Chapter: I

Introduction

In Bangladesh, dairy milk production is one of the major sectors for the national income development. Currently Bangladesh is producing 94.6 lakh metric ton (average) milk yearly and creating great contribution to GDP (DLS, 2017).

However, milk production often does not satisfy the country's milk requirements due to a multitude of associated factors. Mastitis is one of the complex and costly diseases of dairy cows that results from the interaction of the cow and its surrounding environment (Azmi et al., 2008). Mastis is an inflammation of mammary gland that often develops in response to intramammary bacterial infection (Schalm et al, 1971). Mastitis has been known to cause a great deal of loss or reduction of productivity by influencing the quality and quantity of milk yield and culling of animals at an unacceptable age (Vaarst and Envoldsen 1997).

In case of subclinical mastitis, milk can be observed with normal appearance without having visible abnormalities in udder tissues except an elevated Somatic Cell Count (SCC) (McDougall et al., 2001), however causes production loss. If the subclinical mastitis is not detected and treated in time, it may progress and develop into clinical form (Adwan et al., 2005). In clinical form the important components of milk such as lactose, fat, milk protein (casein) are reduced while undesirable components like irons and enzymes are increased (Girma 2001; Shitandi 2004). Thus the milk is considered as unfit for processing and therefore causes nutritional and financial losses.

Different infectious agents are associated with mastitis in cattle. The most common organisms are gram-positive *Staphylococcus aureus* and gram-negative bacteria *Escherichia coli* frequently found in the cow's environment (Mubarack et al., 2012).

Staphylococcus is a Gram-positive coccal bacteria. In this genus, there are forty species, which includes *Staphylococcus aureus, S. intermedius, S. hyicus, S. epidermidis, and S. saprophyticus* (Blood et al., 1989).

E.coli is a member of the family of Enterobacteriaceae. *E. coli* is ubiquitous in the cow's environment. They behave as opportunistic pathogens. Therefore, it is obvious that challenge of *E. coli* udder infection will remain so long as cows produce faeces (Jones T.Q. 1990). *E. coli* serotypes in mastitic milk are similar to the faecal isolates. There are a very large number of *E. coli* serotypes as classified by surface antigens but the inner layer of the cell wall is

common to all serotypes of *E. coli* and Enterobacteriaceae in general (Dosogne H. et al., 2002).

Presence of *Staphylococcus sp* in milk or any food has public health significance (Balaban and Rasooly, 2000). The bacteria may be killed by boiling milk but the toxin remains active at normal cooking temperature (Presscott et al., 2002). Infected udders, teat canals, and teat lesions, and also teat skin, muzzle, nostril 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, milker's hands, wash cloths and flies (Rund et al., 1986; Madsen et al., 1991; Yeruham et al., 1996; Brody et al., 2008).

Different risk factors such as milking hygiene, management practice, stage of lactation were found associated with occurrence of mastitis (East et al., 1986 and Boscos et al., 1996). It is well known that bacterial, environmental or management, and cow factors may change susceptibility of the animals to mastitis. These factors are independent and especially the relative impact of each factor depends on type of pathogen (Tolle A. et al., 1975). Identification of risk factors and taking care of these factors along with proper antimicrobial treatment were proved effective preventing the diseases.

Antimicrobial agents are commonly applied to dairy cattle either to control or to prevent bacterial infections in lactating and dry cows. Thus, the results of *in vitro* susceptibility testing are an important tool to guide the veterinarian in selecting the most efficacious antimicrobial agent(s) for therapeutic and prophylactic interventions. In 2001, a new lincosamide antibiotic, pirlimycin, was approved in Germany for the control of *Staphylococci* and *Streptococci* associated with bovine subclinical mastitis (Lüthje et al., 2006). Therefore, a combination of early detection by field tests followed by identification of pathogens along with their sensitivity to antimicrobial drugs is crucial for prevention of economic losses to this disease. Therefore, the present study was designed to achieve the following objectives:

1. To estimate the prevalence of subclinical mastitis, *Staphylococcus sp* and *E. coli* in healthy udder of cow in the study population.
2. To determine the antibiotic sensitivity of the *Staphylococcus spp* and *Escherichia coli* against commonly used drugs.

Chapter: II

Materials and method

2.1. Study Population and Sample Collection

The study was undertaken on 50 lactating cows for the periods of 8 months from January to August, 2018. Five dairy farms of Chittagong Metropolitan Area (CMA), Bangladesh (Kalurghat, Shikalbaha, Bakolia, Patiya, Karnaphuli area) were visited for sample collection. The sample were collected from healthy cows in 15 ml falcon tubes and sent it to the clinical pathology lab, CVASU for laboratory analysis.

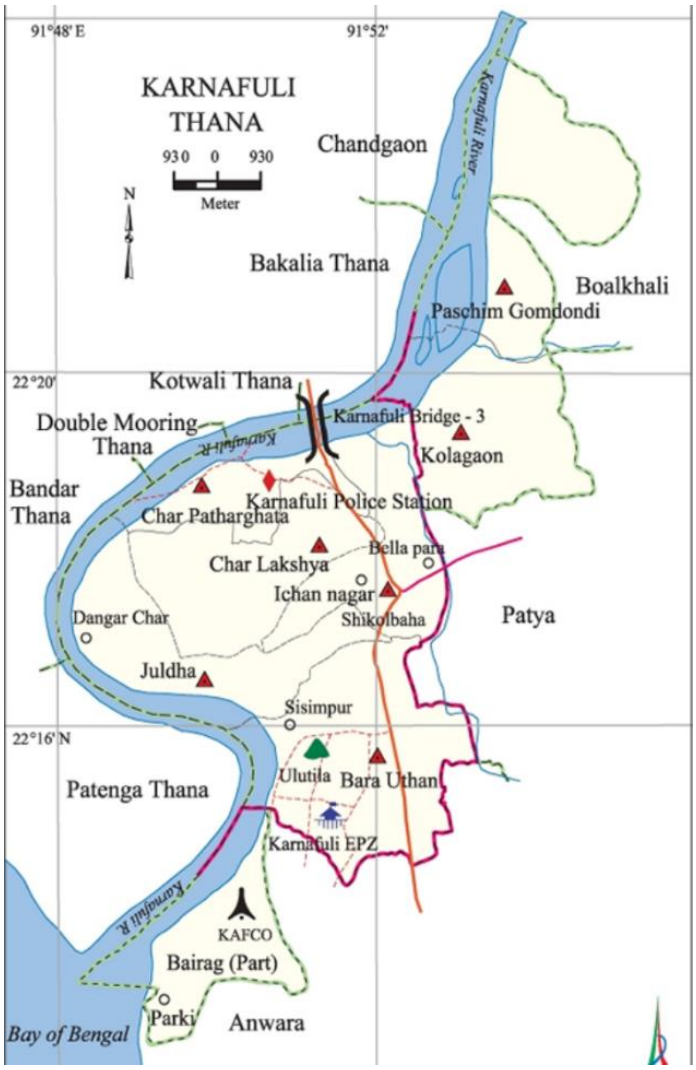


Fig 2.1: Geographical location of sample collection site (Chittagong Metropolitan)

2.2. Collection of data by questionnaire

To collect the data a structured questionnaire was followed during the study period. The questionnaire contained information about farm address, farm size, housing system, rearing system, type of breed, body condition score, milk yield, lactation number, udder cleanliness, floor cleanliness, stage of lactation, history of lameness, history of any reproductive disease, type of drug used. Data collection was done following mixed (open and close ended) questionnaire.

2.3. Experimental design

Total 50 lactating healthy cows were selected randomly from 5 dairy farms. During milk sample collection aseptic procedure was maintained to prevent the microbial contamination by soaking the teat with disinfectant (70% ethyl alcohol) and drying off by cotton. After discarding few drops initially, milk samples were taken from every quarter for CMT (California Mastitis Test). Then the CMT positive 5ml of milk samples (n=25) were taken into sterilized test tubes with rubber cap. After labeling with an identification number, immediately milk samples were transported to the laboratory in ice-box and stored at 4◦C till laboratory analysis. Isolation and identification of the bacterial isolates were done based on their cultural characteristics including pigment production, hemolytic activity etc.

2.4. Determination of subclinical mastitis

CMT (California Mastitis Test) is an important tool for the evaluation of subclinical mastitis by estimating somatic cell concentration.

At first 2ml of milk and 2ml of C.M.T. solution was mixed together in result test paddle/tray. The paddle was rotated to mix the solution properly and changes in color and gel formation was observed within 10 to 15 seconds after mixing. To judge the quality of milk and the state of mastitis, criteria shown in table 1 was followed (Council NM. 2017).

Table 2.1: Judgment of the quality of milk and the state of mastitis:

CMT Score	Observation	Somatic cell level	Interpretation
1 (Negative)	No thickening of mixture	<200,000 cells/ml	Healthy quarter
2 (Trace)	Slight thickening of mixture. Trace reaction seems to disappear with continued rotation of the paddle	150,000 to 500,000 cells/ml	Suspicious
3	Distinct thickening of the mixture, but no tendency to form a gel. If CMT paddle is rotated more than 20 seconds, thickening may disappear	400,000 to 1,500,000 cells/ml	Mastitis positive
4	Immediate thickening of mixture, with a slight gel formation. As mixture is swirled, it moves toward the center of the outer edge.	800,000 to 5,000,000 cells/ml	Mastitis positive
5	Gel is formed and surface of the mixture becomes elevated (like a fried egg). Central peak remains projected even after the CMT paddle rotation is stopped	>5,000,000 cells/ml	Mastitis positive

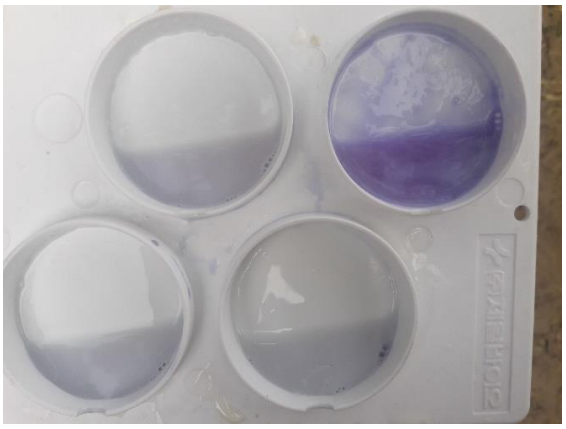
a  b 

Fig 2.2: (a) California Mastitis Test for milk samples, (b) Working in clinical pathology laboratory.

2.5. Bacteriological Investigation

2.5.1: Isolation and identification of *Staphylococcus spp*

According to Singh and Prakash (2008) with slight modification, the isolation of *Staphylococcus sp* was done. Peptone water (PW) (Oxoid Ltd, Hampshire, UK) was used for enrichment. This was done by homogenization of 5ml sample with 45 ml sterile enrichment broth peptone water and enriched for 24 hours at 37°C (Thaker et al., 2013).

The milk samples were streaked onto Mannitol Salt Agar (MSA) and incubated at 37°C for overnight. Golden or Bright yellow coloured colonies after incubation of 24 hrs at 37ºC indicated the isolation of *Staphylococcus spp*. The presumptive colonies of *Staphylococcus spp* were further cultured onto 5% Bovine Blood Agar. 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. To detect the pathogenicity of *Staphylococcus spp* the positive colonies with bright yellow zones (due to mannitol salt fermentation) were then re-inoculated into blood agar. Incubation of all streaked blood agar plates at 37ºC overnight was done to allow hemolysis to occur.

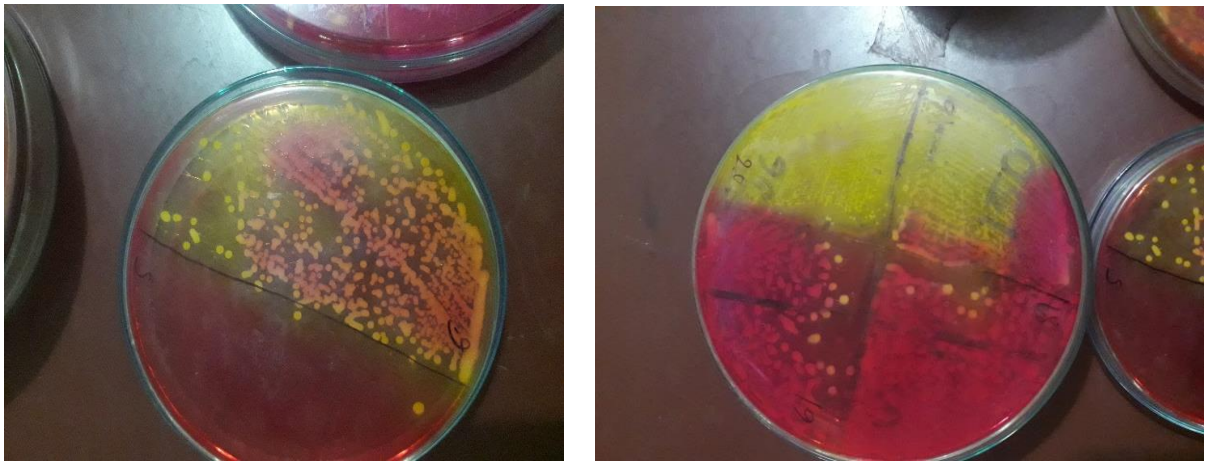


Fig 2.3: Bright yellow colonies indicating the growth of *Staphylococcus spp* on Mannitol salt agar plates.

2.5.2. Isolation and Identification of *E.coli*

The sample was cultured on McConkey Agar (MaC). *E. coli* produces large pink colored colonies after incubation of 24 hrs at 37ºC. The presumptive colonies of *E. coli* were further cultured onto selective medium Eosin Methylene Blue (EMB) Agar and incubated at 37°C for 24 hours. Then the colonies producing metallic sheen were (Priyanka and Alka, 2008)

reinoculated into 5% bovine blood agar to detect the pathogenecity of *E. coli*. All streaked blood agar plates were incubated at 37^{o}C overnight to allow hemolysis to occur.

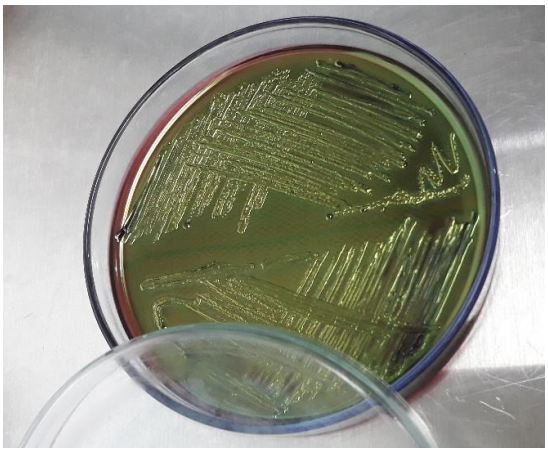
a  b 

Fig 2.4: *E. coli* producing large pink color growth on McConkey agar (a), Metalic green sheen on EMB agar (b).

2.6. Antibiotic Sensitivity test-

To observe the sensitivity of the isolated bacteria to different antibiotics, antibiotic sensitivity test was done. Bauer-Kirby disk-diffusion procedure (Bauer et al., 1966) was followed in this experiment. Muller-Hinton (M173) agar was prepared according to the manufacturer`s instructions (Oxoid). Equivalent of 0.5 Mcfarland R092 (prepared by mixing 0.5 ml of 1.175% barium chloride and 99.5ml of 36N sulfuric acid) standards bacterial turbidity was used as inoculum for each isolate. The antibiotic resistance pattern for the panel of antibiotics was determined considering the zone of inhibition sizes for each of the antibiotics as "resistant (R)", "intermediately resistant (I)", and “sensitive (S)” against the test isolates as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2016) shown in Table 2. After dipping the sterile swab into inoculum rotated against the side of the tube with firm pressure. Then after removing the excess fluid from the swab, the dried surface of MH agar was inoculated by streaking the swab three times turning the plate at 60 degree angle between each streaking. Then the surface of the inoculated agar was ready for antimicrobial disc placement. After that disks were placed carefully on the surface of the agar with a gentle pressure and the agar plate was incubated at 35^{o}C for 20 to 24 hours. At the end of incubation the size of the zone of inhibition around a micro-disk was measured in millimeter scale according to CLSI, 2016.

In the present study the following antibacterial agents (Oxoid) were used: Amoxicillin (AML; 10 µg), Azithromycin (AZM; 15 µg), Pefloxacin (PEF; 5 µg), Enrofloxacin (ENR; 5 µg), Methicillin (ME; 10 µg), Doxycycline (DO; 30 µg), Gentamicin (CN; 10 µg), Streptomycin (S; 10 µg). Interpretation of the test results; sensitive (S), intermediate sensitive (I), and resistant (R) was drew based on CLSI criteria, 2016.

Table 2.2: Diffusion zone break point according to Clinical Laboratory Standard Institute (CLSI), oxoid:

Antimicrobial agent	Disc code	Potency (µ gm)	Bacteria	Test cultures (zone diameters in mm)		
				Resistant	Intermediate	Susceptible
Amoxycilin	AML	10	*E.coli*	≤13	14-17	≥18
			Staphylococcus	≤19	-	≥20
Azithromycin	AZM	15	*E.coli*	-	-	-
			Staphylococcus	≤13	14-17	≥18
Pefloxacin	PEF	5	*E.coli*	≤23	-	≥24
			Staphylococcus	≤23	-	≥24
Enrofloxacin	ENR	5	*E.coli*	≤20	21-30	≤31
			Staphylococcus	≤15	16-20	≥21
Doxycyclin	DO	30	*E.coli*	≤10	11-13	≥14
			Staphylococcus	≤12	13-15	≥16
Gentamycin	CN	10	*E.coli*	≤12	13-14	≥15
			Staphylococcus	≤12	13-14	≥15
Streptomycin	S	10	*E.coli*	≤11	12-14	≥15
			Staphylococcus	-	-	-

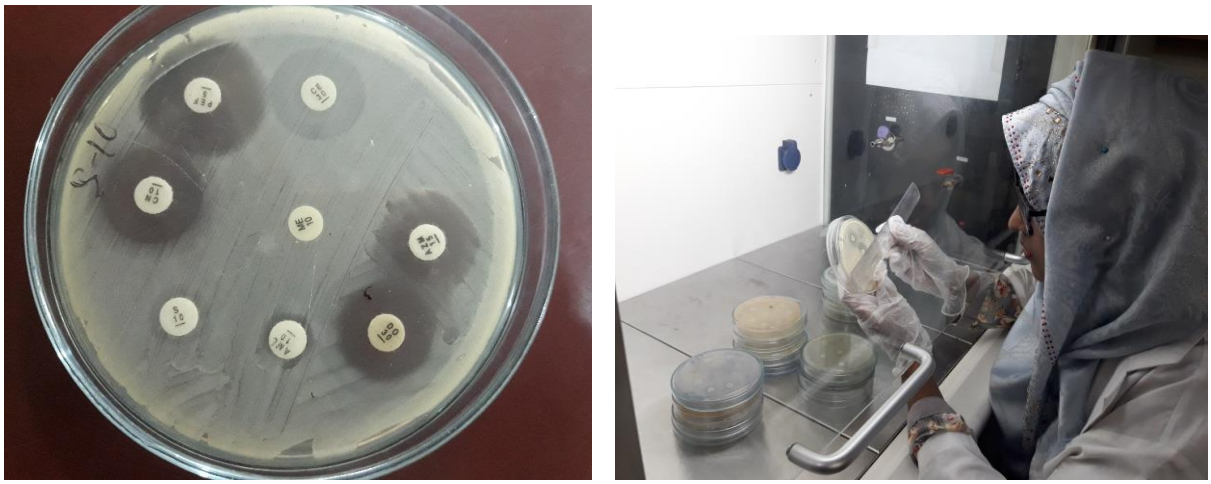


Figure 2.5: Bacterial zone of inhibition

2.7. Statistical Analysis

All the data (from questionnaire and laboratory) were entered into MS excel (Microsoft office excel-2010, USA). Data management and data analysis were done by STATA version-13 (STATA Corporation, 4905, Lakeway River, College Station, Texas 77845, USA). Prevalence of subclinical mastitis, *E. coli* and *Staphylococcus* spp. was estimated by dividing the number of cases with the study population. Distribution of different variables according to infected cows was estimated as frequency and percentage.

Chapter III

Result

Descriptive statistics of the study population:

The study was undertaken on 50 lactating cows from 5 dairy farms of Chittagong Metropolitan Area. Among 50, 25 samples (p: 50%) showed a positive test in commercially available CMT kit. Prevalence of *Staphylococcus* spp. and *E. coli* was 32% each.

Among the positive cows, 8 (32%) were from the farms having 50-80 animals, 5 (20%) from the farms having 81-100 animals and 12 (48%) from farms having more than 110 farm animals.

The variable housing system was consisted of 2 categories: face out and face in stanchion barn. 17 (68%) positive cows were from face out farming system and rest 8 (32%) from face in system. During the study period, 14 (56%) positive cows were in between 1st to 2nd lactation. 9 (36%) cows were in 3rd lactation and rest 2 cows were in higher lactation period. In the breed variation 13 (52%) positive cows were found having 75% Holstein Friesian (HF) blood and 12 (48%) having less than 75% HF blood indicated that cows having high HF blood percentage are having high chance of subclinical mastitis than the cows having less than 75% HF blood.

Among the subclinical mastitis positive cows, 4 (16%) had body condition score (BCS): 2, 18 (72%) cows had BCS score: 3 and rest 3 (12%) cows had BCS score: 4. 16% cows having subclinical mastitis produced in average less than 5 kg milk daily. 9 (36%) of them produced 5-10 kg milk daily, 32% cows produced 10-15 kg milk while 4 (16%) cows produced more than 15 kg milk daily. Cows with the history of avg. 5-10 kg milk production had high prevalence of subclinical mastitis.

During the study period, body weight of 2 (8%) affected cows was less than 250 kg, 10 (40%) cows were in the range of body weight 250-300kg. 11(44%) were in the range of 300-350 kg and rest 8% cows were found having more than 350 kg body weight. Hygienic measures at farm including floor washing, washing of udder regularly etc. were studied. It was observed that 80% mastitic cows were from the farms where less than 2 times floor washing without disinfectant was practiced. More cows (68%) were affected with subclinical mastitis when the udder was observed moderately clean vs. clean udder (32%).

Ninety two percent mastitic cows did not have any reproductive disorder (Table: 3.1).

20% of the mastitic cows were from the farms where mastitic cases were handled by veterinarian, 52%` were from the farms where cases were handled by both veterinarian and veterinary field assistant and the rest 28% from the farms where only veterinary field assistant handled the cases.

Distribution of risk factors in mastitic cows:

Table 3.1: Demography of mastitis affected farms and cows of the study area

Variable	Category	Frequency	Percentage
Farm size	50-80	8	32
	81-110	5	20
	>110	12	48
Housing system	1	17	68
	2	8	32
Lactation number	1-2	14	56
	3	9	36
	>3	2	8
Breed	75% HF	13	52
	<75% HF	12	48
BCS	2	4	16
	3	18	72
	4	3	12
Milk yield	<5kg	4	16
	5-10kg	9	36
	10-15kg	8	32
	>15kg	4	16
Body weight	<250kg	2	8
	250-300kg	10	40
	300-350kg	11	44
	>350kg	2	8
Udder cleanliness	Clean	8	32
	Moderately clean	17	68
Floor cleanliness	Daily washing 2 times with disinfectant	5	20
	<2times washing without disinfectant	20	80
Any reproductive disorder	No	23	92
	Yes	2	8
How mastitis is managed	By vet	5	20
	Vet and VFA	13	52
	VFA	7	28
***Drugs used in farm**	1	8	32
	2	17	68
Staphylococcus status	Negative	17	68
	Positive	8	32

E. coli status	Negative	17	68
	Positive	8	32

*1=gentamycin, amoxicillin, streptopenicillin; 2= gentamycin, amoxicillin, oxytetracycline

Table 3.3: Antibiotic Sensitivity Test for *Staphylococcus spp:*

	Sensitive		**Intermediate**		**Resistant**	
Antibiotic disk	**Frequency**	**Percent (%)**	**Frequency**	**Percent (%)**	**Frequency**	**Percent (%)**
AML	0	0	0	0	8	100
PEF	1	12.5	0	0	7	87.5
DO	3	37.5	1	12.5	4	50
CN	6	75	0	0	2	25
S	1	12.5	1	12.5	6	75
ENR	0	0	0	0	8	100
AZM	0	0	0	0	8	100

The sensitive antibiotics against *Staphylococcus spp* were gentamycin (75%), doxycycline (37.5%), pefloxacin (12.5.5%), streptomycin (12.5%). Intermediate sensitive (I) antibiotics were doxycline (12.5%), streptomycin (12.5%). High percentage of resistant antibiotics were amoxyciline (100%), enrofloxacin (100%), azithromycin (100%), pefloxacin (87.5%) streptomycin (75%), doxycline (50%), gentamycin (25%).

Table 3.2: Antibiotic Sensitivity Test for *Escherichia coli*

	Sensitive		**Intermediate**		**Resistant**	
Antibiotic disk	**Frequency**	**Percent (%)**	**Frequency**	**Percent (%)**	**Frequency**	**Percent (%)**
AML	0	0	0	0	8	100
PEF	1	12.5	0	0	7	87.5
DO	1	12.5	0	0	7	87.5
CN	3	37.5	0	0	5	62.5

S	0	0	1	12.5	7	87.5
ENR	0	0	3	37.5	5	62.5
AZM	0	0	0	0	8	100

The sensitive antibiotics against *E.coli* were gentamycin (37.5%), doxycycline (12.5%) pefloxacin (12.5.5%). Intermediate sensitive (I) antibiotics were enrofloxacin (37.5%), streptomycin (12.5%). High percentage of resistant antibiotics were amoxyciline (100%), azithromycin (100%), pefloxacin (87.5%), streptomycin (87.5%), doxycline (87.5%), gentamycin (62.5%), enrofloxacin (62.5%)

Chapter: IV

Discussion

The occurrence of clinical and subclinical mastitis in the different breeds has been investigated in various parts of the world (Al-Majali et al., 2003).

The study was undertaken on 50 lactating cows from 5 dairy farms of CMA. 25 out of 50 were observed having subclinical mastitis. California Mastitis Test (CMT) was used to diagnose subclinical mastitis. Milk samples of all subclinical mastitic cows were subjected to bacteriological culture for isolation and identification of *Staphylococcus spp*. and *Escherichia coli*. CMT is widely used in different countries in diagnosis of subclinical mastitis in different species. CMT is used widely for diagnosing mastitis (both clinical and subclinical) in large ruminants both in research and field diagnosis purpose (Rahman et al., 2010). The results obtained using CMT revealed a prevalence of the subclinical mastitis of 25.22% in Ethiopia (Almaw G et al., 2009). Bishi (1998), Workineh et al (2002), and Almaw et al (2008) reported 34.30, 38.2 and 34.4%, respectively in Ethiopia. In a study carried out in Tanzania as high as 90.3% (Kivaria et al 2004) subclinical mastitis prevalence was reported. The prevalence of subclinical mastitis was 75·9 % assessed by the CMT inTanzania (Karimuribo et al., 2008).

Coagulase-positive *staphylococci* (30.2%), coagulase-negative *staphylococci* (13.7%), and *Streptococcus dysgalactiae* (9.3%) were predominantly isolated from subclinical mastitis cases (Bortel et al., 2010). *Staphylococcus aureus* was isolated commonly from the subclinical mastitis samples including other bacterial agents (*Escherichia coli, Clostridium perfringens, Streptococcus, Pseudomonas*) in France (Bergonier et al., 2003 and Amin et al., 2011). In our study the overall prevalence of *Staphylococcus spp* was 32% and *E. coli* 32%. Higher prevalence that was observed in our study might be because of association of environmental factors and proper management insufficiency in study population.

In an antibiogram study revealed that most of the isolated bacterial species were sensitive to various antibiotics and resistant particularly to nalidixic acid and streptomycin (Khandkar et al., 2012). In our study we used 7 antibiotics to evaluate the sensitivity. We observed that the most sensitive antibiotics against *Staphylococcus spp* were gentamycin (75%). Other drugs did not show satisfactory level of sensitivity viz doxycycline (37.5%), pefloxacin (12.5.5%), streptomycin (12.5%). A remarkable number of antibiotics showed 100% resistance. High percentage of resistant antibiotics were amoxyciline (100%), enrofloxacin (100%),

azithromycin (100%) followed by pefloxacin (87.5%) streptomycin (75%), doxycline (50%), gentamycin (25%). Only 37.5% isolates were sensitive to gentamycin; other drugs were less sensitive than gentamycin. More than 60% of the drugs were resistant to all the antibiotics in the study where amoxyciline and azithromycin were 100% resistant. The resistant pattern of antibiotics revealed by this study is of concern for dairy farmers as it might lead to non responsive treatment.

Distribution of different factors was observed among mastitic cows in the present study. More mastitic cows were observed in farms with large herd, 1-2 lactation, poor udder management and floor cleanliness that was also reported in other studies **(**Mekibib et al., 2010). High yielding cows were found vulnerable to mastitis in different studies (Rahman et al., 2010).

Chapter: V

Conclusion

Bovine mastitis is a major economic problem in the dairy industry worldwide, with a wide variety of microorganisms involved. In our present study the prevalence of subclinical mastitis in cows in Chittagong Metropolitan area of Bangladesh was 50%. This study also exhibited the presence of multidrug resistant pathogens (*Stephylococcus spp* and *E.coli*) in sampled cow affected by subclinical mastitis. The overall prevalence of *Stephylococcus spp* and *E.coli* in milk sample were 32% and 32% respectively. Our study also showed some risk factors that associated with subclinical mastitis such as lactation number, breed, udder cleanliness, floor cleanliness, housing system age of the animal, parity, length of lactation period and housing system influenced the prevalence of subclinical mastitis in cow population. So, for controlling mastitis, management and hygiene should be maintained and antibiogram analysis, application of sensitive antibiotic prescribed by veterinarian could be an effective way to control the subclinical mastitis.

Chapter VI

Limitation

Small sample size of the present study was a limitation. Data from non affected cows were not collected. Therefore, risk factor analysis was not possible to conduct.

Chapter VII
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ACKNOWLEDGEMENT

All praises are due to the Almighty Allah, the creator.

Accomplishment of this work, as the partial fulfillment of the requirements for the degree of Doctor of Veterinary Medicine (DVM) in (CVASU) have given me the confidence and plesure.

I would like to extend my gratitude to my supervisor, Prof. Sharmin Chowdhury, Phd, Director of One Health Institute, Chittagong Veterinary and Animal Sciences University. My heartfelt thanks to her for valuable guidance, suggestion, supervision and encouragements during the entire period of this study to complete this clinical report.

I would like to express my deep sense of gratitude and thanks to Professor Dr. Gautam Buddha Das, honorable vice chancellor and Professor Dr. Md. Abdul Halim, Dean, Faculty of Veterinary Medicine, CVASU for arranging this type of research work as a compulsory part of this internship program. I express my sincere gratitude and thanks to Professor Dr. A. K. M. Saifuddin, Director of External Affairs, and for his supervision and kind co-operation during the period of internship.

Thanks to DR. Tofazzal Md. Rakib for his kind cooperation, owners of farm, owners of animals, and attendance that have helped me in collecting data for this study and lab work.

Last but not least, I am profoundly grateful to my family members for their endless sympathies, kind co-operation, sacrifices and prayers.

Author

September, 2018

Appendix

Questionnaire survey

1. Farm level baseline Information:

ID no of farm:

Date:

Farm's and Farmer's contact address:

Farm size:

Types of breed:

Housing system:

Rearing system:

Floor cleanliness:

2. Individual level information:

Breed:

BCS:

Age: Body weight:

Milk yield (daily): Duration of lactation:

Lactation no:

Date of occurrence:

Udder cleanliness:

Any history of disorder in & around calving: Yes / No

Any history of lameness: Yes / No

3. General information:

Any knowledge about mastitis: Yes / No

How do you manage problems of your animals: With the help of vet / VFA / Self

What types of drug usually used:

Any idea about CMT: Yes / No

CMT Result:

Brief biography of the student

Jahan Ara passed Secondary School Certificate (SSC) examination from Victory Adarsha High School in 2009 and then Higher Secondary School Certificate (HSC) examination from Govt. Women's College, Chittagong in 2011. Then she admitted to the degree of Doctor of Veterinary Medicine (DVM), Faculty OF Veterinary Medicine, CVASU in 2012-2013 session. She has great interest to work in One Health field.