Subclinical mastitis in water buffalo (*Bubalus bubalis*) in Bangladesh

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David Ericsson

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Supervisor:	**Sofia Boqvist, Swedish University of Agricultural Sciences, Department of Biomedical Science and Veterinary Public Health**
Assistant supervisor:	Ylva Persson, National Veterinary Institute (SVA)
Assistant supervisor:	Md Mizanur Rahman, Chattogram Veterinary and Animal Science University, Bangladesh
Examiner:	Karin Persson Waller, National Veterinary Institute (SVA) and Swedish University of Agricultural Sciences

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Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Science and Veterinary Public Health

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Abstract

Globally, subclinical mastitis (SCM) is an important production disease in the dairy industry, for both dairy cattle and buffaloes, and has a great economic impact due to reduced milk yield, milk quality deterioration, treatment costs, culling and risk for antimicrobial resistance (AMR). A cross sectional study was conducted on 17 randomly selected buffalo farms in coastal areas of Bangladesh during Sep and Oct 2019 to investigate SCM in terms of occurrence, predisposing factors, causing pathogens and AMR. There are just a few small previous studies made on the subject in Bangladesh and there is need for better knowledge level of the situation to enable improvements of the sector. A total of 132 lactating buffaloes were included in this study. The number of lactating buffaloes at each farm varied between 1 and 66. Quarter milk samples were collected before the morning milking procedure from each lactating buffalo and were tested with California mastitis test (CMT). Bacterial culturing was performed from all milk samples with CMT ≥2, which was the definition for SCM in this study. The milk samples were cultured on equine- and bovine blood agar, Mannitol-salt-agar (MSA) and MacConkey-agar (MAC) and agents were identified using visual inspection of the colonies as well as by Gram-staining, biochemical testing and matrix-assisted laser desorption/ ionization (MALDI-TOF). All *Staphylococcus* spp. were tested for production of penicillinase using the clover leaf method.

An average of 56% of the buffaloes had SCM, but the occurrence varied substantially between farms (0 - 100%). The most common pathogen was non aureus staphylococci (59%), followed by *Micrococcus luteus* (9.6%). The average level of penicillinase production within *Staphylococcus* spp. was 33%.

The occurrence of SCM was around the same level compared to results from other recent studies in the region. The causing pathogens were surprising, mainly since *S. aureus* was more common in other recent similar studies. Since *S. aureus* is a species commonly recognized to be hard to eliminate from individual dairy herds this gives positive hope for the future.

Keywords: Staphylococcus spp., *Micrococcus luteus*, Non-aureus *staphylococci*, antimicrobial resistance, mastitis pathogens, prevalence, occurrence

Sammanfattning

Subklinisk mastit (SCM) är en viktig produktionssjukdom inom mjölkproduktion världen över. Sjukdomen har en stor ekonomisk påverkan på grund av minskad mjölkavkastning, försämrad mjölkkvalité, behandlingskostnader, utslagningar och risk för antibiotikaresistens (AMR). En tvärsnittsstudie gjordes på 17 slumpvis utvalda buffelgårdar i kustområden i Bangladesh under perioden september till oktober 2019 för att undersöka SCM med avseende på förekomst, predisponerande faktorer, orsakande patogener och förekomst av AMR. Det finns bara ett fåtal tidigare studier på ämnet i Bangladesh och kunskapsläget behöver stärkas för att förenkla förbättringsarbetet inom sektorn. Totalt inkluderade studien 132 lakterande bufflar. Antalet lakterande bufflar på gårdarna varierade mellan 1 och 66. Mjölkprover från varje juverdel på alla bufflar samlades in före morgonmjölkningen och testades med California mastitis test (CMT). Bakterieodlingar gjordes från alla mjölkprover från juverdelar med CMT ≥2, som också var definitionen för SCM i den här studien. Bakterieodlingarna gjordes på häst- och nötblodagar, Mannitol-salt-agar (MSA) och Mac-Conkey-agar (MAC) och tolkades med hjälp av visuell inspektion av kolonierna, Gram-färgning, biokemiska tester och matrix-assisted laser desorption/ionization (MALDI-TOF). Alla *Staphylococcus* spp., testades med klöverbladsmetoden för pencillinasproduktion.

I genomsnitt hade 56 % av bufflarna SCM. Variationen mellan olika gårdar var betydande (0 – 100%). De vanligaste patogenerna var icke-aureus stafylokocker (59 %), följt *av Micrococcus luteus* (9,6 %). I genomsnitt producerade 33 % av stafylokockerna pencillinas.

Förekomsten av SCM var på ungefär samma nivå som i tidigare studier i regionen. Orsakande patogener var oväntade framför allt för att vi inte hittade *Staphylococcus (S.) aureus*, som varit vanligt förekommande i liknande tidigare studier. Eftersom *S. aureus* är en erkänt svår bakterie att utrota från enskilda besättningar ger detta gott hopp inför framtiden.

Nyckelord: Staphylococcus spp., *Micrococcus luteus*, Non-aureus *staphylococci*, koagulasnegativa stafylokocker, antibiotikaresistens, mastitpatogener, prevalens, förekomst

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Abbreviations

AMR	Antimicrobial resistance
BA	Blood agar
BHI	Brain Hearth Infusion broth
CM	Clinical mastitis
CMT	California mastitis test
CVASU	Chattogram Veterinary and Animal Science University
DCC	DeLaval cell counter
FAO	Food and Agricultural Organization of the United Nations
IMI	Intramammary infection
MAC	MacConkey agar
MALDI-TOF	Matrix-assisted-laser-desorption-ionization-time-of-flight
MSA	Mannitol-Salt agar
MIC	Minimum inhibitory concentration
NAS	Non-aureus staphylococci
NDRI	National dairy research institute of India
PCR	Polymerase chain reaction
SCC	Somatic cell count
SCM	Subclinical mastitis
SLU	Swedish University of Agricultural Sciences
SVA	National Veterinary Institute of Sweden
WHO	World Health Organisation

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1. Introduction

In the 7th national 5-year plan (for year 2016-2020) proposed by the General Economics Division of Bangladesh government it is said that it is important to encourage the research on adaption to climate change and promote non-crop agricultural activities including livestock. Today the development goes the wrong direction, when the average farm size in Bangladesh is getting smaller and smaller which makes each farm more sensible to single years of flood, drought or salinity intrusion. While the national demand for milk is increasing (General Economics Division - Government of Bangladesh 2016), the hot and humid climate makes high producing dairy breed cattle less suitable (Choudhary & Sirohi 2019). The local cattle breeds in Bangladesh are small and have a very low milk yield (Hamid et al. 2016). Crossbred cattle are better suitable for the climate than imported dairy breeds, but still produce under their capacity. (Hamid *et al.* 2016; Choudhary & Sirohi 2019).

The higher milk yield and the ability to better hydrate on saline water makes the buffalo a good choice of livestock to keep in many areas compared to cattle and goats that today accounts for 90 and 6-7 percent, respectively, of the national milk production (General Economics Division - Government of Bangladesh 2016; Hamid *et al.* 2016).

One of the most important challenges in dairy production worldwide is the presence of subclinical mastitis (SCM). The disease is not visible by the human eye but could be detected and measured by easy cow side diagnostic methods or by using advanced laboratory equipment. It is estimated that as many as 90% of dairy cows in the industrialized agricultural world will at least once during their lifetime be affected. The same number for dairy buffaloes is somewhat lower. Most commonly SCM is caused by infectious microorganisms of many different species and subspecies. The primary problem with SCM is that it causes losses in milk yield, but SCM can also have several secondary effects such as, waste of the resources by discarding milk, poor food quality and antibiotic resistance.

The knowledge level about the situation in Bangladesh is low since there are just a few smaller previous studies made on the subject within the country.

This study aims at investigating SCM in domesticated water buffaloes in Bangladesh in terms of occurrence, predisposing factors, causing pathogens and antimicrobial resistance.

2. Literature review

2.1. Bangladesh seen from an agricultural perspective

Bangladesh is a country in South Asia located between India and Myanmar. It became an independent country after the liberation war with West Pakistan in 1971 (CIA 2020). It is one of the most densely populated countries in the world with around 160 million people living on an area of 150 000km^2 (FAO 2017; CIA 2020; The World Bank 2020). The poverty is prominent even though The World Bank ranks Bangladesh as a lower middle income country since 2015 (The World Bank 2020; Utrikespolitiska Institutet 2020). The national wealth is very unevenly distributed in the population and half of the rural population is landless (Utrikespolitiska Institutet 2020). A little more than one third of the population is living in the cities and the rest in the many rural areas (FAO 2017).

The landscape is fertile which makes Bangladesh highly suitable for farming. Still, Bangladesh has got a huge problem with the many monsoon rains that often cover large parts of the landmass in water. More than 70 percent of the land mass is used for farming and out of that 80 percent is used for growing rice. Bangladesh has been almost self-sufficient in food production since around the new millennium, but malnutrition is still considered a large domestic problem (Utrikespolitiska Institutet 2020).

The agricultural sector accounts for about 13 percent of the BNP and employs about 45 percent of the population (FAO 2017; Utrikespolitiska Institutet 2020). That sector also has got the highest poverty reduction rate (FAO 2017). Out of the total national poverty reduction between 2005 and 2010, the agricultural sector accounted for 90 percent (FAO 2017). When people are getting wealthier and get a higher standard of living, they improve their dietary intake, which is reflected by an increased demand for meat and dairy products (General Economics Division - Government of Bangladesh 2016; FAO 2017). Even though Bangladesh claims to be self-sufficient in food production that is only the case when it comes to rice which is the staple food in the country. It is estimated that the national production covers less than 60 percent of the national demand for milk, less than 70 percent of

the demand for eggs and less than 35 percent of meat. Even though the world production of milk has increased with 58% over the last 30 years, and the highest increase has been recorded in South Asia, the milk yield from each buffalo in Bangladesh is lower than that of the neighbouring countries (FAO 2017, 2020). Between 2005 and 2010 the general demand for milk and meat increased by 4 and 13 percent, respectively, while the demand for rice decreased by 5 percent during the same period (Hamid *et al.* 2016).

2.2. Domesticated water buffalo (*Bubalus bubalis*)

The Asian water buffalo can be divided into two different species, the domesticated water buffalo *Bubalus (B.) bubalis* and the wild water buffalo *B. arnee*. Both originate from Asia and should not be confused with the African buffalo (*Syncerus caffer*) or the American Bison (*Bison bison*) which are both also commonly called buffalo (Castelló 2016; Presicce 2017). *Bubalus arnee* still lives as a native wild animal in some Asian countries but is now extinct in Bangladesh (Castelló 2016). It is believed that *B. bubalis* originates from *B. arnee* (Presicce 2017). The Asian domesticated water buffalo is smaller than the wild water buffalo and has a maximum weight of 1000 kilograms. It can be divided into two subspecies; river type buffalo (*B. bubalis* var. *bubalis)* and swamp type buffalo (*B. bubalis* var. *kerabau*) (Castelló 2016). Although the two types have different numbers of chromosomes, 50 (river) versus 48 (swamp), they can mate and get fertile offspring (Castelló 2016; Presicce 2017). Crossbreeding with cattle (60 chromosomes) is not possible (Castelló 2016).

The domesticated water buffalo can be found in large parts of Asia but also in Europe, Australia and North and South America (Presicce 2017). The river type is more prominent in Indochina, Bangladesh, India, Mediterranean, Egypt and America while the swamp type is more common in China, Southeast Asia, and Australia. The swamp buffalo is slightly smaller than the river type (Castelló 2016). The type most common in Bangladesh is the domesticated river buffalo. It weighs 450-1000 kilograms, is usually hairier and darker than the domesticated swamp buffalo, and it has 2-4 times higher milk yield compared to swamp type buffaloes (Castelló 2016; Presicce 2017).

River buffaloes are used as draft power, and also for meat and dairy production (Presicce 2017). In fact, in Asia 20-30 percent of the total draft power in agriculture comes from buffaloes (Campbell & Marshall 2016). There are many different breeds of river buffalo, including Nili ravi, Murrah, Surti, Kundi, Jafarabadi and Mediterranean (Castelló 2016; Presicce 2017). Usually, the males reach sexual maturity at the age of 3-4 years and can obtain good breeding results until they

reach 10 years of age. The buffalo cows usually give birth for the first time at the age of 3 – 4.5 years of age. There are large variations between different countries and individuals (Presicce 2017). It is important that the cow is big enough at first calving, at least 500 kg for a river type Murrah heifer, since the size at first calving will affect the milk yield not only for the first lactation period but also for her ability to reach the maximum milk yield which is usually expected during the fourth lactation (Campbell & Marshall 2016).

2.2.1. Domesticated water buffalo for dairy – worldwide

Worldwide, water buffaloes are the second most important dairy producing animals after dairy cattle. About 5 percent of the global dairy products comes from buffaloes (Campbell & Marshall 2016). As many as 180 million, or more than 90 percent, of the total 194 – 202 million domesticated water buffaloes in the world are found in Asia *(Figure 1)* (Hamid *et al.* 2016; Zhang *et al.* 2020). India, which is one of the neighbouring countries to Bangladesh, has 113 million buffaloes and produces more than two thirds of the buffalo milk globally (Hamid *et al.* 2016).

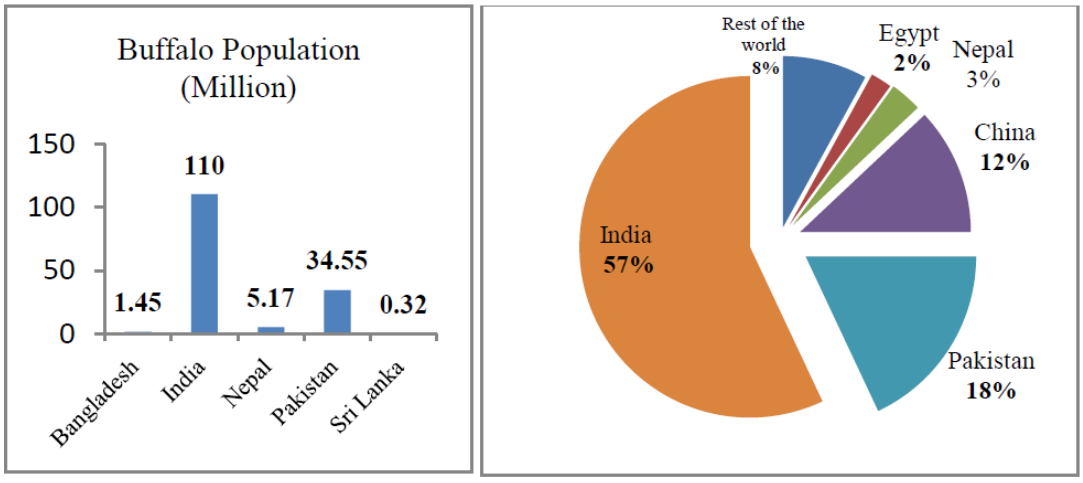

Figure 1. Water buffalo population distribution in Asia and in the world (Siddiky & Faruque 2017). With permission from Md Nure Alam Siddiky (corresponding author).

2.2.2. Domesticated water buffalo in Bangladesh

In 2002 there were 1 million buffaloes in Bangladesh. In 2015 that number had increased by 50 percent to 1.5 million. That can be compared to cattle that only increased by 5 percent or from 22.5 to 23.6 million heads of cattle during the same period of time (General Economics Division - Government of Bangladesh 2016). Even though buffaloes are kept primarily for draft purposes they are also an important source of nutrients for Bangladesh's large rural population (Campbell & Marshall 2016). An indigenous buffalo breed produces between 620 to 1161 kilograms of milk per lactation or 2-3.5 kilograms per day under local conditions, which can be compared to the small (150 kilograms) local cattle breeds with a milk production of 200-250 litres per lactation (Hamid *et al.* 2016). Buffalo milk also contains more protein, fat, lactose and energy than milk from dairy cows (Presicce 2017).

There are mainly three different systems in which buffaloes are kept in Bangladesh: 1. Small scale household farms, 2. Semi-intensive farms, and 3. Extensive (bathan) systems. A small-scale household farm usually keeps 1-3 lactating buffaloes. These animals are usually allowed to graze for 6-7 hours a day backyard or on public land and are fed very little or no extra feed. The semi-intensive farms keep 4-15 lactating animals that are kept grazing when they are needed for ploughing rice paddies. During the remaining year they are kept free-ranging. The extensive or bathan systems consist of large herds of 50-200 lactating buffaloes, or sometimes herds as large as 600 lactating buffaloes. These herds are often kept in the lower coastal areas where the land is muddy. Bathan buffaloes are kept grazing and given no extra feed supply at all. About 40 percent of the domestic buffalo population can be found in the coastal lowland where saline flooding is common (Hamid *et al.* 2016). Another rearing system not mentioned above is the large intensive farms that are common in high income countries. That kind of farms are also present in Bangladesh however still rare (Rahman & Islam 2019).

2.3. Mastitis

Mastitis is a very complex topic. Mastitis means inflammation of the parenchyma of the mammary glands and can be caused by trauma or infection, the latter being the most common (Sandholm *et al.* 1995; Srivastava *et al.* 2015; Presicce 2017). The inflammation can be local in the mammary gland but may also cause systemic responses (Sandholm *et al.* 1995). Clinical signs of mastitis are visible changes in the milk, udder, and/or general symptoms. The severeness of the clinical disease

can range from mild; affecting the milk, moderate; affecting the milk and udder, and severe affecting the milk, udder and the cow in general. Some examples of signs of mastitis are changes in milk appearance such as color change and/or the presence of clots, udder symptoms such as swelling and heat, and systemic signs such as fever (Peek 2017). Mastitis can also be subclinical with changes in milk composition such as an elevated somatic cell count (SCC) but without any visible changes in the milk or other clinical signs as described above (Constable 2017). Mastitis can further be divided into acute, with a sudden onset, and chronic, with a longer duration.

As already mentioned, mastitis can also be classified as infectious or non-infectious. Infectious mastitis can be caused by bacteria, yeast, fungi or in rare cases virus (Sandholm *et al.* 1995). Bacterial infection is most common and the main entry point is the teat canal (Sandholm *et al.* 1995).

2.3.1. Mastitis-causing pathogens

Mastitis-causing pathogens can be divided into contagious and environmental. For contagious pathogens the main source of infection is milk from cows with chronic intramammary infection, and for environmental pathogens the main source of infection is manure, bedding material etc in the close environment. Contagious pathogens are mainly spread during milking while environmental pathogens are mainly spread between milkings. The distinction between contagious and environmental pathogens is not always completely clear since there are some pathogens that could belong to both groups (Sandholm *et al.* 1995; Peek 2017).

Contagious mastitis-causing pathogens in dairy cows include *Streptococcus (Str.) agalactiae, Str. dysgalactiae*, *Staphylococcus (S.) aureus*, and *Mycoplasma* spp. Environmental mastitis-causing pathogens include *Escherichia coli (E. coli)*, *Klebsiella (K.) pneumoniae*, most non-aureus staphylococci (NAS), some streptococci, and *Corynebacterium* spp. There are also pathogens such as *Str. uberis* and some NAS that can be considered both environmental and contagious (Constable 2017; Peek 2017).

Out of the contagious pathogens, *S. aureus* is probably the most problematic one since it causes chronic mastitis that is very difficult to cure (Peek 2017). *Streptococcus uberis* can cause mild to moderate clinical mastitis but more often gives a chronic subclinical form (Constable 2017; Peek 2017). Non-aureus staphylococci are mainly environmental pathogens that can normally be found on the skin of the animal. They have traditionally not been important mastitis-causing pathogens but that have changed in the last couple of decades, and they are now considered important pathogens causing mainly subclinical mastitis. In the Nordic

countries NAS is actually now the most prevalent in cases of subclinical mastitis (Sandholm *et al.* 1995; Constable 2017; Vakkamäki et al. 2017).

Environmental bacteria *E. coli* and *Klebsiella* spp. typically causes acute clinical mastitis that also has a systemic impact with fever and decreased general condition. The systemic effect can be severe and both bacteria can be difficult to treat (Persson Waller & Unnerstad 2004; SVA *E. coli* 2020).

2.3.2. Consequences of subclinical mastitis

Mastitis is the most prevalent disease in dairy animals worldwide and causes large negative effects for both the animal welfare and the economy. Mastitis, in south Asian region carries high costs for the farmers (Srivastava *et al.* 2015). Exactly how high the costs of mastitis are is hard to say since different studies mention different numbers but one estimate is that they go as high as 35 billion US dollars globally each year and that as much as 40% of the total costs of production diseases are caused by mastitis (Srivastava *et al.* 2015). Mastitis is the most common reason for culling a dairy cow (Vakkamäki *et al.* 2017).

The biggest problem for the farms with SCM is that SCM causes the milk yield to drop (Sandholm *et al.* 1995). In dairy cows it has been shown that every twofold increase in the milk SCC above 50 000 cells per millilitre causes a decrease in milk production of 0.5 to 0.6 litres a day (Smith 2015; Peek 2017). A higher SCC is also often correlated with a penalty fee from the dairy plant for poor milk quality, as well as growing costs of veterinarians and medications (Peek 2017). Subclinical mastitis is often considered a larger production problem than CM because it is more prevalent and harder to detect and therefore more difficult to manage properly (Constable 2017).

In the bigger picture treating mastitis, as well as other diseases, with antimicrobials, can cause a growing problem with AMR as well as an increase of pharmacological residues in the environment. Mastitis also causes poor milk quality and constitutes a health risk for people in areas where pasteurization is not done on a regular basis. Poor quality also leads to a bigger amount of food being thrown away. It also causes premature culling of animals, and a decreased milk production per kilogram of feed. This all together are important causes of waste of the earthly resources and driving factors for climate change and global warming (FAO 2020; WHO 2020).

2.3.3. Subclinical mastitis in dairy buffaloes

Although dairy buffaloes are generally considered more resistant to diseases than dairy cows, a highly producing water buffalo is also susceptible to mastitis if not managed properly (Presicce 2017). Many studies have shown that the prevalence of SCM is generally higher than that of CM in water buffaloes (Sharma & Sindhu 2007; Srivastava *et al.* 2015). Even though buffaloes have stronger sphincters and a wider layer of protective epithelium in the teat canal compared to dairy cows, the teat canal is still the main entrance way for infecting microorganisms (Srivastava *et al.* 2015).

The prevalence of SCM in water buffaloes is usually lower than that of dairy cattle (Srivastava *et al.* 2015; Campbell & Marshall 2016). Previous studies on SCM in water buffaloes in Bangladesh shows cow level prevalence reaching from 20% up to 50% (Saiful Islam *et al.* 2016; Talukder *et al.* 2016; Islam *et al.* 2019; Biswas *et al.* 2020). These studies have been small (30 to 70 individuals) and made in other regions of the country. Most common pathogens associated with SCM have been *Staphylococcus* spp. (NAS and *S. aureus*) followed by *E. coli* (Saiful Islam *et al.* 2016; Talukder *et al.* 2016; Biswas *et al.* 2020). Studies from several countries in Asia have shown a clear increase for SCM among buffaloes over the last 25 years. Several studies from India showed the prevalence of SCM in water buffaloes to be around 5-20 percent in 1995 (Srivastava *et al.* 2015). Later studies, conducted on buffaloes in India, have shown prevalence of 45% in 2015, 33.8% in 2018 and 68.3% in 2019 (Srivastava *et al.* 2015; Sharma *et al.* 2018; Kashyap *et al.* 2019). Studies in Pakistan have shown 15.2% in 2011, 38.8% in 2018 and 22.9% and 67.3% in 2019 (Hussain *et al.* 2013, 2018; Khan *et al.* 2019; Maalik *et al.* 2019). Although the prevalence seems to vary in different areas and over the years, most of them indicate a growing problem. Khan *et al.* wrote in 2018 that the rising prevalence of SCM is one of the toughest challenges that faces the development of the Pakistani dairy industry. An Indian study concluded that the cost of SCM in buffaloes was INR 364 per lactation and that about half of that was directly related to decreased milk yield (Sinha *et al.* 2014; Romero *et al.* 2018).

2.3.4. Methods to detect subclinical mastitis – DCC and CMT

The most widely used indicator for SCM is to measure the SCC in milk. The rising SCC is caused by an increased inflow of immune cells (mainly leukocytes) to the milk and can be measured in milk directly either by a direct smear under a microscope or by using a somatic cell counter, e.g., DeLaval cell counter (DCC); or indirectly by using e.g., California mastitis test (CMT) (Constable 2017; Peek 2017; Presicce 2017; DeLaval 2020a, 2020b). Both methods have been proven useful for detection of SCM in buffaloes (Dhakal & Nagahata 2018). Somatic cell count for a healthy dairy cow will be less than 100 000 cells per millilitre of milk (Srivastava, *et al.* 2015). The author has not been able to find a clear definition on normal levels for buffaloes in Bangladesh but several studies have found buffaloes without IMI to have up to 200 000 cells/mL milk (Jorge *et al.* 2005; Dhakal 2006; Dhakal & Nagahata 2018). The National dairy research institute (NDRI) of India recently set the cut off value for healthy Murrah buffaloes to 100 000 cells/mL (National Dairy Research Institute of India 2021).

When using CMT, a few millilitres of milk will be collected in a paddle followed by adding CMT-fluid. The test fluid lysis the cells and causes the DNA in the cells to form a gel (Whyte *et al.* 2005; Peek 2017). The more cells, the stronger gel formation (Whyte *et al.* 2005; DeLaval 2020b). California mastitis test has been proven to be a good method to detect SCC >200 000 cells per millilitre in milk from dairy cows, a level that is often used as a cut-off value in SCM studies (Kandeel *et al.* 2018).

Both DCC (DeLaval Cell Counter) and CMT can be used both on bulk milk and on composite milk from individual cows or on milk from individual udder quarters. The CMT is a cheap and quick cow-side method to use directly at the farm and is most suitable to use for individual quarter milk testing. The DCC is more expensive, and both time and equipment consuming, but gives a more exact SCC number, and is the better choice for testing bulk milk (DeLaval 2020b).

There are also other detection methods for SCM such as testing milk for pH, enzymes and conductivity (Dhakal & Nagahata 2018). Those methods are not as commonly used as the ones mentioned above and have not been used in this study.

2.3.5. Methods to diagnose intramammary infection

There are several methods to determine the presence of intramammary infection (IMI), and to identify different types of microorganisms. Bacterial culturing, polymerase chain reaction (PCR) and Matrix-assisted-laser-desorption-ionization-time-of-flight (MALDI-TOF) are three good methods that can be used.

Bacterial culturing of milk is probably the most common method to diagnose IMI (Peek 2017). Culturing can be done using aseptically collected milk samples taken at bulk, cow, or quarter level. Most commonly, a specified volume of milk is spread on a blood agar plate. After incubation at 37º C for 24-48 hours, the plate is inspected visually for growth of bacterial colonies. If considered growth positive without contamination, generally defined as a certain number of colonies by the same or two different appearances, interpretation can be done visually by size, color, single or double haemolysis etc; by Gram-staining before inspection of the microorganisms in a microscope; and/or by biochemical tests. Further culturing on selective agar might also be an option. Alternatively, species identification can be done by MALDI-TOF, which is used to find a matching microorganism in a reference data base by measuring the length of different proteins in the cultured bacteria. Given that the tested organism exists in the data base this method can give a fast identification of bacterial species or subspecies (Constable 2017).

Analyses using PCR can be done directly on milk, and may give a definitive answer on presence of bacteria based on DNA in a more sensitive and faster way but it is more expensive than routine bacterial culturing and it is not possible to know if present bacteria are viable or dead (Constable 2017). Moreover, this method can only detect pathogens included in the chosen test kit and it is more sensitive to contamination than the methods previously described.

2.3.6. Antimicrobial resistance

Antimicrobial resistance (AMR) is when an organism has mechanisms protecting it from the effects of antimicrobials. There are two main types of resistance, natural and acquired. An example of the former type is that many Gram-negative bacteria have natural resistance to penicillin. Acquired resistance on the other hand develops through mutations or when extra chromosomal DNA transfers between bacteria. An example of that is plasmid-mediated beta lactamase production in staphylococci (Sandholm *et al.* 1995). Antimicrobial resistance is an increasing serious threat to public health globally. Misuse and overuse of antimicrobials; and lack of disease prevention and hygiene in farms being two of the main reasons (WHO 2020). A recent study from Pakistan showed that AMR is common in mastitis-causing pathogens in dairy buffaloes, for example in a recent study where 100 percent of 38 isolates of *S. aureus* were resistant to both penicillin and trimethoprim (Maalik *et al.* 2019). There are few studies from Bangladesh studying AMR in association with SCM in buffaloes but Saiful Islam *et al.* (2016) found that all isolated bacteria (mainly *Staphylococcus* spp. and *E. coli*) from 114 SCM samples in his study were resistant to penicillin and Talukder *et al.* (2016) found *Staphylococcus* spp. from

SCM quarters to be highly resistant to amoxicillin, erythromycin and azithromycin without presenting the exact numbers.

Methods to detect antimicrobial resistance

There are different methods to test if a specific microorganism is resistant to a specific drug. The disc diffusion methods (for example Kirby-Bauer and clover leaf method) are methods where discs with antibacterial agents are placed on agars either pre-prepared with the bacteria or with bacterial colonies added to the agar afterwards. After incubation the zone around the antibacterial disc with lesser or no bacterial growth is then measured to see if the agent in the disc has inhibited the growth (Franklin & Wierup 1982; Carter *et al.* 1990; Bryan & Godfrey 1991). Dilution methods, for example the use of MIC-plates (MIC = minimum inhibitory concentration), use agars or broths with preadded antimicrobials with the addition of dilutions of the tested bacteria to see which concentrations of the tested drug inhibits further growth (Quinn 2011). There are also mechanic specific methods, for example the use of nitrocefin/cefinase discs, where the production of for example beta-lactamase can give a specific reaction e.g. change of color in the test disc. Another type of test that should be mentioned is the PCR test that tests for the specific gene that gives antimicrobial resistance, for example confirmation of Methicillin resistant *S. aureus* (MRSA) (University of Minnesota 2021).

3. Material and methods

3.1. Literature search

The literature review has been made using several online databases such as PubMed, Google Scholar, Web of Science and Scopus.

3.2. Study area and farms

All samples in this study were collected from two areas in Bangladesh – Bagerhat and Noakhali during the period September - October 2019. The weather during the sampling period was warm (25-40 °C), sunny and humid. The laboratory work was conducted at Chattogram (former Chittagong) Veterinary and Animal Science University (CVASU) in Bangladesh and at the National Veterinary Institute (SVA) in Sweden. Bagerhat is located about 300 kilometres or around 15 hours drive west of Chattogram. Noakhali is located just a few hours drive and a couple of hours boat ride northwest of Chattogram. Bagerhat, Chattogram and Noakhali are all marked in the map below (*Figure 2).*

The selection of farms was made mainly on 3 conditions. 1. They had to be located in areas Bagerhat and Noakhali and fairly easily accessible by car or boat. 2. The farmers had to have good relationships with local veterinarians since those were the main connection points between farmers and our research group. 3. The farmers had to willingly accept our visit and to be involved in the study.

Figure 2. Study areas where the milk samples were taken and location of Chattogram Veterinary and Animal Science University (CVASU) where most of the lab work was done. Maps downloaded from Google maps (approved use according to https://about.google/brand-resource-center/products-and-services/geo-guidelines/), CVASU-logo downloaded from CVASU's Facebook page (with permission from Professor Mizanur Rahman. All three figures processed in Microsoft Paint.

We intended to include a total number of 100-150 buffaloes from at least 15 farms from the three farming systems described above 1. household, 2. bathan and 3. semi-intensive (literature review 5.2.2). The number of farms included was based on estimates on how many could be visited during the field work, estimated time for travels and laboratory capacity. The number of farms from different systems were decided on accessibility the days of sampling.

To investigate the potential effect on herd size on occurrence of IMI and SCM, we divided the farms into three categories depending on the number of lactating animals. The different categories were decided on forehand by CVASU personnel and could not be influenced by the author. 1-5 lactating buffaloes was considered a small farm, 6-10 animals a medium sized farm and farms with more than 10 lactating buffaloes were considered large farms.

3.3. Study design

3.3.1. Milk sampling

All the buffaloes in this study were normally milked by hand into a bucket once daily. The udders of the buffaloes in the study were normally rarely cleaned before or after milking. Only lactating and healthy (based on appearance and farmer information) buffaloes with clinical healthy udders were used in this study. The day of sampling, the milk was tested using the CMT and milk samples were collected for bacteriological examination at the start of the milking as described in detail below. The procedure took place between 6:00 and 11:00 AM during the ordinary milking. To minimise the stress for the buffalos and thereby negative impact on milk yield all handling of the buffaloes, including milking and sampling was carried out by the farmers and their staff themselves. All farmers were given the following instructions in Bengali 1. Use the disposable nitrile gloves provided by the research team; 2. Clean and dry the udder with clean water and a clean towel before milking; 3. Discard 2 strips from each teat into the milking bucket; 4. Milk 2 strips of milk from each quarter into each well of the CMT paddle and directly hand the paddle over to a member of the research team standing by. The research team member, most often the author, would then inspect the milk and perform the CMT-test (see below); 5. Take milk samples from selected quarters (see below) as follows; wipe the end of the teat, especially around the orifice of the teat canal, with a cotton ball moisturized with 70% ethyl alcohol, wait 10 seconds after alcohol wipe; milk 1 strip into the test tube before urgently putting the lid back on. CMT testing was done and evaluated as described by Schalm & Noorlander (1957). All quarters with CMT score 2 or higher on a 5-grade scale were defined as having SCM.

Aseptic milk samples were taken in falcon tubes from all SCM quarters and approximately from every fifth healthy (CMT 1). The exact frequency of CMT-1 samples was decided on site based on practical possibilities. Within 10 minutes after sampling, the milk samples were placed in a cooling box with ice packs and then transferred to a -10 to -15°C freezer at a local farm within a maximum of 12 hours. When sampling of all participating farms in a certain area were finished, the samples were transported frozen in a cooling box with ice packs and then restored in a freezer -18°C at the university (CVASU) laboratory.

In addition to the quarter milk sampling at each farm described above, the SCC of one bulk milk sample per farm was tested directly at the farm using DCC. The bulk milk sample was collected as follows; In farms with only one buffalo the milk in the bucket was mixed by moving the bucket in circles. A milk sample was then taken lowering a falcon tube into the bucket. In farms with several buffaloes the individual milking buckets were emptied by the farmer into a barrel. When all buffaloes were milked, the barrel was moved in circles to mix the milk before a bulk milk sample was taken lowering a falcon tube into the barrel the same way as described above. The milk was then directly analysed by the DCC as instructed by the manufacturer (DeLaval 2020a).

3.3.2. Epidemiological data collection

At the farm visits, a pre-formed questionnaire was used to collect information from the farmers about possible predisposing factors, such as size of lactating buffalo herd, the farmers educational level (illiterate, primary-, secondary school or post-secondary school), the breeds held at each farm, parity and time since last calving.

3.3.3. Bacteriology including antimicrobial resistance

Bacteriological culturing

Three different agars were used in the laboratory at CVASU in Bangladesh; blood agar (BA), MannitolSalt agar (MSA) and MacConkey agar (MAC). All agars and brain hearth infusion broth (BHI) were made manually on site using autoclaved reusable petri dishes made of glass, agar base powder (Oxoid) and diluted water. For blood agar, also horse blood from a healthy CVASU pony (See: Appendix 1).

After preparation of agar plates, a few plates from each batch were randomly selected to be incubated for 24 hours at 37 °C. If any of the selected plates showed signs of bacterial contamination the whole batch was discarded. The latter was done to minimize the risk of letting human errors, in the agar preparation procedure, or contaminated blood, distort the results of the study.

The culturing of milk samples was performed within 7 days after sample collection. The milk samples were thawed in room temperature (21 °C). After thawing, the samples were mixed for 10 seconds using a vortex machine. Ten microlitre of the sampled milk was spread on a BA plate using a plastic loop. An initial interpretation based on colony morphology (shape, size and color) and Gram-staining was made after 24 and 48 hours of aerobic inoculation in 37 °C. If growth of 3 or more colonies with the same appearance it was considered an IMI. If two different appearances with at least 3 colonies each it was considered a mixed infection. If 3 or more appearances the sample was considered contaminated (National Mastitis Council 1999; Quinn 2011).

When growth of *Staphylococcus* spp. was suspected based on findings on the BA plate, 1 colony from each agar was also spread on an MSA plate as described above. If there was bacterial growth after 24 hours aerobic inoculation at 37 °C the bacteria were concluded to be NAS unless the pink MSA turned yellow, in that case it was instead considered *S. aureus*. A catalase test was performed of each colony type considered as *Staphylococcus* spp. using a test kit from the manufacturer Oxoid (Quinn 2011).

If after visual interpretation of growth on BA and Gram staining, Gram-negative bacteria were suspected, 1 colony was cultured on MAC for 24 hours at 37 °C. If growth with 3 or more collonies of the same appearance the bacteria was considered to be Gram-negative, most likely *E. coli*.

Transportation of isolates

A sample of each colony being evaluated was also saved for transport to Sweden and confirmation with MALDI-TOF and AMR-testing at SVA. A plastic loop was used to take three colonies from each BA plate of interest and spread on to a new BA. The new BA was then incubated for 24 hours at 37 °C. If no signs of contamination, a plastic loop was then used to take three colonies from the BA followed by mixing the bacterial material with 10 millilitre pre-prepared Brain-hearth infusion broth (BHI) (Oxoid, lot number 1837985) in a glass tube (See appendix 2). The broth tube was then sealed with a cotton ball and incubated for 8 hours in 37 °C. 30 microlitre broth with bacterial growth was then taken using a pipette and added to a small sterile plastic tube, together with 30 microlitre 50% buffered glycerol. The isolate tube was sealed and slowly shaken a few times before being stored in a freezer at -18 °C for 24 hours and then transferred to another freezer (-80 °C) for long term storage.

On the day of transfer to Sweden, the box with frozen isolates was removed from the freezer and stored in a cooling bag with ice packs. At arrival in Sweden 30 hours later, the boxes were frozen at –18 °C. None of the test tubes seemed to be thawed at the visual inspection performed at arrival.

Species identification using MALDI-TOF

The tubes containing the isolates were thawed at 20°C for 1 hour. Then, 10 microliters of material from each tube were spread on 7% horse blood agar with aesculin (SVA) using a sterile plastic loop. Each agar plate was incubated for 24 hours at 37 °C before evaluation of growth. All tubes were expected to contain only one bacterial species. However, if growth of more than one colony type was detected the procedure was repeated. If growth of more than one colony type was detected also on the second agar plate the tube was considered contaminated, and no further testing was done on that sample. An exception from this procedure was done if growth of 2 different colony types was found and one of those was visually

assessed as the bacteria expected and the other as a *Bacillus* spp., the former was used for further testing while the latter was considered a contamination. This last assessment was done due to previous local experience of *Bacillus* spp. being a very common contaminant in frozen isolates in the same laboratory.

From each valid isolate, one colony was collected and spread on a well on a MALDI-TOF test plate. When all isolates had been spread, one drop of matrix was placed on each well. After the matrix fluid had dried, the plate was loaded into the MALDI-TOF machine (Biotyper 3.0 Bruker Daltonics GmBH, Bremen, Germany). All results with score 1.8 or higher of phenotypic identification accuracy was compared with the results from the evaluation at the CVASU lab. If both results were the same that bacterial species was concluded. If the results differed, the SVA agar plate was inspected to see if the colony types were similar as recorded at CVASU. If that was the case, the MALDI-TOF result was considered correct and the earlier manual conclusion to be wrong. If the MALDI-TOF analysis could not give a clear result, i.e., phenotypic identification accuracy score 1.7 or lower, or if the colony on the SVA agar did not match the description recorded at CVASU, the bacterial species was defined as unidentified.

Antimicrobial resistance

All staphylococcal isolates were tested for production of beta lactamase using the clover leaf method according to accredited routines at SVA (ISO/IEC 17025:2017) (Bryan & Godfrey 1991).

In addition, the intention was to test all *S. aureus* isolates for resistance against other antimicrobials using MIC, but no *S. aureus* isolates were found in this study.

3.3.4. Descriptive statistics

All data have been presented descriptively. Statistical analyses were not possible due to a low number of participating farms and individuals. With a few exceptions all tables and diagrams have been made in Microsoft Office Excel 365 and sometimes for aesthetically reasons modified in Microsoft paint.

The occurrence of SCM was presented descriptively and differences in SCM occurrence between geographical regions (Bagerhat, Noakhali) and farms of different size ((small (1-5 animals), medium (6-10 animals), large (>10 animals)) were described in terms of numeric differences. Bulk milk SCC-values (as an alternative SCM marker) were presented descriptively and differences between geographical regions and farms of different size have been described as numeric values only. The proportion of IMI and the numbers of different mastitis-causing pathogens were presented descriptively.

The results of the variables educational level and proportion of staphylococci producing beta-lactamase were presented descriptively. Information on parity, time since last calving and breed was not presented due to inadequate response frequency

and poor data reliability since the information was based solely on the individual farmers knowledge and memory.

4. Results

4.1. Farms and animals

Seventeen farms and 132 animals were included in this study, but 1 farm (18 buffaloes) was excluded at an early stage due to a high proportion (66.7%) of contaminated milk samples. In comparison, the rest of the samples had a contamination proportion of only 2.5%. Thus, 16 farms and 114 buffaloes were included in the results presented in this study.

4.2. Occurrence of subclinical mastitis and somatic cell count

The overall occurrence of SCM on cow level was 56% (n=64 out of 114) and on quarter level 27% (n=124 out of 456). There was a wide range between different farms from 0% occurrence on both cow and quarter level to 100% on both cow and quarter level (*Table 1*).

Bagerhat had a cow level SCM occurrence of 62% (43 out of 69 buffaloes) and quarter level SCM occurrence of 31% (85 out of 276 quarters). The same numbers for Noakhali were 47% (21 out of 45 buffaloes) and 22% (39 out of 180 quarters). The numeric values of occurrence of SCM on both quarter level and cow level was higher in Bagerhat then in Noakhali

The SCM occurrence of different farm sizes were similar (*Table 2).*

Table 1. SCM occurrence on cow- and quarter level and somatic cell count (SCC) on the visited farms in areas Bagerhat and Noakhali. The table is based on 16 farms, 114 buffaloes and 456 quarters. The SCC was measured in bulk milk samples. Farm no. only for reference use in this study. Farm size; Small = 1-5 buffaloes, Medium = 6-10 buffaloes, Large = >10 buffaloes

Farm no. (Size)	**SCM cow level**	**SCM quarter level**	**SCC (cells/mL)**	**Area**
1 (Large)	61%	30%	205000	Bagerhat
2 (Small)	100%	50%	145000	Bagerhat
3 (Small)	100%	50%	188000	Bagerhat
4 (Medium)	86%	43%	250000	Noakhali
5 (Small)	50%	13%	587000	Noakhali
6 (Small)	50%	13%	175000	Noakhali
7 (Small)	50%	38%	213000	Noakhali
8 (Medium)	13%	9.4%	224000	Noakhali
9 (Large)	25%	6.3%	242000	Noakhali
10 (Small)	100%	25%	47000	Noakhali
11 (Small)	0.0%	0.0%	71000	Noakhali
12 (Small)	100%	100%	267000	Noakhali
13 (Small)	100%	25%	153000	Noakhali
14 (Small)	50%	37%	180000	Noakhali
15 (Small)	100%	25%	74000	Noakhali
16 (Small)	100%	25%	113000	Noakhali
All farms	56%	27%	195875	Both areas

Table 2. Occurrence of SCM in 114 lactating buffaloes, 456 quarters at 16 Bengal farms of different sizes in areas Bagerhat and Noakhali. Farm size; Small = 1-5 buffaloes, Medium = 6-10 buffaloes, Large = >10 buffaloes

Farm size	Cows	Quarters	SCM positive cows	SCM positive quarters
Small (n=12)	21	84	14 (67%)	27 (32%)
Medium (n=2)	15	60	7 (47%)	15 (25%)
Large (n=2)	78	312	43 (55%)	82 (26%)

The bulk milk SCC ranged from 47 000 cells/mL to 587 000 cells/mL with an average of 195 875 cells/mL (*Table 1*). The median value was 184 000 cells/mL. As shown in *Figure 3* the bulk milk SCC was lowest in small farms (12 farms) with an average of 184 417 cells/mL followed by large farms (2 farms) with an average of 223 500 cells/mL. The highest SCC was found in medium sized farms (2 farms) with an average of 237 000 cells/mL. The farms in Bagerhat (3 farms) had an average bulk milk SCC of 179 333 cells/mL while the corresponding SCC for Noakhali farms (13 farms) was 199 692 cells/mL.

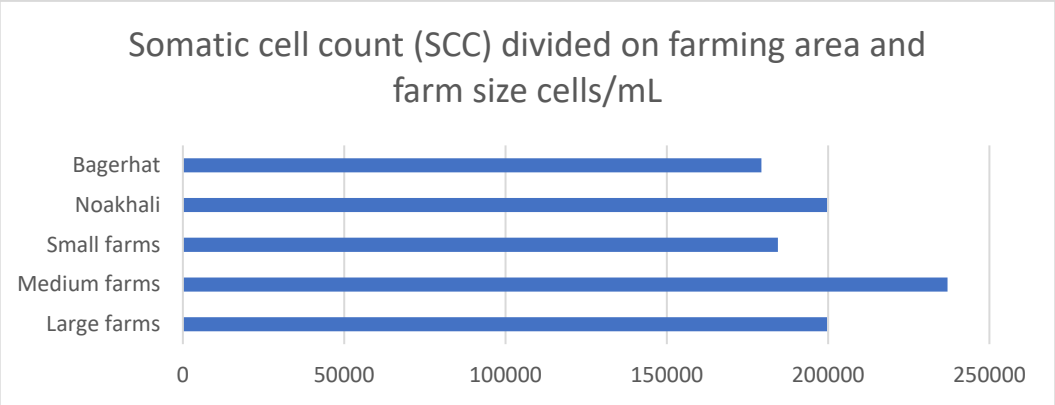

Figure 3. Average bulk milk somatic cell count (SCC) in different farm groups. The bulk milk SCC was analysed in 16 farms having a total of 114 buffaloes. There were 3 farms from area Bagerhat and 13 farms from area Noakhali in this study. Based on the number of lactating cows per farm the farms were divided into small farms (1-5 buffaloes; n=10), medium farms (6-10 buffaloes; n=2) and large farms (>10 buffaloes; n=2).

4.3. Epidemiological data

4.3.1. Geographical area and descriptive data

Three of the farms were from the Bagerhat area and 13 were located in the Noakhali area of Bangladesh. Rearing type wise, 11 of the farms were household, 2 were bathan, and 2 were semi-intensive farms.

Most of the farms in the study were small but most of the animals were kept on large farms (*Table 3*).

Table 3. Distribution of all buffaloes included in the study. A total of 114 lactating buffaloes were kept at 16 farms of different sizes. Small = 1-5 lactating buffaloes, Medium = 6-10 lactating buffaloes, Large = >10 lactating buffaloes

Farm size	Farms	% of farms	Cows	% of cows
Small	12	75%	21	18%
Medium	2	13%	15	13%
Large	2	13%	78	68%

In Bagerhat, almost all of the buffaloes were kept at a large farm and in Noakhali most buffaloes were kept at small farms (*Table 4*).

Table 4. Distribution of buffaloes included in the study. A total of 114 lactating buffaloes were kept in 16 farms in 2 different areas; Bagerhat (n=3 farms) and Noakhali (n=13 farms).

Farm size	Bagerhat	% of Bagerhat buffaloes	Noakhali	% of Noakhali buffaloes
Small	3	4.3%	18	40%
Medium	0	0.0%	15	33%
Large	66	96%	12	27%

In this study, several breeds were included. Based on the research groups own observations small farms had mainly indigenous buffaloes, while the medium and large farms had a mix of indigenous, Murrah and Nili-Ravi buffaloes. All of these breeds belong to the river-type string of the domesticated water buffalo.

As seen in *Table 5* the farmers managing small farms had varied educational level, none of the farmers with medium sized farms had any academic education at all, while the only post secondary-schooled farmer in the study managed a large farm.

Table 5. Educational level of 16 farmers divided into groups depending on the size of their farms. Small = 1-5 buffaloes, Medium = 6-10 buffaloes, Large = >10 buffaloes

Farm size	Illiterate	Primary	Secondary	Post-secondary
Small	3	5	4	-
Medium	2	-	-	-
Large	-	1	-	1

4.4. Intramammary infections

Intramammary infections were found in 40% (50 out of 125) of the milk samples from quarters with SCM. Out of these 50 samples, 21 (42%) samples contained a mixed infection with 2 different bacteria. There was a total of 3 (2.4%) out of 125 contaminated samples among the SCM positive samples from the farms included in this study.

The most common finding among samples from SCM quarters with IMI was NAS (59%), followed by *Micrococcus luteus* (9.6%) and *Corynebacterium* spp. (4.1%). For detailed bacterial results see *Table 6.*

Table 6. Pathogens found in milk samples from SCM quarters with IMI (n=50). 16 farms, 114 buffaloes and 456 quarters were tested.

Pathogens	All farms		Small farms		Medium farms		Large farms	
	N	%	N	%	N	%	N	%
Non-aureus staphylococci	43	59%	13	50%	7	58%	23	66%
Micrococcus luteus	7	9.6%	1	3.8%	1	8.3%	5	14%
Corynebacterium spp.	3	4.1%	-		-		3	8.6%
Streptococcus spp.	2	2.7%	1	3.8%	-		1	2.9%
Bacillus spp.	2	2.7%	-		1	8.3%	1	2.9%
Arcanobacterium pluranimalium	1	1.4%	1	3.8%	-		-	
Klebsiella spp.	1	1.4%	1	3.8%	-		-	
Rothia endophytica	1	1.4%	1	3.8%	-		-	
Moraxella spp.	1	1.4%	-		1	8.3%	-	
Deinococcus wulumuqiensis	1	1.4%	-		1	8.3%	-	
Unidentified bacteria	11	15%	8	31%	1	8.3%	2	5.7%

The most common NAS specie were *S. hyicus* and *S. hominis*, followed by *S. chromogenes*, *S. epidermidis* and *S. sciuri*. The distribution of different NAS-species can be seen in *Figure 4*.

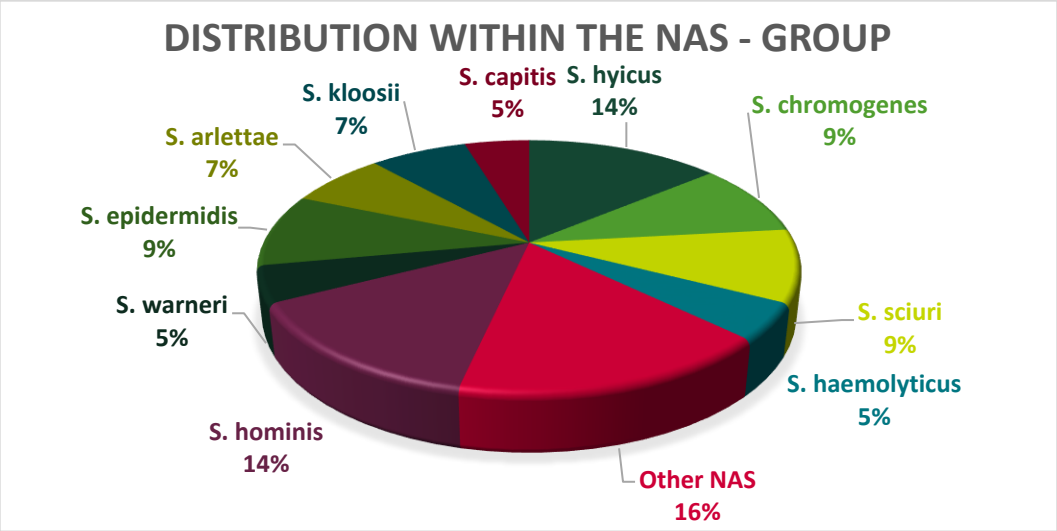

Figure 4. 114 buffaloes and 456 quarters were tested. Out of 73 isolates from SCM quarters with IMI, 43 (58.9%) were non-aureus staphylococci (NAS). The figure shows the distribution between the different NAS species after evaluation by MALDI-TOF.

In addition to the samples from quarters with SCM, 30 randomly selected milk samples from healthy quarters (CMT 1) were taken. Looking at the numbers, there was no obvious visual difference in occurrence of IMI in quarters with SCM and CMT-1 quarters.

Combining results from quarters with SCM and healthy quarters, the overall occurrence of IMI was 39%. The proportion of samples having IMI for each CMT-

score (1-5) can be seen in *Figure 5.* The differences in IMI occurence between healthy quarters (CMT 1) and quarters with CMT 2 and CMT 3 were small. In *Figure 5.* can be seen that the proportion of samples with IMI from quarters with CMT 4 and CMT 5 was clearly higher than that of healthy quarters (CMT 1).

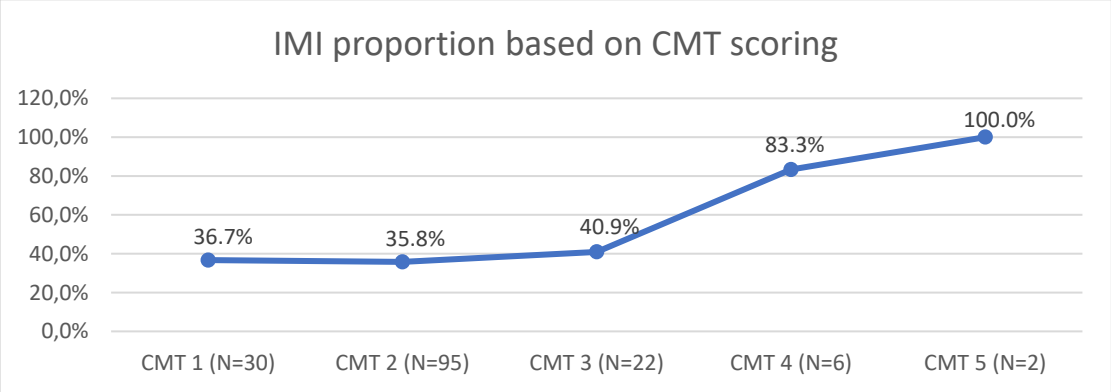

Figure 5. The occurrence of IMI in 155 milk samples with different CMT-scores from 114 buffaloes in Bangladesh.

4.5. Antimicrobial resistance

Out of the 43 NAS isolates found in this study, 14 (33%) were positive for beta-lactamase production. The occurrence of beta-lactamase production varied (0-100%) between species (*Figure 6*). All isolates of *S. arlettae, S. capitis, S. epidermidis and S. kloosi* were beta-lactamase producing while other species did not show any resistance against penicillin.

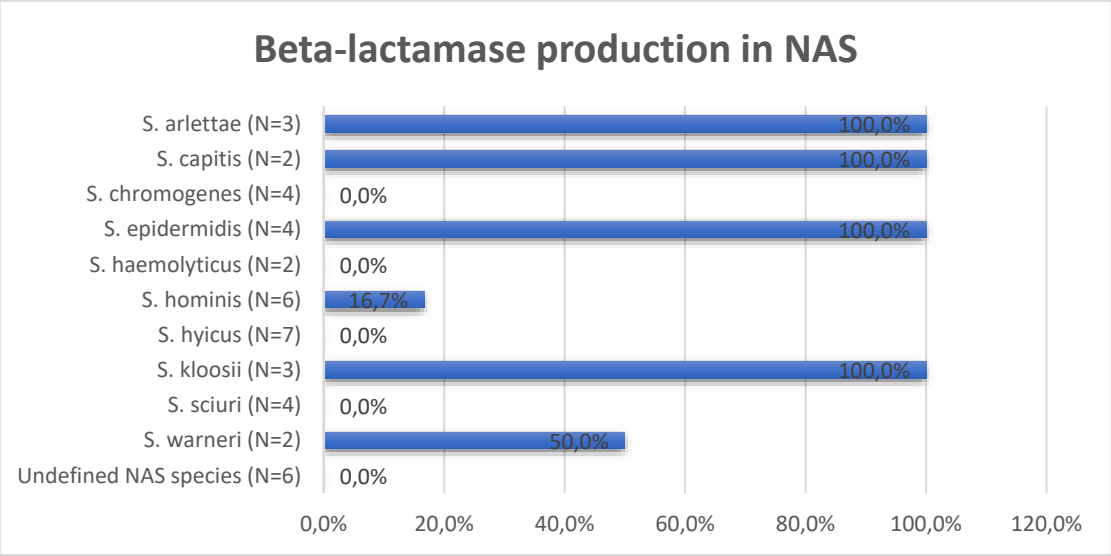

Figure 4. Proportion (%) of isolates producing beta-lactamase production among different species of non-aureus staphylococci (number within brackets) isolated from 155 milk samples from water buffaloes with SCM.

5. Discussion

5.1. Occurrence of subclinical mastitis

5.1.1. Cow and quarter level

The overall occurrence of SCM on cow level in this study was 56% and 27% on quarter level. The cow level occurrence found in this study was high compared to recent studies in Bangladesh, which found it to be between 20% and 50%, while a comparison on quarter level is more difficult to make due to lack of published data (Saiful Islam *et al.* 2016; Talukder *et al.* 2016; Islam *et al.* 2019; Biswas *et al.* 2020). Since the number of previous studies from Bangladesh is low, comparisons were also made with studies in neighbouring countries and compared to those our results both on cow and quarter level aligns well. A study from India on 120 buffaloes in 2019 showed a cow level SCM prevalence of 68.3% and a study from Pakistan in 2013 had a prevalence of 59.6% on cow level (Mustafa *et al.* 2013; Kashyap et al. 2019). Other studies reported lower prevalence where a study from Pakistan in 2018 had a cow level prevalence of only 22.9% and a study from India and Nepal in 2006 had a cow level prevalence of 21.7%. (Dhakal 2006; Maalik *et al.* 2019). Our quarter level occurrence on 27.2% can be compared to 8% in the India and Nepal study, 32.9% in an Indian study and 33.8% in another Indian study (Dhakal 2006; Sharma & Sindhu 2007; Sharma *et al.* 2018). Our results on both cow- and quarter level are between these previous results but in the upper span. However, a comparison between different studies should be made with caution since in some cases the definition of SCM differs from our definition CMT2 or higher; for example using SCC over 200 000 cells/mL and/or presence of IMI as definition of SCM. In yet other studies the authors have not given any clear definitions for classification of SCM samples at all.

5.1.2. Farm level

The occurrence of SCM differed between farms; from 0% occurrence both on cow and quarter level till 100% occurrence for the same in other farms. That can be explained by the small farm size for several individual farms in our study. For example, if a buffalo in a farm with only one animal did not have SCM the occurrence of the whole farm would be 0% both on cow and quarter level and if another buffalo in another farm had SCM in all quarters the occurrence would be 100% on both cow and quarter level.

Measuring of bulk milk SCC is an alternative indirect method to estimate the herd level occurrence of SCM. The SCC in bulk milk, differed a lot between farms. From 47 000 cells/mL to 587 000 cells/mL with an average of 195 875 cells/mL. This can be compared to a study from 2006 that concluded the normal SCC for Murrah buffaloes to be up to 200 000 cells/mL, and to the threshold 100 000 cells/mL for normal Murrah buffalo milk recently set by the National Dairy Research institute of India (Dhakal 2006; National Dairy Research Institute of India 2021). The differences in SCC between farms in Noakhali or Bagerhat divided area wise and farms divided by herd size were both small. There was no obvious association between a high SCM occurrence and a high SCC on farm level.

One explanation to the latter could be to question how reliable the SCC results were. To the authors knowledge there can be 3 sources of errors in the SCC results in this study. 1. Inadequate mixing of the milk prior to taking the bulk milk samples. 2. Since we brought the DCC with us in the field and the weather was warm (25-40 ℃) and sunny, even with our best intent and efforts, it should not be foreseen that the manufacturer's recommended maximum temperature (50 °C) for storage of the DCC cassettes, from time to other could have been exceeded, hence possible cause of malfunction. 3. Several buffaloes had their calves beside them before milking which can be an explanation for a false low SCC if recently having been suckled. Since our definition for SCM was CMT 2 or higher, which is equivalent to a SCC from 150 000 cells/mL, and at the same time a normal SCC count for buffaloes can be up to 200 000 cells/mL, a buffalo could still count as a having SCM, according to our definition, without a severely increased SCC (Schalm & Noorlander 1957; Dhakal 2006).

5.2. Epidemiological data

For result purposes we did not consider which breeds are kept on each farm in this study. Neither did we consider parity or time since last calving. The reasons for this are the low response frequency and poor knowledge about these topics by the farmers themselves.

As for educational levels, there were no obvious differences between managers of farms of different sizes. This was somewhat surprising to us since our hypothesis was that larger farms would have had more well-educated managers. Because there was no difference a comparison with SCM occurrence could not be done.

We also had a hypothesis that there would be differences in SCM occurrence depending on farm size, hence the higher infectious pressure at larger farms but that did not seem to be the case in this study. The reasons for this are somewhat unclear but could be due to a small and skewed sample size.

If there would have been obvious differences in SCM occurrence between the areas Bagerhat and Noakhali, that could have been a good starting point for future studies. Since we did not find any obvious differences and the author has not found any previous studies comparing these two areas no further analysis on the topic can be made based on this study.

5.3. Intramammary infections

5.3.1. Pathogens

Most of our IMI, 59%, were *Staphylococcus* spp. The result was expected since other similar studies in the region came to the same conclusion. For example, a study on 2057 Indian buffaloes found that 48.2% of the IMI cases associated with SCM were caused by *Staphylococcus* spp (Sharma & Sindhu 2007). Another study, also from India, in which 1299 buffaloes were included, *Staphylococcus* spp. was found in 51.7% of the SCM cases with IMI (Sharma *et al.* 2018).

The IMI results were also expected in hand milked farms with low environmental standards and poor hand hygiene, since the transmission of NAS infection can be both by the milkers' hands between different udders and from the environment to the udder.

Not many studies have looked at the distribution between different *Staphylococcus* spp. in buffaloes. In one study that did, 65.7% of the staphylococci found were *S. epidermidis* and the rest, 34.3% were *S. aureus* (Sharma & Sindhu 2007). Unfortunately, the authors of that study failed to communicate how the typing was done, and the fact that they only found 2 different species in their large study of 1338 mastitis cases with *Staphylococcus* spp., makes it hard to trust that their result in all details is reliable. The most common staphylococci in our study, *S. hyicus*, has, to the authors knowledge not been diagnosed in any other similar studies in the region before, possibly due to a lack of diagnosing methods. This species is a skin opportunist, mainly associated with skin diseases in pigs and is also listed as a minor mastitis pathogen in dairy cattle (Constable 2017). *S. aureus* was not found in our study at all, which was rather unexpected since that species

has been a frequent finding in other buffalo SCM studies in the region (Sharma & Sindhu 2007). The author has not been able to find any explanations for this.

Seven (9.6%) of our IMI was caused by *Micrococcus luteus*. This was a surprising find in our hand milked herds since the bacteria has rarely been found in previous similar studies and is most often spread via dairy equipment (Sharma & Sindhu 2007; Campbell & Marshall 2016; Sharma *et al.* 2018).

Almost one fifth of the SCM positive samples found in this study had mixed infections with two different bacterial species. Compared to other buffalo studies in the region this was a high number. For example, Sharma and Sindhu (2007) found 7.8% of the IMI to be mixed infections in a study with both CM and SCM and another SCM study found 17,5% of IMI to be caused by mixed infections (Sharma et al. 2018). It is the authors belief that our results are true and that the reason for our high number compared to others could, at least partly, be explained by our possibility to differ between for example different NAS species (using MALDI-TOF) which the studies mentioned above could not do to the same extent.

5.3.2. Associations between SCM and IMI

The overall occurrence of IMI in samples from SCM quarters was 40.0%, while the same for samples from quarters with CMT 1 was 36.7%. The differences between the two groups were smaller than we had expected.

Comparison with other studies is however hard for 2 reasons. 1. Most other similar studies on buffaloes have not compared the relation between SCM occurrence (as defined by us) with IMI occurrence. 2. In some studies the definition for SCM itself, has been the presence of IMI together with a positive CMT and lack of signs of CM.

Since our definition of SCM starts at CMT 2 (150 000 - 500 000 cells/mL) it is possible that our result is similar to a previous study on 60 buffaloes in 2006, that concluded 94% of buffaloes (cow level) with SCC > 200 000 cells/mL, not to have IMI (Schalm & Noorlander 1957; Dhakal 2006). Several previous studies on dairy cows have found around 80% of CMT-positive quarters to also have IMI, which makes our result somewhat confusing (Godden *et al.* 2017; Kandeel *et al.* 2018). For CMT 4, around 1 million cells/mL, and more the differences in IMI probability were obvious when compared to SCM negative samples.

5.4. Antimicrobial resistance

Almost one third (32.6%) of the NAS isolates found in this study were producing beta-lactamase and therefore resistant to penicillin, however there were big variations from 0 to 100% resistance for different species. Compared to other recent studies on the subject in Bangladesh our results are low since one study found all

isolates from SCM quarters to be resistant to penicillin regardless of bacterial species found. The most common in that study was however *Staphylococcus* spp., and another study found *Staphylococcus* spp. isolated from SCM quarters to be highly resistant to amoxicillin, erythromycin and azithromycin without specifying exact numbers (Sinha *et al.* 2014; Talukder *et al.* 2016). When compared to a review article on studies published in the field of AMR on other dairy producing animals in Bangladesh from 2011-2020 the level of AMR is also fairly low. While we only looked at beta-lactamase production the review article, written by Al Amin *et al.* (2020) reported on different kinds of AMR. Al Amin found alarming AMR-prevalences for the staphylococcal species with 42-100% for amoxicillin, 73-100% for ampicillin, 73-100% for streptomycin and up to 88% for tetracycline while penicillin was not examined (Al Amin *et al.* 2020).

Antibiotic usage for animals is not regulated by law in Bangladesh and more than 80% of the animal owners in rural Bangladesh buy low-cost medicine from their local pharmacy over the counter without prescription, and treat their animal with antibiotics in suboptimal doses on recommendation from unlicensed village health advisors (Al Amin *et al.* 2020). It is widely known that unregulated use of antimicrobials and use due to wrong indications is heavily increasing the AMR-rate (WHO 2020).

When compared to AMR of NAS in a study in Swedish dairy cows with SCM from 2011, the results from our study are, however, not alarming (Persson Waller *et al.* 2011). That study found that 38% of the isolated NAS were producing beta-lactamase. In line with the findings of the present study the resistance patterns of different species varied but not in the same way. The Swedish study found 10%, 40% and 50% of the *S. chromogenes*, *S. epidermidis* and *S. haemolyticus*, respectively, to be resistant to penicillin while in our study it was 0%, 100% and 0%, respectively, for the same species (Persson Waller *et al.* 2011). Since the conditions and species, dairy cows versus buffaloes, are so much different in these two studies a comparison between the two should be made with a big amount of caution.

An explanation for the fairly low penicillin resistance in our study is that penicillin is not a frequently used drug in Bangladesh. A cross-sectional study on self-medication of humans including 1300 people in Bangladesh found the most frequently used antibiotics to be metronidazole, azithromycin and ciprofloxacin (Biswas *et al.* 2014). A review study from 2009 about commonly used antibiotics in agriculture in Bangladesh lists 36 common commercial products none of which a pure penicillin product (Chowdhury *et al.* 2009). The author has not been able to find any official statistics written in the English language on antibiotics used or sold for livestock purposes in Bangladesh.

5.5. Methological considerations

Seventeen farms and 132 buffaloes were included in this study. One farm was excluded from the study directly after first bacterial culturing and interpretation at CVASU lab due to a heavily increased proportion of contaminated samples compared to the other studied farms. Since the now excluded farm would have distorted most of the results, we evaluated that the results of this study would be more trustworthy without it. Therefore, this study should be considered to be based on only 16 farms and 114 buffaloes for all further considerations.

The selection of farms was made mainly on 3 conditions. 1. They had to be located in areas Bagerhat and Noakhali and fairly easily accessible by car or boat. 2. The farmers had to have good relationships with local veterinarians since those were the main connection points between farmers and our research group. 3. The farmers had to willingly accept our visit. Based on point 1, those 2 areas, are to the authors knowledge, representative for coastal areas of Bangladesh and can be considered randomly chosen. However, based on point 2 and point 3 it is the authors opinion that it is possible that we did not get to visit the most poorly managed farms since those could be expected to not have a good relationship with their local veterinarian and not willing to welcome visitors.

It can be mentioned that we visited, and also meant to test, around 30 more buffaloes on 1 more farm but upon arrival to that farm we were not allowed to test any buffaloes there, since the farm was military owned, strictly managed, and we had failed to correctly address our visit on beforehand.

If we would do a similar study in the future, we would consider visiting a higher number of large farms. The time and funds consumed for each tested buffalo and each milk sample in large farms were much lower than in the smaller ones. The low number of large farms makes it possible for the largest farm in the study to affect the results and conclusions made over the whole study group since more than 50% of the total numbers of buffaloes in this study lived on that same farm. Although the larger farms could possibly be more common in the future the small farms visited in this study are very well representative for rural Bangladesh today.

Although the number of buffaloes in our study was low it was still higher than any of the 4 previous studies made in Bangladesh that the authors has been able to find which reached from 30 buffaloes up to 70 buffaloes or 114 samples (Saiful Islam *et al.* 2016; Talukder *et al.* 2016; Islam *et al.* 2019; Biswas *et al.* 2020). The low number of buffaloes in our study however gives little room for statistical analyses and makes it hard to draw valid conclusions on predisposing factors among others.

It would possibly have been better to take samples also from all healthy (CMT 1) quarters for bacterial culturing but that was scrambled in the planning stage due to economic reasons since we expected the proportion of IMI in SCM negative quarters to be low. For a more randomized selection it would possibly also have

been better to take one healthy sample from each udder instead of approximately every fifth healthy quarter. The decision on that however was made by local personnel and out of the author's control.

6. Conclusion

The results of our study regarding SCM occurrence both quarter and cow level align well with previous studies from the region. SCC as an indirect indicator of SCM occurrence on farm level did not show to be a useful method in this study. The definition for SCM in this study; CMT 2 or higher, did not work as an indicator of IMI in this study since the occurrence of IMI in both SCM-quarters and CMT1-quarters reached similar levels.

We found the most common pathogens to be NAS which aligns well with previous studies in the area. The AMR was investigated only in terms of beta-lactamase production in *Staphylococcus* spp. The author has not been able to find previous studies on SCM and buffaloes from the region investigating beta-lactamase production but compared to Scandinavian dairy cow studies and to studies on other types of AMR on buffaloes in the south Asian region, our study found a rather low prevalence.

We also aimed at investigating a few predisposing factors. Area or size did not matter to the occurrence of SCM. Breed, parity and time since last calving, could not be assessed due to lack of data on this in the farms. Educational level of the farmers varied greatly from illiterate till post-secondary school even within farms of the same size, and no conclusions could be drawn about the managers education based solely on farm size.

Since the demand for milk in Bangladesh is much bigger than the production there is a need for increased milk production. Based on what we found in this study and what is already well known within the field that increase must come without increasing the SCM cases and without increasing the antibiotic usage.

References

Al Amin, Md., Hoque, M.N., Siddiki, A.Z., Saha, S. & Kamal, Md.M. (2020). Antimicrobial resistance situation in animal health of Bangladesh. *Veterinary World*, 13 (12), 2713–2727. https://doi.org/10.14202/vetworld.2020.2713-2727

Biswas, D., Hanif, S., Ahmed Rana, E. & Anower, A.M. (2020). A study on udder health management practices, reproductive disorders and subclinical mastitis in buffalo herds in coastal region of Bangladesh. *Turkish Journal of Agriculture - Food Science and Technology*, 8 (8), 1662–1667. https://doi.org/10.24925/turjaf.v8i8.1662-1667.3416

Biswas, M., Roy, M.N., Manik, M.I.N., Hossain, M.S., Tapu, S.T.A., Moniruzzaman, M. & Sultana, S. (2014). Self medicated antibiotics in Bangladesh: a cross-sectional health survey conducted in the Rajshahi City. *BMC Public Health*, 14 (1), 847. https://doi.org/10.1186/1471-2458-14-847

Bryan, L.E. & Godfrey, A.J. (1991). Beta-lactam antibiotics: mode of action and bacterial resistance. In: Lorian, V. (ed.), *Antibiotics in Laboratory Medicine*. 3rd ed. Baltimore: Williams & Wilkins.

Campbell, J.R. & Marshall, R.T. (2016). *Dairy Production & Processing; The Science of Milk and Milk Products*. 1st ed. Long Grove, Illinois; Waveland Press.

Carter, G.R., Cole, J.R. & Carter, G.R. (eds.) (1990). *Diagnostic Procedures in Veterinary Bacteriology and Mycology*. 5th ed. San Diego, California: Academic Press.

Castelló, J.R. (2016). *Bovids of the World - Antelopes, Gazelles, Cattle, Goats, Sheep, and Relatives*. Princeton University Press.

Choudhary, B.B. & Sirohi, S. (2019). Sensitivity of buffaloes (Bubalus bubalis) to heat stress. *Journal of Dairy Research*, 86 (4), 399–405. https://doi.org/10.1017/S0022029919000773

Chowdhury, R., Haque, M., Islam, K. & Khaleduzzaman, A. (2009). A review on antibiotics in an animal feed. *Bangladesh Journal of Animal Science*, 38 (1–2), 22–32. https://doi.org/10.3329/bjas.v38i1-2.9909

CIA (2020). *CIA World Factbook*. https://www.cia.gov/library/publications/the-world-factbook/geos/bg.html [2020-02-05]

Constable, P.D. (2017). *Veterinary Medicine. Volume 1: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats*. 11th ed. St. Louis, Missouri: Elsevier.

DeLaval (2020b). *DeLaval california mastitis test CMT - DeLaval*. https://www.delaval.com/sv/vara-losningar/mjolkning/juverhalsa--hygien/mjolkundersokning/california-mastitis-test-cmt/ [2020-02-18]

DeLaval (2020a). *DeLaval cell counter DCC - DeLaval*. https://www.delaval.com/en-gb/our-solutions/milking/udder-health--hygiene/milk-testing/delaval-cell-counter-dcc/ [2020-02-18]

Dhakal, I.P. (2006). Normal somatic cell count and subclinical mastitis in Murrah buffaloes. *Journal of Veterinary Medicine, Series B*, 53 (2), 81–86. https://doi.org/10.1111/j.1439-0450.2006.00918.x

Dhakal & Nagahata (2018). Evaluation of mastitis related measures & their applications to classify buffalo milk in Chitwan, Nepal. *Journal of Agricultural Science and Technology A*, 8 (2). https://doi.org/10.17265/2161-6256/2018.02.006

FAO (2017). *Evaluation of FAO's Contribution to Bangladesh*. Office of evaluation, Country programme evaluation series. http://www.fao.org/3/BD730/bd730.pdf

FAO (2020). *Gateway to dairy production and products: Milk production.* http://www.fao.org/dairy-production-products/production/en/ [2020-02-17]

Franklin, A. & Wierup, M. (1982). Evaluation of the sensititre method adapted for antimicrobial drug susceptibility testing in veterinary medicine. *Veterinary Microbiology*, 7 (5), 447–454. https://doi.org/10.1016/0378-1135(82)90061-X

General Economics Division - Government of Bangladesh (2016). *7th Five Year Plan FY2016 - FY2020*. https://www.unicef.org/bangladesh/sites/unicef.org.bangladesh/files/2018-10/7th_FYP_18_02_2016.pdf

Godden, S.M., Royster, E., Timmerman, J., Rapnicki, P. & Green, H. (2017). Evaluation of an automated milk leukocyte differential test and the California Mastitis Test for detecting intramammary infection in early- and late-lactation quarters and cows. *Journal of Dairy Science*, 100 (8), 6527–6544. https://doi.org/10.3168/jds.2017-12548

Hamid, M.A., Ahmed, S., Rahman, M. A., & Hossain K. M. (2016). Status of buffalo production in Bangladesh compared to SAARC countries. *Asian Journal of Animal Science*, 10 (6), 313–329. https://doi.org/10.3923/ajas.2016.313.329

Hussain, A., Ahmad, M.-D., Mushtaq, M.H., Chaudhry, M., Khan, M.S., Reichel, M., Hussain, T., Khan, A., Nisar, M. & Khan, I.A. (2018). Prevalence of overall and teatwise mastitis and effect of herd size in dairy buffaloes. *Pakistan Journal of Zoology*, 50 (3), 1107–1112

Hussain, R., Javed, M.T., Khan, A. & Muhammad, G. (2013). Risks factors associated with subclinical mastitis in water buffaloes in Pakistan. *Tropical Animal Health and Production*, 45 (8), 1723–1729. https://doi.org/10.1007/s11250-013-0421-4

Islam, J., Rume, F.I., Liza, I.J., Chaudhary, P.K. & Anower, A.K.M.M. (2019). Assessment of subclinical mastitis in milch animals by different field diagnostic tests in Barishal district of Bangladesh. *Asian-Australasian Journal of Bioscience and Biotechnology*, 4 (1), 24-33.

Jorge, A.M., Andrighetto, C., Strazza, M.R.B., Correa, R. de C., Kasburgo, D.G., Piccinin, A., Victória, C. & Domingues, P.F. (2005). Correlation between the California mastitis test (CMT) and somatic cell count on milk from Murrah buffalo cows. *Revista Brasileira de Zootecnia*, 34 (6), 2039–2045. https://doi.org/10.1590/S1516-35982005000600029

Kandeel, S.A., Morin, D.E., Calloway, C.D. & Constable, P.D. (2018). Association of California mastitis test scores with intramammary infection status in lactating dairy

cows admitted to a veterinary teaching hospital. *Journal of Veterinary Internal Medicine*, 32 (1), 497–505. https://doi.org/10.1111/jvim.14876

Kashyap, D.K., Giri, D.K. & Dewangan, G. (2019). Prevalence of sub clinical mastitis (SCM) in she buffaloes at Surajpur district of Chhattishgarh, India. *Buffalo Bulletin*, 38 (2), 373–381

Khan, A., Durrani, A., Yousaf, A., Khan, J., Chaudhry, M., Khan, M., Habibunnabi, H. & Khan, A. (2019). Epidemiology of bovine sub-clinical mastitis in Pothohar Region, Punjab, Pakistan in 2018. *Pakistan Journal of Zoology*, 51. https://doi.org/10.17582/journal.pjz/2019.51.5.1667.1674

Maalik, A., Ali, S., Iftikhar, A., Rizwan, M., Ahmad, H. & Khan, I. (2019). Prevalence and antibiotic resistance of Staphylococcus aureus and risk factors for bovine subclinical mastitis in district Kasur, Punjab, Pakistan. *Pakistan Journal of Zoology*, 51 (3), 1123–1130

Mustafa, Y.S., Awan, F.N. & Zaman, T. (2013). Prevalence and antibacterial susceptibility in mastitis in buffalo and cow in district Lahore-Pakistan. *Buffalo Bulletin*, 32 (4), 307-314.

National Dairy Research Institute of India (2021). *Cut off value (SCC) for healthy Murrah buffaloes of INDIA*. Personal communication with Prof. Abdul Samad.

National Mastitis Council (1999). *Laboratory Handbook on Bovine Mastitis*. Madison, Wisconsin: NMC Inc.

Peek, S.F. & Divers, T.J. (2017). *Rebhun's Diseases of Dairy Cattle*. 3rd ed. St. Louis, Missouri: Saunders, Elsevier - Health Sciences Division.

Persson Waller, K., Aspán, A., Nyman, A., Persson, Y. & Grönlund Andersson, U. (2011). CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Veterinary Microbiology*, 152 (1–2), 112–116. https://doi.org/10.1016/j.vetmic.2011.04.006

Persson Waller, K. & Unnerstad, H. (2004). Klebsiellamastit - ett potentiellt gissel för mjölkproducenten. *Svensk Veterinärtidning*, 56 (11), 166–185.

Presicce, G.A. (2017). *The Buffalo (Bubalus bubalis) Production and Research*. Bentham Science Publishers.

Quinn, P.J. (2011). *Veterinary Microbiology and Microbial Disease*. 2nd ed. Chichester: Wiley-Blackwell.

Rahman, S.M.R., Islam, N. Harun-ur-Rashid, M,D., Siddiki, M.S.R. & Islam, M.A. (2019). Dairy buffalo production under intensive system in semi arid area of Bangladesh. *Buffalo Bulletin*, 38 (1), 83-98.

Romero, J., Benavides, E. & Meza, C. (2018). Assessing financial impacts of subclinical mastitis on Colombian dairy farms. *Frontiers in Veterinary Science*, 5. https://doi.org/10.3389/fvets.2018.00273

Saiful Islam, K.B.M., Bin Kabir, H., Rahman, H. & Kabir, H. (2016). Status of buffalo diseases in Bangladesh in relation to casual agents and predisposing factors. *International Journal of Life Sciences and Technology*, 9 (5), 44-50.

Sandholm, M., Honkanen-Buzalski, T., Kaartinen, L. & Pyörälä, S. (1995). *The Bovine Udder and Mastitis*. Helsinki: University of Helsinki, Faculty of Veterinary Medicine.

Schalm, O.W. & Noorlander, D.O. (1957). Experiments and observations leading to development of the California mastitis test. *Journal of the American Veterinary Medical Association*, 130 (5), 199–204

Sharma, A., Chhabra, R., Singh, M. & Charaya, G. (2018). Prevalence, etiology and antibiogram of bacterial isolates recovered from mastitis of buffaloes. *Buffalo Bulletin*, 37 (3), 313–320

Sharma, A. & Sindhu, N. (2007). Occurrence of clinical and subclinical mastitis in buffaloes in the State of Haryana (India). *Italian Journal of Animal Science*, 6 (sup2), 965–967. https://doi.org/10.4081/ijas.2007.s2.965

Siddiky, M.N.A. & Faruque, M.O. (2017). Buffaloes for dairying in South Asia: potential, challenges and way forward. *SAARC Journal of Agriculture*, 15 (2), 227–239. https://doi.org/10.3329/sja.v15i2.35167

Sinha, M.K., Thombare, N.N. & Mondal, B. (2014). Subclinical mastitis in dairy animals: incidence, economics, and predisposing factors. *The Scientific World Journal*, 2014. https://doi.org/10.1155/2014/523984

Smith, B.P. (2015). *Large Animal Internal Medicine*. 5th ed. St Louis, Missouri: Mosby, Elsevier Inc.

Srivastava, A.K., Kumaresan, A. & Manimaran, S.P., A. (2015). *Mastitis in Dairy Animals - an Update*. Satish Serial Publishing House.

SVA E.coli (2020). *Mastit orsakad av Escherichia coli hos nötkreatur - SVA*. /djurhalsa/djursjukdomar-a-o/mastit-orsakad-av-escherichia-coli-hos-notkreatur/ [2020-02-20]

Talukder, A.A., Rahman, H.H., Mahmud, S.J., Alam, F. & Dey, S.K. (2016). Isolation, identification and resistance pattern of microorganisms associated with mastitis in buffalo. *Bangladesh Journal of Microbiology*, 30 (1–2), 1–5. https://doi.org/10.3329/bjm.v30i1-2.28445

The World Bank (2020). *The World Bank - Bangladesh*. https://data.worldbank.org/country/bangladesh [2020-02-05]

University of Minnesota (2021). *Microbiology - Testing Methods for Detection of Antimicrobial Resistance*. http://amrls.cvm.msu.edu/microbiology/detecting-antimicrobial-resistance/test-methods [2021-06-09]

Utrikespolitiska Institutet (2020). *UI Landguiden - Bangladesh*. https://www.ui.se/landguiden/lander-och-omraden/asien/bangladesh [2020-02-05]

Vakkamäki, J., Taponen, S., Heikkilä, A.-M. & Pyörälä, S. (2017). Bacteriological etiology and treatment of mastitis in Finnish dairy herds. *Acta Veterinaria Scandinavica*, 59 (1), 33. https://doi.org/10.1186/s13028-017-0301-4

WHO (2020). *Antimicrobial resistance*. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance [2020-02-20]

Whyte, D., Walmsley, M., Liew, A., Claycomb, R. & Mein, G. (2005). Chemical and rheological aspects of gel formation in the California Mastitis Test. *The Journal of Dairy Research*, 72 (1), 115–121. https://doi.org/10.1017/s0022029904000561

Zhang, Y., Colli, L. & Barker, J.S.F. (2020). Asian water buffalo: domestication, history and genetics. *Animal Genetics*,. https://doi.org/10.1111/age.12911

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Appendices

Appendix 1 – Manufacturing of agars

Blood agar

Blood-agar (BA) was made from blood agar base by manufacturer Oxoid, lot no 2192928 with expiration date August 22, and aseptically taken blood from a healthy university (CVASU) horse.

40 grams of Oxoid blood agar base was solved in 1 litre of distilled water. The mix was then heated to boil under intermittent rotations to dissolve the powder completely. The mix was then put in an autoclave for 15 minutes at 121 °C. The mix was then slowly cooled down to 50 °C. At that point 70 millilitre of the collected horse blood was added to the mix. The solution was carefully mixed using movement in eights in varying speeds. The liquid was then poured into petri dishes with lids and put to side in a cupboard to cool down. After 60 minutes the dishes were stored in a refrigerator at 6 °C till needed for bacterial culturing.

Mannitol-Salt agar

Mannitol Salt agar (MSA) was made from agar base by manufacturer Oxoid, lot no 2167943 with expiration date July 22. 111 grams of powder was carefully measured and mixed with 1 litre of distilled water. The mix was heated under intermittent rotations to solve the powder completely. The solution was then put into an autoclave for 15 minutes at 121 °C. After being let to cool to 50 °C the solution was poured into petri dishes with lids. The dishes were put into a cupboard for 60 minutes before being stored in a refrigerator at 6 °C till needed for bacterial culturing.

MacConkey agar

MacConkey agar (MAC) was made from agar base by manufacturer Oxoid, lot number 2543177 with expiration date June 24. 52 grams of powder was carefully measured and mixed with 1 litre of distilled water. The solution was brought to a

boil to solve the powder completely while intermittent rotations were performed. The solution was then put into an autoclave for 15 minutes at 121 °C. After being let to cool down to 50 °C the solution was poured into petri dishes with lids. The dishes were put into a cupboard for 60 minutes before being stored in a refrigerator at 6 °C till needed for bacterial culturing.

Contamination control

Before being used for bacterial culturing a few dishes from each batch were randomly selected to be incubated for 24 hours at 37 °C. If any of the selected dishes showed signs of contamination the whole batch was discarded.

Appendix 2 – Manufacturing of Brain Hearth Infusion broth and preparation of isolates

Brain hearth infusion broth

The Brain hearth infusion broth was made from broth powder by manufacturer Oxoid, lot number 1837985 with expiration date April 21. 37 grams powder was solved into 1 litre of distilled water. After mixing the solution was put in an autoclave for 15 minutes at 121 °C. After cooling down to 50 °C the broth solution was poured into glass tubes. Each broth tube was sealed using cotton balls before incubation in 37 °C for 24 hours. The tubes were then visually inspected. Each tube with any visual signs of growth was considered contaminated and thrown away.

Preparation and storage of isolates

A plastic loop was used to take a few colonies from each BA. Each bacterial sample was mixed with broth in a glass tube before incubation in 37 °C for 8 to 24 hours. After incubation 100 microliters from each broth was taken using a pipette and together with 300 microliters of 50% buffered glycerol deponed in an small sterile plastic isolate tube. The isolate tubes were then sealed, marked and put into a freezer – 18 °C for 48 hours and then transferred, in a cold box with ice packs, for long time storage in a freezer – 80 °C.

Populärvetenskaplig sammanfattning

Mastit betyder inflammation i juvret. Subklinisk betyder att inflammationen inte ger några förändringar som kan ses med blotta ögat. Oftast orsakas sjukdomen av bakterier. Sjukdomen är ett stort problem hos mjölkproducerande djur över hela världen och orsakar stora produktionsförluster. Eftersom djuren inte blir synbart sjuka i den mening att lantbrukaren lätt upptäcker det och kan behandla, så lever djuren ofta i stället länge med sjuka juver som då producerar betydligt mindre mjölkmängd än friska juver.

Sjukdomen kan upptäckas genom att kontrollera om sammansättningen av mjölken förändrats. Celltalen, dvs antalet immunförsvarsceller i mjölken, ökar. Detta kan mätas antingen med laboratoriemaskiner eller med indirekta metoder så som CMT-vätska (California mastitis test). När CMT-vätskan blandas med mjölk med förhöjda celltal blir mjölken geléaktig och trögflytande. Denna förändring sker inte med frisk mjölk.

Mjölkförändringarna gör även att kvalitén på mjölken blir sämre vilket innebär en risk för de människor som dricker mjölken opastöriserad och orsakar även ett ökat matsvinn. Dessutom är sjukdomen ofta svårbehandlad även när upptäckt, vilket leder till ökad utslaktning av djur och risk för en ökad antibiotikaresistens.

Bangladesh är fortfarande ett relativt fattigt land i södra Asien även om levnadsstandarden ökar och sedan 2015 klassas landet som ett ”lägre medelinkomstland” av världsbanken. Bangladesh är till ytan ungefär en tredjedel så stort som Sverige och har 160 miljoner invånare. Ungefär 45 % av befolkningen är verksamma inom lantbruket.

Vattenbufflar är efter mjölkkor det viktigaste mjölkproducerande djuret för människoföda i världen. Ungefär 5 % av mjölk för humankonsumtion i världen kommer från vattenbufflar. Av världens ungefär 200 miljoner domesticerade vattenbufflar finns ungefär 180 miljoner i Asien och ungefär 1,5 miljoner av dessa finns i Bangladesh.

Domesticerade, tama, vattenbufflar hålls för mjölkproduktion i många asiatiska länder då de ofta är bättre anpassade för det varma klimatet än de traditionella mjölkkor som vi ser i bland annat Sverige.

Att ta reda på förekomsten av subklinisk mastit, samt vilka bakterier som orsakar dessa är viktigt för att veta hur lantbrukare ska kunna minska smittoriskerna, minska antibiotikaanvändningen, förbättra mjölkens kvalité som livsmedel, öka

produktionen och därmed minska slitaget på jordens resurser. Att ta reda på graden av antibiotikaresistens är viktigt för att veta hur sjukdomen effektivast bör bekämpas utan att öka resistensen.

I vår studie besökte vi och testade alla mjölkproducerande bufflar på 17 gårdar i Bangladesh. Antalet bufflar på varje gård varierade mellan 1 och 66. Totalt testades mjölken från 132 bufflar i den här studien.

CMT-vätska användes för att testa alla juverdelar på djuren i studien för att se förekomsten av subklinisk mastit, vilket 56 % av bufflarna hade. Mjölkprover togs sedan från de juverdelar som var sjuka och mjölken odlades senare på bakterieplattor för att undersöka vilka bakterier som fanns i den sjuka mjölken. Mjölkprover togs även från ett antal friska juverdelar för att undersöka om de juverdelar som inte gav utslag på CMT-testet och klassades som friska faktiskt inte heller innehöll några bakterier.

3 olika bakterieplattor användes. Blodagarplattor där de allra flesta bakterier växer, Mannitolsalt-agarplattor där bakterier av olika Stafylokock-arter växer, och MacConkey-agarplattor där framför allt bakterier av E. coli och olika Klebsiella-arter växer. Efter odlingarna på plats i Bangladesh bedömde vi bakteriekoloniernas utseende på de olika plattorna samt kollade på bakterierna i mikroskop för en preliminär bedömning av vad som växte. Frusna bakterie-isolat togs även med till Sverige för att testa bakterierna i en laboratoriemaskin som kallas MALDI-TOF. De sammanvägda resultaten visade att de vanligaste bakterierna, 59%, var olika stafylokocker, dock inga *Stafylococcus aureus*, och att den näst vanligaste bakterien var *Micrococcus luteus.*

Alla stafylokocker testades även angående antibiotikaresistens. 33 % av dessa producerade pencillinas som är ett enzym som gör att penicillin inte fungerar för att bekämpa bakterien. Penicillin är den överlägset vanligaste antibiotikatypen som används i Sverige mot juverinflammationer hos djur, i Bangladesh är dock andra antibiotikatyper vanligare. Utöver pencillinasproduktion finns även andra mekanismer för resistens men dessa testades inte i den här studien.

Våra resultat angående förekomsten stämmer väl överens med resultat från tidigare liknande studier i regionen både på individnivå och juverdelsnivå. Tidigare studier har visat att de vanligaste bakterierna oftast är olika stafylokockarter vilket det även var i vår studie men att vi inte hittade någon *Staphylococcus aureus* var förvånande då den stafylokockarten varit vanligt förekommande i flera tidigare liknande studier. Graden av pencillinasproduktion var svår att jämföra då andra jämförbara studier inte tittat på just denna typ av antibiotikaresistens.