ISOLATION AND IDENTIFICATION OF *CORYNEBACTERIUM* SP. FROM THE CASES OF CLINICAL MASTITIS IN COW AT DINAJPUR DISTRICT OF BANGLADESH

A Thesis

By

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05.06.11

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Examination Roll No.: 0905073
Registration No.: 0905073
Session: 2009-2010
Semester: September, 2010 - February, 2011

গ্রন্থাগার

Submitted to the

Department of Microbiology

Faculty of Veterinary and Animal Science
Hajee Mohammad Danesh Science and Technology University

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE (M.S.)

IN

MICROBIOLOGY

Department of Microbiology
Faculty of Veterinary and Animal Science
Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200.

February 2011

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DEDICATED

TO
MY

BELOVED PARENTS

ACKNOWLEDGEMENT

"In the Name of Allah, the most Beneficent, the Most Merciful. All the praises and thanks be to Allah, the Lord of the "Alamin (mankind, jinns and all that exists). - Surnh AI- Fatihah (1-2).

The author always likes 'to bow his head to Almighty Allah for his never ending blessing for successful, completion of the work. At first all praises and deepest sense of gratitude be to Almighty Allah, the supreme creator of this universe, who enabled the author to complete this thesis.

The author would like to express his deepest sense of gratitude, sincere appreciation, profound regards and indebtedness to his reverend teacher and research Supervisor, Dr Md. Khaled Hossain , Assistant Professor, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for his scholastic and dynamic guidance, constant inspiration, cordial consistence, affectionate feeling, utmost desire, sympathetic supervision and constructive criticism in all phases of this study and preparing of the manuscript.

The author finds it a great pleasure in expressing his heartfelt gratitude and immense indebtedness to his honorable and respected teacher and Research Co-supervisor Dr. Md. Fakhruzzaman, Assistant Professor, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, for his sympathy, kind co-operation, inspiration and valuable suggestions, constructive criticism and valuable advice for the completion of the research work and preparation of the thesis.

The author humbly desires to express his profound respect and sincere appreciation to his respectable teacher Dr. Md. Mostafizer Rahman, Associate Professor and Chairman, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for his constant help, cooperation and inspiration to conduct the course work and research smoothly and perfectly.

The author would like to acknowledge his gratitude and sincere appreciation to his respected teachers Dr. Mir Rowshan Ara, Assistant Professor, Department of Microbiology, Hajee

Mohammad Danesh Science and Technology University, Dinajpur, for their valuable advice, constant help, continuous encouragement and contribution in completing the thesis.

Thanks are also extended to Mominul, Sumon, Taposh, Kamrul, Mahfuz, Rumi, Tonni, Mizan for their encouraging attitude in the study period. The author expresses his thanks to all technicians and office staffs of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for their technical assistance during the research.

The author is ever indebted to his beloved parents for their endless sacrifices, heartiest blessings and all-out support throughout his entire life. The author is also grateful to brother and sister, for their heartiest blessings and encouragement through out his academic life.

Finally the author would like to express grate full thanks to his well wishers.

The Author

February 2011

ABSTRACT

The present study was conducted to isolate and identify *Corynebacterium* sp. from the cases of clinical mastitis in cows at Dinajpur district of Bangladesh. For this, a total of 20 milk samples were collected from clinically mastitis affected cows of different dairy farms and rural domestic holdings at Dinajpur district in Bangladesh. The study was carried out during the period of September'2010 to February'2011. The milk samples were collected and brought to the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur and subjected to different bacteriological tests for isolation and identification of bacteria based on morphology, staining, cultural and biochemical properties. Antibiotic sensitivity of the isolated organism was also studied. The isolated bacteria were *Staphylococcus* spp. (25%), *Streptococcus* spp. (15%), *Escherichia coli* (20%), *Corynebacterium* sp. (25%) and 15% were unidentified. On nutrient broth, the *Corynebacterium* sp. showed enough whitish turbidity and on nutrient agar the colonies were grayish in color while on blood agar the colonies were grayish in color about 1mm in diameter and surrounded by a small zone of hemolysis. In Gram's staining technique the *Corynebacterium* sp. revealed Gram positive, violet color, short cocco-bacilli and arranged in bundles or clusters and singly also. In sugar fermentation tests (dextrose, lactose and maltose), the organism produced acid but no gas. In hanging drop slide method, the organism was nonmotile. Results of antibiotic sensitivity showed that *Corynebacterium* sp. was highly sensitive to gentamicin and ciprofloxacin, and some degree of resistance to tetracycline, ampicillin, penicillin and amoxycillin. The present study dictates that the *Corynebacterium* sp. was one of the important agents responsible for the clinical mastitis in cows at Dinajpur district of Bangladesh.

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ABBREVIATIONS USED IN THIS TEXT

AMP	= Ampicillin
AML	= Amoxyciliin .
BA	= Blood agar
BAU	=Bangladesh Agricultural University
CMT	= California mastitis test
CIP	= Ciprofloxacin
ed	= Edition
et al.	= Associates
etc.	= et cetera
Fig.	= Figure
GN	= Gentamycin
gm	= Gram
HSTU	= Hajee Mohammad Danesh Sceince and Technology University
i.e.	= That is
i.u.	= International Unit
mg	= Milligram .
MIU	= Motility Indole Urease
No.	= Number
NA	= Nutrient agar
P	= Penicillin

Pp	= Page number
TE	= Tetracycline
0^0C	= Degree centigrade
%	= Percentage
µg	= Microgram
±	= Plus- minus
+ve	= Positive

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Livestock plays an important role in the subsistence agro-based economy of Bangladesh. Cattle population in Bangladesh is about 24 million (FAO, 2003). Nearly, 85 percent of the populations are engaged in agriculture and livestock sector (Raha, 2000). The number of milking cows in Bangladesh is 3.75 million, which is 35 percent of the total cattle population in Bangladesh. The annual milk production in Bangladesh is nearly 1.62 million metric ton which is very low in respect of our demand which is nearly 9.0 million metric ton (DLS, 1998). The milk production in Bangladesh is very low due to low productive cows and various infectious and noninfectious diseases in the dairy farm. Mastitis is the most important one affecting the cows.

Mastitis is the inflammatory infection of the mammary gland. It is a complex disease involving many factors and is primarily caused by microorganism that enter into the teat canal and mammary glands (Philpot and Nickerson, 1999). Bovine mastitis (both clinical and subclinical) is mainly caused by bacterial infection in the udder (Abdella, 1996) but some determinant factors such as inadequate hygiene and management practices, faulty technique of milking, high milking machine pulsation with vacuumization, pendulous udder with long teats, larger size of teat orifice in high yielding cows, traumatic injury etc. may play an important role and lead to incite developing infection of mastitis.

Clinical mastitis is characterized by sudden onset of pain, heat, swelling, redness and reduced as well as altered milk secretion from affected halves. Abnormal secretion in the form of clots, flakes, or watery milk are the clinical signs (Hinckley and leander,1981). Gangrenous mastitis is characterized by or suspected if the udder is cold, swollen, with an excessive accumulation of fluid and the milk is watery and bloody (Samad, 2008).

The causative organisms of mastitis are ubiquitous in nature and persist long time in the cow yard or barns and there is a chance of constant udder infection under poor hygienic and management systems (Roy *et al.* 1988 and Radostits *et al.* 1994). Epidemiological studies on mastitis revealed that mastitogenic organisms are widespread on different body sites of the cows, milker's hands, milking cans and in the milk samples. Teat apices have been reported to be the most common site (Itagaki *et al.* 1999), from where these organisms have been isolated (Malhotra and Kapur, 1982; Prabhakar *et al.* 1990).

The main etiological agents responsible for mastitis include *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysagalactiae, Streptococcus uberis, Streptococcus bovis, Corynebacterium bovis, Corynebacterium pyogenes, Escherichia coli, Enterobacter foecalis, Klebsiella pneumonia, Mycoplasma bovis* etc. However, Mahbub-E-Elahi *et al.* (1996) isolated and identified *Staphylococci (49.33%), Streptococci (14.0%), Corynebacterium (8.0%), E. coli (6.0%), Bacillus sp. (4.67%)* and *18%* unidentified bacteria from milk of clinical mastitic cows of Manikgonj Milkshed area, Bangladesh Agricultural University Dairy Farm (BAUDF) and BAU Veterinary Clinic. *Corynebacterium* sp. is one of the important etiological agents responsible for the clinical mastitis in cows in Bangladesh.

Considering the above mentioned facts, the present research was undertaken with the following objectives:

I. To isolate and identify the *Corynebacterium* sp. from clinical mastitis.
II. To find out the possible way to prevent the Corynebacterial mastitis.
III. To save the dairy farm from the economic losses due to mastitis.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

For obtaining the distinct evidences and key informations, the whole review of literature on the research title " Isolation and identification of *Corynebacterium* sp. from the cases of clinical mastitis in cow at Dinajpur district of Bangladesh" has been conveniently broad under following four headings. These are outlined below :

1) Isolation and identification of *Corynebacterium* sp.
2) Incidence of *Corynebacterium* sp.
3) Prevalence of *Corynebacterium* sp.
4) Epidemiology of mastitis

2.1 Isolation and identification of *Corynebacterium* sp.

Lee *et al.* (2008) studied a biochip capable of detecting 7 common species of mastitis-causing pathogens, including *Corynebacterium bovis*, *Mycoplasma bovis*, *Staphylococcus aureus*, and the *Streptococcus* spp. *S. agalactiae*, *S. bovis*, *S. dysagalactiae*, and *S. uberis*, within 6 hr was developed. The technique is based on DNA amplification of genes specific to the target pathogens and consists of 4 basic steps: DNA extraction of bacteria, polymerase chain reaction, DNA hybridization, and colorimetric reaction. To examine the accuracy and specificity of this biochip, a preliminary test with 82 random quarter milk samples were analyzed and compared with results from conventional microbiological methods conducted simultaneously. Results from all but 1 sample analyzed by the biochip were in agreement with those analyzed by bacteriology. The biochip could be a feasible tool for rapidly diagnosing mastitis-causing pathogens in milk and providing information for a more effective treatment to cure mastitis.

Pianta *et al.* (2007) performed bacteriologic examinations on 188 milk samples collected from cows from 11 farms for diagnosis of mastitis in three cities of Rio Grande do Sul, Brazil. Among the common causes of mastitis, the most frequent isolates were *Staphylococcus aureus*, followed by *Corynebacterium* sp, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*. Bacteriologic examination of 32 milk samples from one farm didn't show bacteria known as common etiologic agent of mastitis. Six samples of *Arcobacter* spp typed by genotypic tests as *Arcobacter cryaerophilus* (five strains) and *Arcobacter butzleri* (one strain) were isolated from cows' milk of that farm. It is reported the isolation of *Arcobacter* species from the milk of cows in absence of clinical signs of mastitis. This is the first report of the detection of the microorganisms in the milk of dairy cows in Brazil. No previous reports are known from other countries

Hommez *et al.* (1999) investigated a nonlipophilic corynebacteria associated with clinical and subclinical mastitis in dairy cows to belong to four species: *Corynebacterium amycolatum*, *Corynebacterium ulcerans*, *Corynebacterium pseudotuberculosis*, and *Corynebacterium minutissimum*. These species may easily be confused. However, clear-cut differences between *C. ulcerans* and *C. pseudotuberculosis* were found in their acid production from maltotriose and ethylene glycol, susceptibility to vibriostatic agent O129, and alkaline phosphatase. Absence of growth at 20Â°C and lack of Î±-glucosidase and 4MU-Î±-d-glycoside hydrolysis activity differentiated *C. amycolatum* from *C. pseudotuberculosis* and *C. ulcerans*. The mastitis *C. pseudotuberculosis* strains differed from the biovar equi and ovis reference strains and from caprine field strains in their colony morphologies and in their reduced inhibitory activity on staphylococcal Î²-hemolysin. *C. amycolatum* was the most frequently isolated nonlipophilic corynebacterium.

Bhaumik (1997) examined milk samples from 40 quarters of 23 Jersey cross-bred cows, of which only 15 samples revealed bacterial growth on cultural examination of which 8 and 7 samples showed gram positive and gram negative staining characters, respectively. These bacterial isolates were subjected to antibacterial sensitivity test (AST) as per the standard disc diffusion method and on AST all the isolates were sensitive to gentamicin (100%), while doxycycline (86.66%), chloramphenicol (73.33%), streptomycin (46.66%), cephalexin (40%), contrimoxazole (26.66%), ampicillin (26.66%) and cloxacillin (20%) exhibited some degree of resistance.

Mahbub-E-Elahi *et al.* (1996) isolated and identified bacteria from the different quarters of 150 mastitis affected dairy cows in Bangladesh, of which 51 (34.0%) were from cows with one quarter affected, 37 (24.67%) with two quarters affected, 30 (20.00%) with three quarters affected and 32 (21.33%) with all four quarters affected. Bacteria isolated and identified from these samples were *Staphylococcus aureus (31.33%), Staph epidermidis (18.00%), Streptococcus spp. (14.00%), Corynebacterium pyogenes* (8.00%), *Escherichia coli* (6.00%), *Bacillus* spp. (4.67%) and 18.00% remain unidentified.

Fazal-ur-Rehman (1995) examined 400 quarters of 50 buffaloes and 50 cows for isolation of microorganisms. Of the 200 buffalo quarters, 63 and 70 reacted positive to SFMT and CMT, respectively. Similarly, of the 200 cow quarters, 77 and 83 reacted positive to SFMT and CMT. Sixty one and 90 quarters of buffaloes and cows respectively yielded growths of different microorganisms. *Staphylococci* isolates were speciated using API STAPH-Trae system. *Staphylococcus aureus* was the most frequently encountered pathogen (39.1% in buffaloes and 47.3% in cows). Other organisms isolated were *Staphylococcus hyicus* (4.7% in buffaloes and 17.0% in cows), *Staphylococcus epidermidis* (3.1% in buffaloes and 6.4% in cows), *Staphylococcus capitis* (1.6/% in buffaloes), unidentified *staphylococcus* (1.6% in buffaloes and 3.2% in cows, *Micrococcus varians* (3.2% in cows), *Streptococcus dysgalactiae* (10.9% in

buffaloes and 7.4% in cows), *Streptococcus agalactiae* (3.1% in buffaloes and 1.1% in cows), *Streptococcus lactis* (1.1% in cows), *Streptococcus pyogenese* (1.6% in buffaloes, *Corynebacterium bovis* (7.8% in buffaloes and 1.1% in cows), *C. pyogenese* (3.1% in buffaloes and 2.1% in cows), other diphtheroids (4.7% in buffaloes), *E. coli* (6.2% in buffaloes and 1.1% in cows), non-coliform Gram –ve bacteria in buffaloes and 1.1% in cows), *Bacillus spp.* (7.8% in buffaloes and 4.3% in cows), yeast (3.1% in buffaloes and prototheca (1.6% in buffaloes). Mixed infection of 2 organisms was encountered in 1.5 and 2 percent quarters of buffaloes and cows, respectively.

Jha *et al.* (1994) conducted bacteriological examination of milk samples of 18 cows and 27 buffaloes and antibiotic sensitivity test of the isolated organisms. *Staphylococcus* spp. (39.2%) followed by *Escherichia coli* (16.7%) were the most commonly encountered bacteria. Other bacteria included Corynebacterium, Klebsiella, Micrococcus, Proteus, Pseudomonas and Streptococcus. The majority of the isolates were highly sensitive to gentamicin, oxytetracycline and chloramphenicol, but resistant to cloxacillin and ampicillin

Chanda *et al.* (1989) evaluated Bromothymol blue (BTB) card test, Whiteside Test (WST) and California Mastitis Test (CMT) followed by bacteriological examination to determine the subclinical and clinical mastitis in 372 quarter milk samples of 93 crossbred cows in and around Calcutta during 1986-1987. They isolated microorganisms in 113 (30.38%) milk samples, of which 65(57.52%) were Staphylococci, 40(35.40%) were Streptococci, 6(5.31%) were Corynebacterium and 2(1.77%) were *Escherichia coli. All* the isolates were tested on the basis antibiotic disc sensitivity tests for their sensitivity. It ~%as found that gentamicin was the most sensitive followed by ampicillin, tetracycline, chloramphenicol and kanamycin.

Gonalez *et al.* (1980) conducted a research work on microorganisms isolated from sub clinical cases of mastitis in cows included *S. aureus* (43% of samples) *S. epidermidis* (21%), *Str. uberis* (19%), *Str. agalactiae* (13%), *Str. dysgalactiae* (9%), *Corynebacterium pyogenes* (1.3%), *Corynebacterium bovis* (7%) and coliform (1.7%). Mixed streptococcal and staphylococcus infections also occurred.

2.2 Incidence of *Corynebacterium* sp.

Paul *et al.* (2000) analysed a total of 83 milk samples from cows and 49 from buffaloes, suffering from subclinical mastitis to determine the incidence of the disease in and around Chennai farms at Tamil Nadu, India and the prevalent bacteria present in the area. Antibiogram studies were also done with the characterized isolates to determine the drug of choice for the treatment of subclinical mastitis. The incidence of subclinical mastitis was higher in cows (25.78%) than in buffaloes (10.60%). The predominant bacteria in mastitis milk were *Staphylococcus* sp., followed by *Streptococcus* sp., and *E. coll.* Antibiogram pattern of bacterial isolates from 132 milk samples indicated that 96.20% were sensitive to neomycin, gentamicin and chloramphenicol. Oxacillin, bacitracin, penicillin and ampicillin were highly resistant to the bacterial strains. It is concluded that antibiotics like neomycin, gentamicin, chloramphenicol, pefloxacin and norfoplaxacin are effective for controlling subclinical mastitis. It has been suggested that the antibiotic sensitivity test should be conducted prior to treatment of mastitis.

Seddek *et al.* (1999) conducted a study on bacteriological status of subclinical mastitis in Friesian cattle in Assiut Governorate. Subclinical mastitis was diagnosed in 51 of 488 Friesian cows on 3 farms in Assiut Governorate. The total incidence of subclinical mastitis (SCM) in Assiut Governorate was 11.38%, while the incidence on the 3 farms was 7.1, 29 and 14.3%, respectively. Commonest bacterial isolate from affected cattle was *Staphylococcus aureus* (16 cows, 29.1%),

followed by *Escherichia coli* (11, 2%), *Staphylococcus epidermidis* (10, 18.2%), *Corynebacterium bovis* (6, 10.9%), *Streptococcus agalactioe (7,* 12.7%), *Streptococcus dysgalactiae* (3, 5.46%) and *Citrobacter* (2, 3.64%). Most isolates were highly sensitl[i]VC to spectrum, -h sensitive to Ioramphenicol, cephalexin, remectan and gentamicin, moderately sensiti kanamycin and less sensitive to tetracycline and Streptomycin but were resistant to penicillin and amoxicillin.

Singh *et al.* (1994). studied the incidence of subclinical mastitis in machine-milked cows on 8 farms in the Indian Pujab. It was *49.29* and *23.09%* on an individual animal-and quarter basis respectively. Two-quarter infections were more prevalent (50.00%) than 1- (34.29%), 3- (11.43%) and 4- (4.29%) quarter infections. The occurrence of infection was higher in hind quarters than in fore quarters. It was greatest in the left-hind quarter *(29.23%)* and least in-the left-fore quarter *(20.00%).* Staphylococci were the chief causative agents (73.08%) followed by Streptococci (14.61%) and *Escherichia coli* (5.38%). Corynebacterium, Proteus and *Klebsiella spp.* were also isolated. In culture-and sensitivity testing, gentamicin (93.08%), cephalexin (90.00%) and chloramphenicol *(83.85%)* were the most effective drugs and penicillin *(52.31 %)* the least effective

Saxena *et al.* (1993) experimented on incidence and etiology of bovine subclinical mastitis. Of 50 cows tested in Assam 32 (64%) were affected with subclinical mastitis. Among the 191 quarters examined (9 were blind) 74 (38.75%) were infected, with the left front quarter most frequently involved (31.08%). The highest incidence (34.75%) was in the second lactation. Cows in early lactation were most frequently affected (78.13%). From the 74 positive milk samples obtained 145 strains of bacteria were isolated. *Staphylococcus aureus* predominated (40 isolates), followed by coagulasenegative *Staphylococci (34), Bacillus* sp. (21), Streptococcus *uberis (14), Pneumococcus* sp. (12), *Streptococcus* sp. (9), *Corynebacterium* sp. (8),

Micrococcus sp. (6), and *Streptococcus bovis (1).* Mixed infections involving 2 or more organisms were found in 78.33% of the milk samples.

Al-Sha Wabkeh and Abdul Aziz (1987) reported *S. aureus* as the most common cause of both clinical and sub clinical mastitis in cows, followed by coliform, *Corynebacterium* spp., *Proteus* spp., *Streptococcus* spp., and *Pseudomonas* spp.

2.3 Prevalence of *Corynebacterium* sp.

Oliveira *et al.* (2009) conducted to determine the occurrence of mastitis and the microbiological relationship between the laboratory tests and auxiliary tests which were done in the field. Two cattle herds were studied for 18 months and 893 milk samples were collected. The udders were clinically examined, and the milk was tested using the strip cup test and the California Mastitis Test (CMT). Results revealed that *Staphylococcus aureus* was isolated in 196 (21.95%) samples. The coagulase-negative *Staphylococcus* sp. (SCN) was isolated in 104 (11.65%) samples. *Streptococcus agalactiae* was isolated in 67 (7.50%) samples. The esculin-positive *Streptococcus* sp. (ESCPOS) in 20 (2.24%) samples. The esculin-negative *Streptococcus* sp. (ESCNEG) was isolated in 29 (3.25%), leavenings in one (0.11%) sample and *Corynebacterium* sp. in 16 (1.68%) samples. 433 microorganisms were isolated, wherein 350 milk samples were negative and isolation and identification were not possible in 110 (12.32%) samples. The more numerous isolated microorganisms were the pathogenic strains that were higher than the CMT positive test.

Sumathi *et al.* (2008) investigated the current status of clinical mastitis among dairy cattle in and around Bangalore. The prevalence of mastitis was assessed by the results of bacteriological evaluation of milk samples collected from clinical mastitis cases. A total of seventy five bacterial isolates were recovered from sixty clinical cases of mastitis affected cows. The prevalence of major pathogens isolated was twenty four percent for *Staphylococcus aureus* twenty per cent for *Escherichia coli* followed by sixteen per cent

for *Staphylococcus epidermidis* and *Streptococcus* spp and ten per cent and five percent for *Klebsiella* spp and *Corynebacterium* spp respectively. Antibiogram studies were also performed for these isolates and Gentamicin was found to be the most effective drug. It was concluded that microbiological and antibiogram studies are necessary for treatment and control of the disease.

Workineh *et al.* (2006) undertaken to determine the aetiology and prevalence of mastitis in hand-milked cows (n = 186) in two major Ethiopian dairies. The California Mastitis Test and culturing for bacteria revealed that 21.5% of the cows were clinically infected and 38.2% had subclinical mastitis. Most mastitis pathogens isolated from milk samples testing positive by the California Mastitis Test were Gram-positive cocci. Staphylococci constituted 57% of the isolates, of which the predominant cause of bovine mastitis was *Staphylococcus aureus* (40.5%). Other mastitis pathogens isolated include streptococci (16.5%), coliforms (9%) and corynebacteria (5%). Retrospective analysis of farm records indicated that mastitis was the second most important cause of culling and accounted for 27% of the cows removed from these two dairies.

Iqbal (2004) applied Whiteside test and pH – indicator paper technique to 3980 quarter milk samples. A total of 486 (12.21%) samples were positive by Whiteside test as compared to 10.93 percent by pH – indicator paper technique. In most of the animals, only two quarter were affected, while four quarter prevalence was minimum. The prevalence was higher in hind quarters as compared to the fore quarters and slightly higher in right quarters than left one. A total of 190 pooled samples were positive for bacterial microorganisms. Different pathogenic microorganisms isolated were *S. aureus* (32.60%), coagulase negative staphylococci (12.06%), *Str. agalactiae* (16.58%), *Str. uberis* (3.01%), *Str. dysgalactiae* (3.01%), *E. coli* (16.08%), *Pseudomonas aureginosa* (7.50%), *Corynebacteium pyogenes* (3.51%), Bacillus spp. (3.01%) and *Klebsiella* (2.01%).

Larintluanga *et al.* (2003) screened 987 quarters of 248 cows with the help of a modified California mastitis test (MCMT). Quarters (n = 115) reacting positive in this test were examined for mastitis pathogens. Eighty nine (77.39%) of 115 quarter milk samples reacting positive in MCMT yielded growth on bacteriological examination. A total of 98 isolates were recovered. Coagulase-negative Staphylococci (55.1%) were the most predominant isolates followed by *Streptococci* (22.45%), *S. aureus* (7.14%), *Corynebacteria* (6.12%), *E. coli* (3.06%), *Proteus* spp. (20.04%), *Klebsiella* (2.04%) and *Citrobacter* spp (2.04%).

Armenteros *et al.* (2002) reported that out of the *12274* analysed quarters (3069 animals), 18.02% presented chronic mastitis and 3.7% with atrophy; 3.02% showed clinical mastitis; *45.1%* had subclinical mastitis and 76.5% positive to California mastitis test. *Staphylococcus aureus, Corynebacterium bovis* and *Streptococcus agalactiae* were the most frequently isolated microorganisms which showed a prevalence of 30.5, 9.2 and 8.3%, respectively. The investigators demonstrated the necessity to reactivate a Control Mastitis Program in the studied areas.

Oltenacu and Ekesbo (1994) conducted a reserch work on the prevalence of clinical mastitis in Sweden. The incidence rate of clinical mastitis in primiparous Swedish Friesian cows was 15%. The risk of mastitis was greater for calving in July and August and increased with age at calving. Calving disorders and ketosis increased the risk of mastitis 1.8 and 2 fold, while tramped teats and udder injuries increased it 6 and 3 fold, respectively. Herds accounted for 13% of the variation in incidence of mastitis and the risk of mastitis was 1.4 and 1.25 fold greater in high and average producing herds relative to low producing herds. High production predisposed cows for mastitis. Cows with clinical mastitis produced 260 kg less milk and were 2.8 fold more likely to be culled.

Lafi *et al.* (1994) reported the findings of a National cross-sectional study of mastitis in Jordan. Between July 1991 and August 1992, 63 Jordanian dairy farms selected by stratified random sample were visited to identify the major causes and prevalence of

intramammary infections in dairy cows. Of 773 cows examined 60% of all sampled quarters had >283000 cells/mL. The mean value of somatic cell count (SCC) was positively associated with age in lactations and negatively with herd's size. Cows milked by bucket milking machines or in fully automatic parlors had a lower mean SCC than those milked by hand. The most common isolate from clinical cases was *S. aureus* (37.5%). Estimates of prevalence of bacterial pathogens in non-clinical intramammary infections were: coagulase – negative staphylococci (16.04%), *S. aureus* (9.41%), *Klebsiella spp.* (6.17%), *Corynebacterium bovis* (5.35%), and *Brucella melitensis* (4.52%).

Tuteja *et at.* (1993) conducted a study on bovine subclinical mastitis: prevalence and microflora. Of the 178 apparently healthy cows from an organized herd examined for mastitis, 78.1% were found to be culturally positive and 53.9% had somatic cell counts of >500 000 per ml. Of the infected quarters, 42.2% were culturally positive. Of these 19.7% and 22.5% had 'subclinical' and 'latent' mastitis, respectively, as per the International Dairy Federation (IDF) criteria, while 7.7% of the quarters had non-specific mastitis. Among 307 isolates from 697 apparently healthy quarters, *Staphylococci* were predominant (44.3%), followed by Micrococci (30.0%), Streptococci (18.9%), *Corynebacterium* spp. (5.5%), *Bacillus* spp. (1.0%) and Enterococci (0.3%). Serotyping of 37 *Streptococcus agalactiae* strains showed that types R(9), Ib(8), X(5), IIR(4), IbR(4), NT(3), HIR(l), IVR(1), 11(l) and 111X(1) were prevalent in the cows.

Gupta *et al.* (1992) cultured 1223 milk samples and performed their antibiotic sensitivity test of the isolated organisms. They isolated highest percentage of *Staphylococcus* spp. (40.65%), followed by *Streptococcus* spp. (29.35%), *E. coli* (20.02%), *Corynebacterium pyogenes* (4.35%), *Proteus vulgaris* (3.35%), and *Pseudomonas auriginosa* (2.28%). Sensitivity pattern

showed that most of organisms are sensitive to nitrofuradintin, chloramphenicol, gentamicin, kanamycin as compared to ampicillin, oxytetracycline, norfloxine and lincomycin. All strains of *Proteus vulgaris* and *Pseudomonas aeruginosa* were reported to be resistant to ampicillin oxytetracycline and cotetramezole.

Hashmi *et al.* (1980) examined 250 animals (198 buffaloes and 52 cows) for sub clinical mastitis. The prevalence of sub clinical mastitis in buffaloes was 45.45 percent and in cows 50 percent. Quarter infection rate in buffaloes and cows was 23.38 and 25.95%, respectively. The organisms isolated were streptococci (36.82%), staphylococci (43.09%), coliforms (12.15%), pseudomonas (2.92%), corynebacterium (4.18%) and yeast cells (0.44%).

2.4 Epidemiology of mastitis

Rahman *et al.* (2009) observed that the identification of risk factors is important for the design of control programmes for mastitis in cows. Information about farms and management was collected at a farm visit. California Mastitis Test (CMT) was performed to assess sub-clinical mastitis, and cows, udder and milk were examined for clinical mastitis. A total of 347 lactating cows from 83 farms in the dry season (November - February) and 388 lactating cows from 89 farms in the wet season (June – October) were studied. The overall prevalence of mastitis was 19.9% and 44.8% in dry and wet seasons, respectively. The prevalence of mild mastitis was 17.3% and 40.7%, whereas that of moderate mastitis was 2.6% and 4.1% in dry and wet seasons, respectively. The prevalence of mastitis was higher ($P<0.01$) in wet than in dry season. On average, 18.7% quarters had mastitis during the wet season and 6.9% in the dry season. In the dry and wet seasons, respectively, 63.9% and 11.2% had completely dry floors, and the prevalence of mastitis was 22.6% and 30.0%. On the other hand, 88.8% and 36.1% of 83 farms had partly or completely wet and soiled floor and the prevalence of mastitis was 40.0% and 59.5% in the dry and wet seasons, respectively. Udder cleanliness, milk yield

and peri-parturient diseases significantly (P<0.01) increased the risk of mastitis. The prevalence of mastitis is regarded as quite high. Dry and clean floor to keep cow's udder and teat clean would help control mastitis in the dairy farms of Bangladesh. Identification of risk factors is important for the design of control programmes for mastitis in cows. Information about farms and management was collected at a farm visit. California Mastitis Test (CMT) was performed to assess sub-clinical mastitis, and cows, udder and milk were examined for clinical mastitis. A total of 347 lactating cows from 83 farms in the dry season (November - February) and 388 lactating cows from 89 farms in the wet season (June – October) were studied. The overall prevalence of mastitis was 19.9% and 44.8% in dry and wet seasons, respectively. The prevalence of mild mastitis was 17.3% and 40.7%, whereas that of moderate mastitis was 2.6% and 4.1% in dry and wet seasons, respectively. The prevalence of mastitis was higher (P<0.01) in wet than in dry season. On average, 18.7% quarters had mastitis during the wet season and 6.9% in the dry season. In the dry and wet seasons, respectively, 63.9% and 11.2% had completely dry floors, and the prevalence of mastitis was 22.6% and 30.0%. On the other hand, 88.8% and 36.1% of 83 farms had partly or completely wet and soiled floor and the prevalence of mastitis was 40.0% and 59.5% in the dry and wet seasons, respectively. Udder cleanliness, milk yield and peri-parturient diseases significantly (P<0.01) increased the risk of mastitis. The prevalence of mastitis is regarded as quite high. Dry and clean floor to keep cow's udder and teat clean would help control mastitis in the dairy farms of Bangladesh.

Khan *et al.* (2004) used Surf field mastitis test and bacteriological examination of quarter milk samples of 50 buffaloes documented 27, 4, 10 per cent prevalence for sub clinical mastitis, clinical mastitis and blind quarters, respectively.

Qazi *et al.* (1999) surveyed 45 different small livestock units/herds in Lahore (Pakistan) for epidemiologic data on mastitis. Analysis of data showed a prevalence of 8.8% in herds. The prevalence in lactating animals was 8.3%. Of 1000 quarters milk samples,

14.3% were positive for sub clinical mastitis. Highest prevalence of mastitis was recorded in 6-8 year old cows and buffaloes. Out of positive cases the prevalence was highest (53.63%) during early lactation followed by middle (21.97%) and late lactation (24.4%).The prevalence was higher in high yielding animals. Surgically manipulated animals were more prone to disease (4%).

Shpigel *et al.* (1998) performed to determine the clinical, bacteriological and epidemiological aspects of acute clinical mastitis in seven Israeli dairy herds. A total of 1124 clinical mastitis cases were detected by abnormal changes in the milk and udder with concurrent decrease of at least 25% in daily milk production. A total of 1190 quarters were affected with clinical mastitis in 1089 cows. The rear quarters had a higher incidence risk 64.7% of quarter. cases than the front quarters. The annual herd-year-incidence varied from 4.2 to 126.8 casesr100. cowsryear. The whole-lactation incidence risk LIR was 20.8 per 100 lactations. LIR increased from the first to fifth lactation and then decreased. Most clinical mastitis cases were associated with coliform bacteria 60.2% of cases , environmental streptococci 18.6% , coagulase-negative staphylococci 8.7% and samples from which no bacterial growth was detected 8.1% . Most cases of clinical mastitis occurred in the early stages of lactation, with 51.4% of all cases, 52.3% of coliform cases and 54.6% of environmental streptococci mastitis cases occurring during the first 4 months of lactation. The median days in milk at diagnosis was 118 days. The incidence was lower in the dry summer months.

Soomro *et al.* (1997) conducted a study on 785 milk samples collected from 200 buffaloes during 1994-95. The physical examination of the udder of these animals revealed 6% clinical (chronic) and 1.0% congenital abnormalities of udder. Three chemical tests viz., Bromothymol blue test, chloride test and Whiteside test were applied on milk, which respectively revealed 19.7%, 15.9% and 13.85% quarters affected with sub clinical mastitis. In general, 33.3 percent animals had sub clinical mastitis.

Arshad *et al.* (1995) investigated some epidemiological aspects of mastitis in District Gujrat (Pakistan). A total of 4500 smallholder herds were surveyed. Quarter infection rate was significantly higher in hind than in fore quarters in buffaloes. Age and lactation based distribution of mastitis cases varied in different age and lactation groups, being highest in 6-8 year age group. Mastitis prevalence was higher during early stage of lactation than in middle and late stages. The disease was estimated to cause 20.63% decrease in milk production in affected animals.

Bansal *et al.* (1995) investigated milk samples obtained from 154 cows in different herds, and 117 buffaloes in 5 herds in the Indian Punjab province. Sub clinical mastitis was found in 48% of cows and 27.05% cow udder quarters, and 23.93% of buffaloes and 11.32% of buffalo's udder quarters.

Prabhakar *et al.* (1995) studied the incidence of clinical mastitis and its etiologic agents at five farms. The overall monthly incidence was found to be 4.06 per cent. Of the total of 421 buffaloes, 40 were affected with clinical mastitis in 76 quarters. *Staphylococci* were the major causative organisms (34.21% *S. aureus* and 13.16% coagulase-negative *Staphylococci*), followed 11 by *Str. agalactiae* (14.74%), *E. coli* (10.53%), *Pseudomonas* spp (7.89%), *Str. pyogenes* (3.95%), *Klebsiella* spp. (3.95%), *Str. dysgalactiae* (2.63%), *Proteus* spp. (2.63%), *Str. uberis*, Diptheroids and mixed infections (1.31% each). No organism could be isolated from 2.63% quarters.

Zingerser *et al.* (1991) reported the results of a 17- month national survey of clinical and sub clinical mastitis between April 1985 and August 1986. Eighty nine Jamaican diary herds with 10 or more cows were visited. A total of 645 lactating cows were examined using the CMT and 254 composite milk samples collected for bacteriological examination. Widespread management faults were noted especially related to milking machine usage and maintenance and the abuse of antibiotics. Fifty six per cent of all

quarters were found to have CMT scores of one or higher, 0.8% showed clinical mastitis and 3.2% were blind. The most common bacterial pathogen, *Staphylococcus aureus* was recovered from 31% of sampled cows. The resultant milk loss from clinical and sub clinical mastitis was estimated to be 20% of the potential national production.

Sharma (1983) reported that clinical mastitis reduced milk production in cows and buffaloes to the tune of 26% and 19% respectively while sub clinical mastitis affected reductions of 19 and 12%, respectively. Most of the *Staphylococcal* and *E. coli* infections occurred within one week of calving. New quarter infection was predominantly associated with Staphylococcal and Streptococcal infections. Whereas in dry period corynebacterial infections predominated.

Kalara and Dhanda (1964) in the rural areas of the North West India, the overall incidence of clinical mastitis was 7.5% in buffaloes and 8.80% in cows. In urban areas, the corresponding value was 10.23% in buffaloes and 11.08% in cows.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

The research was dealt with isolation and identification of *Corynebacterium* sp. from the cases of clinical mastitis in cows at Dinajpur district of Bangladesh. A total of 20 milk samples were collected from the different dairy farms and rural domestic holdings of Dinajpur district. This study was conducted at the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur during the period from September/2010 to February/2011.

3.1 Materials

3.1.1 Sample: Milk samples were collected from the clinically mastitis affected cows of different dairy farms and rural domestic holdings at Dinajpur district in Bangladesh.

Table 1: Areas of sample collection.

SL No.	Name of farm	No. of samples collected
1	Milk Vita dairy farm, Chirirbandar, Dinajpur	4
2	Mimima dairy farm, Sadar, Dinajpur	3
3	Dairy farm, HSTU, Dinajpur	3
4	Upazila livestock office, Sadar, Dinajpur	2
5	Rural domestic holdings	8

3.1.2 Media:

Different bacteriological culture media and reagents were used for isolation and identification of bacteria. The culture media and reagents that were used in this experiment are mentioned below.

3.1.2.1 Nutrient Agar(NA) Medium (HI-MEDIA, India)

3.1.2.2 Nutrient broth(NB) (HI-MEDIA, India)

3.1.2.3 Blood Agar (BA) Medium (HI-MEDIA, India)

3.1.3 Media used for biochemical test

For biochemical characterization of *Corynebacterium* sp. the following media were used.

3.1.3.1 Sugar media such as dextrose, lactose and maltose.

3.1.3.2 MIU (Motility Indole Urease) broth

3.1.4 Chemical, reagents and solutions

The chemical and reagents used during the study were

3.1.4.1 Gram's staining reagents (0.5% Crystal violet, Gram's iodine, acetone alcohol and 2% safranin).

3.1.4.2 Other chemicals and reagents as per require requirement during conducting the experiment.

3.1.5 Glass wares and other appliances

The different kinds of glass wares and appliances used during the course of the experiment were as follows:

a) Test tubes (with or without Durham's fermentation tube and stopper)
b) Conical flask
c) Petridishes
d) Pipette

e) Slides
f) Cover slips
g) Hanging drop slide
h) Glass rod spreader
i) Test tube stand
j) Inoculating loop
k) Water bath
l) Centrifuge tubes and machine
m) Spirit lamp
n) Electric heater
o) Ice box
p) Incubator
q) Autoclave
r) Refrigerator
s) Hot air oven
t) Compound microscope
u) Electronic pH meter etc.

3.1.6 Antimicrobial sensitivity discs

To determine the drug sensitivity patterns of *Corynebacterium* sp. with different types of antimicrobial discs. Commercially available antimicrobial discs (Benex Ltd., USA; Oxoid Ltd, UK) were used. The method allowed for the rapid determination of the efficacy of the drugs by measuring the diameter of the zone of inhibition that resulted from different diffusion of the agent into the medium surrounding the disc. The following are the antibiotics that were tested against selected organism with their disc concentration.

Table 2: Antimicrobial agents with their disc concentration

Name of antibiotics	Disc concentration (µg/ disc)
Penicillin (P)	10
Gentamycin (GN)	10
Ciprofloxacin (CIP)	05
Ampicillin (AMP)	10
Amoxycillin (AML)	10
Tetracycline (TE)	30

3.2 Methods

The following methods were used for the isolation and identification of bacteria.

3.2.1 Experimental design

The milk samples were collected directly from clinically mastitis affected cows of different dairy farms and rural domestic holdings at Dinajpur district for the isolation and identification of *Corynebacterium* sp. by morphology, staining and cultural characteristics. Characterization of bacteria was done by cultural amd biochemical reactions. The isolated *Corynebacterium* sp. was tested for the sensitivity and resistance patterns to different antibiotics.

EXPERIMENTAL DESIGN

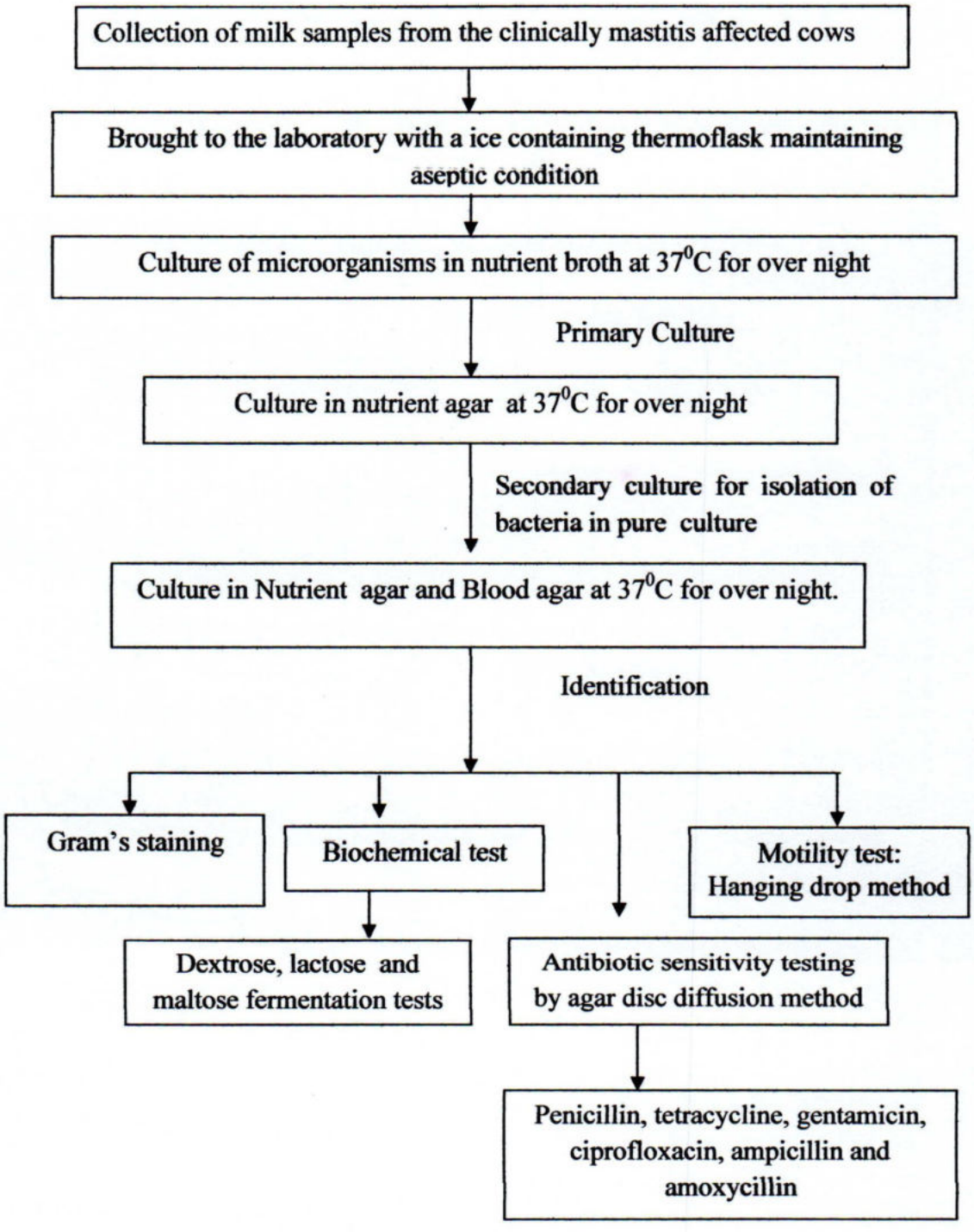

Fig. 1. Flow- chart of Experimental Procedure

3.2.2 Collection of milk sample

Milk samples from cows were collected aseptically in separate sterile screw cap vials as described by Rosenberger (1979) and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur with necessary precautions for bacteriological examination.

Before taking the milk samples, a teatful of milk was milked into a tube and later discarded. This was to flush out any bacteria that might be present in the teat canal. The teat and teat tips were disinfected with a swab soaked in 70% alcohol.

Milk samples were collected aseptically from individual quarter of udder at the time of morning milking. Milk samples were carried out in a wide mouth thermos flask and kept in the refrigerator at 4˚C in the laboratory for an hour before testing.

3.2.3 Preparation of culture media and broth:

3.2.3.1 Nutrient broth medium

Nutrient broth was prepared by dissolving 13g of dehydrated nutrient broth (Hi-Media, India) into 1000ml of distilled water and was sterilized by autoclaving at 121˚C under 15lbs pressure per square inch for 15 minutes. Then the broth was dispensed into tubes (10ml per tube) and store at 4˚C in the refrigerator until used.

3.2.3.2 Nutrient agar medium

Twenty eight grams of nutrient agar powder(HI-MEDIA) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile Petridish and allowed to solidify. After solidification of the medium in the Petridishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use.

3.2.3.3 Blood agar medium

Forty grams of Blood agar base (HI-MEDIA, India) powder was suspended in 1000 ml of distilled water and boil to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm ^{2} pressure and 121' C for 15 minutes and cool to 45° C. Then 5-10 % sterile defibrinated sheep blood was added to the medium aseptically and distributed to sterile petridishes and allowed to solidify. After solidification of the medium in the petridishes; these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use.

3.2.3.4 Sugar media

The medium consists of peptone water of which fermentable sugar was added to the proportion of 1%. One gram of Bacto peptone (HI-MEDIA) and 0.5 grams of sodium chloride were added in 100 ml of distilled water. The medium was boiled for 5 minutes, adjusted to PH 7.0, cooled and then filtered through filter paper. Phenol red, an indicator at the strength of 0.2% solution was added to peptone water and then dispensed in 5 ml into cotton plugged test tubes containing Durham's fermentation tubes and placed invertedly. These were then sterilized by autoclaving at 1.2 kg/cm ^{2} and 121 ° C for 15 minutes. The sugars used for fermentation were prepared separately as 10% solution in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A little heat was necessary to dissolve the sugar. The sugar solutions were sterilized in Arnold steam sterilizer at 100° C for 30 minutes for 3 consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tube containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at 37°C for 24 hours.

3.2.3.5 MIU (Motility Indole Urease) Broth

18.00gms of powder of MIU agar based (HiMedia, India) was added to 950ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium

was then sterilized by autoclaving at 121°C maintaining a pressure of 15lbs/square inch for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to decrease its temperature. After this the medium in the test tubes were allowed for incubating at 37°C for overnight to check their sterility and stored in refrigerator for future use.

3.2.4 Culture of milk sample

Milk samples earlier put into transport media were divided and inoculated separately in Nutrient agar (NA) and Blood agar (BA) to promote growth of bacteria. Each group of these media was incubated at 37° C for over night. The colonies on primary cultures were repeatedly sub-cultured by streak plate method (Cheesbrough, 1985) until the pure culture with homogenous colonies were obtained. Media such as Blood agar, Nutient agar were used for sub-cultures and were incubated at 37° C for 24 hours for growth.

3.2.5 Staining methods

Gram's staining method was followed to study the morphological and staining characteristics bacteria and to provide information about the presumptive bacterial identification as per recommendation of Cowan and Steel (1979).

3.2.6 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into Nutrient agar media by streak plate technique to obtain isolated colonies as per methods suggested by poindexter (1971).

Step 1. A loopful aliquot was taken from the selenite broth culture and was spread on a small area of the medium in the petridish.

Step2.T'he loop was sterilized by being heated as red hot in a flame.

Step3. The inoculum was spread over the remainder of the plate by drawing the cooled, sterilized loop across the part of the inoculated area, then streaking in a single direction in each parallel line.

This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.2.7 Maintenance of stock culture

The stock culture was maintained following the procedures of choudhury *et al.* (1987). Nutrient agar slants were used for the maintenance of stock culture for each of the bacterial isolates. One slant was used for an individual isolate. After growth of the organism in the slant, the sterile mineral oil was overlaid and the culture was kept at room temperature for further use as seed.

3.2.8 Isolation and identification of *Corynebacterium* sp.

Diluted samples were inoculated on blood agar and incubated at 37°C for 24 to 48 hours. The growth of gram positive bacteria form small (1mm) grayish colony indicated *Corynebacterium*. In Gram's staining, encapsulated and unsporulated bacteria arranged in pair suggested *Corynebacterium* and subsequently identified according to the Bergey's manual of determinative bacteriology (1994).

3.2.8.1 Culture into different media

Loopful aliquot was taken from the Nutrient broth culture and streaked on Nutrient agar and Blood agar media to get isolates in pure culture. All inoculated media were kept at 37° C for overnight in an incubator.

3.2.8.1.1Nutrient broth(NB)

1ml milk samples were inoculated into NB and incubated at 37°C for overnight. After incubation enough whitish turbidity was observed.

3.2.8.1.2 Nutrient agar (NA)

Materials from Nutrient broth tubes were inoculated into Nutrient agar plates and incubated at 37°C for overnight.

. 3.2.8.1.3 Blood agar (BA)

Materials from Nutrient broth were inoculated into Blood agar plates and incubated at 37°C for overnight.

3.2.8.2 Gram's staining method

Gram's staining was performed for morphological characterization of bacteria according to the method describe by Cowan and Steel (1979).

3.2.8.3 Motility test

The Motility test was performed according to the method described by Cowan (1985) to differentiate motile bacteria from the non-motile one. Before performing the test, a pure culture of the organism was allowed to grow in NB. A drop of fresh broth culture was placed on the cover slip and was placed invertedly over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the concave depression of the hanging drop slide for better attachment of the cover slip to prevent air current and evaporation of the fluid. The hanging drop slide was then examined carefully under 100X objective of a compound microscope using immersion oil. The motile and non-motile organisms were identified by observing motility in contrasting with to and fro movement of bacteria.

3.2.8.4 Biochemical test

The suspected isolated organisms were subjected to different biochemical tests, such as sugar fermentation test for acid or acid and gas production. Standard methods were followed for conducting these tests as described by Cowan (1985) during the experiment.

The sugar fermentation test was performed by inoculating a loop full of overnight Nutrient broth culture of the organism into the tubes containing three basic sugars (dextrose, lactose and maltose) and incubated into the Bacteriological incubator for 24 hours at 37°C to observe their sugar fermentation capability.

3.2.8.4.1 MIU (Motility Indole Urease) Broth

Materials from Nutrient broth tubes were inoculated into MIU media and observed for any changes for identification of motile and nonmotile organism.

3.2.8.5 Antimicrobial sensitivity test

Antimicrobial sensitivity test was performed according to the procedures suggested by Choudhury *et al.* (1987) to determine the sensitivity and resistance patterns of *Corynebacterium* sp. isolated from milk. The antimicrobial used were penicillin, tetracycline, gentamicin, ampicillin, amoxicillin and ciprofloxacin.

An amount of 0.5 ml freshly grown pure broth culture of the bacteria was poured on agar plates and allowed to spread gently over the entire surface with a sterile glass rod spreader. After 1-2 minutes the antimicrobial discs were placed on the inoculated plates keeping a distance of about 1 cm. apart and incubated at 37° C

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

The results describe the isolation and identification of bacterial flora emphasizing on the *Corynebacterium* sp. from the cases of clinical mastitis in cows at Dinajpur district of Bangladesh.

Frequency of bacteria isolated from the milk of clinically mastitis affected cows:

The result of frequency of different bacterial isolates are presented in Table-3. A total of 20 milk samples were examined for the isolation of bacteria.

Table -3: Frequency of isolates in milk samples.

Name of isolated organism	No. of isolated organism	Percentage (%) of isolated organism
Staphylococcus spp.	5	25
Streptococcus spp.	3	15
Escherichia coli	4	20
Corynebacterium sp.	5	25
Unidentified	3	15
Total	20	100

Table-4: Cultural and staining characteristics

SL No.	Name of isolated organism	Nutrient broth	Colony characteristics		Morphology	Staining characteristics
			Nutrient agar	Blood agar		
1	*Staphylococcus* spp.	Enough whitish turbidity	Large, circular, convex, glistening with an entire edge and golden yellow color	Not done	Spherical cocci 1.0 µm in diameter and arranged in groups or grape- like clusters.	Gram positive, violet color.
2	*Streptococcus* spp.	Enough whitish turbidity	Small, smooth, glistening dew drop like and finely granular.	Not done	Spherical or ovoid, 1.0 µm in diameter and arranged in pairs or chains varying in length from 2-12 or more cocci.	Gram positive, violet color.
3	*Escherichia coli*	Enough whitish turbidity	Smooth, circular and white to grayish white	Not done	Short rod shape, arranged in single or pair	Gram negative, pink color.
4	*Corynebacterium* sp.	Enough whitish turbidity.	Small, smooth, circular and grayish in color.	Grayish in color, about 1mm in diameter and surrounded by a zone of hemolysis.	Short cocco bacilli or rods with a maximum length of 2µm and arranged in bundles or clusters and singly also.	Gram positive, violet color.

4.1 Isolation and identification of *Corynebacterium* sp.

4.1.1 Results of Cultural characteristics

a) Nutrient broth

Enough whitish turbidity was observed which was the characteristic for the growth of *Corynebacterium* sp. (Plate 1).

b) Nutrient agar

In nutrient agar the colonies were small, smooth, circular and grayish in color (Plate 3).

c) Blood agar

On blood agar the colonies were grayish in color about 1mm in diameter and surrounded by a small zone of hemolysis (Plate 8).

4.1.2 Results of Staining characteristics (Gram's staining)

The organism revealed Gram positive, violet color, short cocco-bacili and arranged in bundles or clusters and singly also (Plate 7).

4.1.3 Results of Motility test (Hanging drop slide method)

The isolated organism was non motile in Hanging drop slide method (Plate 9).

4.1.4 Results of Biochemical test

For identification of *Corynebacterium* sp. the different biochemical tests were performed with the isolated Gram positive cocco-bacilli organism. Various sugar fermentation tests were performed namely dextrose, lactose and maltose. The organism produced acid but no gas in all sugar fermentation tests (Plate 10 & Plate 11). The color of the biochemical

media was also changed. MIU test was also performed in this study and recorded negative reaction which demonstrate nonmotile *Corynebacterium* sp. (Plate 12).

Table-5: Biochemical test

Dextrose fermentation	Lactose fermentation	Maltose fermentation	MIU
Positive reaction and acid production without gas.	Positive reaction and acid production without gas.	Positive reaction and acid production without gas	Negative reaction indicates the organism was nonmotile

4.1.5 Results of Antibiotic sensitivity test

On nutrient agar plate culture the clear zone of growth inhibition was found around the antibiotic discs and *Corynebacterium* sp was highly sensitive to gentamicin and ciprofloxacin but some degree of resistance to tetracycline, amoxycillin, ampicillin and penicillin. (Plate 13 &14).

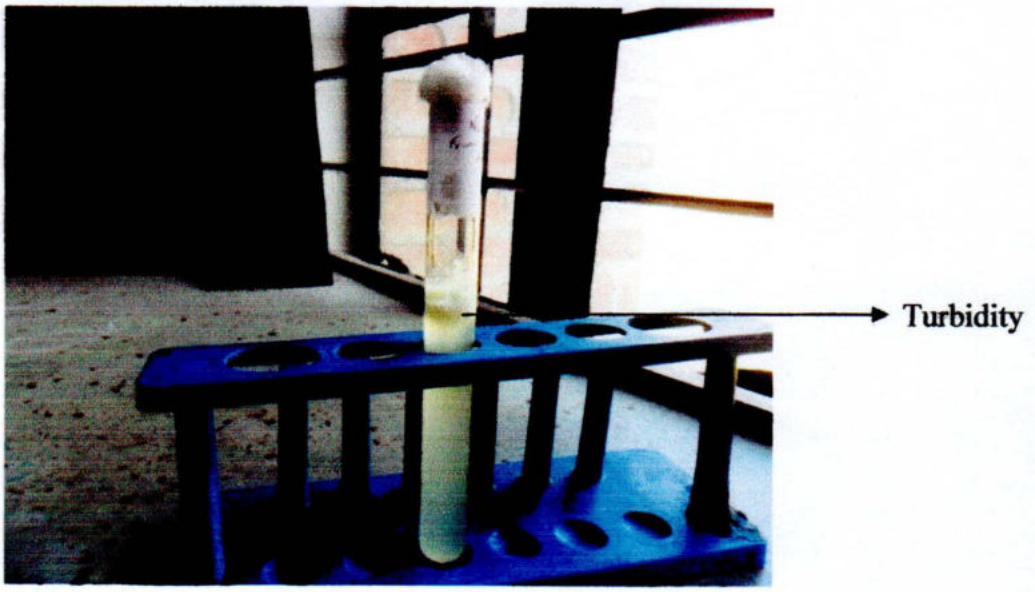

Plate 1: Culture of milk sample in Nutrient broth.

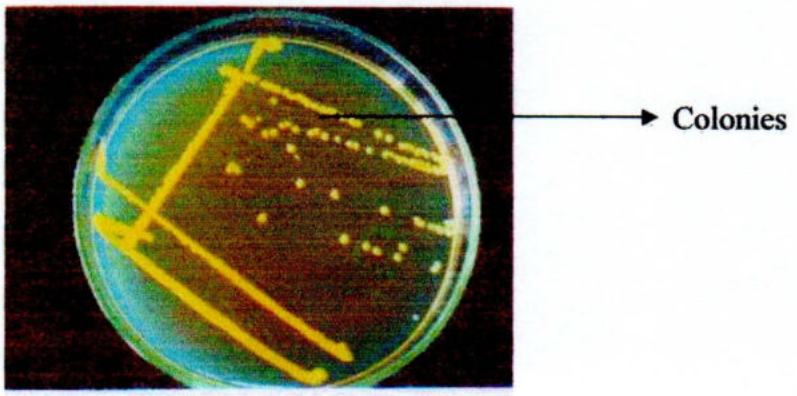

Plate 2: Culture of *Staphylococcus* sp. in Nutrient agar.

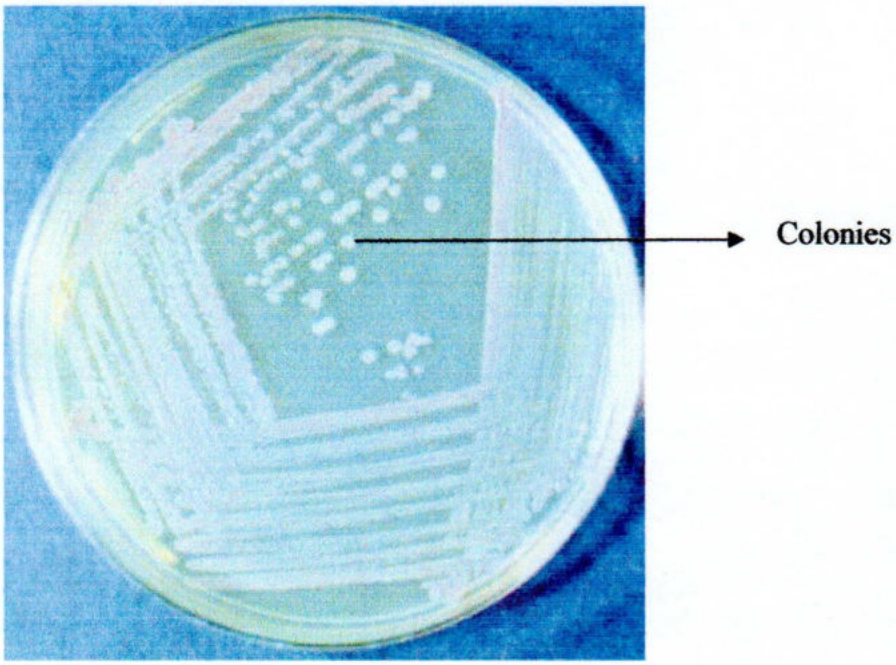

Plate 3: Culture of *Corynebacterium* sp. in Nutrient agar.

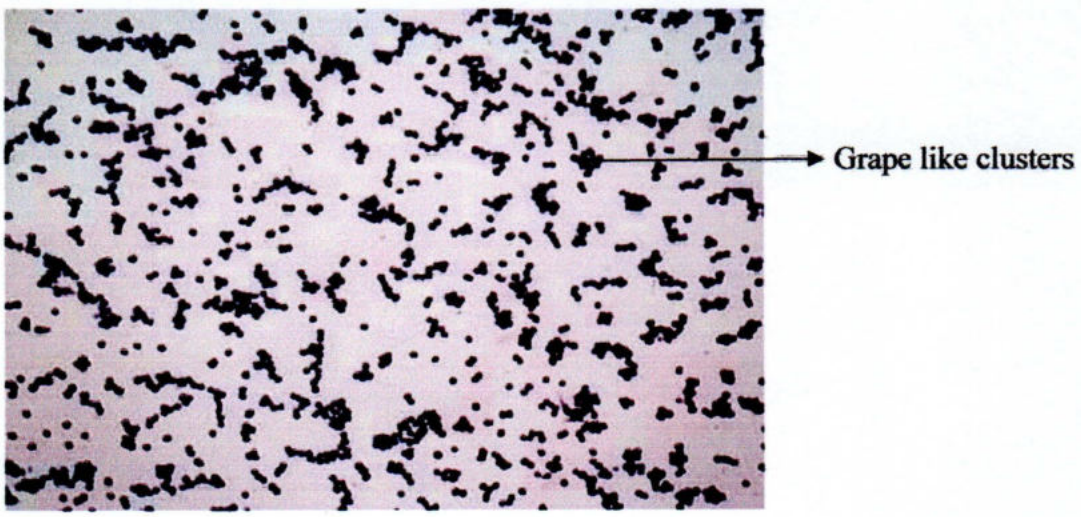

Plate 4: *Staphylococcus* sp. showing Gram positive (violet color) Spherical cocci and arranged in groups or grape- like clusters at 100X (Gram's staining).

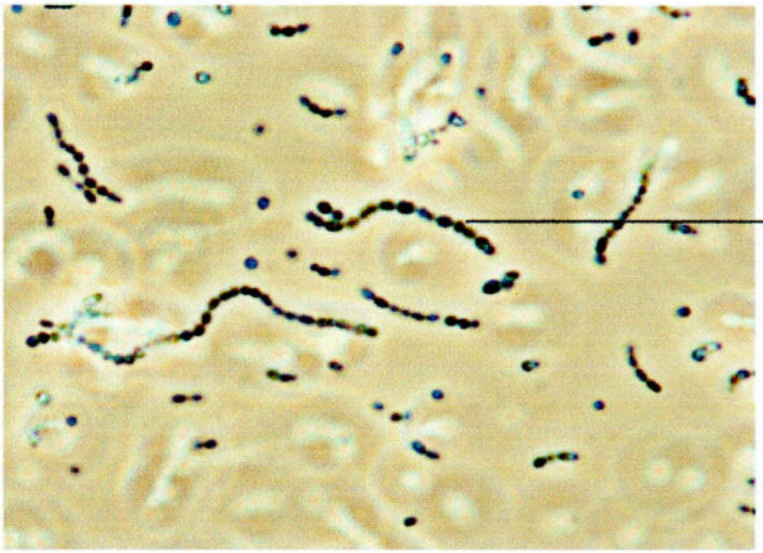

Plate 5: *Streptococcus* sp. showing Gram positive (violet color) Spherical or ovoid and arranged in pairs or chains at 100X (Gram's staining).

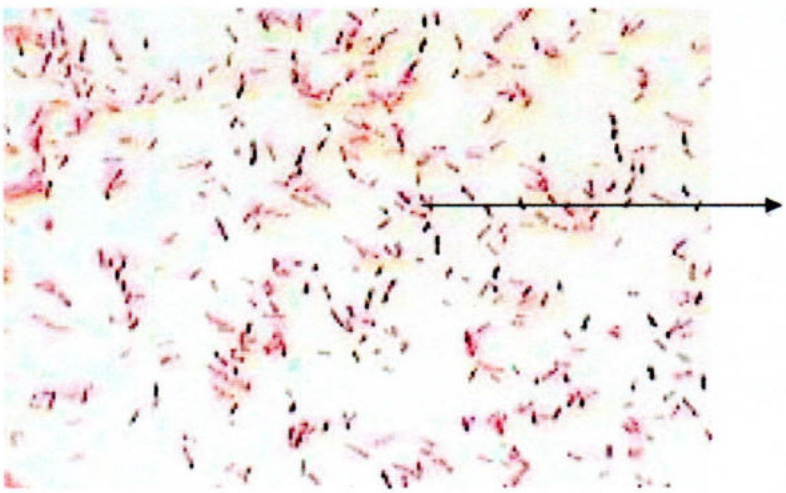

Plate 6: *Escherichia coli* showing Gram negative (pink color) Large rod shape, arranged in single or pair at 100X (Gram's staining).

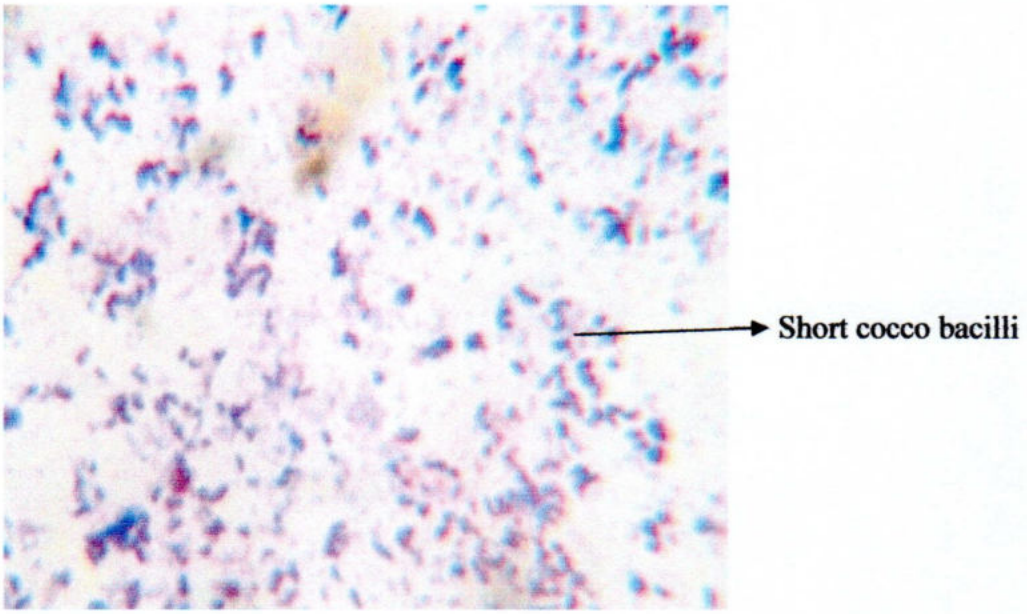

Plate 7: *Corynebacterium* sp. showing Gram positive (violet color) short cocco-bacilli and arranged in bundles or clusters and singly also at 100X (Gram's staining).

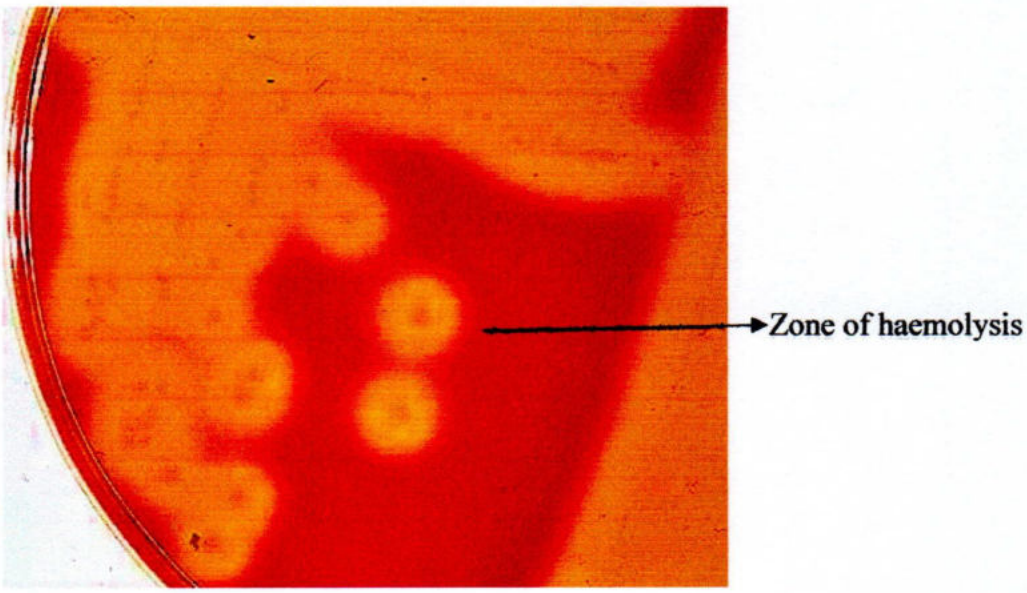

Plate 8: Culture of *Corynebacterium* sp. in Blood agar.

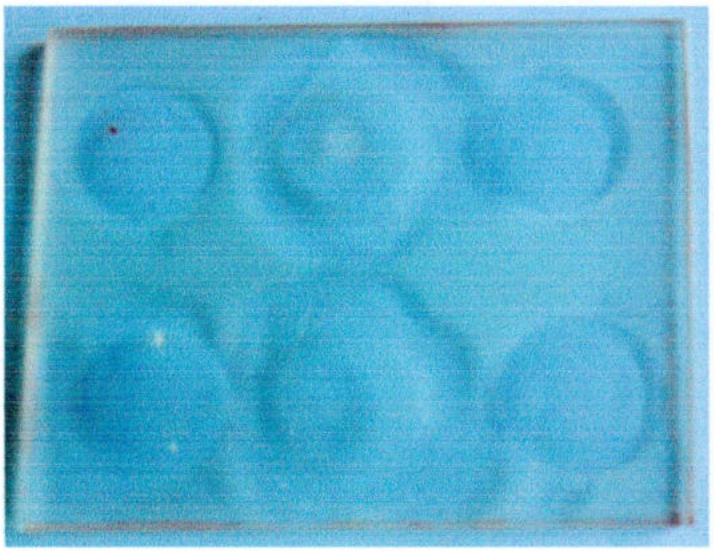

Plate 9: Hanging drop slide.

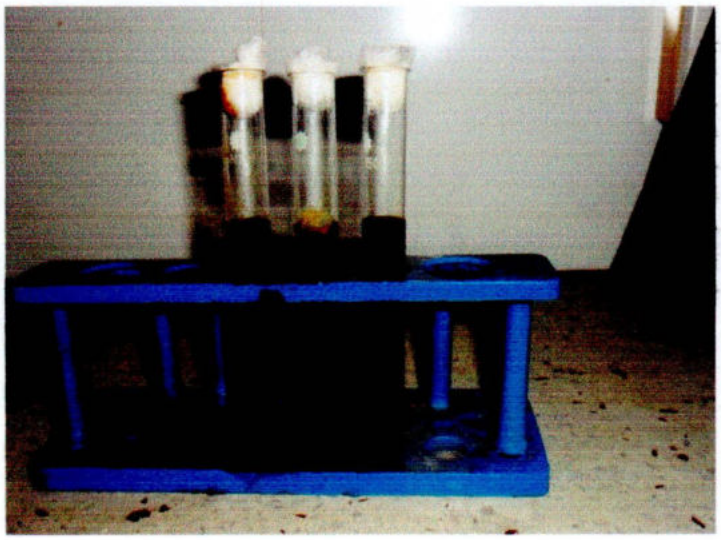

Plate 10: *Corynebacterium* sp. in dextrose fermentation test showing acid production without gas.

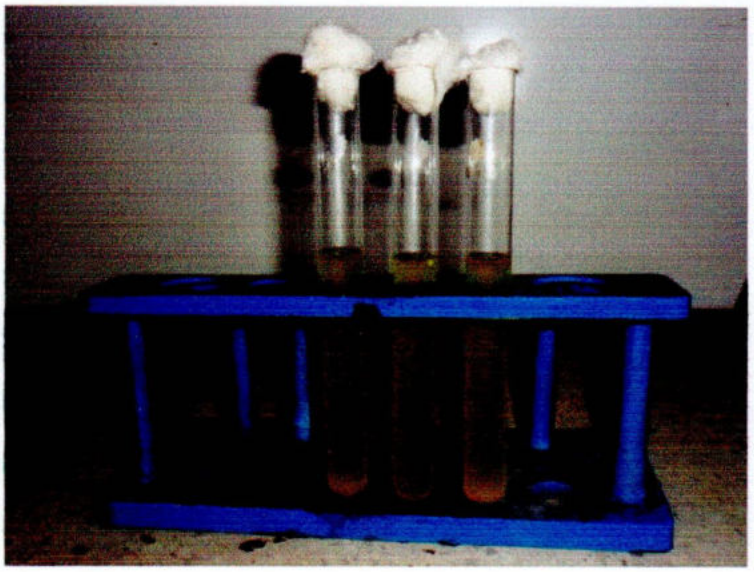

Plate 11: *Corynebacterium* sp. in lactose fermentation test showing acid production without gas.

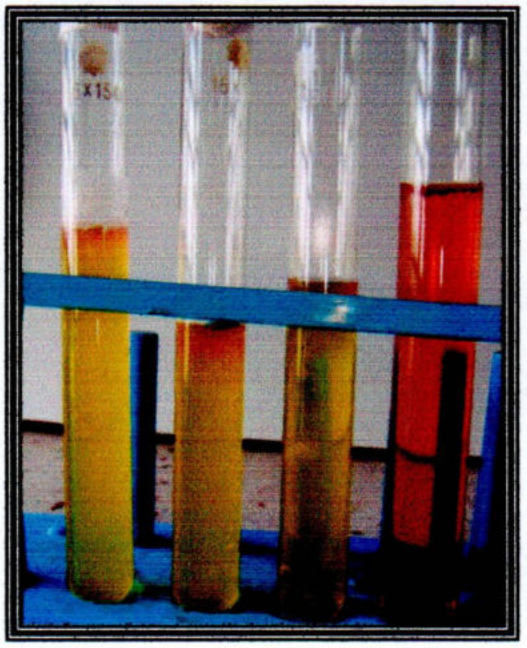

Plate 12: *Corynebacterium* sp. in MIU medium showing negative reaction.

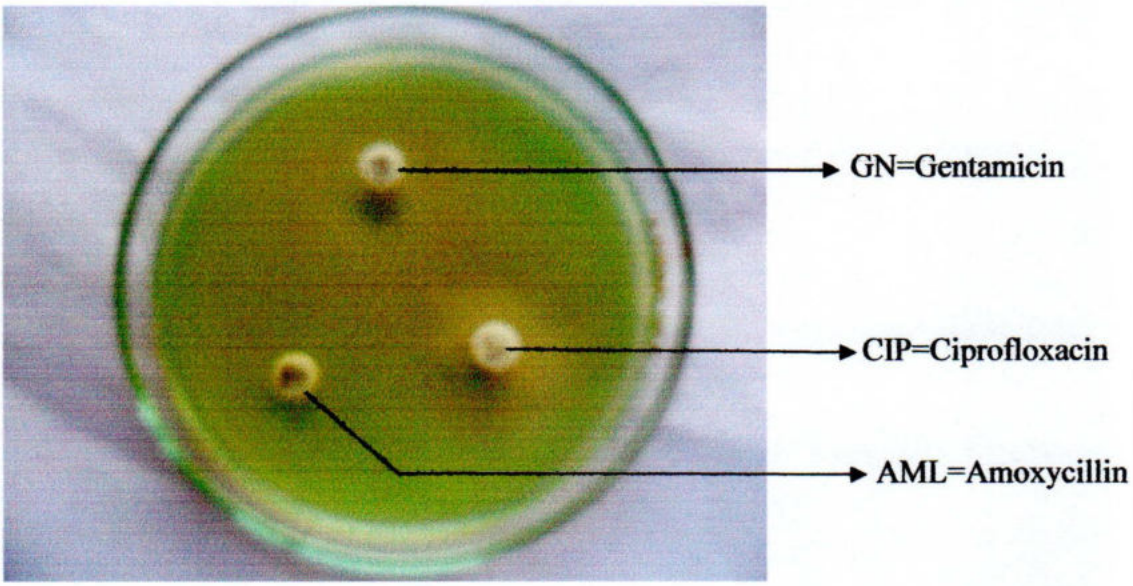

Plate 13: Antibiotic sensitivity pattern of *Corynebacterium* sp. on nutrient agar.

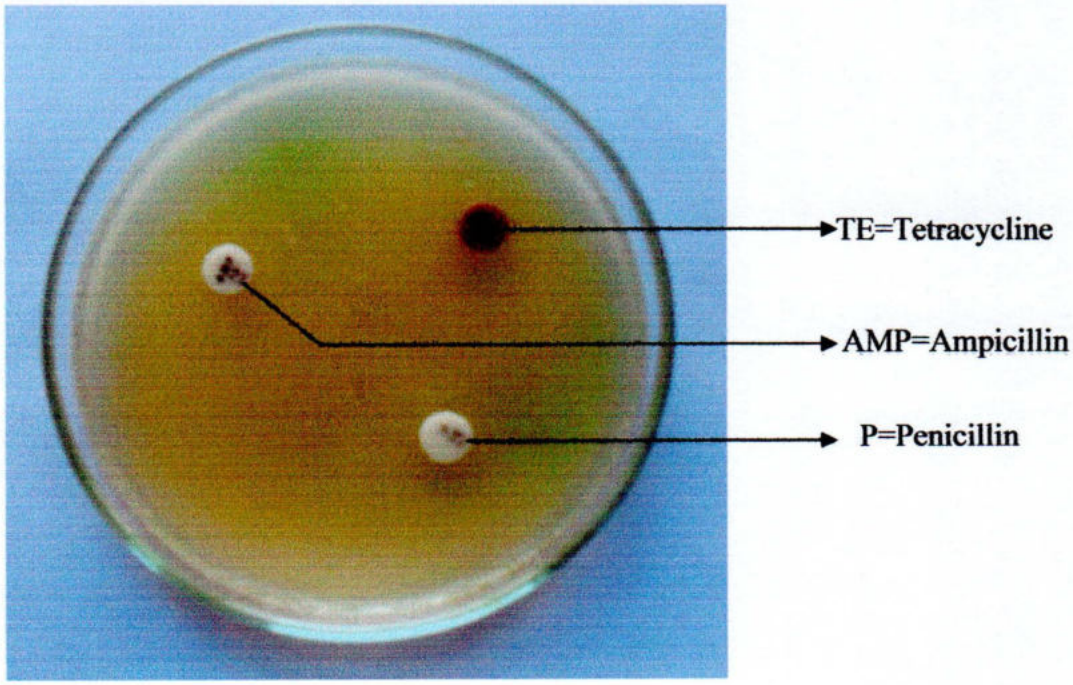

Plate 14: Antibiotic sensitivity pattern of *Corynebacterium* sp. on nutrient agar.

CHAPTER 5

DISCUSSION

CHAPTER 5

DISCUSSION

The present study was reflected on the isolation and identification of *Corynebacterium* sp. from clinically mastitis affected cows at Dinajpur district of Bangladesh. To isolate the *Corynebacterium* sp. from milk samples were subjected to cultural, staining, motility and different biochemical tests.

Four different types of bacteria were isolated from a total of 20 milk samples which were collected from clinically mastitis affected cows. The isolated bacteria were *Staphylococcus* spp. (25%), *Streptococcus* spp. (15%), *Escherichia coli* (20%), *Corynebacterium* spp. (25%) and 15% were unidentified. The incidence of different types of bacteria isolated from milk sample, correlate with the findings of Misra *et al.,* (1993) with slight variation.

In this study, the milk samples were subjected to cultivate in different bacteriological culture media to observe the cultural characteristics of the *Corynebacterium* sp. On nutrient broth, the *Corynebacterium* sp. showed enough whitish turbidity, on nutrient agar the colonies were grayish in color and on blood agar colonies were grayish in color about 1mm in diameter and surrounded by a small zone of hemolysis. In Gram's staining technique the *Corynebacterium* sp. revealed Gram positive, violet color, short cocco-bacilli and arranged in bundles or clusters and singly also. Gabbar (1992) and Shaikh (1999) both found the similar morphological and staining properties of *Corynebacterium pyogenes* as recorded in this study.

In the present study, the isolated *Corynebacterium* sp. revealed non motile in motility test when subjected to MIU and Hanging drop slide technique which has an agreement with Buxton and Fraser (1977). The *Corynebacterium* sp. showed identical result in

different biochemical test such as sugar fermentation (Dextrose, lactose and maltose). The present findings are in agreement with the findings of Shaikh (1999).

In vitro antibiotic sensitivity of *Corynebacterium* sp. to six different types of antibiotics such as gentamicin, amoxycillin, ciprofloxacin, tetracycline, ampicillin and penicillin was studied by Agar disc diffusion method. It was highly sensitive to gentamicin and ciprofloxacin but some degree of resistant to tetracycline, amoxycillin, ampicillin and penicillin was observed in this study. The findings of antibiotic sensitivity are in agreement with the result of Abid *et al.* (2007), Das *et al.* (2005), Neleesh *et al.* (2007) and Mahmoued *et al.* (2008).

According to the above context it can be mentioned that the future study can also help in the following ways:

a) Characterization of the *Corynebacterium* sp. by serological tests.

b) Molecular characterization of the *Corynebacterium* sp. by using molecular diagnostic technique.

CHAPTER 6

SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUSION

The present study was conducted for the isolation and identification of the *Corynebacterium* sp. from clinically mastitis affected cows. A series of tests were performed for the isolation and identification of *Corynebacterium* sp. and also determine the sensitivity patterns to different antibiotics. Twenty (20) samples were examined and isolated organisms were *Staphylococcus* spp. (25%), *Streptococcus* spp. (15%), *Escherichia coli* (20%), *Corynebacterium* sp. (25%) and 15% were unidentified.

On nutrient broth, the *Corynebacterium* sp. showed enough whitish turbidity and on nutrient agar the colonies were grayish in color while on blood agar the colonies showed a zone of hemolysis. In Gram's staining technique the *Corynebacterium* sp. revealed Gram positive, violet color, short cocco-bacilli and arranged in bundles or clusters and singly also. In sugar fermentation tests (dextrose, lactose and maltose), the organism produced acid but no gas. Results of antibiotic sensitivity in this study showed that *Corynebacterium* sp. was highly sensitive to gentamicin and ciprofloxacin, and some degree of resistance to tetracycline, ampicillin, penicillin and amoxycillin.

Clinical mastitis in a dairy herd sends alarm to a farmer and treatment is given immediately to control it. Most mastitis are man made. The incidence of mastitis in dairy herds should be minimized or prevented by providing good environment, good management and good sanitation practices. Period screening tests should be done for udder health and identification of organism causing mastitis should be conducted. Check list for mastitis should be prepared and accordingly proper treatment should be recommended.

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APPENDICES

A. Composition of different media

1. Nutrient Broth

Peptic digest of animal tissue	5.0 gm
Sodium chloride	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Distilled water	1000 ml
Final pH at 25°C	7.4 ±0.2

2. Nutrient agar

Peptic digest of animal tissue	5.0 gm
Sodium chloride	5.0 gm
Beef extract	3.0 gm
Agar	20 gm
Distilled water	1000 ml
Final pH at 25°C	7.4 ± 0.1

3. Blood agar

Peptic digest of animal tissue	10.5 gm
Sodium chloride	5.0 gm
Beef extract	10 gm
Agar	15 gm
Distilled water	1000 ml
Final pH at 25°C	7.4 ± 0.2

B. Composition of reagents

1. Solutions for Gram's staining

a. Stock Crystal violet Solution

Crystal violet	10.0 m
Ethyl alcohol 95%	1000 ml

b. Gram's iodine

Iodine crystal	1.0 gm
Potassium iodide	2.0 gm
Distilled water to make	300 ml

c. Acetone alcohol

Ethyl alcohol	250 ml
Acetone	250 ml

d. Safranine working solution

Safranine dye	2.5 ml
Ethyl alcohol 95%	100 ml
Distilled water	300 ml