Chapter-1: Introduction

Bulk milk is the total amount of milk from all cows that is intended for sale. A bulk milk sample is the small proportion collected from this. Milk is a highly nutritious food and an ideal medium for growth of microorganisms (Pereira et al., 2010). High bacterial count in bulk milk decreases milk quality and shelf life and can be a health hazard for the consumer. The bacteria can come from the udder, but more often as a contaminant during or after milking and handling. Thus, increased bacterial counts can affect the quality of pasteurized milk and milk products (Barbano et al., 2006). The presence *Staphylococcus* (*S*.) *aureus* in raw milk is very common. *Staphylococcus aureus* can produce heat-stable enterotoxin and may be present even in pasteurized milk (Zinke et al., 2012) and thus milk having high bacterial counts (having *S. aureus* specially) may not be safe for human consumption (Hoekstra et al., 2018). Zoonotic pathogens related with bulk milk can be transmitted to humans through direct contact with animals, farm workers, or through contaminated farm environment (Leitner et al., 2003). To ensure safe milk for consumption as well as to decrease transmission of zoonotic pathogen, bulk milk evaluation is necessary.

Milk contains very low number of microorganisms when it leaves the udder of healthy cows (Wallace et al., 2009). Raw milk can be contaminated at farm level through the milking machine, external surface of udder and teats (through bacterial contamination) and within the udder from mastitis organisms (Murphy and Boor, 2000). External surface of the cow's udder and teats may contribute microorganisms as natural inhabitants and can originate from the environment where the cows are housed and milked (Wallece et al., 2009). Cow's with heavily soiled teats may contribute >10,000 CFU/mL of milk. Healthy udders contain less than 1,000 colony forming units (CFU) of total bacteria per mL of milk while cows affected with mastitis may shed large number of microorganisms into milk (Wallace et al., 2009).

Herd hygiene, udder health of cows, milking conditions, equipment, transportation to selling point as well as health status of workers are related to the microbiological quality of raw milk. (Fonseca and Santos, 2000; Holm et al., 2004). The degree of cleanliness and the sanitizing process during milking can influence the bacterial counts in bulk milk (Wallace et al., 2009). Coliform bacteria can be found due to poor sanitation around shed, improper handling, and contamination of sample by unhygienic water (Sanderson et al., 2015).

Total bacterial count (TBC) is the count of the number of bacterial colony-forming units present in the milk sample. It is a quantitative measurement and the most useful method for estimating the total number of bacteria in milk (Ruegg & Reinemann, 2002; Biyani et al., 2018). Hygienic and cleaning conditions in milk production can be measured by TBC as well as handling milk in the farm and adequate refrigeration can also be detected (Guerra et al., 2013). It has been reported that the sanitary requirements during production and processing of milk are not satisfactory in Bangladesh (Addo et al., 2011; Marjan et al., 2014). Due to poor hygiene proliferation of pathogens in milk and milk products, it has become an important concern. Lack of drainage system, absence of floor cleaning, limited use of foot bath are responsible for contamination of bulk tank milk by *S. aureus* as well as other mastitis pathogens through infected udders during handling of raw milk (Jorgensen et al., 2005; Scherrer et al., 2004).

The highest TBC found was 1.2×10^6 CFU/mL and the lowest TBC was 3.6×10^5 CFU/mL in different brands of pasteurized milk in Bangladesh (Ahmed et al., 2019). In south India the highest TBC was found in the eastern region with 13.9×10^6 CFU/mL and the lowest value was detected in the western region with 11.7×10^6 CFU/mL of milk (Lingathurai & Vellathurai, 2010). In Nepal, the maximum TBC was 13.3×10^6 CFU/mL and the minimum value was 2.8×10^6 CFU/mL in milk at different milk pocket areas (Dahal et al., 2010). According to Sri Lanka Standard Institute (1983), the expected standard for TBC is less than 30×10^4 CFU/mL (Gunasena et al., 2021).

Bulk milk may contain contagious as well as environmental pathogens such as *Streptococcus agalactiae, Streptococcus dysgalactiae, S. aureus, Mycoplasma* spp., *Escherichia coli, Klebsiella* spp. and *Corynebacterium bovis* (Jayarao and Wolfgang, 2003). *Staphylococcus aureus* is a common microorganism isolated from bulk milk in cases of mastitis and after contamination. *Staphylococcus aureus* can easily be transmitted through the food produced from raw milk handled in poor hygienic conditions (De Buyser et al., 2001).

Prevalence (percentage of the herds) of *S. aureus* from bulk milk was 23.5% reported in a study in Bangladesh (Jahan et al., 2015). In a study of India, 68% herd prevalence was recorded (Bharathy et al., 2015). Herd prevalence of *S. aureus* estimated from bulk milk was 84% found in a study of USA (Haran et al., 2012) where as in Hungery, 70% and in Korea 5.6% of prevalence was reported for *S. aureus* (Peles et al., 2007; Moon et al., 2007).

Methicillin resistant *Staphylococcus aureus* (MRSA) is multi-drug resistant and identified as an emerging pathogen that can be isolated from bulk tank milk. People working on dairy farms are at risk of getting infected with MRSA while handling cattle as well as personnel with MRSA can infect the cows and contaminate the milk (Juhász-Kaszanyitzky et al., 2007; Spohr et al., 2011). Veterinarians can get infection from animals while handing them at farms, clinics and hospitals and they can transmit infections to contacting persons and their patients as well (Saleha and Zunita, 2010). So far, limited information is available on the prevalence of MRSA in dairy herds in Bangladesh. Eight raw milk sample collected from different areas of Dhaka city contained MRSA (Nusrat et al., 2015). Previous studies from northwest India and Chennai reported the MRSA positive percentages as 13% and 10.9% respectively (Kumar et al., 2011; Chandrasekaran et al., 2014). A study from Minnesota found 4% prevalence for MRSA in dairy herds (Haran et al., 2012).

Somatic cells are mostly cells of body defense mechanism, and their presence indicates inflammation (Schukken et al., 2003; Sordillo et al., 1997). The level of inflammation in the udder can be measured by the somatic cell count in milk. To get a rough estimate of the herd udder health, the bulk milk somatic cell count (BMSCC) can be measured (Wilson et al., 1997). Season, housing system, stage of lactation, age of cows, drying off, infection status of the cows´ udder, hygienic management in the herd can influence the BMSCC (Nyman et al., 2007; Peeler et al., 2002; Barkema et al., 1999). Milk produced by mastitic cows often contains more than 100,000 cells/ml of milk (Kehrli and Suster, 1994). Increased levels of somatic cells and bacteria are associated with increased enzyme activity which can result in impaired milk quality (Ismail and Nielsen, 2010). For ensuring good quality milk and milk products, BMSCC standards have been set by different countries. For example, standard BMSCC for dairy products requires <400,000 cells/mL in New Zealand, EU and Australia, <750,000 cells/mL in the USA and <500,000 cells/mL in Canada. (Sargeant et al., 1998; Norman et al., 2000; Van Schaik et al., 2002). There are no such benchmarks for Chattogram as well as Bangladesh. In a recent study, BMSCC was more than 400,000 per mL of milk in 50% of the farms (Singha et al., 2019).

In Bangladesh, limited studies are available on somatic cell count and total bacterial count and no established BMSCC for raw milk has been set. A few studies on prevalence of *S. aureus* as well as MRSA are reported for bulk milk in Bangladesh. This study will provide information about levels of BMSCC, TBC, *S. aureus* and MRSA in bulk milk from dairy farms in Chattogram, a region in

Bangladesh. The results will hopefully give the Bangladeshi dairy industry tools for benchmarking milk quality control to ensure food safety of milk and milk products.

1.1.Objectives:

i. To determine quality of bulk milk by measuring SCC and bacteriological examination.

ii. To estimate prevalence of *S. aureus* in bulk milk.

iii. To determine the proportion of MRSA isolated from *S. aureus.*

iv. To estimate the relation between BMSCC and farm factors.

Chapter-2: Review of Literature

Relevant literature has been reviewed in this section. This chapter contains development of dairy farming in Bangladesh, bulk tank milk analysis and available screening tests, somatic cell count (SCC) associated risk factors at various level, monitoring udder health, contagious mastitis pathogens and their prevention at herd level, zoonotic importance, and emergence of methicillin resistant *Staphylococcus aureus* (MRSA) and its incidence in Bangladesh with relevant literatures. The objectives of this chapter is to relate the previous studies and to define the importance of conducting the present study.

2.1. Dairy Farming in Bangladesh

2.1.1. Development of dairy sector

Bangladesh is a densely populated country and there is a major gap between demand and production of milk for the people of this country (Shamsuddoha and Edwards, 2000). As a result, dairy farming has attracted young, educated people in Bangladesh. To produce more milk, dairy farming in Bangladesh is now established as an emerging sector and around 85% people of Bangladesh are involved in the agriculture and livestock sector (Raha, 2000). Agricultural sector contributes 14.77% of GDP and livestock sector contributes 1.66% of GDP (BBS, 2017). Bangladesh has a cattle population of about 23.1 million. The population of dairy cows is about 6 million, 10-15% are cross breeds and 85-90% are local or indigenous (Barua et al., 2018). Cow milk contributes about 90% of milk produced in Bangladesh (Datta et al., 2019) and smallholder dairy farms dominate about 70% of the existing farms (Uddin et al., 2012). Due to a rapid increase of the population, demand for milk as well as dairy farms are increasing in Bangladesh.

2.1.2. Dairy cattle rearing and milk production

Several breeds of cattle are reared in backyard and commercial settings of dairy farming in Bangladesh such as: Sindhi, Sahiwal, Jersey, and Holstein Friesian breeds (Miazi et al. 2007). According to Bangladesh Dairy Farmers' Association (BDFA) there are about 1,200,000 dairy farms in Bangladesh (The Dhaka Tribune, 2019) where 58,590 farms having 10 or more cows are registered with DLS (Department of Livestock Services) (The Daily Star, 2018). Traditional milk

production areas are Sirajganj, Pabna and in last decades, Gazipur, Savar, Rangpur, Jashore have been introduced as new dairy zones (Figure 1). Most farmers use rice straw, jamboo and napier grass as roughage and bran, broken maize, and til oil cake as concentrate feed. Some farmers also use ready feeds for dairy cows (Talukder et al, 2019). In recent years, milk production increased from 7.0 million metric ton in 2014-2015 to 9.9 million metric ton in 2018-2019 (Annual Report, Ministry of Fisheries and Livestock, 2019) (Figure 2). Artificial insemination is extensively practiced in Bangladesh to transform local indigenous cows to more high yielding cross-bred dairy cows to meet demand of milk (Livestock Directory, 1992-93.).

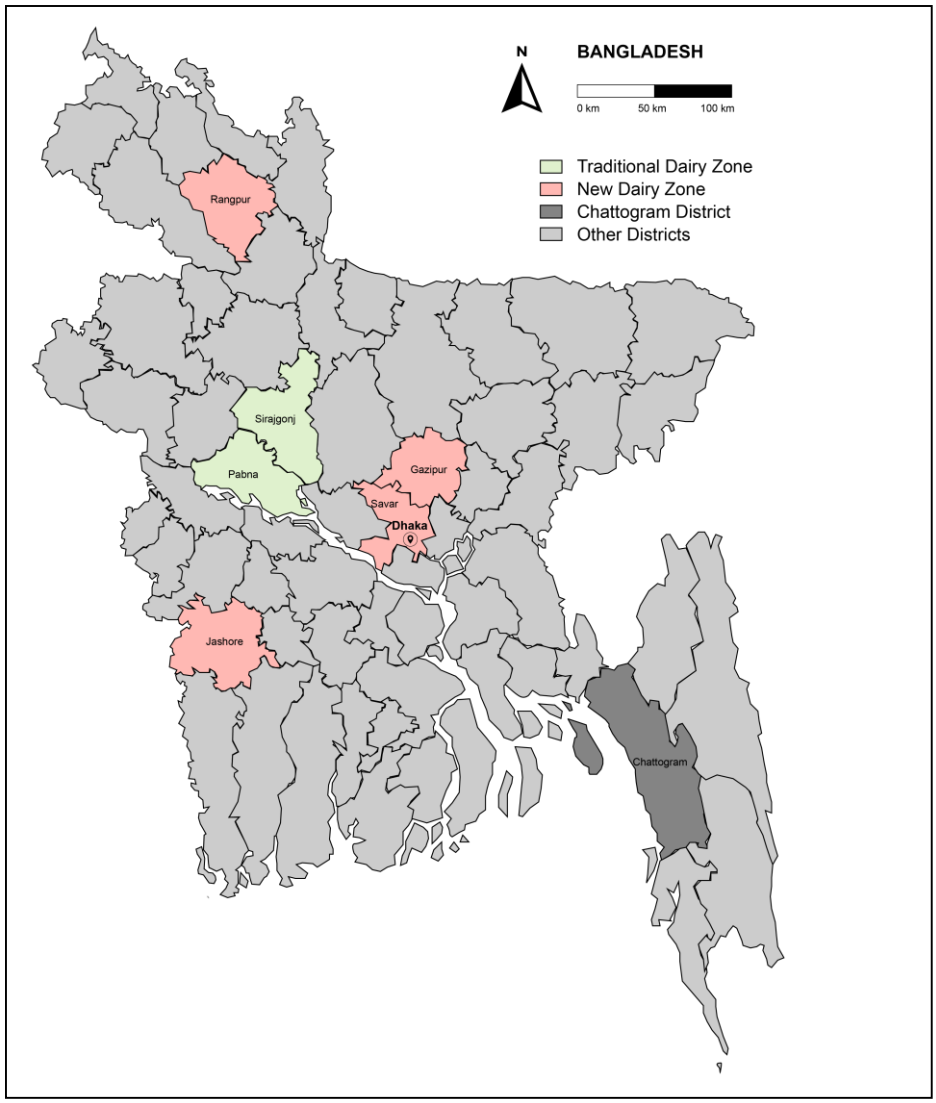

Figure 1: Major milk producing areas in Bangladesh (Talukder et al, 2019).

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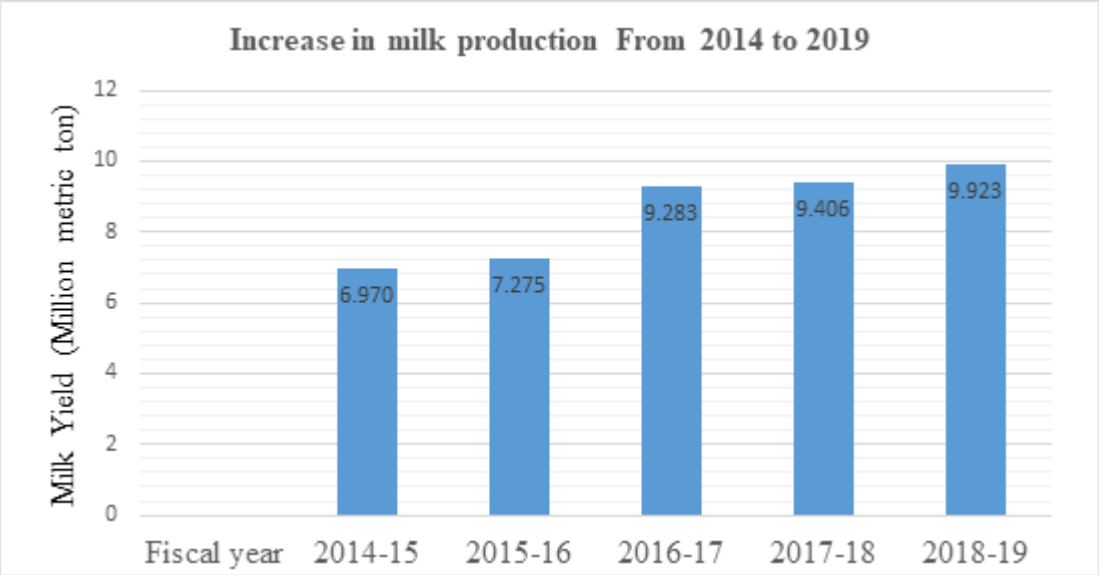

Figure 2: Milk production trends in Bangladesh in recent years (Annual Report, Ministry of Fisheries and Livestock, 2019).

2.1.3. Dairy farming and employment opportunities

To reduce unemployment as well as poverty in Bangladesh, dairy cattle rearing has been intensified. It became a good source of income for landless, smallholders and marginal farmers (Saadullah et al., 2002). Around or more than 60% of the families are involved in dairying as subsidiary occupation (Shamsuddoha and Edwards, 2000). More than 70% of the farmers in Bangladesh are smallholder and they produce 70-80% of the country's total milk (Uddin et al., 2011). Presently, 47% of the farmers in Chattogram and 53% farmers in Mymensingh have chosen dairy farming as their main source of income (Khan et al., 2010).

2.1.4. Milk production and quality in Chattogram

Chattogram district of Bangladesh is one of the major commercial dairy hubs of Bangladesh. In the early 1990's there were some expert veterinarians and local elite people who motivated people to come forward with dairy farming. In the continuation, in Chattogram district there are at least 1,551 dairy farms harboring 0.3 million dairy cattle heads that represent 1.2% of the cattle population in

this country (BBS, 2011). The commercial dairy producers mainly sell the unpasteurized farm produced fluid milk (FPFM) on farm or home delivery and fewer farmers provide their milk through branded and non-branded packaged raw milk (PRM) to Milk vita (Govt. supported milk producers co-operative union) and others mainly to Pran dairy, Arong dairy and Farm fresh milk. About 90% branded packaged milk are processed into pasteurized and 10% processed into milk powder, ghee, and butter (Uddin et al., 2011). However, FPFM were found superior in quality compared to PRM in terms of milk shelf life, solid-not-fat, total solids and usage of preservatives (Haque, 2009). Small holder dairy producers sell milk directly to consumers or neighbors, tea stalls, sweetmeat shops and local markets (Staal, 2006; Uddin et al., 2011). Milk and dairy food products contaminated with MRSA are sold at local markets of Mymensingh. Raw milk and cheese sample collected from different areas in Dhaka city can be a great concern for public health especially for children (Nusrat et al., 2015; Haque et al., 2018).

According to the author's knowledge, there are only a few reports available in small scale microbiological assessment of branded pasteurized milk quality. Ahmed et al., 2019 reported the total bacterial count was between 3.6×10^5 to 1.2×10^6 CFU/mL which exceeded the acceptable zone (<20,000 CFU/ml milk set by the Bangladesh Standard and Testing Institution) (BSTI, 2002; Ahmed et al., 2019). Unacceptable bacterial contaminations were previously reported in Bangladeshi pasteurized milk ranging from 1.8×10^4 to 9.8×10^4 CFU/ml (Hasan et al., 2015). But further largescale studies are needed including physical and microbiological parameters for conclusive reflection of milk quality.

2.2. Bulk milk analysis

2.2.1. Importance of bulk milk analysis

Raw milk may contain a large number of pathogenic microorganisms as it is an ideal medium for growth of bacteria (Oliver et al. 2009). Bacterial contamination may occur at different stage of milk production: inside the udder, outside the udder and through the surface of instruments used during milk handling and storage (Wallace, 2009). Unacceptable levels of hygiene index, inappropriate pasteurization, and presence of antimicrobial residues in BM and adulteration of milk can also increase the opportunity of entering pathogens into BM (Addo et al., 2011). Increased drug resistant pathogens in BM can be a great concern for treating foodborne disease outbreaks (Marjan et al.,

2014). Multiplication of spoilage bacteria in raw milk makes it unsuitable for consumption and further processing in terms of safety and satisfaction of consumer's demand (Nanu et al., 2007). For production of good quality pasteurized milk as well as milk products, good quality raw milk is important (Murphy and Boor, 2000). *S. aureus, Streptococcus (Str.) agalactiae* and *Mycoplasma* spp. are contagious mastitis pathogens that can be found in bulk milk (BM) and their presence can indicate intramammary infection (IMI) in the herd. Environmental mastitis causing pathogens such as coagulase negative staphylococci, streptococci, coliforms and non coliforms can also be found in BM (Jayarao and Wolfgang, 2003; Fox et al., 2005). *Salmonella* spp. is an unusual mastitis pathogen and more important as a fecal contaminant from infected animals and human (Ponce et al., 2008). *Salmonella* spp., *Listeria* spp., *Campylobacter* spp., cause mastitis and are often contaminants from the environment (Fox LK et al 2005). *Staphylococcus aureus* may enter bulk milk both from the udders, the milkers' hands and the milking equipment etc. (Daka and Yihdego, 2012). Identifying *S. aureus* is one of the targets of the present study. This pathogen has been reported to cause subclinical mastitis (SCM) as well as clinical mastitis (CM) in dairy cows (Keefe et al., 1997), sometimes it takes longer time to cure and could lead to chronic mastitis (Peton and Le Loir, 2014). This organism has been reported to be responsible for milk borne diseases in humans (Fadel, 2015). Staphylococcal toxin can cause nausea, abdominal cramp, vomiting and diarrhea in humans (Japoni et al., 2004, Morandi et al., 2010). In this perspective analysis of BM can be useful to observe milk quality and causative pathogens at herd level (Godkin and Leslie, 1993; Olde Riekerink et al., 2006) to ensure milk safety as well as good udder health.

2.2.2. Available screening tests for bulk milk analysis

Bulk milk analysis is mainly based on SCC on farm sites, TBC and bacteriological culture. The other name of TBC is standard plate count (SPC) which is useful to detect how many bacteria are present in bulk milk. Bacterial culture has been used as a quantitative test for identification as well as isolation of causative mastitis pathogens from bulk milk (Gonzalez et al. 1986; Riekerink et al., 2010). Real time PCR assay is also helpful to diagnose mastitis organisms from BM (Bi et al., 2016). Moreover, screening tests are used for antibiotic residues in milk (Yamaki et al., 2004). When antibiotic residues exceed the maximum residue limits in animal originated food, the quality become poor for human health. Their presence in milk may generate antibiotic resistance in human as well as may cause hypersensitivity in sensitive individuals (Schenk and Callery, 1998).

2.2.2.1. Identification of bacteria

2.2.2.1.1. Bacterial culture

Bulk cultures has high test specificity for contagious mastitis pathogens and that is why BM analysis is more suitable for contagious mastitis pathogens than environmental. There are several methods for bacterial culture such as broth culture, agar plate, stab cultures etc (Old and Duguid, 1970; Uruburu, 2003). Bacteriological culture of BM samples can be used for diagnosis of IMI at herd level (Bartlett et al., 1990; Godkin & Leslie, 1993; Gonzalez et al., 1986). It helps to prevent transmission at an early stage through diagnosis of contagious pathogens (Lam et al., 2009). Though it is more time consuming, it is trustworthy and documentation of ensuring milk-quality protocol practiced on the farm (Jayarao and Wolfgang, 2003). Due to low sensitivity of bacterial culture for *Mycoplasma* spp. from a single BM sample, repeated test is recommended for diagnosis (Krick et al., 1997). Some authors emphasized on frequent sampling to detect true estimate of contagious mastitis pathogens (Godkin and Leslie, 1993; Ruegg and Reinemann, 2002).

2.2.2.1.2. Polymerase chain reaction

Mastitis organisms can be identified rapidly through real time PCR having predefined cycle threshold (ct) values and internal controls. These criteria are helpful to differentiate truly positive and negative results (Koskinen et al., 2009). It has high sensitivity and specificity compared to conventional culture and can also identify growth inhibited and dead bacteria. Thus, it became a trustworthy test for identifying pathogenic microorganisms from BM samples (Koskinen et al., 2009). However, PCR has yet to attain extended use due to its high cost in laboratory analysis (Berger et al. 2004; McDonald et al. 2005).

2.2.2.1.3. Matrix assisted laser desorption/ionization

Matrix assisted laser desorption/ionization (MALDI) is a laser energy absorbing matrix used to produce ions through large molecules and they are ionized with minimal fragmentation (Hillenkamp et al., 1991). Due to large mass range time-of-flight mass spectrometer (TOF) is broadly used with MALDI. In case of TOF measurement, pulse laser takes particular shots instead of working in

continuous operation, thus it is suited with MALDI ionization (Xian et al., 2012). MALDI-TOF is used to analyze DNA, proteins, peptides, and sugar as well as large organic molecules and other macromolecules. It is also applicable to identify bacteria where a portion of a microbial colony is taken in a target sample and superimposed with a matrix. The mass spectra are produced which are analyzed by dedicated software and assimilated with stored profiles in enriched databases. MALDI-TOF is based on biochemical tests which are cost effective, quicker as well as more accurate procedure for identification of species of bacteria in microbiological laboratories (Bahr et al., 1992; Sandrin et al., 2013). Furthermore, not only organisms but also antibiotic susceptibility of bacteria can be foretold by MALDI-TOF. Methicillin resistant *S. aureus* (MRSA) can be suspected with the help of a single mass spectral peak (Rhoads et al., 2016).

2.2.2.2. Somatic cell count

Generally somatic cells present in milk in a certain amount. Somatic cell count can be performed through direct microscopic SCC, the Fossomatic and Coulter Counter somatic cell counting method (Lintner et al., 1984). Proportion of SCC in milk may vary with the change of udder status (Östensson et al., 1988). Large number of SCC predominantly neutrophils may accumulate due to microbial action in udder tissue (Harmon, 1994; Kehrli & Shuster, 1994). At the time of IMI, SCC increases as a result of influx of leucocytes into milk (about 90% polymorphonuclear leucocytes) (Jones and Bailey, 2006). In mastitc cows, SCC exceeds 1000, 000 cells/ml in milk (Kehrli & Shuster, 1994; Östensson et al., 1988). The use of SCC is a quick and widely used screening test and became early a useful, trustworthy, and explicit test for diagnosis of SCM (Kitchen, 1981). Bulk milk SCC (BMSCC) is a rough indicator of the herd´s udder health and is a common determinant of milk quality on farms (Elmoslemany et al. 2010; Jayarao et al., 2004).

Subclinical as well as clinical mastitis are indicated by increased SCC (NMC, 2001). But higher prevalence of SCM has more relevance to increased BMSCC, often caused by *S. aureus* and *Str. agalactiae*. (Wilson et al. 1997).Food safety risk may arise from ingestion of bacterial toxins, human pathogens and antibiotic residues; factors that are associated with high SCC in milk (Hogan, 2005).

2.3. Presence of *Staphylococcus aureus* in bulk milk

2.3.1. Sources of *Staphylococcus aureus* in bulk milk

In dairy ruminants, *S. aureus* is one of the most common pathogens causing IMI and mastitis resulting in high economic losses to farmers (Katsuda et al., 2005; Reshi et al., 2015). The udder is the primary reservoir of *S. aureus*. The organism colonizes the skin of teats and thrives in the lesions like wounds, cuts, scabs, frostbites, and warts (Jayarao et al., 2004). Contamination of bulk tank milk due to *S. aureus* mainly occurs via direct secretion infected quarters (clinical or subclinical mastitis), from the skin surface of the udder and teats, from milking equipment or during handling of raw milk (Jørgensen et al., 2005; Scherrer et al., 2004).

2.3.2. Effect of *Staphylococcus aureus* on humans

The world's third most important food borne diseases is caused by *S. aureus* (Normanno et al., 2005; Noskin et al., 2005). Staphylococcal toxins ranging from 20 to < 1000 ng can result in food poisoning and may cause abdominal cramps, diarrhea, nausea, vomiting like symptoms in humans (Morandi et al., 2010).

2.3.3. Emergence of MRSA

The first known MRSA isolates were mentioned in 1961 in a British study. Sporadic hospital outbreaks from 1961 to 1967 were reported in Western Europe and Australia (The University of Chicago Medical Center, 2010). In 1975 MRSA was first isolated from bovine specimens (Devriese and Hommez, 1975). Wide range of use of antibiotics at hospital as well as dairy farms results in evolution of MRSA (Oliveria et al., 2009). They are multidrug resistant including all beta (β)-lactam antibiotics, thus MRSA results in increased rate of hospitalization, increased hospitalization cost and longer treatment times (Noskin et al., 2005; Cosgrove, 2006).

2.3.4. MRSA in animals and personnel working with animals

The presence of MRSA in bovine milk and dairy environments poses a potential risk to farm workers, veterinarians (Lee, 2003; Juhász-Kaszanyitzky et al., 2007), and other farm animals that are exposed to affected cattle (Haran et al., 2012). Currently >20% of all MRSA infections in the human population in The Netherlands are of animal origin (Van Loo et al., 2007). Direct

transmission between cows and humans in Hungary was reported by Juhasz Kaszayitsky et al., (2007).

2.3.5. Public health concern of MRSA

Methicillin resistant *S. aureus* is an important pathogen responsible for mastitis and can be present in BM (Spohr et al., 2011). Contamination of raw milk with MRSA can transmit to farmers as well as people who consume raw milk or can cause an initial contamination in the production chain of raw milk products (Oliver et al., 2009). It was reported by Kluytmans et al., (1995) that 5 out of 21 patients died of the first food borne outbreak of MRSA.

2.4. Somatic cell count in bulk milk

2.4.1. Factors associated with elevated somatic cell counts in bulk milk

2.4.1.1. Physiological factors

Age and number of lactations can influence SCC in milk (Harmon, 1994). When lactation progresses, SCC also increases (Blackburn, 1996; Schultz, 1977). Somatic cell count remains high after parturition and within 4-5 days after calving it starts to decrease at normal level (Barkema et al., 1999). Hamann, (2001), observed that SCC increases if the milking intervals is 4 hours and less than 4 hours. At older cattle and/or at the end of lactation, SCC increases due to increased prevalence of infection and glandular damage from previous infection (Barlett et al., 1990). There are different opinions regarding rise of SCC in milk from healthy cows before drying off. Some authors found increasing SCC and decreasing milk production below 4 kg/day (Bodoh et al., 1976), while others have not found a rise in SCC at drying off (Duttschaever et. al, 1972). Some authors reported the lowest SCC during winter and the highest during summer season (Bodoh et al., 1976; Wegner et al., 1976).

2.4.1.2. Poor udder health

Most inflammatory response in the udder occurs due to entry of pathogenic microorganisms into the mammary gland which may lead to increased BMSCC and later may results in mastitis (Schukken et al, 2003). With the increase of age, prevalence of SCM increases as well (Reichmuth, 1975;

Westgarth, 1975). In multiparous cows, the risk of developing CM increases with increasing parity (Steeneveld et al., 2008).

2.4.1.3. Management

Housing systems of cows have a great effect on somatic cell content in milk. Higher SCC is found in cows maintained in groups in loose housing when the groups are mixed. Somatic cell count increased 4 days after mixing in mixed group from 175 000 to 420 000 cells/mL (Kay et al, 1977). An elevated SCC was observed in cows due to induced stress when they were enclosed in an area (paddock) and /or chasing them with a dog.

2.4.1.4. Technical aspects

Milk samples collected from bulk tanks for SCC can be influenced by hygiene, transportation (farm to laboratory), storage temperature and methods used for counting cellular content (Greer and Pearson, 1976; Sweetsur and Phillips, 1976).

2.4.1.5. Relationship between bulk tank somatic cell count and mastitis prevalence

Concentration of somatic cells in normal milk is less than 100,000 cells/ml excreted through healthy quarters (Harmon, 1994). Increased number of SCC in milk can be used as an indirect measure of mastitis (Reneau, 1986) and significant relationship between organisms responsible for mastitis and increased SCC in bulk milk was studied by many authors (Barkema et al., 1998; Zadoks et al., 2004). They emphasized higher BMSCC in BTM samples containing *Str. agalactiae*, *S. aureus*, and *Str. dysgalactiae* compared to pathogen-free BTM samples. A high BMSCC is often associated with mastitis caused by *Str. agalactiae, S. aureus*, and *Str. dysgalactiae* (Barkema et al., 1998). Significant association was obtained between occurrence of CM and elevated level of SCC (Barkema et al. 1998). Somatic cell count often exceeds 100 000 cells/ml in milk produced by mastitic cows (Kehrli & Shuster, 1994). Clinical mastitis caused by *S. aureus*, *Str. agalactiae* and coliforms are responsible for increased SCC (Eberhart et al., 1979). On the other hand, *Corynebacterium bovis* and non-aureus staphylococci are responsible for moderate increase in SCC (Harmon, 1994). Somatic cell count of 200,000 cells/mL in bulk tank milk indicates 6% quarter level infection (NMC, 2016). According to NMC, 2016, a herd with a BTSCC of 500,000 cells/mL is associated with 16% infected quarters with 6% loss in milk yield. This value was compared with

a herd having a bulk tank SCC of 1,000,000 where 32% of quarters could be infected and milk yield loss was estimated at 18% (Bramley et al., 1996).

2.4.2. Effects of high BMSCC

2.4.2.1. Milk production

The relation between SCC and milk production became an asset to measure loss of milk production due to udder diseases (Bartlett et al., 1990). High SCC due to inflammation in udder tissue results in reduced milk production and causes huge loss to the milk producer. It is advantageous for farmers as well as milk processors if they can maintain a maximum low level of SCC in milk. According to Jones (1986), 0.6 to 1 million cell count/mL milk is related with a reduction of 8% to 12% milk production in herd. Nielsen (2009) found an association with SCC of 5 million cell count/mL with daily milk production loss from 0.7 to 2.0 kg milk in primiparous cows and from 1.1 to 3.7 kg milk in multiparous cows. Changes in milk quality and composition are associated with additional loss. On the other hand, good quality milk and higher milk yield can be obtained through maintaining lower SCC (Jones, 1986).

2.4.2.2. Milk composition and quality

Due to mastitis, milk secretory cells lose cellular functions which can interfere with lactose, fat, and protein synthesis (Schällibaum, 2001). Thus, milk becomes less suitable for consumption and processing and remains unsuccessful to meet the consumer's satisfaction (Nanu et al., 2007). When SCC and bacteria increase in raw milk, enzyme activity also increases which results in deterioration in milk products. Somatic cell count above 100,000 cells/mL causes reduced cheese yield and more than 400,000 cells/ml will cause defects of texture and flavor in cheese and other products (Murphy et al., 2016). An effect of SCC was found on characteristics of manufactured yogurts (Le Maréchal et al., 2011) where some studies found that increased SCC can lower sensory quality of manufactured yogurt (Murphy et al., 2016). So, to maintain excellent shelf life of manufactured yogurt, SCC level should not exceed 400,000 cells/mL of milk (Andreatta et al., 2007). Rise in whey protein and decrease in casein associated with higher SCC leads to lower cheese yields. Moreover, increased lipolysis in milk is related with mastitis or high SCC due to lipoprotein lipase activity.

Lipolytic activity in milk also results in increased free fatty acids (FFA) which may cause rancidity and flavor defects of milk and dairy products (Murphy et al., 2016).

2.4.3. Somatic cell count and monitoring udder health

For monitoring udder health, a particular goal for SCC should be set. There must be available data of SCC in the farm so that results can be measured. The farmer should be motivated about the importance of record keeping as well as setting SMART goal for SCC as he/she has to work for. If bulk milk SCC remains around 350,000 cells/ml for many years, it is not possible to set SCC <100,000 cells/ml for upcoming years (Lam et al., 2011). When goals become unrealistic to achieve, it could be a demotivation and loss for the farmers (Schukken et al., 2003). Acceptable threshold level of SCC should be set as well as achievable time for the result should be detected (Lam et al., 2011). Proposed goal for bulk milk SCC can be set less than 200,000 where mastitis incidence is below 20% (Schukken et al., 2003).

2.4.4. Guidelines for monitoring BMSCC and other data

2.4.4.1. Entry into the milking herd

Before entering a milking herd, new heifers as well as cow of several parity must be monitored to ensure entry of clean animals into the herd. Culture results from them should be evaluated for monitoring. Contagious pathogens such as *S. aureus*, *Str. agalactiae* and *Mycoplasma* spp. should be monitored as preventive udder management (Neave et al., 1969). To evaluate udder health status of new animals into the herd, SCC data in the first milking after calving can be used. Preferred goals can be set as SCC over 200 000- 250 000/ml milk (in <10% new heifers). For further analysis of udder health issues, housing systems as well as hygiene status of heifers and dry cows can be observed (Peeler et al., 2000).

2.4.4.2. New infections

New infection data can be obtained through recording and sampling of CM cases which can be enlisted as the number of cows with at least one clinical case per lactation. For monitoring SCM, SCC data can be monitored repeatedly (Becker, 1989; Schepers et al., 1997). Risk factors data obtained from new infections: milking procedure observation, teat end quality and cow udder

hygiene score, evaluation of culling animals having chronic infection and evaluation of herd environment (Schukken et al., 2003) should be critically evaluated.

2.4.4.3. Cure

Treated animals should also be monitored for success. Cows those were containing high SCC during previous test day and were treated at lactation should have at least a 50% chance of having a low SCC at current test day (Sol et al., 1994). Susceptibility patterns of bacteria isolated from infections (clinical and subclinical) must be monitored to evaluate the treatment protocols and existing antimicrobial resistance situation. Recent cases of clinical mastitis data including cow ID, lactation number, current days in milk, date of calving, mastitis date and treatment remark can be recorded to monitor cure rate from bacterial infection (Sol et al., 1994; Sol, 2002).

2.4.4.4. Culling

Culling can be evaluated by assessment of culling data and reason for culling, estimating the mean SCC. In a herd, length of high SCC record of cows in the current and previous lactation is also useful for culling. Cows having negative economic value as well as having chronic mastitis should be considered for culling (Schukken et al., 2003).

2.5. Bulk milk analysis in Bangladesh

Limited studies have been performed in Bangladesh on bulk milk analysis. Paul et al., (2018) studied total staphylococcal count at Sylhet city corporation area was 642.67 CFU/ml and 610.23 CFU/ml in rural areas. The highest and lowest TBC were 1.2×10^6 and 3.6×10^5 CFU/mL in different brand of pasteurized milk in Bangladesh (Ahmed et al., 2019). Hasan et al., 2015 found high bacterial counts at raw (1.3×10^6 to 7.4×10^5 CFU/ml) and pasteurized (1.8 x104 to 9.8 x104 CFU/ml) milk at selected areas of Dinajpur. Overall microbial findings of raw milk were unsatisfactory and poor hygiene found among handlers (Hasan et al., 2015). Our study will be helpful to figure out the present condition of milk quality at Chattogram.

2.6. Conclusion

In Bangladesh there is no formal milk value chain, and people are not aware of the quality of the milk. To evaluate the milk produced, present status of dairy farming in Bangladesh, necessity of bulk milk analysis, contagious and environmental pathogens related with BTM, different screening tests for BM analysis as well as their pros and cons, risk factors associated with increased SCC, how mastitis affects milk production, SMART goals for udder health monitoring, etiology, prevalence, and public health importance of *S. aureus* as well as MRSA were discussed to understand the knowledge gaps. Knowledge gaps are hygienic standard maintained in farm, TBC for pasteurized as well as raw milk, prevalence *S. aureus* and MRSA in the milk produced, relation between BMSCC and factors associated with CM caused by *S. aureus*.

Chapter-3: Materials and Methods

3.1. Experimental design

A comprehensive list of 102 dairy farms having at least 3 lactating cows was previously developed during the Swedish project: "Development of udder health control program in dairy cows in Bangladesh" under Chattogram district. Among the 24 farms, a manageable cohort with 72 samples (3 samples from each farm) was selected for bulk milk sample collection. The separate milk sampling was performed at each 2 months' interval for this study during June to October 2018. Epidemiological data were recorded using a questionnaire (**Appendix-I)** prior to the start of main study and filled up through face-to-face interviews. The questionnaire contained both farm and animal level data including animal source, housing, feeding management, udder hygiene etc.

3.2. Sample collection, preservation and transportation

Composite raw milk sample (containing milk from all lactating cows of a herd) (Reyher and Dohoo, 2011) from bulk milk tank on each farm was aseptically collected using sterile screw capped 50mL falcon tube. The collected milk samples were transported within 3 hours of milk sample collection using an ice box and shifted to Udder Health Bangladesh bacteriological laboratory located at Department of Medicine and Surgery, Chattogram Veterinary and Animal Sciences University (CVASU). Sample collection was performed following NMC protocol (2017) with modifications described and illustrated in **Appendix-II.**

3.3. Bulk milk somatic cell count

Somatic cell counts (SCC) were measured by a digital somatic cell counter (DeLaval Group, Stockholm, Sweden; Sensitivity: 88% and Specificity: 80%) (NMC, 2017) immediately after transportation. After that samples were transferred to Poultry Research and Teaching Centre (PRTC), CVASU and stored at -80°C for further bacteriological examination.

3.4. Bacteriological examination of bulk milk

A total of 72 samples from 24 farms (N=24) were undertaken and the bulk milk samples were examined for total bacterial count (TBC), total staphylococci count (TSC), *S. aureus* count, total

environmental streptococci count (TESC) and total coliforms count (TCC). Mannitol salt agar (HIMEDIA® Ltd., Mumbai, India) was used to determine the presence of *S. aureus*. The numbers of TES in BM samples were estimated using modified Edward's base (Himedia® Ltd., Mumbai, India. For determination of TCC, MacConkey agar (HIMEDIA® Ltd., Mumbai, India) was used (NMC, 2017; Jayarao et al., 2004).

Colonies suggestive of *S. aureus* from MSA were randomly (1-2 colonies) selected, streaked on 5% bovine blood agar, and incubated for 48 h at 37°C. The isolates were examined for hemolysis, catalase production, and coagulase production (NMC, 2017; Jayarao et al., 2004).

Protocol for the biochemical tests for detecting *S. aureus* are described in **Appendix-III.** All isolates were further examined for Gram's reaction and catalase production (NMC, 2017). Isolates were further examined for biochemical tests using oxidase production, citrate utilization, indole, Methyl red & Voges-Proskauer (MR-VP) test, and motility test (Rysanek et al., 2009).

Total bacterial count

Spot plate technique was followed for TBC (Wang et al., 2017). The milk samples were mixed thoroughly by gently inverting the milk vial 20 to 25 times (Marshall, 1992). One milliliter of milk was transferred to a sterile tube containing 9 mL of 0.9% Sodium Chloride solution (EMPLURA®, Mumbai, India). The samples were vortexed at 1500 rpm (revolution per minute) for 15 seconds. After that 1 mL of milk sample was transferred from 1st test tube to 2nd test tube and continued up to 6th (last one) and finally 1 mL of diluted milk was discarded from the last test tube (10^{-6}). The 10-fold diluted samples were vortexed at 1500 rpm again and from each dilution 20μL was plated on Nutrient agar (Oxoid, Basingstoke, UK) media using a micropipette (NMC, 2017; Jayarao et al., 2004). After that, plates were incubated at 32°C for 48 h. Bacterial counts between 30 to 300 colonies at the last countable dilution was considered as result and expressed as CFU/ml by using following formula:

Colony count at the final countable dilution × dilution factor/mL of milk = CFU/mL of original culture

Total staphylococcal count

The TSC was determined using similar technique as followed for TBC. Bulk milk samples were diluted up to 10^{-6} and from each dilution 20μL was plated on Mannitol salt agar (HIMEDIA® Ltd., Mumbai, India) and incubated at 37°C for 48 h. Counts between 30 -300 colonies at the last countable dilution was considered as result and CFU/ml expressed by:

Colony count at the final countable dilution × dilution factor/mL of milk = CFU/mL of original culture

Total environmental streptococci count

The TESC was determined using similar technique as followed for TBC. Bulk milk samples were diluted up to 10^{-6} and from each dilution 20μL was plated on modified Edward's base (Himedia® Ltd Mumbai, India) and incubated at 37°C for 48 h. Counts between 30-300 at the last countable dilution was considered as result and CFU/ml expressed by:

Colony count at the final countable dilution × dilution factor/mL of milk = CFU/mL of original culture

Total coliform count

The TCC was determined using similar technique as followed for TBC. Bulk milk samples were diluted up to 10^{-6} and from each dilution 20μL was plated on MacConkey's agar (HIMEDIA® Ltd., Mumbai, India) and incubated at 37°C for 48 h. Bacterial colonies between 30-300 at the last countable dilution was considered as result and CFU/ml expressed by:

Colony count at the final countable dilution × dilution factor/mL of milk = CFU/mL of original culture

3.5. Preservation of isolates

Then Isolates were preserved at -80°C with 50% glycerol for storage and further use. Preserved samples were streaked in bovine blood agar, incubated at 37°C for 24 hours. After that 2-3 colonies were transferred to Brain Heart Infusion broth (BHB) (Oxoid, Basingstoke, UK) and incubated at

37°C for 24 hours. Following enrichment in BHB, 700µL pure culture was transferred in a sterile cryovial and 300µL 50% glycerol added and preserved at -80°C before shipment to National Veterinary Institute (SVA) for MALDI-TOF.

3.6. Matrix associated laser desorption/ ionization- time of flight

Staphylococcus aureus isolates were transferred to National Veterinary Institute (SVA), Uppsala, Sweden using Copan Transsystem® (Capon, Brescia, Italy) transport media tube for species confirmation using Matrix-Associated Laser Desorption/Ionization- Time of Flight (MALDI-TOF) Biotyper 3.0 (Bruker Daltonics GmbH, Bremen, Germany) for validation (Score > 1.8) of phenotypic identification accuracy. Part of single colony were touched with a sterile toothpick and smeared on sample plates for MALDI-TOF. For repeated testing of the isolate, single sample was smeared in two separate wells. Matrix solution is then added over each well in sample plate and inserted into MALDI-TOF device. Best matched organism with high spectra was detected and matched with micro-organisms in database.

3.7. Minimum inhibitory concentration

For antimicrobial susceptibility testing, Minimum Inhibitory Concentration (MIC) using micro-dilution method done from preserved isolates of *S. aureus* as described by VetMIC CLIN staf/strept (SVA) (Ver. 2016-05; Lot no. 168/16; Art no. E395129, Statens veterinärmedicinska anstalt, Uppsala, Sweden). To validate the test, *S. aureus* CCUG 15915 which is an analogue to *S. aureus* ATCC 29213 was used as control. Details are attached in **Appendix-IV**.

3.8. Statistical analysis

Findings of BTM including SCC, TBC, TSC, TESC, TCC and coagulase positive *S. aureus* and MIC findings were entered into Microsoft Excel 2013. Before exporting to STATA IC 13 (StataCorp, 4905, Lakeway Drive, College Station, Texas 77845, USA) data cleaning, coding and integrity checking were done in the Excel program.

3.8.1. Descriptive statistics

The mean value of BMSCC (cells per mL), TBC (CFU/mL), TSC (CFU/mL), TESC (CFU/mL), TCC (CFU/mL) of 3 consecutive measurements (with a 2-month interval) per farm was calculated

before conducting summary of log transformed quartile estimates of the mean BMSCC, TBC, TSC, TESC, and TCC, of all studied farms. The results were presented as means with Log10 transformed values. Data of BMSCC, TBC, TSC, TESC and TCC were not normally distributed. Therefore, log transformation was done.

The prevalence of *S. aureus* and MRSA was estimated by dividing the number of samples positive by total number of samples tested. The results were presented as total number of farm and percentage and 95% confidence interval (CI).

A Pearson correlation test was performed to examine potential correlation between bacterial counts for the purposive bacteria of this study. This test was performed by using Log 10 values of BMSCC, TBC, TSC, TESC and TCC. The r- value and p-values were estimated to interpret potential significance. The results were expressed as frequency, percent (%), mean, minimum, maximum, percentile and correlation co-efficient (r). The significant cut off 0.05 or less was used.

3.8.2. Risk factors analysis

Risk factors were analyzed for mean BMSCC with farm level factors.

Univariable analysis

Farm level factors were recorded into 2 to 3 categories based on a meaningful number of frequencies. For the variables, either t-test or 1-way ANOVA was carried out to test the association between BMSCC and each of 19 selected farm level factors. These factors included farm size, year of establishment, educational qualification, age of farmer, number of employees, number of milking cows, housing system, source of animals, water refreshed per day, footbath, floor cleanliness, farm environment cleaning frequency, floor cleaning frequency per day, draining score, milking system, calf sucking, number of milkers, environmental temperature and humidity. Some quantitative factors were categorized into 2 or 3 based on their percentiles for ensuring optimum frequencies under each category of respective factors. For developing multivariable regression models, significant factors at $p \leq 0.2$ level of significance were used.

Multivariable linear regression model

The model was constructed by backward selection through application of the maximum likelihood estimation procedure and the statistical significance of the contribution of individual factors (or

group of factors). The interaction between factors were assessed by creating two-way interaction product for the significant main effect factors in the model. Changes in the coefficients examined and *p*-values were included. More than 10% coefficient change was considered to indicate confounding. Variance inflation factors (VIF) for the factors and the Cook–Weisberg test was used to examine collinearity and the homogeneity of variance and to check whether the overall data fitted the model. The results were presented in correlation co-efficient, 95% confidence interval (CI) and *p*-value (0.05 or less).

Chapter-4: Results

4.1. Dairy cattle herd characteristics

Herd size varied from 26 to 353 in which lactating cows ranged from 9 to 235. Farmers allowed the entry of new animals in the case of 79.2% farms from both of its own stock and also from other sources. Shed surroundings of farms (79.2%) were cleaned 1-4 times per day and 20.8% farmers cleaned on a weekly basis or even after a long period. A water refreshing schedule of once or twice per day was maintained by 54.2% farms. No disinfectant foot bath was used in 87.5% farms at the entry point as a regular hygienic practice. Shed floor of farms was cleaned 4 to 6 times a day in 75% farms. Hand milking system was performed by the majority of the farms (75%) where at least 50% of the farms had 1 to 4 milkers. In 20.8% farms farmers allowed calf sucking where in 50% farms, calf sucking was strictly avoided and in 29.2% farms both were maintained based on individual animals. Average temperature and humidity within farms were between 27.2°C to 39.8°C and 62% to 82.2%, respectively. Diverse educational background found among farmers from secondary or less (33%) to graduation or higher than that (67%).

4.2. Assessment of somatic cell count and bacterial contamination in bulk milk

The mean of various parameters (min-max) that indicate the level of SCC and bacterial contamination was as follows: 291,000 - 1,156,670 cells/mL of milk (BMSCC), 400-1,890,567 CFU /mL of milk (TBC), 161-200,776 CFU /mL of milk (TSC), 78-189,223 CFU /mL of milk (TESC), 33-1,500 CFU /mL (TCC) (Table 4.1).

Table 4.1. Mean, min-max and quartile estimates of BMSCC and bacterial contaminants (TBC, TSC, TESC and TCC) in bulk milk samples from 24 dairy herds in Chattogram, Bangladesh

	N	Mean/ Median	Min-Max	25%	50%	75%
BMSCC	24	765,389/ 777,500	291,000-1156,667	597,000	777,500	932,333
TBC	24	92901.3/ 7784.3	400.11-1890567	3036.6	7784.3	11711.1
TSC	24	23090.4/ 3167.8	161.2-200776.7	499.4	3167.8	9102.2
TESC	24	31483.3/ 9133.2	77.7-189223.3	2596.1	9133.2	55052.2
TCC	16	386.6/ 125	33.3-1500	66.665	125	583

##BMSCC= Bulk milk somatic cell count; TBC=Total bacterial count; TSC= Total staphylococcal count; TESC=Total environmental streptococcal count; TCC=Total coliform count

Log transformed quartile estimates and more details of SCC and other variables of this study is depicted in Table 4.2.

Table 4.2. Summary statistics (mean, min-max and quartile estimates) of somatic cell count and milk contaminants (TBC, TSC, TESC and TCC) in bulk milk samples from 24 dairy herds in Chattogram, Bangladesh, using Log10 transformed values

	N	Mean	Min-Max	25%	50%	75%
BMSCC	24	5.9	5.5-6.1	5.8	5.9	6
TBC	24	3.9	2.9- 6.3	3.5	3.9	4.1
TSC	24	3.5	2.2- 5.3	2.7	3.5	3.9
TESC	24	4	2- 5.2	3.4	4	4.7
TCC	16	2.2	1.5- 3.2	1.8	2.1	2.7

##BMSCC= Bulk milk somatic cell count; TBC=Total bacterial count; TSC= Total staphylococcal count; TESC=Total environmental streptococcal count; TCC=Total coliform count

4.3. Correlation among the variables

Significant negative correlation was estimated for the following pairs: (TSC vs. BMSCC, r=-0.71; p=0.07) (Table 4.3)

Table 4.3. Pair-wise correlation matrix (Pearson's correlation) of bacterial contamination in bulk milk samples

	Log Mean BMSCC	Log10TBC	Log10TSC	Log10TES	Log10TCC	P
Log Mean BMSCC	1.0					
Log10TBC	-0.42	1.00				0.53
Log10TSC	-0.71	0.65	1.00			0.07
Log10TES	0.14	0.49	0.15	1.00		0.47
Log10TCC	0.16	0.23	-0.08	0.25	1.00	0.56

##BMSCC= Bulk milk somatic cell count; TBC=Total bacterial count; TSC= Total staphylococcal count; TESC=Total environmental streptococcal count; TCC=Total coliform count

4.4.Prevalence of pathogens isolated from bulk milk

Through bacteriological culture, 4 isolates of *S. aureus* were confirmed using MALDI-ToF. Among these 4 isolates, 2 isolates were molecularly confirmed as MRSA (Table 4.4).

Table 4.4. Prevalence of *Staphylococcus aureus* and MRSA in bulk milk from 24 dairy farms in Chattogram, Bangladesh

Pathogens	N	Positive (%)	95% CI
S. aureus	24	4 (16.7)	4.7 to 37.4
MRSA	24	2 (8.3)	1.0-27.0

4.5. Minimum inhibitory concentration determination of selected *S. aureus*

Four isolates of *S. aureus* underwent antimicrobial susceptibility testing to determine the minimum inhibitory concentration. Among the panel of antibiotics, two isolates of *S. aureus* were found sensitive to the tested antibiotics. Another two isolates were methicillin resistant *S. aureus* (MRSA) which was also confirmed by PCR and found resistant against 5 antibiotics (penicillin, oxacillin, cefoxitin, gentamicin and tetracycline) (Table 4.5).

Table 4.5. Resistance[1] (numbers in brackets) and distribution of MIC for *Staphylococcus aureus* (*n*=4) from 24 dairy farms in Chattogram, Bangladesh

Test agent	Resistance (N)	Distribution (n of MICs (µg/mL)											
		0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
Penicillin	4						4						
Cefalotin	0						3	1					
Oxacillin+2 %NaCl	3				1		3						
Cefoxitin	2								2	2			
Enrofloxacin	0				4								
Fusidic acid	1					3	1						
Erythromycin	0					4							
Clindamycin	0					4							
Gentamicin	4								4				
Nitrofurantoin	0										4		
Tetracycline	2				2				2				
		Distribution (n) of MICs (mg/L)											
		0.25/4.75		0.5/9.5		1/19		2/38		4/76			
Trimethoprim +0 Sulfamethoxazole		4											

[1]Resistance percent was calculated as the number of isolates identified as resistant according to the cut off divided by total number of isolates tested for a particular type of bacteria tested (*S. aureus*/NAS); **White fields denote range of dilutions tested for each substance. In the MIC plate, there was a designed well for Trim-sulfa contained combination of 0.25 µg/mL Trimethoprim and 4.75 µg/mL Sulfamethoxazole. MIC of above range is given as the concentration closest to the range. Bold vertical lines indicate available epidemiological cut off values from EUCAST (https://eucast.org/) (Cefalothin, Nitrofurantoin), CLSI, 2018 M-100 (S-28) (https://clsi.org/) (Penicillin) and Swedres-Svarm 2018 (Oxacillin, Cefoxitin, Clindamycin, Enrofloxacin, Erythromycin, Fusidic acid, Gentamicin, Tetracycline, Trimethoprim + Sulfamethoxazole). When no cut-off values were available, bacteria were not categorized as sensitive or resistant.

4.6. Risk factors analysis

4.6.1. Univariate analysis

Mean BMSCC significantly ($p \leq 0.2$) varied by farm size, source of animals, floor cleanliness, farm environment cleaning frequency and v) humidity (%) (Table 4.6).

Table 4.6. Univariable associations between the farm level factors and mean bulk milk somatic cell count of lactating cows in Chattogram during the period of 6 months (n=24 farms)

Variable	Categories	n	Mean BMSCC	*P (t-test or 1-ANOVA)*
Farm size	26 - 65	13	712.9	0.3
	66 - 353	11	847.7	
Year of establishment	1978 - 2004	12	724.5	0.7
	2004 - 2016	12	824.9	
Number of employees	3 - 8	14	762.6	0.8
	9 - 58	10	791.5	
Age of farmer (years)	24 - 40	14	769.0	0.6
	41 - 60	10	782.5	
Educational qualifications	Secondary or less	6	856.6	0.04
	Higher secondary	2	683.3	
	Graduation or higher	16	755.4	
Number of milking cows	9 - 24	14	757.3	0.9
	25 - 235	10	799.0	
Housing system	Face-in	7	687.9	0.9
	Face-out	10	855.1	
	Both	7	746.6	
Source of animals	Both	19	778.8	0.9
	Own stock	4	659.6	
	Purchase	1	1156.7	
Water refreshed per day	1-2	13	806.7	0.9
	3-6	9	739.4	
	Auto drinker	2	725.5	
Footbath	Yes	3	803.8	0.6
	No	21	770.5	
Floor cleanliness	Dry	3	561.2	0.8
	Wet	21	805.2	
Farm environment cleaning frequency	1-4 times per day	19	808.9	0.2
	Weekly or more	5	644.7	
Floor cleaning frequency per day	2 - 3 times	6	787.6	0.8
	4 - 6 times	18	770.4	

Draining score	Excellent and very good	13	754.4	0.6
	Good and poor	11	798.6	
Milking system	Manual	18	781.6	0.1
	Manual and Machine both	6	753.8	
Calf sucking	Yes	5	795.1	0.9
	No	12	784.3	
	Both	7	743.5	
Number of milker	1 - 3	12	737.6	0.7
	4 - 16	12	811.8	
Within farm temperature (°C)	27.2 - 30	12	809.2	0.9
	30.1 - 39.8	12	740.2	
Within farm humidity (%)	62 - 75	11	813.1	0.07
	75.1 - 82.2	13	742.1	

n: Frequency number; Mean BMSCC: Mean of bulk milk somatic cell count; SD: Standard deviation; t- test: when an exposure variable have 2-categories; 1- ANOVA: when an exposure variable have more than 2-categories

4.4.2. Multivariable linear regression model for bulk milk somatic cell count

Neither confounding nor interaction was detected during the model building process. Variance inflation factors remained below 10, indicating the factors were not collinear. The Cook–Weisberg test p-value of 0.9 suggested the model fitted well. According to the final model, own stock as replacement (p=0.09) compared to both purchased and own stock animals, dry floor (p=0.06) compared to watery floor and weekly or more cleaning (p=0.1) compared to 1-4 times cleaning per day were associated with lower level of BMSCC (Table 4.7).

Table 4.7. Outputs of multivariable linear regression between the farm level factors and mean bulk milk somatic cell count of lactating cows during the period of 6 months (n=24 farms)

Variables	Categories	β	95% CI	p-value
Source of animals	Both	Ref		
	Purchase	295.1	-130.9 to 721.1	0.2
	Own stock	-202.0	-437.4 to 33.4	0.09
Floor cleanliness	Watery	Ref		
	Dry	-244.4	-502.9 to 14.2	0.06
Farm environment cleaning frequency	1-4/day	Ref		
	Weekly or more	-168.0	-382.2 to 46.1	0.1

β: Correlation of co-efficient; 95% CI: 95% confidence interval; *p*: probability

Chapter-5: Discussion

Bulk milk analysis is for milk quality testing and is mainly based on SCC, TBC and bacteriological culture for mastitis and zoonotic pathogens. There are scattered studies found on raw and pasteurized milk done across Bangladesh (Ahmed et al., 2019; Nusrat et al., 2015; Paul et al., 2018) but not in an organized way. The present study was conducted to fill the gaps and with the target of determination of SCC, TBC occurrence of *S. aureus* along with MRSA from bulk milk studies. This chapter discusses important findings, implications, limitations, conclusions, recommendations, and future directions.

5.1. Farm characteristics

In this study, 24 farms were sampled and interviewed concerning the general condition, background history and management system of the farm. Most of the farms had different farm sizes, indigenous and crossbred cows for herd, face in as well as face out system, lack of good drainage system, poor floor cleaning, low percentage of foot bath and antiseptic usage, tube well for water supply mostly and limited grazing facilities for cows which agreed with other studies in other parts across Bangladesh (Rahman et al., 2009; Datta et al., 2015; Sultana et al., 2015). Poor drainage system, lack of floor cleaning, absence or limited use of foot bath are favorable for contamination of bulk tank milk by *S. aureus* as well as other mastitis pathogens through infected udders during handling of raw milk (Jorgensen et al., 2005; Scherrer et al., 2004).

5.2. Somatic cell count and bacterial contamination in bulk milk

Bulk milk somatic cell count

In the present study, the lowest mean BMSCC was 291,000 cells/mL and the highest mean was 1,156,670 cells/mL of milk. According to European Union, the threshold level for SCC raw milk is 400,000 cells/mL of milk (EU, 2004). In this study, for 25% farm the BMSCC was 597,000 cells/mL of milk which was higher than EU threshold. Only four farms contained good quality milk satisfying EU threshold. The lowest mean of this study agrees with Jayarao et al. (2004) as well as the legislative limit in many other countries. The threshold standard of BMSCC in China is 300,000

cell/mL (Alhussein and Dang, 2018), in Europe 400,000 cells/mL (USDA, 2013), in Canada 500,000 cells/mL and in USA 750,000 cells/mL (Sargeant et al., 1998).

Total bacterial count

The lowest mean TBC ranged from 400 CFU/mL which indicates good quality milk, and the highest mean was 1,890,567 CFU /mL of milk in our study. Total bacterial counts ranged from 360,000 CFU/mL to 1,200,000 CFU/mL in a study on different brands of pasteurized milk in Bangladesh (Ahmed et al., 2019). According to Sri Lanka Standard Institute 1983, expected standard is less than 30,000 CFU/ml (Gunasena and Siriwardhana, 2021). In this study most of the farms (more than 75%) contained lower TBC than expected standard of Sri Lanka. In Myanmar TBC ranges from 6,000 to 3,000,000,000 CFU/mL of milk (Naing et al., 2019). The lowest TBC was found in western region with 11,700,000 CFU/mL and the highest value was found with 13,900,000 CFU/mL of milk in eastern region of south India (Lingathurai & Vellathurai, 2010).

Total coliform count

Enteric bacteria are mostly coliforms which are important mastitis pathogens (Hogan and Smith, 2003). Internationally, the acceptable limits for coliforms in the raw milk are less than 100 CFU/mL milk (Mubarack et al., 2010; Salman and Hamad, 2011). In this study, the lowest TCC was found to be 33 CFU /mL whereas the highest value was 1,500 CFU /mL of milk which is not in agreement with the findings of neighboring countries. Findings of 25% farms contained satisfying TCC. In Myanmar, the lowest TCC was 10 CFU /mL and the highest value was 8,400,000 CFU/mL of milk (Naing et al., 2019). Coliform counts of 10 CFU/mL or less are desirable and can be achievable. In New York, bulk milk samples contained less than 10 CFU/mL (10%) and below 66 CFU/mL (70%) of milk (Jones and Sumner, 1999).

5.3. Correlation with bulk milk somatic cell count with different bacterial counts

Significant negative correlation (r=0.71) was seen for TSC vs. BMSCC which is not agreement with Jayarao et al. (2004). A positive significant correlation between TBC and TSC was found (r=0.65) which was also reported in several studies (Jayarao et al., 2004; Pyz-Łukasik et al., 2015). Weak negative correlation estimated for TBC (r=-0.42) with BMSCC which is statistically non-significant. Similar finding also observed by Pyz-Łukasik et al. (2015). This result could be due to small sample

size. In present study, all other correlations between bacterial counts were statistically non-significant.

5.4. Prevalence of *S. aureus* and MRSA

Staphylococcus aureus

Among 24 farms, almost one fifth of the samples (16.7%) were confirmed as *S. aureus,* and it has public health importance due to antimicrobial resistance and zoonotic potential (Haran et al., 2015). Our findings are consistent with Jahan et al. (2015), which reported a prevalence of *S. aureus* from raw milk of 23.5%. But much lower than in a study from India where a 68% prevalence was recorded (Bharathy et al., 2015) and 84% found in a study of USA (Haran et al., 2012) and 70% in Hungary (Peles et al., 2007). Our results were higher than from a study in Korea which reported a 5.6% prevalence of *S. aureus* (Moon et al., 2007).

Methicillin resistant *Staphylococcus aureus*

In developing countries like Bangladesh more than 70% of contagious bacteria have been termed as multi drug resistant strains (Jilani et al., 2008; Dutta et al., 2013). The isolates of *S. aureus* that were confirmed as MRSA was found to be resistant against multiple antibiotics. In our study the prevalence of MRSA was 8.3%. There were limited number of isolates and therefore it is difficult to make any definite conclusion. Eight raw milk samples collected from different areas of Dhaka city contained MRSA (58.8%) (Nusrat et al., 2015). A study from Minnesota found 4% prevalence for MRSA in dairy herds (Haran et al., 2012). Present study unveiled the existence of MRSA and oxacillin resistant *S. aureus* in raw milk and the percentage is lower than the findings of Nusrat et al (2015).

5.5. Risk factors associated with bulk milk Somatic cell count

There was a positive relationship between larger herd size and BMSCC. The result of this study is in contrast with other studies that mentioned that larger farm size was a risk factor for increased BMSCC (Elmoslemany et al., 2010; Jayarao et al., 2004). A similar relationship, as in our study, was found in Skrzypek et al. (2004). There is no good explanation for our discrepant findings.

In this study, dry floor condition, compared to wet floor conditions, significantly related with lower level of BMSCC which is agreed with Santman-Berends et al. (2016) who found increased incidence of CM at herds where floor cleaning is done once daily compared to cleaning 4 times a day. It can be explained as the presence of moisture is helpful for growth and survival of microorganisms. Wet bedding materials may transmit microorganisms through adhering with cow's skin and can be associated with increased levels of BMSCC (Naing et al, 2019).

Higher BMSCC was associated with animals purchased from other farms, compared to replacement from own stock which agrees with Schukken et al., (2011). Purchase of animals is one of the most important factors for introducing new infections to a herd. Newly purchased animal may carry chronically affected cows. Own stock animals are more consonant in preventing infection due to strong herd immunity (Schukken et al., 2011).

5.6. Limitation of the study

1. Statistical assumption and selection bias: Only 24 farms were selected based on accessibility of the farmers, but no statistical assumptions were followed for estimating the number of farms required. Selection may have caused a bias during taking convenience samples, probably selecting more motivated farmers.

2. Diagnostic error: All the BMSCC, TBC was done by one person. There may be some random error and also some systematic error, which would be misclassification bias. Bacteriological cultures were used for isolation of bacteria from all the samples and few isolates were retested using MALDI-ToF. The BMSCC testing has a sensitivity and specificity of 88% and 80%. This might also intend some minor false positive error.

3. Confounding bias: There may be some more extraneous factors at various levels (farms, cow and quarter level) which may confound the significant association of potential risk factors with *S. aureus*.

4. Loss of follow-up: No loss of follow up was subjected in the present conducted study. Although loss of follow up is common phenomena for cohort study, this was possible due to the small number of farms considered and study period was reasonably shorter.

5.7. Conclusions

- The present study deals with the quality assessment of bulk milk samples procured from subunits (upazillas/thanas) in Chattogram district.
- Somatic cell count in 16.7% of the farms were within the international acceptance level (equal or less than 400, 000 cells/mL of milk). It can be improved by regular monitoring of BMSCC as well as reducing factors responsible for elevated BMSCC.
- Most of the farms found lower level of TBC (30, 000 CFU/mL of milk according to Sri Lanka Standard Institute).
- A few farms had MRSA in the bulk milk. It can be reduced through maintaining good milking hygiene and biosecurity, together with prudent use of antibiotics.
- Dry floor and weekly or more cleaning were factors associated with lower levels of BMSCC.
- Introduction of newly purchased cows in farms correlated with higher BMSCC.

Chapter- 6: Recommendations and Future Directions

6.1. Recommendations

I. Bulk milk somatic cell count estimates varied from low to very high among farms. In 25% of the farms, the value is ≤597,000 cells/ml of milk which is higher than the EU threshold of 400,000 cells/ml of milk. Primarily a particular goal for BMSCC should be set which can be achievable in Bangladesh context. Low BMSCC is associated with good udder health. Dairy herds should therefore be encouraged to enter an udder health program.

II. To ensure the hygienic status and public health safety, milk quality parameters such as TBC, TSC, TESC and TCC should be checked before the milk reaches the final consumers. Routines for good hygiene should be implemented in all aspects of dairy herding, from cleanliness of cows and good hygiene around them to hygienic milking and milk handling.

III. Among 24 farms, 2 isolates of *Staphylococcus aureus* were molecularly confirmed as MRSA. Risks of MRSA entering the bulk milk can be minimized by maintaining good udder health and good biosecurity within and between farms as well as by implementing good hygiene and biosecurity during milking and milk handling. Prudent use of antibiotics is also an important factor for reducing the occurrence of MRSA.

IV. Livestock trade is the largest source of disease transmission between farms and buying live animals is therefore not recommended. Own stock replacement is much better from a biosecurity point of view. Still, if needed, newly purchased animals should be kept in isolation shed as they can carry contagious pathogens. From own stock, comparatively resistant cows to mastitis should be selected as future stock and cows with chronic illness should be culled.

6.2. Future directions

I. The study area was limited to the Chattogram district. To investigate bulk milk quality across different dairy zones of Bangladesh, similar studies should be conducted.

II. A longitudinal study should be conducted considering a large number of farms. This will help to investigate risk factors associated with BMSCC as well as to obtain long term data on BMSCC and other bacterial counts. This will allow estimating the present status of BMSCC in Bangladesh.

III. Further studies should be conducted to confirm identification of isolated organisms at species level together with molecular testing like MALDI-TOF and their antibiogram.

IV. Further studies on isolation of zoonotic bacteria from bulk milk and their route of transmission along with public health significance should be considered.

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Appendix-I

Questionnaire on Evaluation of bulk milk collected from selected dairy farms in Chattogram

A. Baseline Information

1. Date of recording/interview [**DATE**]_____/_____/_____

2. Farm identity number [**FID**]_____/_____/_____/_____

3. Name of the farm [**NF**]:_____

4. Type of the dairy farm [**TDF**]

1=Small (_____); 2=Medium (_____); 3=Large (_____)

5. Village [**VIL**]:_____

6. Union [**UN**]:_____

7. Ward [**WD**]:_____

8. Upazilla [**UP**]:_____

9. Longitude [**LON**] and Latitude [**LAT**] of the farm location:_____;_____

10. Year of establishment of the farm [**YEF**]_____

11. Number of farm employees [**EMPLOY**]_____

12. Farmer's demography

12.1. *Name of the farmer [**NF**]*_____

12.2. *Age of the farmer [**AF**]*_____

12.3. *Sex of the farmer [F**SEX**]*_____

12.4. *Education of the farmer [**EF**]*_____

12.5. *Religion of the farmer [**RF**]*_____

12.6. *No of family members [**NFM**]*_____

13. Interviewee's demography (If not the farmer)

13.1. *Name of the farm manger or farm vet [**NM or FV**]*_____

13.2. *Age of the farm manger or farm vet [**AFM or AFV**]*_____

13.3. *Sex of the farm manger or farm vet [FM**SEX or FVSEX**]*_____

13.4. *Education of the farm manger or farm vet [**EFM or EFV**]*_____

13.5. *Religion of the farm manger or farm vet [**RFM or RFV**]*_____

14. Farm's composition

	Types	Total numbers	Breed	Blood percentage
14.1.	*Milking cow[**MC**]*			
14.2.	*Pregnant cow [**PC**]*			
14.3.	*Pregnant heifer [**PH**]*			
14.4.	*Dry cow [**DC**]*			
14.5.	*Non pregnant heifer [**NPH**]*			
14.6.	*Heifer bull [**HB**]*			
14.7.	*Calf [**CALF**]*			
14.8.	*Total [**TOTAL**]*			

15. Housing system of the farm [**HOUSE**]: 1=Face-in; 2=Face-out 3=Both

16. Source of the animal [**SA**]: 1=Own stock; 2=Purchase 3=Both

17. Source of water [**SW**]: 1=Tube well; 2=Deep tubewell; 3=Any other (Pl mention_____)

17.1. *How often water is refreshed? [**WREF**] 1=Three times/day; 2=Four times/day; 3=Any other schedule (Pl mention_____)*

17.2. *Average amount of water provided per lactating cow per day: 1st stage of lactation_____; 2nd stage of lactation_____; 3rd stage of lactation_____*

18. Does the farm have footbath at the entrance? [**FOOTB**] 1=Yes; 2=No

19. Cleanliness of farm environment (Gutter, floor etc.)? **[CLENV]** 1=Watery; 2=Dry

19.1. *How often farm environment is cleaned? [**HOCLEAN**]_____*

20. Does the floor of farm suitable for cleaning and disinfecting? [**CLDIS**] 1=Yes; 2=No

20.1. If yes, frequency of cleaning [***FCLEAN***]----------

21. Does the farm have isolation/quarantine shed? [**ISO/QUA**] 1=Yes; 2=No

22. Overall drainage system of the farm [**DRAIN**]:

1=Excellent; 2=Very good; 3=Good; 4=Poor; 5=Very poor

23. Does the farm have boundary wall? [**BOUND**] 1=Yes; 2=No

24. Does the farm have any pest control measure in place? [**PCONT**] 1=Yes; 2=No

25. Feeding system **[FSYST**]. 1=Stall-feeding; 2=Open-feeding; 3=Both

26. Does the farmer have pasture land? [**PAST**] 1=Yes; 2=No

27. Farm milking system [**MILKSY**]: 1=Manual; 2=Machine; 3=Both

27.1. *If manual, sucked by calf [**SUCK**]: 1=Yes; 2=No*

27.2. *If manual, types of milker [TMILKER] 1=New; 2=Old*

28. No of milkers [**MILKER**]:_____

29. Climatic parameters

29.1. *Ambient temperature [**ATEM**]_____℉*

29.2. *Humidity [**HUM**]_____*

29.3. *Rainfall [**RAIN**]_____*

29.4. *Radiation [**RAD**]_____*

30. Farmer's behaviour

30.1. *What do you do about mastitis? [**DOMAST**]_____*

30.2. *How often do you check the milk? [**CHMILK**]_____*

30.3. *Do you cull animal because of poor udder health? [**CULL**]* *1=Yes;* *2=No*

30.3.1. If yes, when do you exactly cull?[TCULL]----------

30.4. *Do you think clinical mastitis is problematic? [**CMP**]* *1=Yes;* *2=No*

30.5. *What do you do with the milk obtained from cows affected by clinical mastitis?*

*[**CMMILK**]_____*

31. Climatic parametrs

Variables	May	July	September
*Ambient temperature ℉ [**ATEM**]*			
*Humidity [**HUM**]*			
*Rainfall [**RAIN**]*			
*Radiation [**RAD**]*			
BMSCC/mL of milk			

Appendix-II

Sample collection procedures

Materials

- Sterile falcon tube
- Tube holding rack
- 70% Alcohol
- Cotton for Alcohol soak
- Ice Box
- Disinfectants for cleaning teats (70% Alcohol, 0.5% w/v Chlorhexidine)
- Permanent marker, gloves and mask

Detailed Procedure

1. Falcon tubes were labeled using permanent markers with Date, Farm ID, Cow and Quarter.
2. Teats and udder washed and loose dirt, hairs removed from teats.
3. 1-2 streams of milk discarded from the affected quarter.
4. All quarters were dipped (at least 30 seconds) with disinfectant solution.
5. Alcohol soaked cotton swab is used to clean teat prior to sample collection.
6. Test tubes were held at a 45° angle holding on the left hand.
7. 5- 50ml of milk collected.
8. Affected quarters were disinfected.
9. Collected milk samples were stored with a rack in an ice box.

Appendix-III

For different biochemical tests manual of clinical microbiology was followed (Warren, 1985).

3.3.1. Catalase test

Small amount of the colony was placed on a fresh, clean and grease free slide. One drop of 3% H_2O_2 poured onto the colony, a cover slip was placed, and bubble formation was indicated as a positive result.

3.3.2. Coagulase test

3.3.2.1. Horse plasma collection

Whole blood from horses was collected into commercially available sterile tubes containing EDTA to perform the test. Then blood was centrifuged at 2600 rpm (rotation per minute) for 10 minutes using a refrigerated centrifuge device. The resulting supernatant, the plasma, was then immediately transferred to a sterile 1.5 ml Eppendorf tube using sterile tips and stored at -20ºC for future use.

3.3.2.2. Tube coagulase test

From each tube cultivated in BHIB, 50 µL was transferred to sterile tubes containing 50 µL of horse plasma. The incubation was done at a temperature of 37ºC 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.

3.3.3. Indole test

Pure bacterial culture was grown in sterile Brain Heart Infusion Broth (BHIB) for 24 hours. Following incubation, 4-5 drops of Kovac's reagent was added to the culture broth. A positive result was reflected by the presence of a red or red-violet color in the surface layer of the broth. A negative result appears yellow. A variable result can also occur, showing an orange color as a result.

3.3.4. Oxidase test

A piece of filter paper was placed in a clean petri dish and 2 drops of oxidase reagent was added to filter paper. A colony of test organisms was removed using a wire loop (not an oxidized wire loop) and rubbed onto treated filter paper. A color change to blue/ dark purple within 5 to 10 seconds was considered a positive test. Microorganisms were oxidase negative if the color did not change.

3.3.5. Methyl red test

Using a light inoculum, tubes of MR-VP media were inoculated with 24-hour pure cultures of test organisms. Then tubes were incubated aerobically at 37°C for a minimum of 48 hours. After incubation, 2.5 ml of culture was transferred into a new sterile culture tube and 5 drops of the methyl red reagent was added to the tube. Red color at the surface of the medium as a result of high acid production and a decrease in the pH of the culture medium was indicated as a positive test. Yellow color at the surface of the medium was indicated as a negative test.

3.3.6. Voges-Proskauer (VP) test

0.6 ml (or 12 drops) of Barritt's reagent A was added to the remaining 2.5 nil (after MR test) of culture grown in MR-VP broth. Then 0.2 ml (or 4 drops) Of Barrit’s reagent B was added and carefully shaken the tube for 30 seconds to expose the medium to atmospheric oxygen. Then the tube was allowed to stand for at least 30 minutes. Red coloration on top of the culture was considered as VP positive. Yellowish color at the surface of the medium was considered as VP positive.

3.3.7. Preservation of stock culture

1. Fifty percent sterile buffered glycerin was made by mixing 50 parts of pure glycerin and 50 parts of buffered glycerol saline.

2. Pure culture of isolated bacteria from blood agar was incubated overnight in Brain heart infusion broth.

3. Then the bacterial culture was mixed with 50% sterile buffered glycerin in 1.5 ml Eppendorf tubes (700µl broth culture and 300µl glycerol).

4. Then preserved at -80°C for long term use.

Appendix-IV

3.5.1. Minimum Inhibitory Concentration (MIC)

For MIC user instruction provided by VetMIC CLIN staf/strept was followed (VetMIC – SVA, 2019).

3.5.1.1. Preparation of inoculum

a) 3-5 colonies of bacterial isolates from blood agar (<48 hours) were suspended with the help of 1 µl loop in 4 ml sterile 0.9% saline, to obtain a concentration of about 108 CFU/ml.

b) From this suspension around 20 µl was transferred to 10 ml Cat-ion Adjusted Muller Hinton broth (CAMHB) (SIGMA ALDRICH) for a final inoculum density of approximately (5 x 105) CFU/ml {accepted interval is (2 x 105) – (8 x 105) CFU/ml}.

c) The inoculum density was verified by viable counts. 10 µl of the final inoculum was taken and diluted in 10 ml 0.9% saline.

d) From this dilution 100 µl was spread on blood agar. After incubation of 24 hour at 37°C CFU counted.

3.5.1.2. Inoculation and incubation

50 µl of the inoculum suspended to each well. The wells were sealed with a transparent covering tape and the panels were incubated for 16-18 hours at 35-37°C. After that each panel was placed on a viewing device and the result was recorded.