Chapter I

Introduction

Milk is a very nutritional food that is rich in carbohydrate, proteins, fats, vitamins and minerals. The quality of milk may be lowered by a numbers of factors such as adulteration, contamination during and after milking and the presence of udder infections (Esron et al., 2005). Pathogenic organisms in milk can be derived from the cow itself, the human hand or the environment (Bradely, 2002). Mastitis, inflammation of the mammary gland, is a highly prevalent problem in dairy cattle and is one of the most important threats affecting the world's dairy industry (Wallenberg et al., 2002). *Staphylococcal* mastitis is the commonest and economically the greatest concern wherever dairy farming is practiced.

Staphylococcus aureus is the most notorious pathogen that resides in the world with its wide genetic diversity and wide host range and different pathologies associated with mild to severe infections. The bacterium is frequently found associated with subclinical mastitis in dairy cattle (Adesiyun et al., 1998) and may be present in milk and other dairy products (Capurro et al., 2010).

Mastitis in cattle caused by *S. aureus* can either be subclinical or clinical. According Bachaya et al. (2011), sub-clinical mastitis is of global importance in the dairy industry. It shows no noticeable alterations in the appearance of the milk or the udder, but there is decrease in milk production. The symptoms of clinical mastitis include swelling, hardness, redness, heat, and pain.

Presence of *S. aureus* in milk or any food is very important in public health ground (Balaban and Rasooly, 2000). On heating at normal cooking temperature, the bacteria may be killed but the toxins remains active (Presscott et al., 2002). In food stuffs it thought to be more resistant than in a laboratory culture medium (Bergdoll, 1983). *S. aureus* is reported to produce enterotoxins and involved in food poisoning (Beckeret et al., 2011). Therefore, milk of cow could also be a possible source of Staphylococcal infection responsible for food intoxication and toxico-infection.

S. aureus is a normal inhabitant of the healthy lower reproductive tract especially in vagina and vulva in female animals, urinogenital tract and various intra-abdominal organs (Murray et al., 2008; Ateba et al., 2010. Infected udders, teat canals, and teat lesions, and also teat skin, muzzles, nostrils and vagina act as major reservoirs of the micro-organisms causing mastitis and the bacteria tend to spread to uninfected parts by teat cup liners, milkers hands, wash cloths and flies (Rund et al., 1986; Madsen et al., 1991; Yeruham et al., 1996; Brody et al., 2008). At times even aerosols play a role in transmission of bacteria to uninfected parts. Usually Staphylococci do not colonize on healthy teat skin, but readily colonize teat canal if there are lesions present. The organisms multiply in infected lesions and enter into the udder causing the eradication procedure to be more severe.

Considering the aforementioned points, our investigation was conducted with an objective to isolate S. aureus and to estimate the prevalence and identify risk factors associated with it.

Chapter II

Materials and method

Study area

The study was conducted in Chittagong district, which is located about 250 km south east of Dhaka. Its annual temperature ranges from 16^{o}C - 38^{o}C.

Study animals

The study animals included 628 lactating cross breed cows from 102 small to large scale dairy farms.

Study type

The study was a cross-sectional study in which 628 lactating cows were tested for the presence of clinical and sub clinical mastitis.

Sample size determination

The sample size was determined from the cluster of 102 dairy farms which are found in and around Chittagong. The sampling frame from the study site indicated that the farms were small to large scale dairy farms having an average of 15 lactating cows each. Therefore, all the lactating cows from the 102 dairy farms were considered for this study which consisted of a total of 1481 lactating cows.

Study methodology

Data regarding the different potential risk factors (age, parity, lactation stage, housing conditions and previous history of mastitis) were collected from 628 lactating cows from farm records when available and by interviewing the farm owners. The udder, screening was done using the California mastitis test (CMT) and bacteriological examination were also carried out.

Clinical inspection of the udder

Udders of the cows were examined by visual inspection and palpation for the presence of any lesion, pain, heat and swelling. In addition, milk from each quarter was withdrawn and checked for any change in colour and consistency.

California mastitis test (CMT)

The California mastitis test was conducted to diagnose the presence of subclinical mastitis and it was carried out according to procedures given by Quinn et al. (1994). A squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the reagent was added. A gentle circular motion was applied in a horizontal plane. Positive samples show gel formation within a few seconds. The result was scored based on the gel formation and categorized as negative if there was no gel formation, or positive if there was gel formation ranging from 1-2. If at least one quarter was positive by the CMT then the cow was considered positive. Therefore, a cow was considered mastitic if one or more quarters were CMT positive with or without isolation of microorganisms.

Milk sample collection

Milk samples were collected by following aseptic measure. Every quarter was washed with tap water and dried (in cases when there was a considerable amount of dirt to be removed). Approximately 10 ml of milk was then collected aseptically from every four quarter of clinical and subclinical (CMT positive) mastitic cows into sterile universal bottles after discarding the first three milking

streams. Samples from each quarter were transported on ice to PRTC laboratory of Chittagong Veterinary and Animal Sciences University, where they were immediately cultured or stored at -204°.

Bacteriological Investigation:

Isolation and identification of Staphylococcus aureus

Isolation of *Staphylococcus aureus* was attempted according to Singh and Prakash (2008) with slight modification. Enrichment was carried out in Peptone water (PW) (Oxoid Ltd,

Basingstoke, Hampshire, UK). 5ml sample was homogenized with 45 ml sterile enrichment broth peptone water and enriched for 24 hours at 37°C (Thaker et al., 2013).

Both Mannitol salt agar (MSA) and Blood agar base were prepared according to the instructions of manufacturer (Oxoid Ltd, Basingstoke, Hampshire, UK). Blood agar was prepared by adding 5% citrated-bovine blood in the blood agar base. A loop full of inoculum from enrichment were streaked on Blood Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 hours for detection of hemolysis. Growth of yellow colonies on MSA (Oxoid Ltd, Basingstoke, Hampshire, UK) surrounded by yellow zones as a result of fermentation of mannitol after 24 hours of incubation at 37°C indicated a positive result (Kateete et al., 2010). The pure cultures were streaked on Nutrient agar (Oxoid Ltd, Basingstoke, Hampshire, UK) and incubated for 24 hours at and were further subjected to catalase and coagulase tests for biochemical confirmation of *Staphylococcus aureus* (Monica, 1991).

Morphological Characteristics:

Grams staining was done for identification of morphology and staining characters of microorganisms.

Suspected colony from MSA (Staphylococcus sp) was stained as described by manual of veterinary investigation laboratory Technique (OIE, 2000).

The procedure was as follows: A small colony was picked up with a bacteriological loop, smeared on clean grease free microscopic glass slide and fixed by gentle heating. Then we used crystal violet solution on smear to stain for two minutes and then washed with running water. Few drops of Gram's iodine were then added to act as mordant for one minute and poured off excess fluid. Acetone alcohol was then added for few seconds who act as a decolorizer. After washing with running water, safranin was added as counter stain on smear and allowed to stain for 2 minutes. The slides were then washed with water, blotted and dried in air and then examined under microscope with high power objective (100X) using immersion oil.

Smear revealed Gram positive, spherical cells arranged in irregular clusters resembling to bunch of grapes.

Biochemical examination:

Biochemical tests were performed to confirm Staphylococcus sp using Catalase test and Coagulase test.

Catalase test

The catalase test is used to detect presence of catalase enzymes by the decomposition of hydrogen peroxide to release oxygen and water. Hydrogen peroxide is formed by the some bacteria as an oxidative end product of anaerobic breakdown of sugars. Hydrogen peroxide being highly toxic should be eliminated from the bacteria or else it will result death of the cell. Catalase usually degrades hydrogen peroxide and does not show any effect on other peroxides.

a) Slide catalase test

A small amount of pure growth culture was transferred with a bacteriological loop from the MSA into clean grease free microscopic glass slide, and then a drop of catalase reagent (3% H_2O_2) was added. The evolution of gas bubbles indicates a positive result (Hogan et al., 1999; Macfaddin, 2000).

b) Tube catalase test

Nutrient agar slant was prepared according to the instructions of manufacturer (Oxoid Ltd, Basingstoke, Hampshire, UK). Suspected bacterial colonies inoculated into agar slant and incubated at 37°C for 24 hours. After that 1 ml of Catalase reagent (3% H_2O_2) was added and rapid ebullition of gas considered as positive reaction of Staphylococcus sp (Hogan et al., 1999).

Coagulase test

There are two methods for identifying *S. aureus* by coagulase test. One is tube coagulase test and other is slide coagulase test. Slide test is also known as latex agglutination test. Coagulase is an enzyme which can clot blood plasma and convert into gel like consistency. On this basis, microorganisms can be classified as coagulase positive or coagulase negative.

For coagulase test, whole blood from horse was collected into commercially available sterile tubes containing EDTA to perform the test. Then blood was centrifuged at 2600 rpm for 10 minutes using a refrigerated centrifuge device. The resulting supernatant, the

plasma, was then immediately transferred to a sterile 1.5 ml Eppendorf tube using a sterile tip and stored at -20°C for future use.

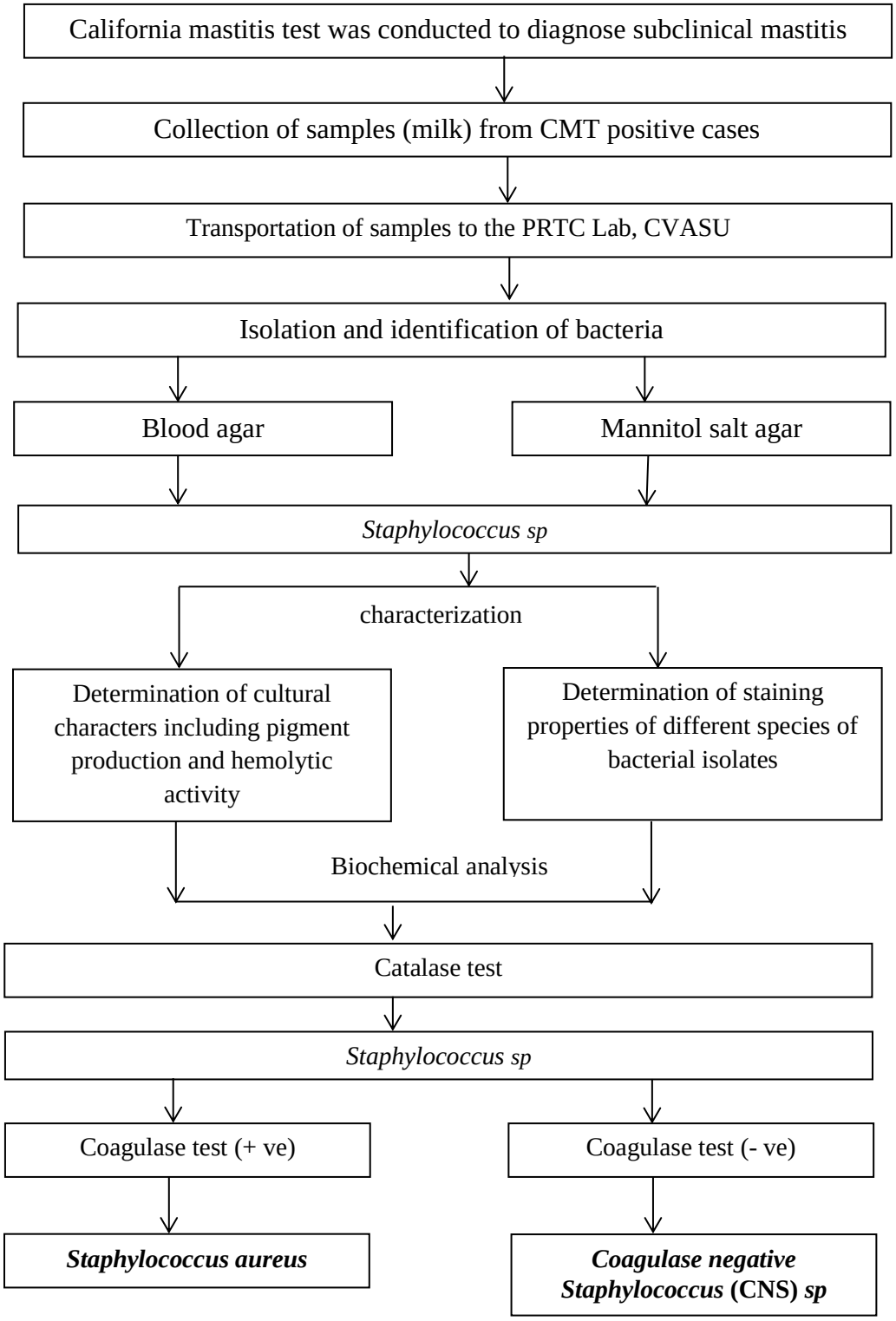
a) Tube coagulase test

From each tube cultivated in brain heart infusion broth (BHIB), 50 µL was transferred to sterile tubes containing 50 µL of horse plasma. Then, it was incubated at 37°C for 6 hours. The presence of coagulases was justified, considering large organized coagulation and coagulation of all the contents of the tube which do not come off when inverted (Brasil, 2003).

b) Slide coagulase test

One a clean grease free microscopic glass slide, test microorganisms are applied at two ends and a drop of horse plasma is added on to it. Clot formation observed were noted within 5-10 seconds is considered to be positive for coagulase (Macfaddin, 2000).

EXPERIMENTAL SCHEME



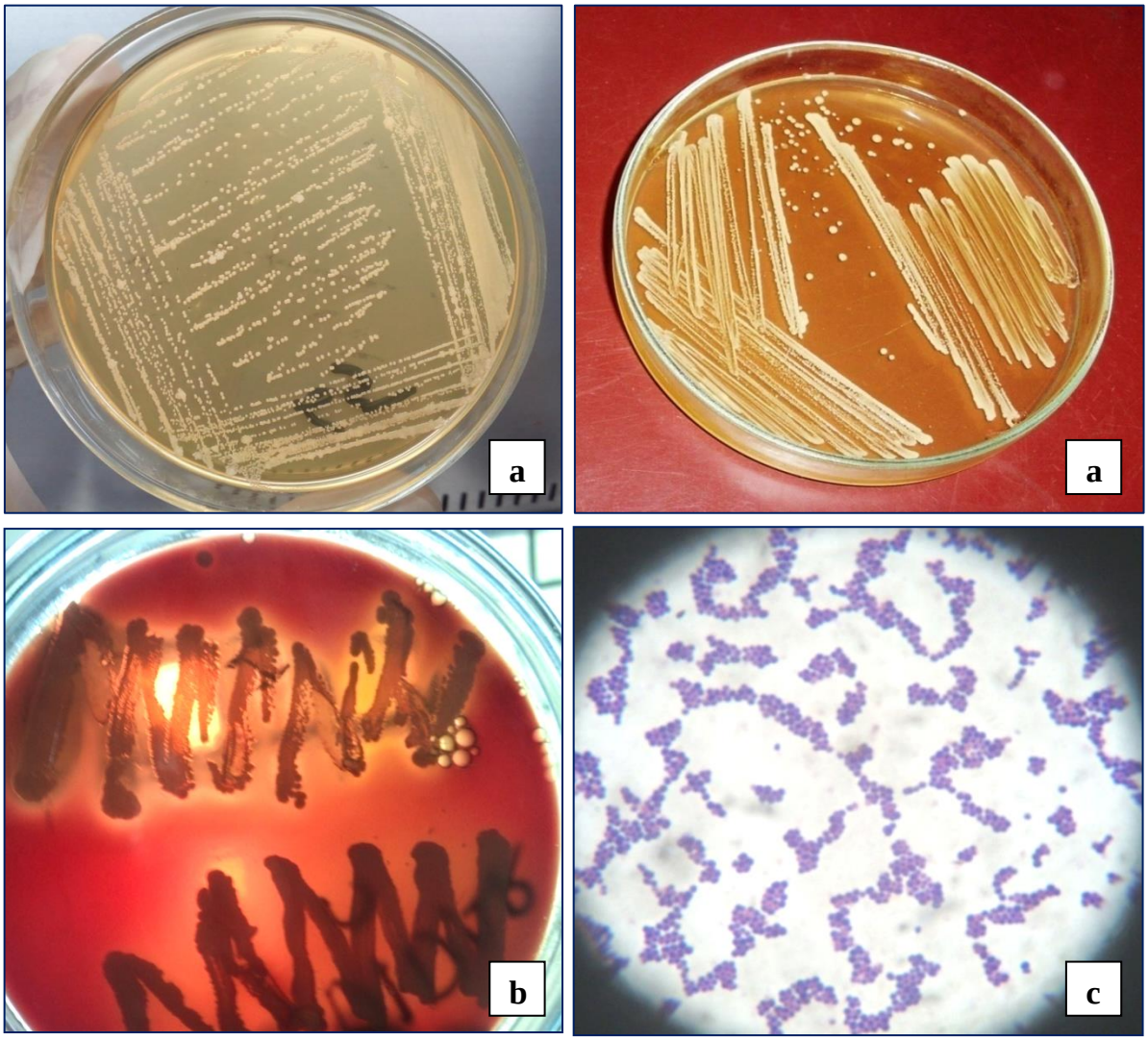


Fig: Bright yellow colonies indicating the growth of Staphylococcus sp on Mannitol salt agar plates (a) and hemolysis on blood agar plates (b) and grape like cluster under microscope in Gram's staining (c).

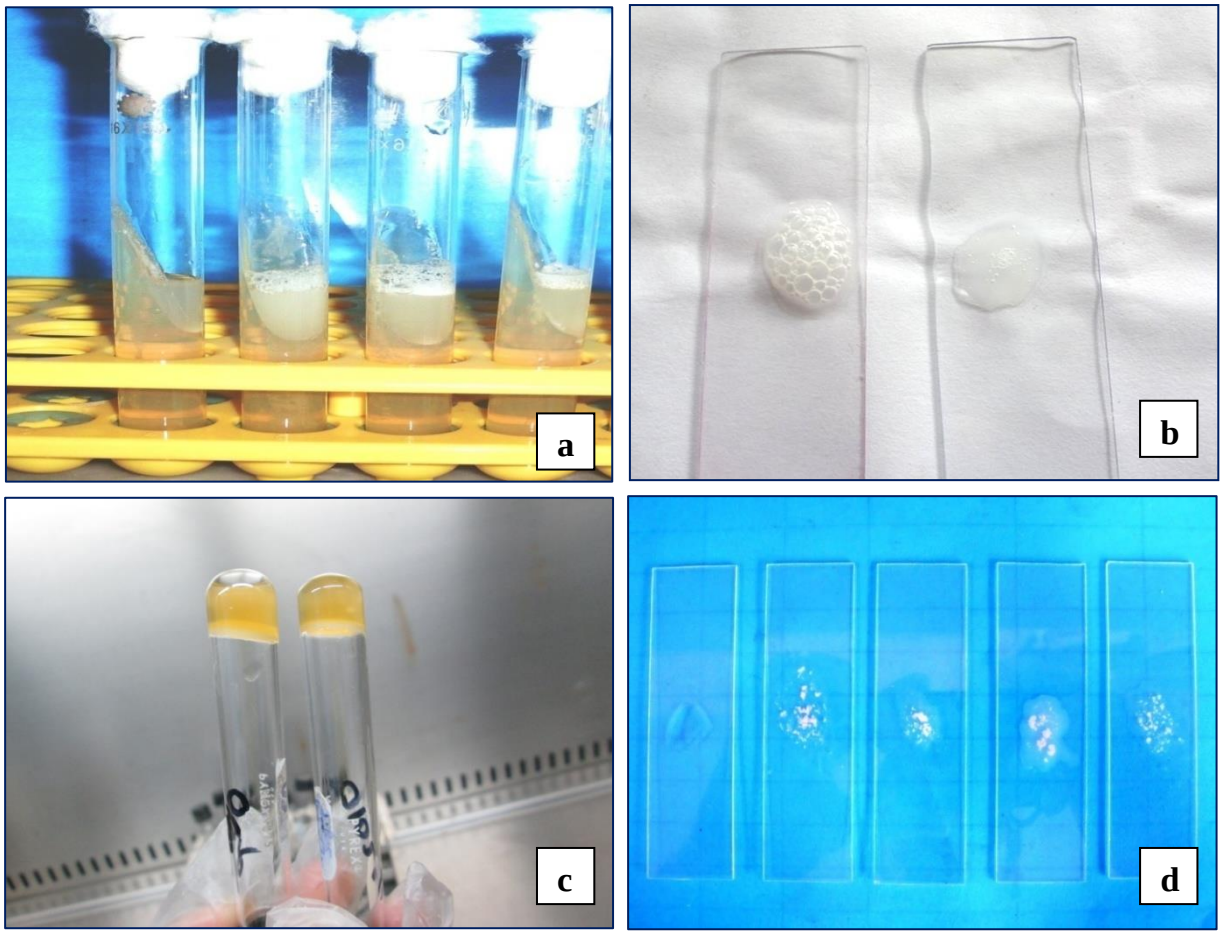


Fig: Tube catalase test for confirmation of Staphylococcus aureus (a) Slide catalase test for confirmation of Staphylococcus aureus (b) Tube coagulase test for confirmation of Staphylococcus aureus (c) Slide coagulase test for confirmation of Staphylococcus aureus (d).

Statistical analysis

All the data like Somatic cell count of bulk milk, cows forstripped before milking, milker wash udder before and after milking, disinfectants used before and after milking, udder dried before milking, Milking procedure Udder hygiene score, useing Mastitis vaccine and floor cleanliness score were entered into MS excel (Microsoft office excel-2007, USA). Data management and data analysis were done by STATA version-13 (STATA Corporation, 4905, Lakeway River, College Station, Texas 77845, USA). The association of bacterial isolates with different variables was evaluated by using chi-square ($\chi 2$) test. $P<0.05$ was set for significance.

Chapter III

Result

Properties of *Staphylococcus aureus* in different culture media:

Each collected sample was cultured on different culture media. The colonies which reflected various morphological characteristics were identified based on their staining, cultural, morphological and biochemical properties.

Following Gram's staining technique, the smear of the slide that revealed as gram positive, cocci, spherical cells and irregularly arranged in grapes like cluster depicted as Staphylococcus sp .

In non-selective media such as in blood agar colonies of Staphylococcus sp were circular, small, smooth raised with gray white or yellowish in color. On blood agar β-hemolysis was seen for S. aureus .

Staphylococcus sp showed different colonies on the mannitol salt agar (MSA). *Staphylococcus aureus* fermented MSA with the production of small to large yellowish colonies .

Result of Biochemical analysis:

Both slide and tube catalase tests were performed to differentiate Staphylococci (catalase producer) from Streptococci (non-catalase producer). Hydrogen peroxide was breakdown into water and oxygen. Production of oxygen was reflected by the bubble formation. All coagulase positive Staphylococcal isolates were found to be positive in catalase tests .

Positive isolates on MSA were further subjected to coagulase test and 13 isolates of them were found to be positive in both the tube and slide coagulase tests. Cultures which showed agglutination were recorded as positive in slide test [(Figure 3.2) (b)] and those which showed heavy coagulation of all the contents of the tube and did not come off even after inverting the tube upside down were recorded as positive for coagulase test .

Prevalence of *Staphylococcus aureus*:

A total number of 13 samples were found positive out of 101 collected healthy milk samples for S. aureus. The numbers were confirmed by their colonial growth characteristics on blood agar mannitol salt agar and biochemical test. The samples were further characterized by coagulase test and got the positive result for *Staphylococcus aureus.*

The farms that collected heifers from their own farm and from market both had a higher prevalence (63%) than the farms which collected the new cows from their own farm only (25%)

The farms that practiced stimulating the cow for milking by warm cloth had a higher prevalence (29.41%) relatively than the farms practiced by calf suckling (8.82%), hand (0%) and by warm water (0%).

Some farms practice forstrip before milking. These farms have a higher prevalence (29.41%) than the farms do not practice forstriping before milking (9.52%) and it was statistically significant ($p<0.05$).

The prevalence of *Staphylococcus aureus* is higher in the farms, where disinfectant is used before and after milking (15.63%) rather than the farms where it is not practiced (11.59%). But, this was not statistically significant.

Drying of udder was practiced in some farms where prevalence of subclinical mastitis was higher (18.51%) with *Staphylococcus aureus,* but was not statistically significant.

The prevalence of *Staphylococcus aureus* in case of clean udder, slight dirty udder, mostly dirty udder and completely dirty udder is respectively 6.06%, 26.92%, 28.57% and 0%.. Mostly dirty udder has a higher prevalence than the other variable. it was statistically significant ($p<0.05$).

Milking frequency of the farms are once daily, twice daily and trice daily. The prevalence is respectively 0%, 13.18% and 50%. Three times milking per day has a higher prevalence than the others.

Machine milking practicing farms have a higher prevalence (40%) than the farms practiced hand milking and it was also statistically significant ($p<0.05$).

Some farms apply dry cow therapy in some cows of the farm. The prevalence *Staphylococcus aureus* in those farms is 16.27%. On the other hand, the prevalence in the farms that apply dry cow therapy to all the cows and the farms dose not practice it at all , has a prevalence 10.52% and 9.68% respectively.

Prevalence of *Staphylococcus aureus* in mastitis vaccine applied farms is 11.11% which is lower than the farms where mastitis vaccine is not used (13.25%).

The estimated proportionate prevalence of *S. aureus* in muddy, concrete and brick made floor is respectively 11.95%, 25% and 0%.But, it was not statistically significant ($p>0.05$).

Rubber mattress and other materials are used as bedding. The prevalence of *S. aureus* in rubber mattress bedding is 12.28% and other than rubber mat it is higher (14.28%).

In 100% dry floor (no feces), almost 70%% dry floor (no feces), maximum 50% dry floor (feces and mud) and completely muddy floor covered by feces , the prevalence of *S. aureus* is respectively 5.56%, 16.27%, 13.33%, and 28.57%. The dirty floor has a higher prevalence in deed.

Table: Prevalence of Staphylococcus aureus in sub-clinical astatic milk in relation to different variables

Variables	Category	N	Positive	Negative	Prevalence (%)	P-Value
Animal source	Farm	29	4	25	13	
	Farm and market	72	9	63	12.5	0.86
Stimuli	Calf/suckling	34	3	31	8.82	
	Hand	6	0	6	0	0.512
	Warm water	1	0	1	0	
	Warm cloth	60	10	50	29.41	
Forstripped before milking	Yes	17	5	12	29.41	
	No	84	8	76	9.52	0.026
Disinfectants used before and after milking	yes	32	5	27	15.63	0.574
	No	69	8	61	11.59	
udder dried before milking	Yes	27	5	22	18.51	0.306
	No	74	8	66	10.81	
Udder hygiene	Clean	66	4	62	6.06	
	Slight dirty	26	7	19	26.92	0.027
	Mostly dirty	7	2	5	28.57	
	Completely dirty	2	0	2	0	
Milking frequency	1 time/day	8	0	8	0	
	2 time/day	91	12	79	13.18	
	3 time/day	2	1	1	50	0.094
Milking process	Machine milking	5	2	3	40	
	Hand milking	96	11	85	11.45	0.063
Dry cow therapy	Practiced in all cows	19	2	17	10.52	
	Practiced in some cows	43	7	36	16.27	0.664
	Don’t practiced	31	3	28	9.6811.	

Variables	Category	N	Positive	Negative	Prevalence (%)	P-Value
Mastitis vaccine	Used	18	2	16	11.11	0.806
	Don't used	83	11	72	13.25	
Floor	Muddy	92	11	81	11.95	
	Concrete	8	2	6	25	0.531
	Brick	1	0	1	0	
Bedding	Rubber mat	57	7	50	12.28	
	Straw	2	0	2	0	0.824
	Other	42	6	36	14.29	
Floor cleanliness	100% of the floor is dry & no feces	36	2	34	5.56	
	At least 70% of the floor is dry & no feces	43	7	36	16.27	
	Maximum 50% of the floor is covered with mud & feces	15	2	13	13.33	0.295
	Floor totally covered with mud & feces	7	2	5	28.57	

Chapter IV

Discussion

The present study was designed for the isolation, identification and characterization of *S. aureus* from raw milk samples from dairy cattle. Our results indicated that 13 samples were positive for *S. aureus* that means prevalence rate is 12.87% (n=13/101). Various studies have been conducted to evaluate the degree of contamination of milk with *S. aureus;* obtained from commercial farms. In most cases, milk containing *S. aureus* were obtained from animals with subclinical mastitis. Jahan et al. (2015) found 25.53% prevalence of *S. aureus*. The findings in this study has similarities with other studies (Shitandi and Sternesjö, 2004; Gündoğan et al., 2006). Based on observations made throughout the collection of samples, we concluded that the improper hygiene practice, poor management before and during milking, milking frequency and milking process might have contributed to the contamination of milk with *S. aureus,* and the rural farms are more vulnerable in this case. The higher prevalence of *S. aureus* is alarming both for dairy farming and for public health. The presence of *S. aureus* in the milk sample is an important finding of this study. *S. aureus* was resistant to multiple antibiotics which can cause serious health problems (Tenover, 2006)**.**

Investigation in other countries revealed similar results obtained in this study. Farhan and Salk (2007) studied 130 milk samples in Palestine and found 48 (36.9%) samples were positive for *S. aureus.* Ekici et al. (2004) found 18.18% of the milk samples positive for S aureus in Turkey. In Morocco, Bendahou et al. (2008) recorded 40% of the milk samples positive*;* while in India, 61.7% of the raw milk contained this organism (Lingathurai and Vellathurai, 2010).

The high incidence of *S. aureus* is indicative of poor hygienic measures during production, handling and distribution, stated in the findings of Zakary et al. (2011). The proper heat treatment followed by the refrigeration can minimize the chance of contamination with *S. aureus*. In our country it is commonly noticed that during heat treatment of milk, the temperature don't rise up to the boiling point many a time or even if it reaches, the boiling duration is not enough. El-Malt et al. (2013) stated that the difference in the prevalence rates of *S. aureus* between the examined products may originate from the method of manufacture, storage and handling or use of unhygienic utensils. The lowest prevalence

rate of *S. aureus* was recorded in yogurt might be attributed to the effect of heating and then freezing during manufacture which inhibits the multiplication of organism.

The table shows the prevalence of *S. aureus* based on risk factors associated with subclinical mastitis in the lactating cows. There is a relation between Udder hygiene (P = 0.027) and the prevalence of *S. aureus* isolated from subclinical mastitic milk samples. Schreiner et al. (2003) also found a significant relationship between occurrence of mastitis and udder hygiene. Our study showed that 15% cows have clean and dirt free udder, 35% slight dirty udder, 34% cows has mostly dirty udder and 16% have completely dirty udder. Cows, having clean udder, have very low prevalence of *S. aureus.*The prevalence increased gradually according to the degree of dirt present on udder. The inside of a healthy udder is free of bacteria. The teat canal closes in between milking times and also when the cow is dry. It acts as a seal that protects the inside of the udder from the outside. Mastitis bacteria invade the udder almost always through the teat canal. Dirty udder facilitates the invasion as well. Low hanging udders and very long teats are more exposed to dirt. Cleanliness of the udder is thought to influence the quantity and type of bacteria present on teat surfaces, and dirty teats and udders are considered to be a source of environmental bacteria in milk (Galton et al., 1982; Guterbock, 1984). In a review article, Pankey (1989) reported that bacterial numbers in milk increased when udder and teats are inadequately cleaned and dried.

Forestripping consists of the removal of several streams of milk per quarter through manual compression of the teat. With regards to Forstripped before milking as a risk factor, it was showed that there is significant relationship between lactation stage (p=0.026) and the prevalence of *S. aureus* isolated. Forestripping is recommended to check for clinical mastitis and as a means of premilking stimulation (Rasmussen et al., 2000). Weiss and Bruckmaier (2005) indicated that forestripping would increase stall capacity if full udders were milked and that prolonged stimulation might be beneficial when milking udders that are not full. Again, a summary of studies from the past 30 year indicated that stimulation of at least 20 sec reduced milking unit on-time and increased the average flow rate when compared to no stimulation (Reneau and Chastain, 1995). Earlier researchers have suggested that the amount of milk in the udder before milking may influence milking performance (Williams and Mein, 1978). Forestripping is an important

part of many premilking routines and should continue to be recommended because it is the only way that farmers can detect mild clinical mastitis and divert abnormal milk.

Chapter V
Limitations

The study was conducted based on biochemical analysis, not on molecular test.

Chapter VI

Conclusion

S. aureus adapts very well in the udder and establishes chronic and subclinical infections. From there it is shed into the milk, which serves as a source of infection for healthy cows during the milking process. Of the 101 farms were examined, 13 were positive for subclinical mastitis, which may be an indication of a future mastitis problem at the region. Therefore, further extensive experiment is required for the identification of possible risk factors of the organism which will help in taking possible intervention measures.

Chapter VII

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Author

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Appendex

Peptone water

Composition	gm/liter
Peptone	10.0
Sodium chloride	5.0
Disodium phospahte	3.5
Potassium dihydrogen phosphate	1.5

Nutrient broth

Composition	gm/liter
Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

Mannital Salt Agar (MSA)

Composition	gm/liter
Casein	5.0
Animal tissue	5.0
Beef extract	1.0
D-mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

Biography:

Name	Abdullah Al Sattar
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Educational background and Year	H.S.C in 2011, Govt. Hazi Mohammad Mohshin College, Chittagong; S.S.C in 2009, SSGB High School, Chittagong.
No. of publication	No
Research interest	Develop artificial womb