RectalTemp: °F

Milking of Doe: Yes/No If yes sanitary measures used during milking: Yes/No

Antiseptic used during milking: yes/no If yes what:

Date of occurrence of mastitis: .../...../..... Type: Clinical/ gangrenous/ subclinical

Symptoms:

.....

Any previous treatment used for current illness: yes/no

If yes which drug used:

Previous history of mastitis: Yes/No

Date of occurrence: Treatment:.....

History of Periparturient disease: Yes/No

If yes what:.....

Drug used for periparturient disease:

.....

History of udder edema: Yes/No

History of previous other diseases:

Treatment of animal by the help of : Vet/VFA/Self

Udder cleanliness: 1/2/3/4 ; Thigh cleanliness: 1/2/3/4; Leg cleanliness: 1/2/3/4

Body Length: cm Body Height:..... cm Body Width:cm

Udder Information

Udder base: Above hocks/below hocks Right Teat place: vertical/outward

Left Teat place: vertical/outward

Right Teat size: normal/larger than feast Left Teat size: normal/larger than feast

Distance of teat from floor: Left.....cm Right.....cm Distance of udder base from floor:
.....cm

Teat end callosity thickness: None/Thin/ Moderate/ thick /extreme ring

Right Teat end callosity roughness: Smooth/rough ring

Left Teat end callosity roughness: Smooth/rough ring

Rt Teat end shape: rounded/pointed / flat/inverted Lt Teat end shape: rounded/pointed / flat/inverted

RtTeat shape: Cylindrical/bottle/conical/short/collapsed/fleshy/pencil

RtTeat shape: Cylindrical/bottle/conical/short/collapsed/fleshy/pencil

Any defects of udder: redness/swelling/warmth/painfulness/firmness/fibrous tissue

Unevenness: Right Quartert.....Left quarter.....

Skin problems on udder:

Grading of CMT Right: 1/2/3/4/5 Left: 1/2/3/4/5

Longitude: N.....

Latitude: E.....

Appendix-III

Bacteriological examination of samples

Bacteriological examination was performed to isolate and identify microorganisms by culture of samples, staining reaction and motility test and biochemical properties of organisms.

Inoculation of samples into different culture media

About 100 µl of milk samples was inoculated into nutrient broth (NB) and incubated under aerobic condition at 37°C overnight for growth of organisms. After that, culture and subculture was performed into different media gradually such as Blood agar (BA), Mannitol salt agar (MSA), MacConkey agar, Eosin-methylene blue (EMB) agar and Brilliant Green (BGA) agar as described by Cheesbrough (1981).

Isolation and identification of organisms from milk sample

Isolation and identification of the bacteria was performed on the basis of culture characteristics, microscopic examination for motility, morphology and staining reactions and on the basis of biochemical tests. Bacterial isolation was performed according to (Cheesbrough, 1981); Quinn et al. (2011).

Staphylococcus aureas

Each milk sample was first inoculated into NB and incubated at 37° for 24 hours. Loopful aliquot was taken from nutrient broth and streaked on different media. All inoculated media were incubated at 37° C for overnight. On blood agar, colonies of *Staphylococcus aureas* were golden yellow with beta (β) hemolysis. *Staphylococcus aureas* produce growth of yellow colonies with yellow zones on mannitol salt agar (Kateete et al., 2010). On nutrient agar colonies were grey white to yellow. They were Gram-positive cocci in clusters resembling bunches of grapes and non-motile. Bacteria were catalase test positive. Coagulase test was used to differentiate *Staphylococcus aureas* which produces the enzyme coagulase, from coagulase negative Staphylococci. *Staphylococcus aureas* produces acid from glucose, lactose, maltose, mannitol, sucrose and glycerol but not from salicin, raffinose and insulin.

Coagulase negative staphylococci (CNS)

Each milk sample was first inoculated into NB and incubated at 37°C for 24 hours. Loopful aliquot was taken from nutrient broth and streaked on different media. All inoculated media

were incubated at 37°C for overnight. On blood agar, colonies of CNS were moderately-sized (up to 4 mm in diameter) white or golden color. Most species of CNS were non-hemolytic. Coagulase negative Staphylococci produced small red colonies with no color change to the medium on mannitol salt agar. They were Gram-positive cocci in clusters resembling bunches of grapes and non-motile. Bacteria were catalase test positive and coagulase test negative.

Coagulase test

Whole blood from horse was collected into commercially available sterile tubes containing EDTA to perform the test. Then blood was centrifuged at 2600 rpm for 10 minutes using a refrigerated centrifuge device. Resulting supernatant, the plasma, was then immediately transferred to a sterile 1.5 ml eppendorf tube using a sterile tip and stored at -20°C for future use (Hogan et al., 1999).

Tube coagulase test

From each tube cultivated in Brain Heart Infusion Broth, 50 µL was transferred to sterile tubes containing 50 µL of horse plasma. The incubation was done at a temperature of 37°C for 6 hours. The presence of coagulates was justified, considering large organized coagulation and coagulation of all the contents of the tube which do not come off when inverted. A control tube also was placed to validate the result.

Slide coagulase test

Staphylococcus sp (which were confirmed by tube coagulase test) were further confirmed by slide coagulase test. One drop of the horse plasma was placed on a clean grease free glass slide. A loopful of suspected culture was mixed with plasma separately and checked for agglutination. The cultures showing agglutination were recorded as positive for coagulase test and thus were confirmed as *Staphylococcus sp*.

Tube catalase test

Nutrient agar slant was prepared according to the instructions of manufacturer (Oxoid, England). Suspected bacterial colonies inoculated into agar slant and incubated at 37°C for 24 hours. After that 1 ml of 3% H₂O₂ was added and rapid ebullition of gas considered as positive reaction of *Staphylococcus sp*.

Slide catalase test

Small amount of colony was placed on a fresh, clean and grease free slide. One drop of 3% H₂O₂ poured onto the colony, a cover slip was placed and bubble formation was indicated as positive result.

Streptococcus spp.

After inoculation and incubation of milk sample into NB for 24 hours, loopful aliquot was taken from NB and streaked on different media. All inoculated media were incubated at 37° for overnight. The organisms were alpha or beta hemolytic on blood agar. Most streptococci produce small colonies (about 1 mm after 48 hours incubation) and in the case of the beta haemolytic streptococci the colonies appear translucent. Gram-positive cocci and characteristic chains occur in broth cultures are seen at staining. The streptococci are catalase negative which helps to distinguish them from the catalase positive staphylococci.

CAMP test:

A culture of a *Staphylococcus aureus* with a wide zone of partial haemolysis (betahaemolysin), is streaked across the centre of a sheep or ox blood agar plate. A streak of the suspect Group B streptococcus is made at right angles to, and taken to within 1 to 1.5 mm of the staphylococcal streak. The plate is incubated at 37°C for 18-24 hours. A positive CAMP test is indicated by an arrow-head of complete haemolysis. The Group B streptococci produce a diffusible metabolite that completes the lysis of the red cells, only partially haemolysed by the beta-haemolysin of the staphylococcus.

Bacillus spp.

After inoculation and incubation of milk sample into NB for 24 hours, loopful aliquot was taken from NB and streaked on different media. All inoculated media were incubated at 37° for overnight. On blood agar, colonies were appeared dull, opaque, grayish-white with an irregular border from which long strands of cells are seen in parallel arrangement, giving the typical “ medusa head ” appearance. The organisms were beta hemolytic on blood agar. On Gram staining, large, Gram-positive rods, cells occur singly, in pairs or in long chains. They were catalase test positive and oxidase-negative.

Eshcherichia coli

Pre-enrichment of *E. coli* was done in NB broth (Oxoid ltd, Basingstoke, Hampshire, UK). A loopful of culture inoculates on MacConkey (Oxoid ltd, Basingstoke, Hampshire, UK) agar. Pink colonies obtained from MacConkey agar were taken and inoculated on Eosin methelene blue (EMB) (Oxoid ltd, Basingstoke, Hampshire, UK) agar to verify whether the bacterial population was *E. coli*, or not. Dyes Eosin and Methylene Blue react with products released by *E. coli* from lactose or sucrose as carbon and energy source, forming metallic green sheen regarded as positive isolate. Various biochemical tests were performed for confirmation of *E. coli*.

Enterobacter spp.

Each milk sample was first inoculated into NB and incubated at 37° C for 24hours. Loopful aliquot was taken from nutrient broth and streaked on different media. All inoculated media were incubated at 37°C for overnight. On MacConkey agar the colonies were large, mucoid and surrounding medium was pink due to acid production from lactose. The colonies were large, mucoid, blue-black on EMB agar. A yellow (acidic) color in the slant and butt of the TSI agar tube was observed with gas (CO₂) and without H₂S production indicated *Enterobacterspp*. Microscopic examination revealed Gram-negative, pink color, motile rod bacterium. They were oxidase negative, indole test negative, MR test negative and VP test positive. The bacteria fermented dextrose, sucrose, lactose, maltose and mannitol with the production of acid and gas.

Pseudomonas spp.

Each milk sample was first inoculated into NB and incubated at 37° C for 24hours. An abandon growth occurred in nutrient broth with the formation of a thick pellicle, dense turbidity and heavy sediment. The medium usually became green which changes to brown as the culture ages. . Loopful aliquot was taken from nutrient broth and streaked on different media. All inoculated media were incubated at 37° for overnight. Growth of the *Pseudomonas aeruginosa* on nutrient agar plate shown bluish green coloration due to the pigment Pyocyanin. Lactose-negative, pale colonis were developed on MacConkey agar. On EMB agar colonies were irregular colorless or pinkish mucoid. No color change of butt and slope of TSI agar tube. Microscopic examination showed medium-sized, Gram-negative rods, actively motile by polar flagella. They were catalase positive, oxidase positive, indole, MR and VP tests negative.

Brief Biography

Sazeda Akter passed Secondary School Certificate Examination (SSC) with GPA 4.88 in 2005 and Higher Secondary Certificate Examination (HSC) with GPA 4.50 in 2007. DR. Sazeda Akter achieved her Doctor of Veterinary Medicine Degree with GPA 3.83 in 2012 (held in 2014) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, she is a candidate for the degree of MS in Medicine under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU. She has boundless interest to work in mastitis in food animal.