A STEP TOWARDS DEVELOPMENT OF VACCINE AGAINST BOVINE MASTITIS

A THESIS

BY

MD. SALAUDDIN
REGISTRATION NO.: 1705429
SEMESTER: JULY–DECEMBER, 2018
SESSION: 2017-2018

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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DECEMBER, 2018

DEDICATED
TO MY
BELOVED PARENTS
&
BROTHER

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ABSTRACT

Mastitis is the ultimate threat to the dairy industry throughout the world. It causes a major economic loss of dairy farmers. There are so many pathogenic organisms like *Streptococcus agalactiae, Staphylococcus aureus, E. coli, Klebsiella pneaumoniae* other *Streptococcus* sp. responsible for it. Indigenous as well as crossbreeds are frequently affected by mastitis. The principle objective is to detect major pathogenic organisms from mastitis milk and development of vaccine candidate. All the mastitis milk samples are tested by CMT for the confirmation of mastitis. Organisms are isolated and identified by the cultural and biochemical test. After that molecular identification is done by PCR and RT-PCR. Gene amplification is done by 16S rRNA and 23S rRNA with reference primer that helps in their specification. Besides, phylogenetic analysis precisely specified the study isolates. Antimicrobial susceptibility test of the positive isolates performed by the disc diffusion method against fifteen (15) antimicrobial agents. Antimicrobial patterns of isolates were also subjected to study by using house fly (*Musca domestica*). Among the isolates, *E. coli* and *Staphylococcus aureus*; methicillin resistance (MRSA) appeared in all cases. *Klebsiella pneumonia, Streptococcus agalactiae* and other *Streptococcus* sp. appeared about 50% or less from the isolates. Antibiogram study reveals that most of the isolates were MDR (Multi-Drug Resistance). Housefly (*Musca domestica*) were showed complete resistance in the case of *Staphylococcus aureus* and *Klebsiella* sp. but showed good response in case of *E. coli* and *Streptococcus agalactiae.* Mastitis causing most important organisms that are characterized were used in the production of Polyvalent Formalin Inactivated Vaccine (PFIV) seed where colony forming unit (CFU) maintained. Antibiogram study helps to choose specific drug and formulated vaccine seed helps to immunize the dairy cows as well as protect the farmers from massive economic loses.

Key words: Bovine mastitis, Pathogenic bacteria, PCR, RT-PCR, Phylogenic study and PFIV seed

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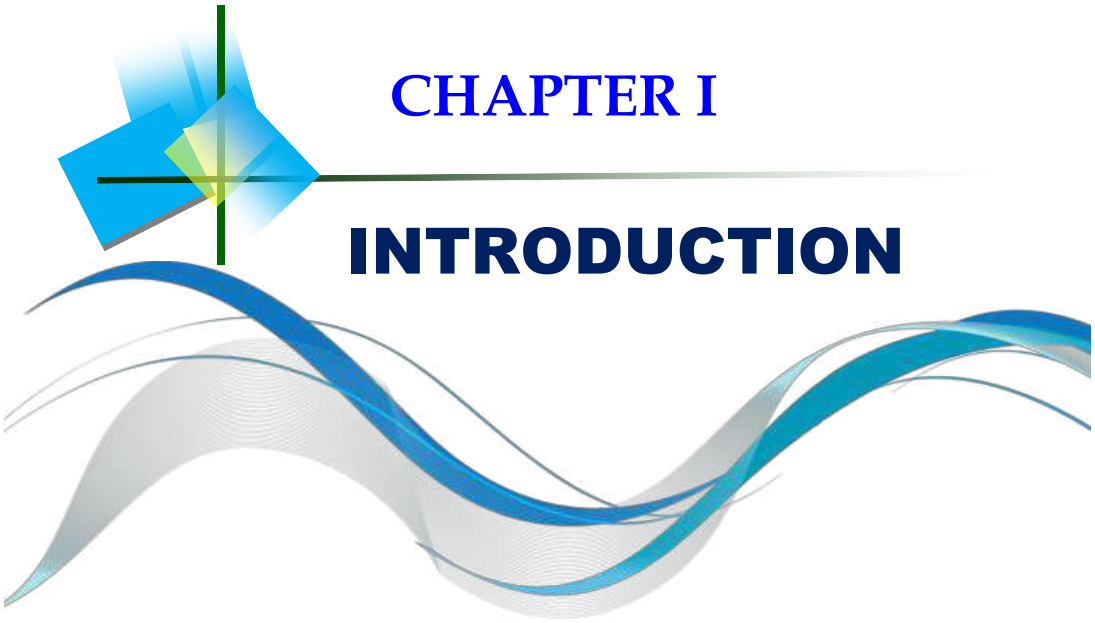
LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
#	:	Identifying number
%	:	Percentage
@	:	At the rate of
+	:	Positive
µg	:	Microgram
µl	:	Microliter
^{0}C	:	Degree of Celsius
Ag	:	Antigen
Assist	:	Assistant
BA	:	Blood Agar
BD	:	Bangladesh
BGA	:	Brilliant Green Agar
EMB	:	Eosin Methylene Blue
ER	:	Erythromycin
et al.	:	Associated
etc	:	Etcetera
FAO	:	Food and Agricultural Organization
Gm	:	Gram
H.S	:	Haemorrhagic Septicemia
H_2O_2	:	Hydrogen Peroxide
H_2S	:	Hydrogen Sulphide
HSTU	:	Hajee Mohammad Danesh Science and Technology University
i.e.	:	That is
Ltd	:	Limited
M.S	:	Master of Science
MC	:	MacConkey Agar
MI	:	Milliliter
MIU	:	Motility Indole Urease
MR	:	Methyl Red
NA	:	Nutrient Agar
NB	:	Nutrient Broth

No.	:	Number
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase Chain Reaction
PM	:	Post Mortem
Prof.	:	Professor
PSS	:	Physiological Saline Solution
RPM	:	Rotation Per Minute
SC	:	Subcutaneous
SE	:	Standard Error
SL	:	Serial number
Sp	:	Species
SSA	:	Salmonella Shigella Agar
v/v	:	Volume by volume
VP	:	Voges-Proskauer
w/v	:	Weight by volume



CHAPTER I

INTRODUCTION

CHAPTER-I

INTRODUCTION

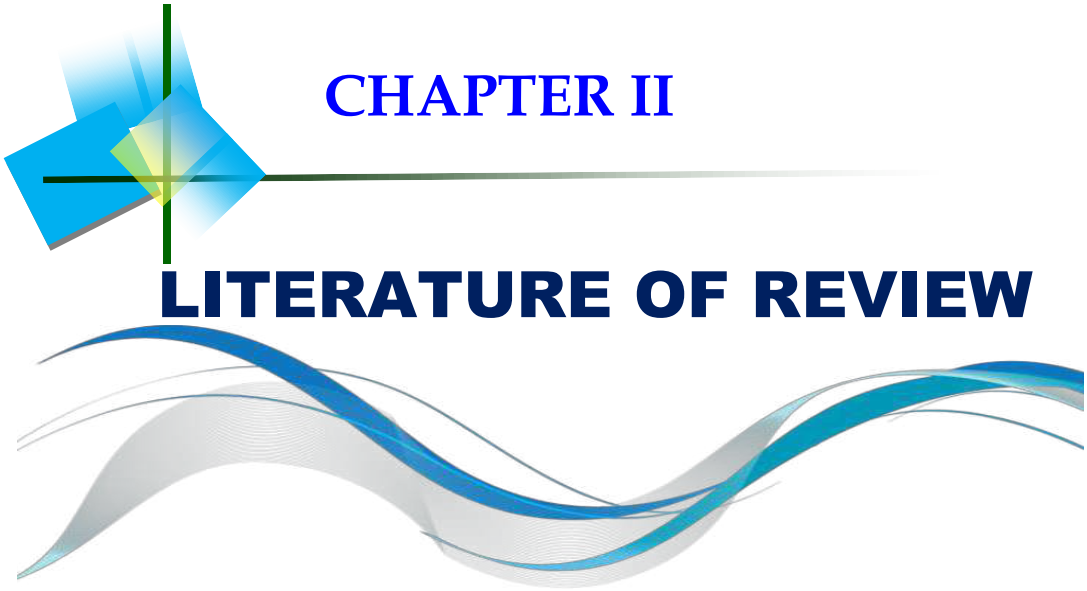
Dairy industry is one of the important sub-sectors of livestock. It provides milk, which is the important source of nutrition for human. But one of the major problems of dairy industry is mastitis. It is caused by variety of pathogenic organisms (Zaragoza *et al.*, 2011); which causes inflammation in mammary gland (Pal *et al.,* 2017). Resulting vital economic losses, damages growing dairy industry, milk production fallen, increase veterinary costs, culling of chronically infected cows and occasional deaths (Zaragoza *et al.*, 2011; Lopez *et al.,* 2006; Abebe *et al.,* 2016). Not only indigenous breed but also cross breed cows are highly susceptible to mastitis (Pal *et al.,* 2017). About 140 pathogenic causal agent isolated some of them shedding toxin in milk and some others having zoonotic importance, causes serious human health hazard (Pal *et al.,* 2017; Abebe *et al.,* 2016). It's a matter of great regret that to protect mastitis extensive use of animal as well as human drug causes and produce antibiotic resistance microorganism that causes public health issue (Anderson *et al.,* 2014). Mainly *E. coli, Streptococcus* spp. (*S. agalactiae*, *S. dysgalactiae*, *S. uberis*), *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Corynebacterium* spp. are responsible for mastitis (Chaneton *et al.,* 2011; Lange *et al.,* 2015). Among them *E. coli* is most notorious throughout the world (Blum *et al.,* 2008). Intramammary infections (IMI), mucosal membrane damaging most frequently caused by coagulase negative *Staphylococcus aureus.* Among 45 species and 21 subspecies some are considered as commensals, under some conditions (Hosseinzadeh *et al.,* 2014; Krol *et al.,* 2016). Besides *S. agalactiae*, *S. dysgalactiae*, *S. uberis* frequently isolated from mastitis animal and are exclusively human pathogen but now-a-days they are very common in human due to drug abuse (Lange *et al.,* 2015; Rato *et al.,* 2013).

Streptococcus agalactiae or other *Streptococcus* sp. commonly known as fundamental mastitis causing organism and one of the major threat Pang *et al.,* (2017). Their specific detection method is not available at field level. Molecular methods were used by using specific primers with the help of PCR, that's why these are easily identified (Riffon *et al.,* 2001). Forward and Reverse primer sequencing and phylogenetic analysis also done based on 16S rRNA and 23S rRNA to detect all the available bacteria in the current study more precisely and specifically.

The prevention and treatment of mastitis present a serious hurdle to producers and it is always the primary concern of the dairy industry. In Bangladesh an imported vaccine-named Mastivac® used to control mastitis which is manufactured by Ovejero laboratories, Spain, and Eskayef Pharmaceuticals Ltd. The vaccine contains *Streptococcus agalactiae, S. dysgalactiae, S. uberis, S. pyogenes, Staphylococcus aureus, Escherichia coli* (strains Bov-13, Bov-14, Bov-15, Suis-21 and J5) and *Arcanobacterium pyogenes.* But the vaccine is not effective to control mastitis in Bangladesh. This may be due to some additional causal agents who are not incorporated in the imported vaccine, or due to differences of the antigenic properties of the causal agents. Therefore, to save the dairy industry in Bangladesh, it is important to isolate and identify the causal agent of bovine mastitis, and preparation of effective vaccine using the local isolates. Hence, the current research is also attempted to determine the following objectives.

Objectives

1. To isolate and identify the bacterial agents causing mastitis in cattle.
2. To characterize the identified isolates by using PCR and phylogenetic study.
3. To develop experimental vaccine against bovine mastitis.



CHAPTER II

LITERATURE OF REVIEW

CHAPTER-II

REVIEW OF LITERATURE

2.1 Isolation and identification of organisms

Pal *et al.* (2017) worked with a total of 780 quarters of teats from 195 apparently healthy cows were screened by using Californian Mastitis Test (CMT). Commonly recovered organisms were *Staphylococcus aureus*, *Streptococcus agalactae*, *Streptococcus uberis*, *Streptococcus intermidius*, *Proteus* spp., *Escherichia coli*, *Corynebacterium bovis*, *Corynebacterium pyogenes*, *Micrococcus* spp. and *Bacillus* spp. Among the isolated pathogens, *S. aureus*, *Corynebacterium bovis*, *Corynebacterium pyogenes* and *E. coli* were the most prevalent that accounted 15.9%, 11.9%, 12.7% and 11.1%, respectively.

Zaragoza *et al.* (2011) stated that there are 1,095 milk from clinical and subclinical mastitis samples. Mastitis confirmed by Californian Mastitis Test (CMT). Bacteria and Yeasts identification was carried out by morphology and biochemical methods. Twenty different species of Candida were identified out of 282 (25.75%) milk samples. The most frequently identified species are candida. The possible role that *Candida* species other than *C. albicans* may play in mycotic mastitis in cows.

Jose *et al.* (2006) determined mastitis causing organisms by the uptake and survival in a primary culture of bovine mammary epithelial cells (BMEC) and isolated from a healthy lactating cow and characterized by their morphology, immunostaining for cytokeratin and the detection of *b*- and *j*-casein mRNAs. Ten bacterial isolates comprising the staphylococcal species *Staphylococcus aureus* (3), *Staphylococcus epidermidis* (1), *Staphylococcus haemolyticus* (1), *Staphylococcus equorum* (2), *Staphylococcus xylosus* (1) and *Brevibacterium stationis* (2) obtained from raw milk of cows with mastitis from backyard farms were assayed for their ability to invade BMEC. Only two *S. aureus* and one *S. epidermidis* isolates were able to invade BMEC, at similar levels to the *S. aureus* control strain ATCC 27543. In conclusion, using the in vitro model of infection used in this study, differences in bacterial invasion capability may be detected.

Blum *et al.* (2008) worked with clinical bovine mastitis cases where 11 *E. coli* from the cowshed environment and mastitic cows. The mastitic and environmental strains could not be distinguished according to O antigen and antibiotic sensitivity. All mastitic

isolates showed significantly ($P < 0.0001$) faster growth in milk and faster lactose fermentation than most (64%) environmental strains, but growth rates in nutrient broth did not differ. Results suggested that clinical bovine mastitis *E. coli* isolates may form a subset of the general.

Chaneton *et al.* (2011) isolated pathogenic microorganisms such as *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli* from the mammary gland. Observed innate and adaptive immune mechanisms based on lactoferrin (86-kDa protein) with antibacterial activity that plays a role in the mammary gland's defense against infection. β-Lactoglobulin (β-LG) is an 18-kDa protein that is present in most mammals but is notably absent in humans, rodents, and lagomorphs. Analysis of the genetic variants of β-LG showed that β-LG A has higher inhibitory activity against *Staph. aureus* and *Strep. uberis* than β-LG B. Coincubation of β-LG and lactoferrin resulted in an augmented antibacterial activity against *Staph. aureus*, suggesting an additive effect of the proteins. This result, along with the proteins' complementary spectrum of action, suggests that β-LG and lactoferrin may complement each other in the mammary gland's defenses against bacterial infection.

Hosseinzadeh *et al.* (2014) revealed that staphylococcal mastitis is a major and costly problem of dairy cattle all over the world. About 71.5% staphylococcal species identified based on of cultural and biochemical features as well as by genus specific PCR. Then, species level identification of staphylococcal isolates was carried out using restriction fragment length polymorphism (RFLP) analysis of the *gap* gene (933 bp). On the basis of polymerase chain reaction-RFLP, 10 different patterns were identified. This study demonstrated that CoNS, especially *S. haemolyticus* and *S. chromogenes*, were predominant and thus be considered as emerging pathogens causing mastitis in the North West of Iran. Our results also revealed that the *gap* PCR-RFLP was useful for identifying staphylococcal isolates derived from bovine mastitis at species level.

Krol *et al.* (2016) worked with multiple udder infections in a dairy herd caused by *Staphylococcus microti*. Over a 22-month period, eleven *S. microti* isolates from milk samples from 9 cows were collected. The animals experienced subclinical (with one exception) intramammary infections with a high self-cure rate. The identification of the microorganism was carried out by means of two independent approaches: nucleotide sequence analysis of the 16S rRNA gene, as well as some housekeeping genes (*sodA*,

rpoB, *dnaJ*), and matrix-assisted laser desorption ionization—time of flight mass spectrometry. Reported to the first ever case of bovine mastitis caused by *S. microti* and the first instance of isolation of this microorganism from domesticated animals.

Carla *et al.* (2015) studied with isolation and identification of *Staphylococci* from bovine milk and not classified as *Staphylococcus aureus* whose are coagulase-positive and coagulase-negative. About 95% isolates were successfully identified at the species level and about 99% identity with 16S rRNA sequences deposited in Gene Bank. 16S rRNA sequencing was an objective and accurate method for the proper identification of *Staphylococcus* species isolated from bovine mastitis. Additional target genes could be used in non-conclusive cases for the species-level identification of these microorganisms.

Rato *et al.* (2013) stated that *Streptococcus agalactiae* (Group B *Streptococcus*, GBS), *Streptococcus dysgalactiae* subsp. *dysgalactiae* (Group C *Streptococcus*, GCS) and *Streptococcus uberis* are relevant mastitis pathogens, a highly prevalent and costly disease in dairy industry due to antibiotic therapy and loss in milk production. The aims of this study were the evaluation of antimicrobial drug resistance patterns, particularly important for streptococcal mastitis control and the identification of strain molecular features. Antimicrobial resistance was assessed by disk diffusion against amoxicillin–clavulanic acid, cefazolin, cefoperazone, pirlimycin-PRL, rifaximin, streptomycin, chloramphenicol, erythromycin-ERY, gentamicin, tetracycline TET and vancomycin.

Hang *et al.* (2016) reported *Staphylococcus aureus* is the leading pathogen involved in bovine mastitis, but knowledge about antimicrobial resistance, virulence factors, and genotypes of *Staphylococcus aureus* resulting in bovine mastitis in Ningxia, China, is limited. Therefore, antimicrobial susceptibility, virulence gene, and randomly amplified polymorphic DNA (RAPD) analyses of *Staph. aureus* were carried out. A total of 327 milk samples from cows with clinical and subclinical mastitis in 4 regions of Ningxia were used for the isolation and identification of pathogens according to phenotypic and molecular characteristics. Antimicrobial susceptibility against 22 antimicrobial agents was determined by disk diffusion. There was great variation in genotypes of *Staph. aureus* isolates, not only among different farms, but also within the same herd in Ningxia province. The study showed a high incidence of *Staph. aureus* with genomic variation of resistance genes, which is matter of great concern in public and animal health in Ningxia province of China.

Hussein *et al.* (2008) studied in Sulaimani district with bovine clinical mastitis were investigated for their bacteriological causative agents; 76 milk samples were cultured on primary and selective media and the isolated bacteria were tested for their susceptibility to antimicrobial agents used in commercial intramammary infusion products. *Escherichia coli* was the most common bacteria followed by *Staphylococcus aureus, Streptococcus agalactia* and coagulase–negative staphylococci isolated based on cultural and biochemical properties. Two other bacterial species (*Pseudomonas aeruginosa* and *Streptococcucs uberis*) were also isolated but in a lower proportion. Antibacterial susceptibility testing showed that the use of florfenicol, cephalexin and gentamicin may be useful for the treatment of clinical mastitis cases in cows.

Vazquez *et al.* (2013) aimed to detect the prevalence of subclinical mastitis and to investigate the major udder pathogens in Jalisco State, western Mexico. Milk samples were subjected to California Mastitis Test (CMT) to differentiate clinical cases. Bacteriological identification of the causative agents revealed the presence of a major group of pathogens including the Coagulase negative staphylococci (CNS), *S. aureus, S. agalactiae*, *Corynebacterium* spp. and Coliform bacteria. Other pathogens could be detected in the investigated milk samples such as *S. dysgalactiae* (0.4%), *S. uberis* (0.37%), *Bacillus* spp. (1%), *Nocardia* spp. (0.6%) and *Candida* spp. (0.1%). Meanwhile, others were present in a negligible ratio.

Anjali *et al.* (2017) focused on development of a rapid and efficient method for detecting major pathogens of bovine clinical mastitis- *E. coli, S. aureus, P. aeruginosa, K. pneumoniae, S. agalactiae, S. dysgalactiae* and *S. uberis* simultaneously without the need of cultivation of bacteria. mastitic milk samples were processed for isolation and biochemical identification of bacteria which were further confirmed genotypically by PCR. However, *S. aureus* and *S. agalactiae* were not obtained by multiplexing that might be due to incompatibility of primers or differences in annealing temperatures of primers. Conclusively, molecular methods specifically multiplex PCR can be used as an efficient tool for detecting major pathogens of mastitis in one run with high accuracy and in short period of time.

Abebe *et al.* (2016) estimated the prevalence of mastitis, identify the cow-and herd-level potential risk factors and isolate *Staphylococcus aureus*, one of etiological agents for contagious mastitis, from cows positive for mastitis. California mastitis test (CMT) for

sub-clinical mastitis and positive cows were cultured for isolation of *S. aureus*. Based on CMT result and clinical examination, the prevalence of mastitis at herd-level was 74.7% (95% CI: 64.5, 82.8). The corresponding cow-level prevalence was 62.6% (95% CI: 58.3, 66.7), of which 59.2 and 3.4% were sub-clinical and clinical mastitis cases, respectively. *S. aureus* was isolated from 51.2% of the milk samples cultured and 73.2% of the herds affected with mastitis. In the multivariable logistic regression model, the herd-level factors significantly associated ($p < 0.05$) with the presence of mastitis were herd size, bedding material, and milking mastitic cows last, while at cow-level, breed, parity, stage of lactation, udder and leg hygiene, and teat end shape were noted to have a significant effect on mastitis occurrence.

2.2 Molecular characterization of isolates

Zadoks *et al.* (2014) observed that mastitis is commonly caused by intramammary infection with bacteria, which can be detected by bacterial culture or PCR. PathoProof is a commercially available real-time PCR system for the detection of bovine mastitis pathogens. The aim of this study was to evaluate whether the PathoProof system was suitable for detection of mastitis pathogens in sheep milk. species identified by culture, the diagnosis was confirmed by species-specific conventional PCR or by sequencing of a housekeeping gene. The majority of samples were negative by culture (74.4% of 219 samples) and real-time PCR (82.3% of 192 samples). Agreement was observed for 138 of 192 samples. Thirty-four samples were positive by culture only, mostly due to presence of species that are not covered by primers in the PCR system (e.g., *Mannheimia* spp.). Two samples were positive for *Streptococcus uberis* by culture but not by PCR directly from the milk samples. This was not due to inability of the PCR primers to amplify ovine *Streptococcus uberis*, as diluted DNA extracts from the same samples and DNA extracts from the bacterial isolates were positive by real-time PCR. Samples that were negative by either method had lower bacterial load than samples that were positive for both methods, whereas no clear relation with species identity was observed. This study provides proof of principle that real-time PCR can be used for detection of mastitis pathogens in ovine milk.

Tark *et al.* (2017) aimed to assess trends in antimicrobial resistance and to investigate the characteristics of extended-spectrum β-lactamase (ESBL)-producing isolates from bovine mastitic milk. *Escherichia coli* isolates were analyzed. Multidrug resistance was

observed in 15.5% of isolates. Among these isolates, 15 (4.0%) carried one or more *bla*CTX-M and AmpC ESBL genes from 11 different farms. It is suggested that ESBL might spread by both clonal and horizontal spread in dairy farms in South Korea. Although no significant changes occurred in the antimicrobial resistance of *E. coli* during the 4-yr study period, the resistance rates and presence of ESBL were high compared with those in other countries. Thus, these findings suggest the importance of control measures for *E. coli*, particularly ESBL-producing bacteria, on dairy farms to reduce treatment failure and transmission to human.

Schmidt *et al.* (2017) stated that *Staphylococcus aureus* is one of the most common etiological agents of contagious bovine mastitis worldwide. The purpose of this study was to genetically characterize a collection of *S. aureus* isolates recovered from cases of bovine mastitis and nasal swabs of close human contacts in the dairy environment. Isolates were screened for a combination of clinically significant antimicrobial and virulence gene markers whilst the molecular epidemiology of these isolates and possible inter-species host transmission was investigated using a combination of genotyping techniques. Bovine and human isolates from three sampling sites clustered together and were genotypically indistinguishable. Two of the isolates, ST97 and ST352 belong to the common bovine lineage CC97, and their isolation from close human contacts suggests zoonotic transfer. The detection of indistinguishable *S. aureus* isolates from bovine and human hosts at three of the sampling sites is suggestive of bacterial transmission and supports the need for vigilant monitoring of staphylococcal populations at the human-animal interface.

Shome *et al.* (2012) investigated the occurrence and virulence characteristics of streptococci from bovine milk and to assess the molecular epidemiology and population structure of the Indian isolates using multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). *Streptococcus* spp. were isolated from 29 milk samples. species-specific PCR and partial 16S rRNA gene sequence analysis identified 17 *Streptococcus agalactiae* arising from herd A and 13 *Streptococcus uberis* Unique sequence types identified for both *S. agalactiae* and *S. uberis* were found to be herd specific. Since all the isolates of both the species belonged to novel sequence types, their epidemiological significance in global context could not be ascertained, however, evidence suggests that they have uniquely evolved in Indian conditions.

Saei *et al.* (2010) performed polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of the *aroA* gene for the characterization of *Staphylococcus aureus* from clinical and subclinical mastitis milk samples. Amplification of the *aroA* gene resulted in a single amplicon with a size of approximately 1,153 bp from all isolates of *S. aureus*. To obtain the RFLP patterns of the isolates, the PCR products were digested with *Taq*I restriction enzyme and the fragments separated by gel electrophoresis. The results demonstrate that strain variations of *S. aureus* could occur within and between herds and also between different regions, although a few genotypes of *S. aureus* were predominant in bovine mastitis. This study also indicated that PCR amplification of the *aroA* gene is specific for *S. aureus* identification.

Kuang *et al.* (2009) observed that the bovine mastitis-associated bacteria were host-specific. *Klebsiella pseudomoniae*, *Lactococcus lactis*, *Staphylococcus aureus* and members of the *Escherichia* genus were found to be widely distributed and diagnosed by using the classical microbiological method. Furthermore, more than one pathogen known to cause mastitis was found to be present in some milk samples. These pathogens are not only potential etiological agents but may also play a role in disrupting the natural microbial ecology in mastitic cows. This finding highlights the limitation of the traditional identification and characterization strategy, and the PCR–DGGE are shown to be a powerful tool for describing the bacterial flora and especially etiological agents in mastitic milk.

Ismail. (2017) studied the molecular characteristics, antibiogram and prevalence of multidrug resistant *Staphylococcus aureus* (*S. aureus*) (MDRSA) isolated from acute clinical mastitis milk samples. The bacteria were identified using colony morphology, Gram staining and biochemical characteristics. *S. aureus* isolates were then subjected to molecular characterization using PCR targeting 16S rRNA and *mec*A gene to identify Methicillin Resistant *S. aureus* (MRSA). The antibiogram of all isolates was performed using the Kirby–Bauer disk diffusion method against 10 commonly used antibiotics in dairy farms. All MRSA isolates were completely resistant to all tested antibiotics and were positive for the presence of the *mec*A gene. MRSA may pose a potential health risk to the public, farm workers and veterinarians.

Gurjar *et al.* (2012) highlighted the application in several real farm case studies of routinely used molecular techniques for primary diagnosis and epidemiologic investigation of IMI caused by major mastitis pathogens, such as *Staphylococcus aureus*, *Mycoplasma bovis*, *Streptococcus uberis*, and *Enterobacter* spp.

Eisenberger *et al.* (2017) determined the rate of extended-spectrum *b*-lactamase (ESBL)-producing microorganisms among *Escherichia coli* isolates causing bovine mastitis, including molecular characterization of these isolates. *E. coli* isolates from milk samples of dairy cows with mastitis were investigated for ESBL production by antimicrobial susceptibility testing, PCR-based detection, and sequencing of ESBL encoding genes, which were identified 4.5% of total. Moreover, resistance to the fluoroquinolones enrofloxacin and marbofloxacin occurred in 15 of 22 ESBL-producing isolates 68.2%. The rate of ESBL-producing *E. coli* associated with cattle mastitis was 4.5%. Furthermore, a high proportion of fluoroquinolone coresistance could be detected. Therefore, careful and continuous surveillance of ESBL-producing *E. coli* in cattle and consequent implementation of prevention measures are needed to avoid a further spread of these multidrug-resistant bacteria.

Sun *et al.* (2017) Bovine mastitis caused by *Streptococcus agalactiae* continues to be one of the major veterinary and economic issues in certain areas of the world. The more prevalent *S. agalactiae* strains that cause bovine mastitis in China dairy farms belong to a number of bovine-adapted sequence types (STs) ST67, ST103 and ST568. However, it is unknown why these STs can emerge as highly prevalent clones in bovine dairy farms. Here, to determine if a variety of virulence characteristics were associated with these highly prevalent STs, the molecular and virulence characterization of 116 strains isolated from bovine, human, fish and environment were analyzed. Obtained data showed that all bovine-adapted strains could be assigned to capsular genotype Ia or II, and carried pilus island 2b, and lactose operon. Importantly, we demonstrated that the growth ability in milk, biofilm formation ability and adhesion ability to bovine mammary epithelial cells (BMECs) were significantly higher for all bovine-adapted strains compared to strains from other origins.

Riffon *et al.* (2001) stated that a rapid and specific test for the detection of the main pathogens of bovine mastitis is not actually available. Molecular probes reacting in PCR with bacterial DNA from bovine milk, providing direct and rapid detection of

Escherichia coli, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, and *Streptococcus uberis*, have been developed. Two sets of specific primers were designed for each of these microorganisms and appeared to discriminate close phylogenic bacterial species (e.g., *S. agalactiae* and *S. dysgalactiae*). In addition, two sets of universal primers were designed to react as positive controls with all major pathogens of bovine mastitis. The sensitivities of the test using *S. aureus* DNA extracted from milk with and without a pre-PCR enzymatic lysis step of bacterial cells were compared. The detection limit of the assay was 3.1253×10^2 CFU/ml of milk when *S. aureus* DNA was extracted with the pre-PCR enzymatic step compared to 5.3×10^3 CFU/ml of milk in the absence of the pre-PCR enzymatic step. This latter threshold of sensitivity is still compatible with its use as an efficient tool of diagnosis in bovine mastitis, allowing the elimination of expensive reagents.

2.3 Detection of *Streptococcus agalactiae* by RT-PCR

Jahan *et al.* (2015) was intended for identification and characterization of *Staphylococcus aureus* isolated from raw cow milk. A total of 487 milk samples were collected from Sheshmore, Shutiakhali and Bangladesh Agricultural University Dairy Farm, Mymensingh. Using bacteriological, biochemical and PCR-based identification schemes, 12 (25.53%) isolates were confirmed as *S. aureus*. All the isolates showed β-hemolysis on 5% sheep blood agar. *S. aureus* specific *nuc* gene (target size 279-bp) was amplified in the cases of all isolates along with antibiogram study performed.

Taponen *et al.* (2009) revealed that clinical and subclinical bovine mastitis causing bacteria fail to grow even after 48 h of conventional culture. The "no-growth" samples are problematic for mastitis laboratories, veterinarians, and dairy producers. This study provides the first investigation of the bacteriological etiology of such samples, using a real-time PCR-based commercial reagent kit. Of the 79 samples, 34 (43%) were positive for 1 (32 samples) or 2 (2 samples) of the target bacteria. The positive findings included 11 *Staphylococcus* sp. (Staphylococci other than *Staphylococcus aureus*), 10 *Streptococcus uberis*, 2 *Streptococcus dysgalactiae*, 6 *Corynebacterium bovis*, 3 *Staph. aureus,* 1 *Escherichia coli*, 1 *Enterococcus,* and 1 *Arcanobacterium pyogenes*. The diagnosis of clinical mastitis and indicated that real-time, PCR-based bacterial findings are able to reveal bacteriological etiology.

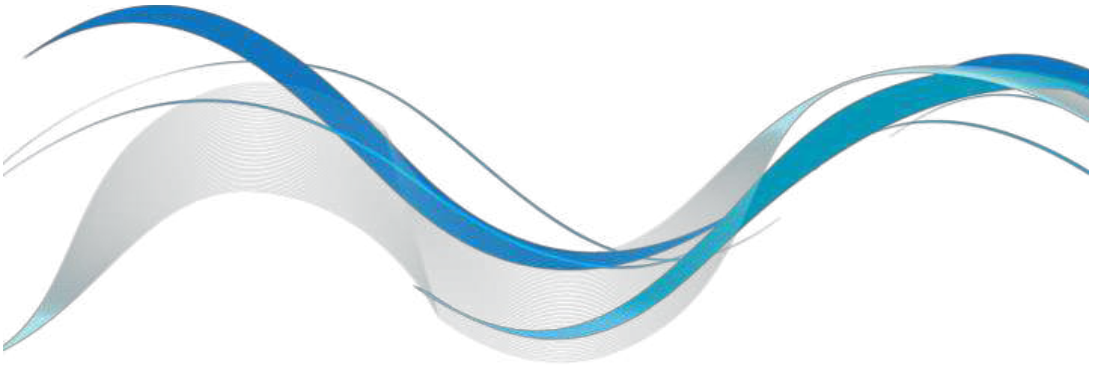
2.4 Development of Vaccine Seed

Sayed *et al.* (2015) prepared and evaluated the effectiveness of a locally prepared polyvalent vaccine against mastitis from the most common causes of mastitis. *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* were the most prevalent bacteria recovered from clinical and subclinical mastitis. Montanide ISA-206 adjuvant inactivated polyvalent vaccine containing the three strains was prepared. Twenty pregnant cows were inoculated intramuscularly with the prepared polyvalent vaccine two months prior to calving and boostering at day 21 from the primary injection. Serum samples from vaccinated and non-vaccinated cows were collected at the 1st, 3rd, 8th, 12th, 16th, 20th and 24th weeks post vaccination and evaluated immunologically using ELISA. The results showed that immune response was significantly higher in the vaccinated group than that of controls. These results could be indicated the safety and effectiveness of the vaccine in reduction of incidence and severity of clinical cases of mastitis but further studies should be done to elucidate the possibility of field application and effectively.

Giraudo *et al.* (1997) developed against bovine mastitis based on inactivated, highly encapsulated *Staphylococcus aureus* cells; a crude extract of *Staph. aureus* exopolysaccharides; and inactivated, unencapsulated *Staph. aureus* and *Streptococcus* sp. cells. This vaccine was tested on 30 heifers during a 7-mo period. The 30 heifers were randomly assigned to three groups of 10 heifers each. The prepartum group received two injections of the vaccine at 8 and 4 wk before calving, and the postpartum group received two injections at 1 and 5 wk after calving. The control group received two injections of a placebo at 8 and 4 wk before calving. The vaccine or the placebo was administered subcutaneously in the brachiocephalicus muscle of the neck. The frequencies of intramammary infections caused by *Staph. aureus* were reduced from 18.8% for heifers in the control group to 6.7 and 6.0% for heifers in the prepartum and postpartum groups, respectively. This protective effect was maintained for at least 6 mo. The relative risk of mastitis caused by *Staph. aureus* was 0.31 and 0.28 for heifers in the prepartum and postpartum groups, respectively, compared with that for heifers in the control group. The results of the trial indicated the effectiveness of the vaccine in decreasing the incidence of intramammary infections caused by *Staph. aureus*. A slight but non-significant increase occurred in fat production in the milk of vaccinated cows. The vaccine had no observable effect on somatic cell count or streptococcal infections.

CHAPTER III

MATERIALS AND METHODS



CHAPTER-III

MATERIALS AND METHODS

The research work was conducted during the period from September-2017 to December-2018 at the Bacteriology and Molecular Biology Laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. The detailed outline of the materials and methods are given below:

3.1 Materials

3.1.1 Study Area and Period

The research work was carried out at Rangpur Division with selective mastitis cow's milk samples. The laboratory works were conducted in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur.

Figure 3.1: Study area (Rangpur Division)

3.1.1.1 Sample Collection

A total 48 samples were collected from pre-selected mastitis dairy cows of different aged groups. The milk samples were collected with the help of pre-sterilized corkscrew tube and immediately transferred into pre-sterilized container. On the other hand, as soon as possible samples were transferred to the ice box containing plenty of ices then brought to the department of microbiology for laboratory analysis.

3.1.2 Media for Culture

The media and reagents that have been used for the isolation and identification of the bacteria are mentioned below.

3.1.2.1 Solid Media

- Nutrient Agar Medium, (HI-MEDIA, India)
- Eosin Methylene Blue, (EMB) (HI-MEDIA, India)
- Motility, Indole, Urease Medium, (HI-MEDIA, India)
- Blood Agar Medium, (HI-MEDIA, India)
- MacConkey Agar Medium, (HI-MEDIA, India)
- Triple Sugar Iron (TSI) Agar Slant, (HI-MEDIA, India)
- Staphylococcus Agar No. 110 media, (HI-MEDIA, India)
- Manitol Salt Agar, (HI-MEDIA, India)
- Streptococcus Agalactiae Selective Agar Base (HI-MEDIA, India)

3.1.2.2 Liquid Media

- Nutrient broth, (HI-MEDIA, India)
- Methyl Red-Voges Proskauer (MR-VP) broth, (Hi-MEDIA, India)
- Luria Bertani Broth, Miller (HI-MEDIA, India)

3.1.2.3 Data Analysis

Data were analyzed using SPSS version 22.

3.1.2.4 Chemical and Reagents

- Gram's staining reagent: Crystal violet, Gram's iodine, Acetone alcohol and Safranine
- Catalase test reagent: Hydrogen peroxide (3% solution)
- Kovac's reagent
- Rabbit serum

- Ethyl alcohol (70% and 95%)
- Sugar Media (Dextrose, Maltose, Lactose, Sucrose and Mannitol)
- Gel loading dye
- Ethidium bromide
- PCR master mix, DNA ladder, forward and reverse primer and other chemicals and reagents as when required during experiment.

3.1.3 Glassware's and Appliances

The different kinds of glassware's and appliances used during the course of the experiment were as follows:

- Test tube (with or without stopper)
- Conical flask
- Inoculating loop
- Petridishes
- Pipette
- Cover slips
- Hanging drop slide
- Glass rod spreader
- Test tube stand water bath
- Ice box
- Autoclave
- Refrigerator
- Hot air oven
- Compound microscope
- Micropipette
- Centrifuge tube
- Spirit lamb
- Slides
- Gel documentation
- Gel electrophoresis
- Thermal cycler
- Micro centrifuge
- Gel casting tray

- Microwave oven
- PCR tube

3.1.4 Materials used for Bacterial Genomic DNA Isolation

- 1.5ml microcentrifuge tubes
- Water bath, 80°C
- Water bath, 37°C
- Isopropanol, room temperature
- 70% ethanol, room temperature
- Water bath, 65°C (optional; for rapid DNA rehydration)
- 50 mM EDTA (pH 8.0) (for gram positive bacteria)
- 10 mg/ml lysozyme (Sigma Cat.# L4919) (for gram positive bacteria)
- 10 mg/ml lysostaphin (Sigma Cat.# L7386) (for gram positive bacteria)
- 10% (w\v) Sodium dodecyl sulfate (SDS)
- 20 mg/ml protinase k (stored in small single-use aliquots at -20°C)
- 3 M Sodium Acetate, pH 5.2
- 25:24:1 Phenol/Chloroform/Isoamyl alcohol
- 70% Ethanol
- 95% Ethanol
- Followed wizard-genomic-DNA-purification-kit-protocol (Promega, USA)

3.1.5 Materials used for Polymerase Chain Reaction

Table 3.1: PCR Reaction Mixture:

PCR Master Mix	25 µl
Forward Primer	1.5 µl
Reverse Primer	1. 5 µl
Nano Pure Water	18 µl
DNA	4.0 µl
Final Volume	50 µl

Legends: DNA used as 35X concentration, primer for PCR used 20 pmol and for sequencing primer used as 10 pmol in concentration

- Primers used for PCR:

Table 3.2: Primer and their Specification:

Organism	Primer Name	F/R	Sequence (5' – 3')	% GC Content	Amplicon Size	References
E. coli	Eco 223	F	ATC AAC CGA GAT TCC CCC AGT	52.4%	232 bp	Riffon *et al.* (2001)
	Eco 455	R	TCA CTA TCG GTC AGT CAG GAG	52.4%		
Staphylococcus aureus	Sau 234	F	CGA TTC CCT TAG TAG CGG CG	60%	1267 bp	Riffon *et al.* (2001)
	Sau 1501	R	CCA ATC GCA CGC TTC GCC TA	60%		
Streptococcus agalactiae	Sag 40	F	CGC TGA GGT TTG GTG TTT ACA	47.6%	405 bp	Riffon *et al.* (2001)
	Sag 445	R	CAC TCC TAC CAA CGT TCT TC	50%		
Streptococcus sp.	Uni 1870	F	TGG AAG GTT AAG AGG AGT GG	50%	438 bp	Riffon *et al.* (2001)
	Uni 2308	R	GCC TCC GTT ACC TTT TAG GA	50%		
Klebsiella sp.	27F	F	AGAGTTTGATCCTGGCTCAG	50%	1492 bp	Lane *et al.* (1991)
	1492R	R	TACCTTGTTACGACTT	50%		

- Thermal Cycler (Thermo cycler, ASTEC, Japan)
- 1.7 % agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask
- Electrophoresis apparatus (Biometra standard power pack P 2T)
- 100 bp DNA size marker
- 50 bp DNA size marker
- Bromphenicol blue of loading bufter
- Ethidium bromide (0.5 µg/ml)
- Distilled water
- UV trans-illuminator

3.1.6 Materials used for Real Time Polymerase Chain Reaction

Table 3.3: Primer and their Specification:

Organism	Primer Name	F/R	Sequence (5' – 3')	% GC Content	Amplicon Size	References
Streptococcus agalactiae	Sag 40	F	CGC TGA GGT TTG GTG TTT ACA	47.6%	405 bp	Riffon *et al.* (2001)
	Sag 445	R	CAC TCC TAC CAA CGT TCT TC	50%		

- Follow wizard-genomic-DNA-purification-kit-protocol (Promega)
- Probe (Go Taq, Promega Co. Ltd.) Follow the recommended protocol.
- RT-PCR machine (Eco 48 PCR MAX).

3.1.6.1 Real Time Polymerase Chain Reaction Condition

Table 3.4 RT-PCR Condition

Sl. no	Steps	Temperature	Duration	Cycle
1.	Initial denaturation	95°C	2 min	01
2.	Denaturation	95°C	15 Sec	40
3.	Annealing and Extension	60°C	1min	

3.1.6.2 Real Time Polymerase Chain Reaction Master mix

Table 3.5: RT-PCR Master Mixture

qPCR Master Mix (Go taq probe)	10 µl
Forward Primer	1µl
Reverse Primer	1 µl
Nano Pure Water	6 µl
DNA	2 µl
Final Volume	20 µl

Legends: DNA used as 35X concentration, primer for RT-PCR used 20 pmol.

3.1.7 Media for Culture

3.1.7.1 Liquid Media

3.1.7.1.1 Nutrient Broth

Thirty grams of Nutrient broth add with 1000 ml of distilled water and dissolve it completely. Then autoclave was performed for sterilization at 121°F, 15 lb pressure for 15 minutes (HI-MEDIA, India). This medium is used in accordance with the official recommended procedures for the bacteriological analyses of water, milk, dairy products and feces of clinical samples, and as a base to prepare media supplemented with other nutrients. Nutrient Broth is used in many laboratory procedures as it is or with added indicators, carbohydrates, organic liquids, salts, etc. Gelatin peptone and Beef extract provide nitrogen, vitamins, minerals and amino acids essential for growth (Carter, 1979).

3.1.7.1.2 Luria Bertani Broth, Miller

Luria Bertani Broth is used for the general cultivation of microorganisms in water, feces and other materials. This medium is used in accordance with the official recommended procedures for the bacteriological analyses of water, milk, dairy products and feces of clinical samples, and as a base to prepare media supplemented with other nutrients. Luria Bertani Broth is used in many laboratories. Mainly used for DNA extraction (HI-MEDIA, India).

3.1.8 Semi Solid Media

3.1.8.1 Motility Indole Urease (MIU) Media

It is a semi solid media used to determine motility indole formation and of the test organisms (HI-MEDIA, India; Carter (1979).

.

3.1.9 Solid Media

3.1.9.1 Nutrient Agar Medium

Nutrient agar is used for cultivating of non-fastidious microorganisms according to Cheesbrough (1985) and Carter (1979).

3.1.9.2 MacConkey Agar Medium

A differential medium for the isolation of coliforms and intestinal pathogens in water and biological specimens. Mac-Conkey agar is a culture medium designed to selectively grow Gram-negative bacteria and differentiate them for lactose fermentation. Lactose-fermenting organisms grow as pink to brick red colonies with or without a zone of precipitated bile. Non- lactose fermenting organisms grow as colorless or clear colonies (Cheesbrough, 1985).

3.1.9.3 Eosin Methylene Blue

EMB contains dyes that are toxic for Gram positive bacteria and bile salt which is toxic for Gram negative bacteria other than coliforms. EMB is the selective and differential medium for coliforms. Escherichia coli: Blue-black bulls eye; may have green metallic sheen. *Eseherichia coli* colonies grow with a metallic sheen with a dark center, *Aerobacter aerogenes* colonies have a brown center, and non-lactose-fermenting gram-negative and *Klebshella* sp. bacteria appear pink (Cheesbrough, 1985).

3.1.9.4 Staphylococcus Agar No. 110

Staphylococcus Agar No. 110 (HI-MEDIA, India) was used as a selective medium for Staphylococci which causes enhancement of the growth of Staphylococcus while inhibiting the growth of other contaminating organisms and shows typical colony characters (Cheesebrough, 1984).

3.1.9.5 Blood Agar Medium

For the isolation, cultivation and detection of hemolytic reaction of fastidious microorganisms. Blood Agar Base (HI-MEDIA, India) is suitable to isolate and cultivate

a wide range of microorganisms with difficult growth. Upon adding blood, it can be utilized for determining hemolytic reactions *Staphylococcus aureus* gives beta hemolysis. *Streptococcus pneumoniae* gives alfa hemolysis. *Streptococcus pyogenes* gives beta hemolysis and also followed by Merchant and Packer (1967); Buxton and Fraser (1977) and OIE (2004).

3.1.9.6 Streptococcus Agalactiae Selective Agar Base

Streptococcus Agalactiae Selective Agar Base (HI-MEDIA, India) was used as a selective medium for *Streptococcus agalactiae* which causes enhancement of the growth of *Streptococcus agalactiae* while inhibiting the growth of other contaminating organisms anaerobically with recommended procedure.

3.1.9.7 Simmons Citrate Agar Medium

Simmon's citrate agar (HI-MEDIA, India) is used for determination the ability of bacteria to ferment citrate as a sole source of carbon.

3.1.9.8 Triple Sugar Iron Agar

Triple sugar iron agar (HI-MEDIA, India) is used for identification of gram negative bacteria that capable of fermenting sugar. Triple sugar iron agar contains lactose, sucrose and glucose.

3.1.10 Media for Biochemical Test

Sugar fermentation broth, indole broth, methyl red broth, Voges-Proskauer broth, Simmon's citrate agar, triple sugar iron agar and motility indole urease (M1U).

3.2 Methods

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

3.2.1 Laboratory Preparations

All items of required glassware including test tubes, pipettes plate, slides, cylinder, flasks, conical flasks, glass and vials soaked in a household dishwashing detergent solution ('Trix' Recket and Colman Bangladesh Ltd) overnight Contaminated glassware was disinfected with 2% sodium hypochlorite solution prior to cleaning. The glassware was then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized either by dry heat at 160°C for 2 hours or by autoclaving for 15 minutes at 121°C under 15 lbs pressure per sq inch. Autoclaved items were dried in a hot air oven over at 50°C. Disposable plastic (items e.g. micropipette tips) was sterilized by autoclaving according to Merchant and Packer (1967); Buxton and Fraser (1977) and OIE (2004). All the glassware was kept in oven at 50°C for future use.

3.3 Experimental Layout

The experimental work was divided into following steps: The steps were performed for the isolation and identification of the organisms of the collected sample using cultural, staining and biochemical characteristics and Antibiogram study. The Molecular characterization by PCR, RT-PCR was done later Phylogenetic study performed.

Experimental Layout

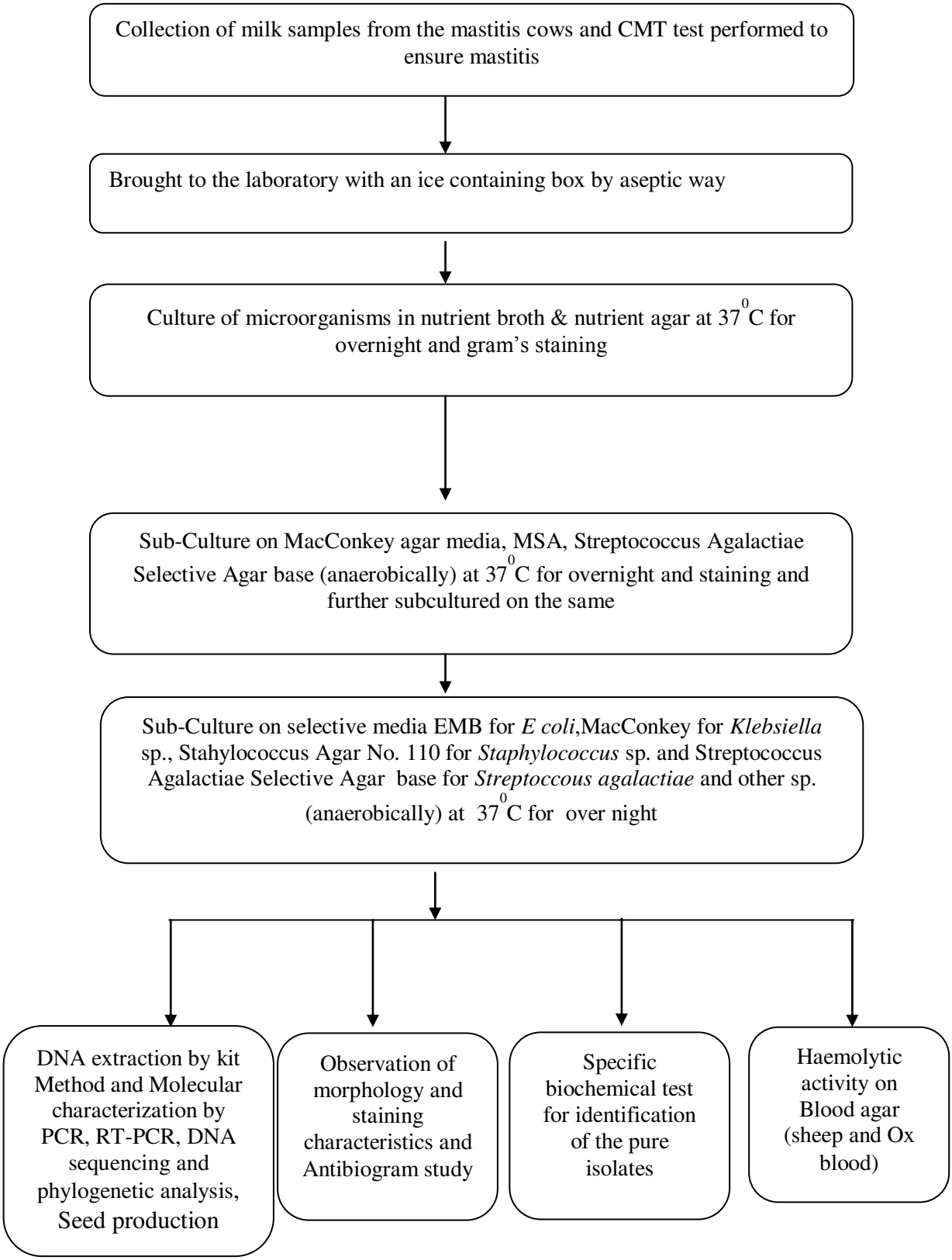
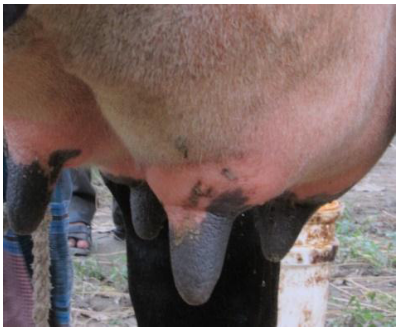


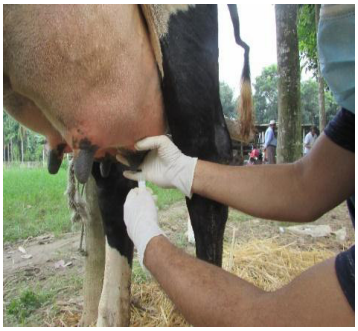
Figure 3.2: The Schematic Illustration of the Experimental Layout

3.4 Collection of Milk Samples

Total of 48 mastitis milk samples were randomly collected from Rangpur division. Samples are mainly collected from Dinajpur, Rangpur, Thakurgaon and Gaibandha district and soak the teat were soaked with 70% ethanol and drying off by tissue paper, one to two drops of milk was discarded and then 10 ml of milk were taken from infected udder and teat. After collection CMT test performed according to Schalm and Noorlander (1957). A labeled sterilized test tubes with rubber cap using to transfer samples in the laboratory in an ice box contained ice and processed for the isolation and characterization of bacteria subsequently and kept in incubator at 37°C for 24 hours for the isolation and identification of bacteria by morphology, staining and cultural characteristics. Characterization of bacteria was done by biochemical reactions according to Merchant and Packer (1967), Buxton and Fraser (1977) and OIE (2004). The remaining samples were stored at 4°C for further use.

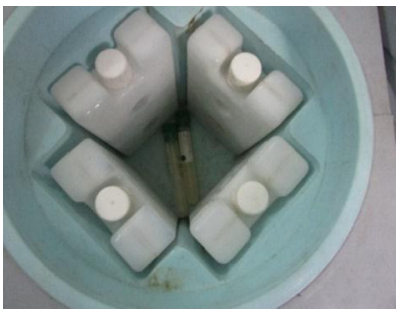


Infected udder and teat



Collection of infected milk sample

Samples after in sterilized tubes



Samples in ice box

CMT test kit

CMT positive jelly like appearance

Figure 3.3: Sample Collection, Preparation and CMT Test

3.5 Preparation of Sample

In the laboratory mastitis milk samples were stored in 4°C until the preparation of inoculation media. All the samples were inoculated within the clean bench by maintaining aseptic procedure. From collection to inoculation was done within 24 hours.

3.6 Preparation of Culture Media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer guidelines.

3.6.1 Nutrient Broth Media (NB Media)

Thirteen grams of dehydrated nutrient broth (HI-MEDIA, India) was suspended into 1000ml of distilled water and boil to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121^0C and 1.2 kg/cm^2 pressure for 15 minutes. The sterility of the medium was checked by incubating at 37°C for overnight and stored at 40°C in aerator for future use.

3.6.2 Nutrient Agar Media (NA Media)

Twenty-eight grams of nutrient agar powder (HI-MEDIA, India) was dissolved in 1000 ml of cold distilled water in a flask. 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 at 4°C refrigerator for future use.

3.6.3 MacConkey Agar (MAC Media)

51.5 grams MacConkey agar base (HI- MEDIA, India) powder was added to 1000 ml of distilled water in a flask and heated until boiling 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. After autoclaving the medium was put into water bath at 45-50°C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass Petridishes (large sized) to form thick layer in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petridishes partially removed. The

sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. 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.

3.6.4 Eosin Methylene Blue Agar (EMB Media)

Thirty-six grams of EMB agar base (HI-MEDIA, India) was added to 1000 ml of water in a flask and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121°C for 15 minutes shake the medium in order to oxidize the metbylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petridish sized 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 petridishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use.

3.6.5 Blood Agar Media (BA Media)

Forty grams of Blood agar base (HI-MEDIA, India) powder was dissolved in 1000 ml of distilled water. The medium was sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes and 45°C. Then 5-10 % sterile defibrinated blood was added to the medium 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 refrigerator for future use.

3.6.6 Mannitol Salt Agar Media (MSA)

111.02 grams of Mannitol salt agar powder (HI-MEDIA, India) was suspended in 1000 ml of distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. The precipitate was resuspended by gentle agitation to avoid bubbles and pour the plates while the medium is hot and allowed to solidify. Alternatively, the medium was cooled to 45-50°C. After solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.6.7 Staphylococcus Agar No. 110

149.6 grams of Staphylococcus Agar (HI-MEDIA, India) powder 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. Re-suspend the precipitate by gentle agitation to avoid bubbles and pour the plates while the medium is hot and allowed: o solidify. Alternatively, cool the medium to 45-50°C and add blood or egg yolk if desired. Staphylococcus agar no. 110 may also be used without sterilization; it should be boiled for 5 mins used at once. After solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.6.8 Streptococcus Agalactiae Selective Agar Base

Suspend 34.34 grams in 940 ml distilled water. Heat to boiling to dissolve the medium completely. No need to autoclave. Cool to 45-50°C and add 60 ml defribinated blood and 25ml Staphylococcus ß-toxin. Mix well and pour into sterile Petri plates (HI-MEDIA, India).

3.6.9 Mueller Hinton Agar

Mueller Hinton Agar (HI-MEDIA, India) is used in antimicrobial susceptibility testing by the disk diffusion method. 38 grams of Mueller Hinton agar powder was suspended in 1000 ml of distilled water. It was mixed well. It was heated agitating frequently and boiled for about one minute. It was dispensed and sterilized in autoclave at 116 - 121°C (15 lbs. sp) for 15 minutes. It was cooled to 45° or 50°C (Carter, 1979).

3.6.10 Antibacterial discs

The sensitivity of isolates to antibiotics was determined by disc diffusion technique (Bauer *et al.,* 1966), according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI 2013; Cappuccino 2005). The isolates were cultured into peptone water and incubated at 37°C for two hours. A Petri dish containing Muller hinton Agar (MHA) medium, was put in the incubators for 30 minutes to dry and then inoculated with 2 ml volume of the test culture. The inoculated culture was evenly distributed by rotation, the excess inoculums was withdrawn by sterile Pasteur pipette and the plate was left to dry at room temperature for 15 minutes. To determine the drug

sensitivity pattern of different isolated bacteria different types of commercially available antibiotic discs (Oxoid Ltd., UK) were placed on the surface of the inoculated medium with a sterile forceps and pressed gently to ensure good contact with the surface of the medium. The plates were then incubated at 37°C. The followings are the antibiotics that were tested against the selected organism. In this research also studied by using house fly on MHA media and observed their antimicrobial activity. On the other hand, Park *et al.* (2010) studied with house fly maggot extracts but here we applied whole fly.

Table 3.6: Antimicrobial Agents with their Disc Concentration

Sl. No.	Name of antibiotics	Disc concentration (µg/disc)
1.	Gentamicin (GEN)	10 µg/disc
2.	Amoxicillin (AMX)	30 µg/disc
3.	Chloramphenicol (C)	30 µg/disc
4.	Ciprofloxacin (CIP)	5 µg/disc
5.	Bacitracin (B)	10 µg/disc
6.	Azithromycin (AZM)	30 µg/disc
7.	Erythromycin (E)	15 µg/disc
8.	Mithicillin (MET)	5 µg/disc
9.	Novobiocin (NV)	30 µg/disc
10.	Vancomycin (VA)	30 µg/disc
11.	Norfloxacin (NX)	10 µg/disc
12.	Tetracyclin (TE)	30 µg/disc
13.	Levofloxacin (LE)	5 µg/disc
14.	Nalidixic acid (NA)	30 µg/disc
15.	Cephradine (CH)	25 µg/disc
16	House fly	-

Legend: µg = Microgram

3.7 Preparation of Reagents

3.7.1 Methyl-Red Solution

The indicator Methyl- Red (MR) solution (HI-MEDIA, India) solution was prepared by dissolving 0.1 gm of Bacto methyl- red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water.

3.7.2 Methyl Red

A quantity of 17 gms of MR-VP medium (HI-MEDIA, India) was dissolved in 1000 ml of distilled water, dispensed in 2 ml amount in each tube and the tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight o check their sterility and then in refrigerator for tutored use.

3.7.3 Alpha-Naphthol Solution

Alpha- naphthol solution was prepared by dissolving 5 gm of Alpha- naphthol in 100 ml of 95% ethyl alcohol (HI-MEDIA, India).

3.7.4 Potassium Hydroxide Solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals indoor ml of cooled water (HI-MEDIA, India).

3.7.5 Phosphate Buffered Saline Solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate Na_2HPO_4, 12 H_2O), 0.2 gram of potassium chloride (KC1) and 0.24 gram of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled for the preparation of phosphate buffered saline solution. The solution was heated to dissolve completely. Then the solution was sterilized by autoclaving at 1.2 kg / cm^2 pressure and 121° C for 15 minutes and stored for future use (HI-MEDIA, India).

3.8 Indole Reagent

3.8.1 Kovac's Reagent

This solution was prepared by dissolving 25 ml of concentrated Hydrochloride acid in 75 ml of amylalcohol and to the mixture 5 grams of paradimethyl amino- benzyldehyde

crystals were added. This was then kept in a flask equipped with rubber cork for future use (HI-MEDIA, India).

3.9 Cultivation and Isolation of Organisms

Samples were collected and each of the samples was directly inoculated into nutrient agar. Then the petri dishes were marked properly and incubated at 37°C for 24 hours aerobically and anaerobically in bacteriological incubator, then sub-cultured onto the MacConkey, EMB, Manitol Salt Agar, Staphylococcus Agar No. 110, Streptococcus Agalactiae Selective Agar base and by streak plate method (Cheesbrough, 1985) to observe the colony characteristic colony morphology of *Escherichia coli, Staphyloccus* sp.*, Streptococcus* sp., *Corynebacterium* sp. Then was repeatedly sub-cultured onto Mac-Conkey, EMB agar, Staphylococcus Agar No. 110 and Streptococcus Agalactiae Selective Agar base until the pure culture morphology (shape, size, surface texture, edge and elevation, color, opacity etc.) were obtained.

3.9.1 Morphological Characterization by Gram's Staining Method

The most widely used staining procedure in microbiology is the gram stain, discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884, Gram staining is a differential staining technique that differentiates bacteria into two groups: gram- positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple Gram-positive bacteria are not decolorized by alcohol and will remain as purple after decolonization step, and a counter stain is used to impart a pink color to the decolorized gram- negative organisms.

3.9.2 Preparation of Gram's Staining Solution

The four solutions needed for the Gram staining procedure and followed by (Cuppuccion and Sherman, 1996).

- Crystal violet
- Gram's iodine
- 95% alcohol
- Safranin

3.9.3 Gram Staining Procedure

1. First, the clean glass slides were obtained.
2. Smears of each of the organisms were prepared using sterile technique. It was done by placing a drop of distilled water on the slide, and then by transferring each organism separately to the water drop with a sterile, cooled loop. Organisms were mixed and speeded by means of a circular motion of the inoculating loop.
3. Smears were allowed to air dry and then were subjected to heat fixation in the usual manner.
4. The smears gently flooded with crystal violet and let stood for I mm and gently washed with tap water.
5. Smears were gently flooded with Grams iodine mordant and let stood for 1 mm then gently washed with tap water.
6. Then decolorized with 95% ethyl alcohol and gently washed with tap water.
7. Counter staining was done with safranin for 30 sec.
8. Then gently washed with tap water.
9. Finally examined under microscope with oil immersion.

3.10 Biochemical Examination

Isolated organism with supporting growth characteristics of suspected identified by biochemical test are performed, Catalase test, Indole test, MR Test, Voges-Proskauer test, Simmons citrate, Triple sugar iron agar (TSI), Motility Indole Urease (MIU) test.

3.10.1 Catalase Test

This test was used to differentiate bacteria which produce the enzyme catalase. To perform this test, a small colony of good growth pure culture of test organism was smeared on a slide. Then one drop of catalase reagent (3%H_2O_2) was added on the smear. The slide was observed for bubbles formation. Formation of bubble within few seconds was the indication of positive test while the absence of bubble formation indicated negative result (Cheesbrough, 1985).

3.10.2 Coagulase Test

Coagulase test performed (HI-MEDIA, India) by using rabbit serum with manufacturing protocol. Clumping formation show positive and no clumping indicates negative test result.

3.10.3 Methyl Red Test (MR)

Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°c for 24 hours, if the organism would ferment glucose via the mixed acid fermentation pathway like lactic, acetic, which decreases the PH, hence upon the addition of the indicator methyl red the broth becomes red in color and yellow color indicated a negative result (Cheesbrough, 1985).

3.10.4 Voges-Proskauer Test (VP)

Voges-Proskauer test- if the organism would ferment glucose via the butylenes glycol pathway, an intermediate product, acetyl methyl carbinol or acetrone which is neutral is Converted to diacetyl upon the addition of the VP - Reagent -B (40% KOH with 0.3% creatine in the presence of VP-Reagent -A (5% alpha- naphthol in abs. methyl alcohol). Diacetyl is red in color. Negative is yellow color (Cheesbrough, 1985).

3.10.5 Indole Test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°c for 48 hours. Kovac's reagents (HI-MEDIA, India) (0.5m1) were added, shake well and examined after 1 minute. A red color in the reagent layer indicated Indole test positive. In negative case there is no development of red color (Cheesbrough, 1985).

3.10.6 Simmon's Citrate Agar (SCA)

This tube medium is used to identify Gram negative enteric bacilli based on the ability of the organisms to utilize citrate s the sole source of carbon. (Citrate utilization test). The organism which utilizes citrate as its source of carbon degrades it to ammonia and subsequently converts it to ammonium hydroxide. The pH of the medium is then increased and this is indicated by a change in color from green to blue (Cheesbrough, 1985).

3.10.7 Triple Sugar Iron Agar (TSU)

This tube medium is used to identify Gram negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985):

- Glucose fermentation — indicated by yellow butt
- Lactose fermentation — indicated by yellow slant
- Hydrogen sulfide production — indicated by blackening of the medium
- Gas production — indicated by presence of a crack, bubble or gas space
- pH indicator — phenol red
- Hydrogen sulfide indicator — ferric ammonium citrate with sodium thiosulfate.

3.10.8 Motility Indole Urease (MIU) Test

MIU medium (HI-MEDIA, India) is a semisolid medium used in the qualitative determination of motility, production of indole and ornithine decarboxylase. MIU medium is used for the differentiation of the Family Enterobacteriaceae. The organisms tested must ferment glucose for proper performance of the medium. MIU medium contains dextrose as fermentable sugar, ornithine as an amino acid, bromcresol purple as pH indicator, casein peptone as a source of tryptophan, and other essential nutrients for growth. The medium contains a small amount of agar allowing for detection of motility. Whole procedure followed according to Cheesbrough, (1985); Carter (1979).

3.10.9 Haemolytic Activity

To determine the haemolytic property of isolated bacteria, the colonies of bacteria were inoculated on to blood agar media and incubated at 37°c for 24 hours. Various types of haemolysis were observed after development of bacterial colony on the blood agar media. The hemolytic pattern of the bacteria was categorized according to the types of hemolysis produced on BA and this was made as per recommendation of Carter (1979) and was listed as mentioned below:

3.10.9.1 Alpha (α) Hemolysis

A zone of greenish discoloration around the colony manifested by partial hemolysis.

3.10.9.2 Beta (β) Hemolysis

Complete clear zone of hemolysis around the colony.

3.10.9.3 Gamma (ɤ) Hemolysis

No detectable hemolysis

3.11 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 suggested by Leadbetter and Poindexter (2013); Cowan and Steel (2003).

- **Step 1.** An inoculum was picked up with a sterile inoculating loop and was spread on an area of the medium in the petridish.
- **Step 2.** The loop was sterilized by being heated as red hot in a flame.
- **Step 3.** 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.

3.12 Techniques for the Isolation and Identification of *Escherichia coli*

3.12.1 Culture into Different Media

Sterilized platinum loop was used for streaking the lactose broth culture on MacConkey and EMB agar to get isolates in pure culture. All inoculated media were kept at 37°C for overnight in an incubator.

3.12.2 MacConkey Agar

Inoculum from lactose fermentation tubes was inoculated into MacConkey agar plates which after incubation, if positive for *Escherichia coli* showed rose pink color colonies.

3.12.3 Eosin Methylene Blue (EMB) Agar

Inocutum from lactose fermentation tubes was inoculated into EMB agar plates which after incubation, showed smooth circular colonies with dark centers and metallic sheen if *Escherichia coli*.

3.12.4 Microscopic study for Identification of *Escherichia coli* Suspected Colonies by 'Gram's Staining Method

Gram's staining was performed to determine the size, shape, and arrangement of bacteria. Gram staining reaction was performed according to the methods described by Cowan and Steel (2003). The organism if *Escherichia coli* revealed Gram negative, pink color, large rod shape appearance, arranged in single or paired.

3.13 Identification of *Escherichia coli* by Biochemical Test

Sugar fermentation test was performed to identify of *Escherichia coli.* For sugar fermentation on the tubes containing different sugar media such as sucrose, maltose, dextrose, lactose and mannitol were inoculated with a hopeful of broth culture of the isolated and incubated at 37°C for 18 hours. The isolates if positive, ferment five sugar viz, dextrose, maltose, lactose, sucrose, and mannitol, the organism's acid and gas in' all cases, Acid production was indicated by the change of the color reddish to yellowish in the medium and gas production was noted by the appearance of gas bubble in the inverted Durham's tube.

3.14 Identification of *Staphylococcus* sp. by colony morphology gram's staining and biochemical test

Gram's staining was performed to determine the size, shape, and arrangement of bacteria. Gram staining reaction was performed according to the methods described by Cowan and Steel (2003). After that biochemical tests are done according to Merchant and Packer (1967), Buxton and Fraser (1977) and OIE (2004).

3.15 Identification of *Streptococcus* sp. by colony morphology gram's staining and biochemical test

Gram's staining was performed to determine the size, shape, and arrangement of bacteria. Gram staining reaction was performed according to the methods described by Cowan and Steel (2003). After that biochemical tests are done according to Merchant and Packer (1967), Buxton and Fraser (1977) and OIE (2004).

3.16 Identification of *Klebsiella* sp. by colony morphology, gram's staining and biochemical test

Gram's staining was performed to determine the size, shape, and arrangement of bacteria. Gram staining reaction was performed according to the methods described by Cowan and Steel (2003). After that biochemical tests are done according to Merchant and Packer (1967), Buxton and Fraser (1977) and OIE (2004).

3.17 PCR, DNA Sequencing, 16S rRNA and 23S rRNA Gene Amplification and Phylogenetic Analysis of Isolated Organisms

3.17.1 Basic protocol of Bacterial Genomic DNA Extraction (Gram positive and Gram negative).

- Bacteria cultured for overnight on Luria Bertani Broth. After that follow the recommended procedure (wizard-genomic-DNA-purification-kit-protocol (Promega, USA).

Procedure

1. Added 1ml of an overnight culture to a 1.5ml micro centrifuge tube.
2. Centrifuged at 13,000–16,000 × g for 2 minutes to pellet the cells. Remove the supernatant. For Gram Positive Bacteria, proceed to Step 3. For Gram Negative Bacteria go directly to Step 6.
3. Re-suspend the cells thoroughly in 480µl of 50mM EDTA.
4. Added the appropriate lytic enzyme(s) to the resuspended cell pellet in a total volume of 120µl, and gently pipet to mix. The purpose of this pretreatment is to weaken the cell wall so that efficient cell lysis can take place.

Note: For certain Staphylococcus species, a mixture of 60µl of 10mg/ml lysozyme and 60µl of 10mg/ml lysostaphin is required for efficient lysis. However, many Gram Positive Bacterial Strains (e.g., Bacillus subtilis, Micrococcus luteus, Nocardia otitidiscaviarum, Rhodococcus rhodochrous, and Brevibacterium albidium) lyse efficiently using lysozyme alone.

5. Incubated the sample at 37°C for 30–60 minutes. Centrifuge for 2 minutes at 13,000–16,000 × g and remove the supernatant.
6. Add 600µl of Nuclei Lysis Solution. Gently pipet until the cells are resuspended.
7. Incubated at 80°C for 5 minutes to lyse the cells; then cool to room temperature.

8. Added 3µl of RNase Solution to the cell lysate. Invert the tube 2–5 times to mix.

9. Incubated at 37°C for 15–60 minutes. Cool the sample to room temperature.

10. Added 200µl of Protein Precipitation Solution to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate.

11. Incubated the sample on ice for 5 minutes.

12. Centrifuged at 13,000–16,000 × g for 3 minutes.

13. Transferred the supernatant containing the DNA to a clean 1.5ml micro centrifuge tube containing 600µl of room temperature isopropanol.

Note: Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

14. Gently mix by inversion until the thread-like strands of DNA form a visible mass.

15. Centrifuged at 13,000–16,000 × g for 2 minutes.

16. Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.

17. Centrifuged at 13,000–16,000 × g for 2 minutes. Carefully aspirate the ethanol.

18. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 minutes.

19. Added 100µl of DNA Rehydration Solution to the tube and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.

20. Stored the DNA at 2–8°C.

3.17.2 PCR Amplification and Sequencing

PCR Condition:

Table 3.7: Condition of PCR

Sl. no	Steps	Temperature	Duration	Cycle
1.	Initial denaturation	94°C	2 min	01
2.	Denaturation	94°C	45 Sec	35
3.	Annealing	**Table 3.8**	1 min	
4.	Extension	72°C	2 min	
5.	Final extension	72°C	10 min	01
6.	Holding	4°C	hold	

Table 3.8: Annealing Temperature for Specific Primer

Sl. no.	Organism	Primer	Annealing Temperature
1.	*E. coli*	Eco 223 Eco 455	64°C
2.	*Staphylococcus aureus*	Sau 234 Sau 1501	58°C, 60.5°C, 60.9°C,61.3°C
3.	*Streptococcus agalactiae*	Sag 40 Sag 445	60°C, 61°C, 62.5°C, 63.2°C
4.	*Streptococcus* sp.	Uni 1870 Uni 2308	58°C, 59.1°C
5.	*Klebsiella* sp.	27F 1492R	62°C

3.17.3 Electrophoresis

Process of Electrophoresis: According to The Biotechnology Education Company®, www.edvotek.com. Follows-

- Preparation of gel.
- Sample application in the gel.
- Adjustment of voltage or current (gel-electrophoresis about 70-100 volts).
- Set up run time about 30-60 minute

- When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
- The gel stained in ethidium bromide (0.5 µg/ml) for 10 minutes, in a dark place.
- The gel was distained in distilled water for 10 minutes. The distained gel was placed on the imaging system in the dark chamber of the image documentation system.
- The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in an USB flash drive.

3.17.4 Nucleotide Sequence Accession Number and BLAST Analysis

The nucleotide sequence 16S rRNA and 23S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogenic tree, primer pairs were designed from NCBI database search tool

3.17.5 Chain-Termination Methods (Sanger Sequencing) Steps of Sanger Sequencing Using ABI 3130 Genetic Analyzer

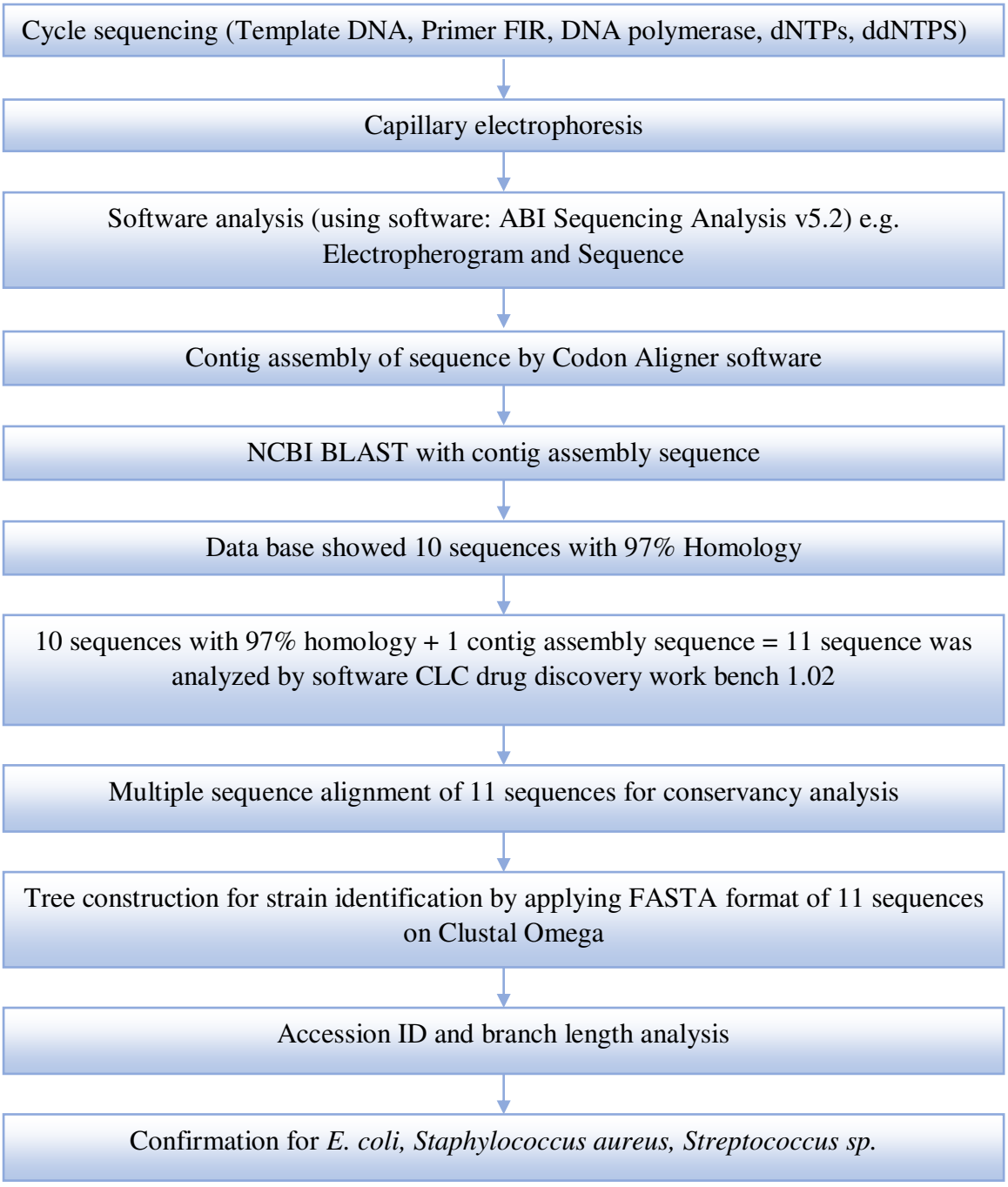


Figure 3.4: Steps of Sanger Sequencing Using ABI 3130 Genetic Analyzer

3.18 Maintenance of Stock Culture

The stock culture was maintained following the procedures of Angshumanjana *et al.,* (2016). 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 organisms in the slant, the sterile mineral oil was overlaid and the culture was kept at

room temperature for further use as seed. During the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of the isolated *E. coil.* Were stored in sterilized 80% glycerin and used as stock culture. The equal volume of 80% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at 37°C. The isolated organisms were given code name for convenience.

3.19 Statistical Analysis

Data were analyzed using SPSS version 22**.**

3.20 Development of Vaccine Seed

After isolation, identification and molecular characterization of pathogenic bacteria, we formulate vaccine seed based on Sayed *et al.,* 2015 and Giraudo *et al.,* 1997. Procedure is given bellow-

- Organisms are grown in Brain Heart Infusion Broth (BHIB) at 37°C for 24 hours; For *E. coli* Tryptic Soy Broth Medium used.
- Each sample taken @ 5ml in a tube
- Cells are in activated by 0.4% V/V formalin and centrifuge @ 7000 rpm for 20 min at 4 °C
- Suspended with normal saline (0.9% NaCl), pH 7.0
- Taken equal volume of sample 1×10^{10} cfu/ml *Staphylococcus aureus,* 4×10^{9}cfu/ml *Streptococcus agalactiae* and 1×10^{9}cfu/ml *E. coli* and *Klebsiella pneumonia. Streptococcus* sp. taken 4×10^{9}cfu/ml taken in a same tube.
- Equal amount of adjuvant taken in that tube.

3.20.1 Calculation of CFU (Colony Forming Unit)

Samples were collected and each samples were diluted with PBS as 10^{-1}, 10^{-2}, 10^{-3} 10^{-4} 10^{-5}. Then 50 µl samples were taken and spread in nutrient agar plate following the spread-plate method and incubate at 37ºC for 24 hours. The number of organisms per ml of original culture is calculated by multiplying the number of colonies counted by the dilution factor (Colony Forming Units cfu/ml Calculation Bio Resource, www. technologyinscience.blogpost.com)

Number of cells per ml=number of colonies × Dilution factor

3.20.1.1 CFU count

For each dilution, the number of colony forming units (CFU) should be counted on the plates. Typically, numbers between 30 and 300 are considered to be in the range where one's data is statistically accurate. If the number of CFUs on the plate are greater than 300, this might be recorded in the table as TNTC (too numerous to count). Alternatively, if the CFU numbers are greater than 300 and diluted bacterial suspension was distributed on the surface of the plate uniformly and the CFUs can be discerned as individual colonies; By the help of digital colony counter we count colony in HSTU microbiology laboratory. If the number of CFUs on the plate is below 10, the number of CFUs should be recorded, but using this data in the calculations is not needed (Scott 2011).

3.20.1.1.1 CFU/ml Calculating by Following Formula

cfu/ml = (No. of colonies × dilution factor) / volume of culture plate

3.20.2 Adjuvant Adding

In newly developed polyvalent formalin inactivated vaccine (PFIV) candidate Montanide@ISA 206 adjuvant was added at equal volume according to Acres *et al.* (1979). This adjuvant is nontoxic referred by Barnett *et al.,* (1996).

3.21 Quality Control Testing of Vaccine Candidate (vaccine seed)

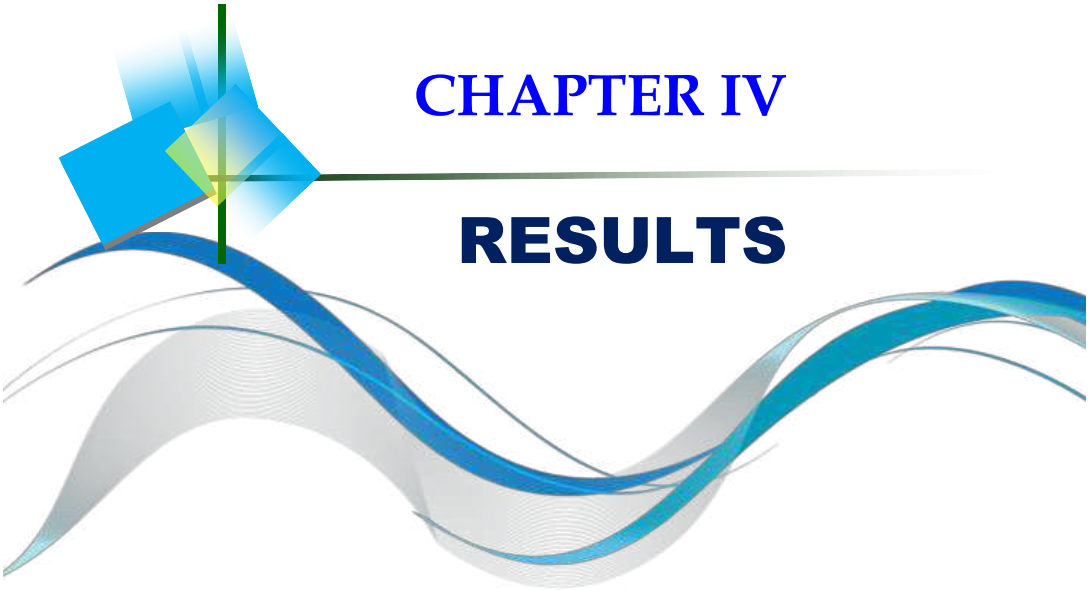
3.21.1 Sterility Test of Vaccine Candidate

Sterility test of the newly prepared PFIV candidate from foreign contaminants (aerobic and anaerobic bacteria and fungi) was carried out according to OIE (2013). Though it is difficult to maintain, we tried our level best to maintain the sterility of vaccine candidate. We were taken specific agar media for each isolates and inoculated on it. Finally observed their colony and staining characteristics.

3.21.2 Safety Test of the Vaccine Seed

Safety of the prepared vaccine seed was tested according to OIE (2013). PFIV candidate is safe or hazardous to animal was observed by inoculation of double dose intramuscular in cows which kept under daily observation for 14 days.

Figure 3.5: Vaccine Candidate Preparation and Store



CHAPTER IV

RESULTS

CHAPTER-IV

RESULTS

The results describe the isolation, identification, molecular characterization of bacteria isolated from mastitis milk samples. The study also presents antibiogram of obtained bacteria. The results are presented bellow in detail:

4.1 Results of Bacteria Isolated from Mastitis Milk at Different Area of Rangpur Division

The results of frequency of different bacterial isolates are presented in Table 4.1. A total of 48 milk samples were examined for the isolation of bacteria. The present research work mainly focused on 4 (four) types of bacteria. However, *Corynebacterium* sp. and *Bacillus* sp. also found at very lower in number.

Table 4.1: Frequency of Different Isolates in Milk Samples

Sample collection Area	Number of samples tested	Positive number of sample for *E. coli* (%)	Positive number of sample for *Staphylococcus* sp. (%)	Positive number of sample for *Streptococcus* sp. (%)	Positive number of sample for *Klebsiella* sp. (%)
Chirir bandar	12	100	100	58.34	50
Sadar Livestock office Dinajpur	8	100	100	62.5	62.5
Nandigram, Birganj	5	100	100	60	60
Birol, Dinajpur	10	100	100	70	60
Sadullahpur, Gaibandha	2	100	100	100	50
Kornai, Baserhat	2	100	100	-	100
Thakurgaon, Sadar	2	100	100	100	50
Thakurgaon, Haripur road	4	100	100	50	75
Birganj Livestock office	3	100	100	66.67	100
Total	48	48	48	28	30

4.2 Results of Isolation and Identification of Organisms

The cultural characteristics of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Streptococcus* sp. including *Streptococcus agalactiae* on various selective media are presented in Table 4.2.

Table 4.2: Isolation of Bacteria from Different Milk Samples by Using Cultural Characteristics

Name of the culture media used	Suspected organisms			
	Escherichia coli	***Staphylococcus aureus***	***Klebsiella pneumoniae***	***Streptococcus* sp. and *Streptococcus agalactiae***
Nutrient agar	Large, circular, low convex, grayish, white, moist colonies were found.	Circular, small, smooth, convex and gray-white colonies were produced	Large, circular, smooth, convex, colonies. were found	Circular, small smooth, convex, and golden, yellowish colonies
MacConkey agar	Bright, pink colored, smooth colonies were found.	—	Round, pink, slightly raised, translucent and mucoid colonies were found.	—
EMB agar	Smooth and green Metallic sheen colonies were formed.	—	Round, pink, slightly raised, translucent and mucoid colonies were found.	—
MSA	—	Golden yellow color pigment and sometimes Pale yellowish colonies appeared	—	Pale yellowish colonies and
Staphylococcus Agar No. 110	—	Pale opaque in color colonies and sometimes Golden yellowish colonies	—	Opaque in color colonies and sometimes red in color
Streptococcus Agalactiae Selective Agar Base	—	—	—	Dark brown in color, sometimes dove blue in color and Blackish red also appeared
Blood agar	—	Complete hemolytic zone (β hemolysis) in sheep and ox blood agar. Sometimes partial hemolysis appeared.	—	Complete hemolytic zone (β hemolysis) in sheep and ox blood agar.

All the isolates produced whitish, opaque, smooth or fuzzy colony on the nutrient agar. On the other hand, *Escherichia coli* and *Klebsiella pneumonia* produced pink colonies on MacConkey agar due to lactose fermentation. *Escherichia coli* and *Klebsiella pneumoniae* produced metallic green sheen and dark pink colonies on eosin methylene blue agar respectively. In MSA *Staphylococcus aureus* produced golden yellowish pigment. Whereas *Streptococcus* sp. produced pale yellow and sometimes red in colony. In Staphylococcus Agar No. 110 pale and opaque, small circular colony appeared in case of *Staphylococcus aureus.* Streptococcus Agalactiae Selective Agar Base media used along with blood and anaerobically for *Streptococcus agalactiae.* They were given dark brown color colony also black red which are sticky. Sheep and Ox blood agar used for both *Streptococcus agalactiae* and *Staphylococcus aureus*. Both of them produce complete hemolysis.

4.3 Identification of Isolated Organisms by Using Morphological Characteristics

Table 4.3: Identification of Different Isolates by Using Morphological Characteristics

Staining and morphological characteristics	**Suspected organisms**
Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain.	***Escherichia coli***
Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain, non-motile, encapsulated	***Klebsiella pneumoniae***
Gram positive, Violet colored, cocci shaped organisms arranged in clusters. They may also appear as single, paired and short chains of three or four bacteria.	***Staphylococcus aureus***
Gram positive, Violet colored, cocci shaped organisms arranged in short chain. They may also appear as long chain when grown in liquid medium.	***Streptococcus agalactiae***

The isolates were identified as *E. coli* (Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain), *Klebsiella* sp. (Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain, non-motile, encapsulated), *Staphylococcus* sp. (Gram positive, Violet colored, cocci shaped organisms arranged in clusters. They may also appear as single, paired and short chains of three or four bacteria) and *Streptococcus* sp. (Gram positive, Violet colored, cocci shaped organisms arranged in short chain. They may also appear as long chain when grown in liquid medium) by observing their morphological characteristics.

4.4 Characterization of Field Isolates by Using Different Biochemical Techniques

Table 4.4: Biochemical Properties of the Isolated Organisms

Organisms	SC	IT	TSI	MR	VP	C
E. coli	—	+	S- Yellowish with colony, B-No change, Gas +, H_2S —	+	—	—
Klebsiella sp.	+	—	S- Yellowish with colony, B-No change, Gas +, H_2S —	—	+	+
Staphylococcus sp.	—	—	S- Yellowish with colony, B- No change, Gas —, H_2S —	+	+	+
Streptococcus sp.	—	—	S- Pinkish with colony, B- No change, Gas —, H_2S —	—	—	—

(**Legends: S=**Slant, B=Butt, SC = Simmon's Citrate Test, IT = Indole test, TSI = Triple sugar iron test, MR = Methyl-Red test, VP = Voges-Proskauer test, C= Catalase Test**,** + = Positive reaction, — = Negative reaction).

Biochemical Test showed in Plate 13, 14, 15 for *Streptococcus* sp.; Plate 16, 17 for *Staphylococcus* sp.; Plate 18 for *Klebsiella* sp.; Plate 19 for *E. coli*

Catalase test was performed by placing a drop of hydrogen peroxide on slides and mixing the colony of the bacteria to be tested thoroughly. Presence of bubbles indicates the positive result. Indole test was performed by inoculating the buffered peptone water broth with the bacteria to be tested, followed by incubation for 24 hours at 37°C. The next day tubes were observed after adding Kovac's reagent. Formation of cherry red colored ring on the surface of the broth medium indicates positive result. Methyl red test was performed by inoculating the target organisms into MR medium and incubating for 48 hours at 37°C. Formation of red colored ring on the surface of the broth medium indicates positive result. Voges-Proskauer test was performed by inoculating the target organisms into VP medium and incubating for 72 hours at 37°C. Formation of red colored ring on the surface of the broth medium indicates positive result. The organisms to be tested were inoculated onto Simmon's citrate agar medium slant and incubated for 24 hours at 37°C. The turning of the media slant into Prussian blue color indicates the positive result. TSI agar slant was inoculated by the organisms to be tested and incubated for 24 hours at 37°C. Glucose fermentation is indicated by yellow butt, lactose fermentation by yellow slant and H_2S production by blackening of the medium and gas production by presence of bubble or gas space.

4.5 Characterization of field isolates by using coagulase test

Staphylococcus show positive and *Streptococcus* show negative result

Figure 4.1: Coagulase test of *Streptococcus* sp. and *Staphylococcus* sp. by rabbit serum

4.6 Result of PCR, Amplification of 23S rRNA Gene with following Primers of *E. coli*

Out of 48 samples of *E. coli* was present in 48 cases and the percentage was 100%, 23S rRNA gene region was amplified with the Primer Eco 223 (F); Eco 445 (R)

PCR Amplification band was found at 232 bp.

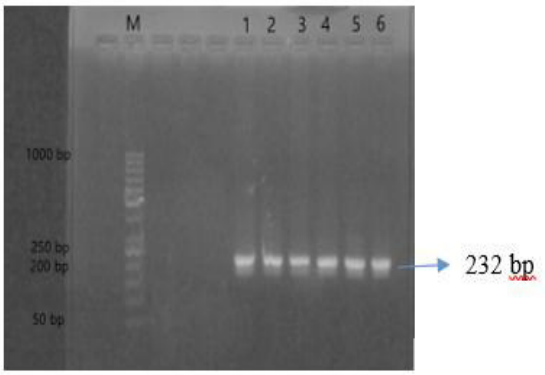


Figure 4.2: Results of PCR of *E. coli*

Note: M: Marker 100 bp Plus DNA ladder

PCR: Polymerase Chain Reaction

4.6.1 Sequence of *Escherichia coli* Forward and Reverse Primer

4.6.1.1 Forward Primer Sequence

>E_F

GRRRMGGGGGCRCAGCCCAGAGTCTGATCAGCTTGTGTGTTAGTGGAACG

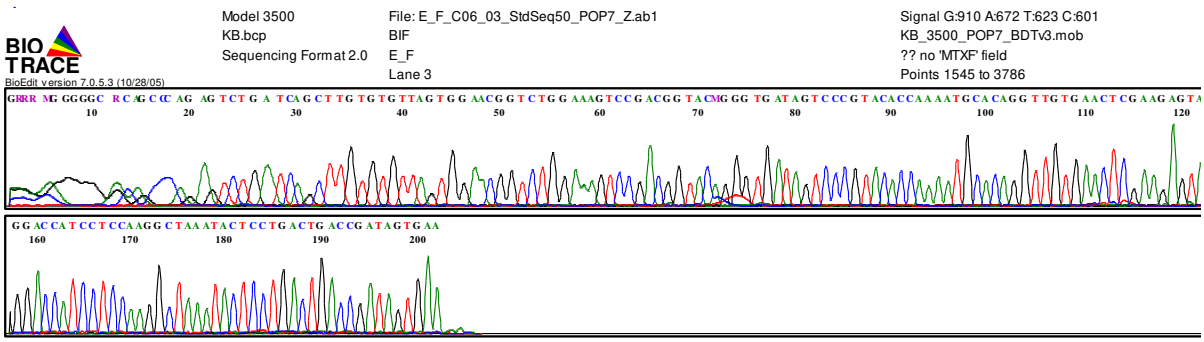
GTCTGGAAAGTCCGACGGTACMGGGTGATAGTCCCGTACACCAAAATGCA

CAGGTTGTGAACTCGAAGAGTAGGGCGGGACACGTGGTATCCTGTCTGAA

TATGGGGGGACCATCCTCCAAGGCTAAATACTCCTGACTGACCGATAGTG

AA

4.6.1.2 Chromatogram of Forward (F) Primer



4.6.1.3 Reverse Primer Sequence

>E_R

CTKGGAGGAWGGGTCCCCCCATATTCAGACAGGATACCACGTGTCCCGCC

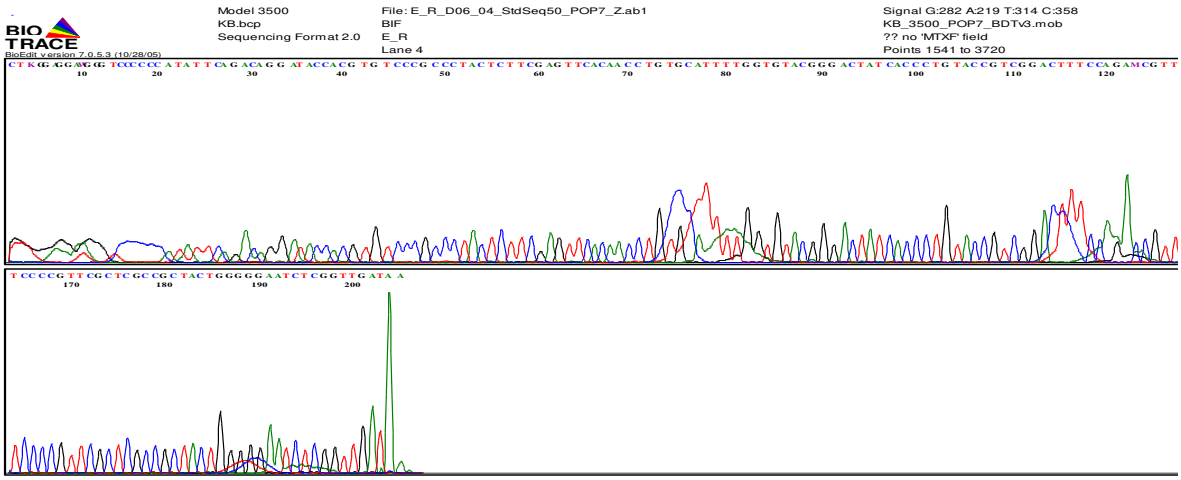
CTACTCTTCGAGTTCACAACCTGTGCATTTTGGTGTACGGGACTATCACC

CTGTACCGTCGGACTTTCCAGAMCGTTCCACTAACACACAAGCTGATTCA

GACTCTGGGCTGCTCCCCGTTCGCTCGCCGCTACTGGGGGAATCTCGGTT

GATAA

4.6.1.4 Chromatogram of Reverse (R) Primer



4.7 Characterization of *Staphylococcus aureus* by DNA Amplification, Sequencing of *Staphylococcus aureus* with 23S rRNA Gene Amplification

Out of 48 samples of *Staphylococcus aureus* was present in 48 cases and the percentage was 100%, 23S rRNA gene region was amplified with the Primer Sau 234 (F); Sau 1501 (R).

PCR Amplification band was found at 1267 bp.

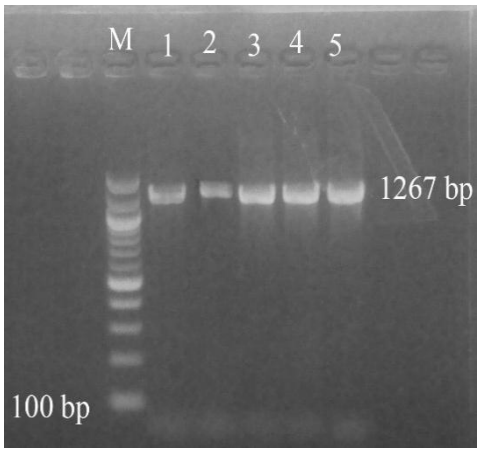


Figure 4.3: Results of PCR of *Staphylococcus aureus*

Note: M: Marker 100 bp Plus DNA ladder

PCR: Polymerase Chain Reaction

4.7.1 Sequence of *Staphylococccus aureus* forward and Reverse Primer

4.7.1.1 Forward Primer Sequence

>13-F

CRRRSRKCCAWWMSAWSGAGCTTGCTTGTTGGGGTTGTAGGACACTCTAT

ACGGAGTTACAAAGGAATAAATTAKACGAATCATCTGGAAAGATGAATCA

TAGAAGGTAATAAYCCTGTAGTCGAAAGTTTATTCTCTCTTGAGTGGATC

CTGAGTACGACGGAGCACGTGAAATTCCGTCGGAATCTGGGAGGACCATC

TCCTAAGGCTAAATACTCTCTAGTGACCGATAGTGAACCAGTACCGTGAG

GGAAAGGTGAAAAGTACCCCGGAAGGGGAGTGAAATAGAACTTGAAACCG

TGTGCTTACAAGTAGTCAGAGCCCGTTAATGGGTGATGGCGTGCCTTTTG

TAGAATGAACCGGCGAGTTACGATTTGATGCAAGGTTAAGCAGTGAATGT

GGAGCCGTAGCGAAAGCGAGTCTGAATAGGGCGTTGAGTATTTGGTCGTA

GACCCGAAACCAGGTGATCTACCCATGACCAGGCTGAAGTTCAGGTAACA

CTGAATGGAGGGCCGAACCGACTTACGTTGAAAAGTGAGCGGATGAGTTG

TGGGTAGCGGAGAAATTCCAATCGAACCTGGAGATAGCTGGTTCTCTCCG

AAATAGCTTTAGGGCTAGCCTCAAGTGATGATTATTGGAGGTAGAGCACT

GTTTGGACGAGGGGCCCTTCTCGGGTTACCGAATTCAGACAAACTCCGAA

TGCCAATCAATTTAACTTGGGAGTCAGAACGCGGGTGATAAGGTCCGTGT

TCGAAAGGGAAACAGCCCAGACCACCAGCTAAGGTCCCAAAATATATGTT

AAGTGGAAAAGGATGTGGCGTTGCCCAGACAACTAGGATGTTGGCTTAAA

GCAGCCATCATTTAAAGAGTGCGTAATAGCTCACTAGTCGAGTGACACTG

CGCCGAAATGTACCCGGGGCTAAAMATATTACCGAAGCTGTGGATTGTCC

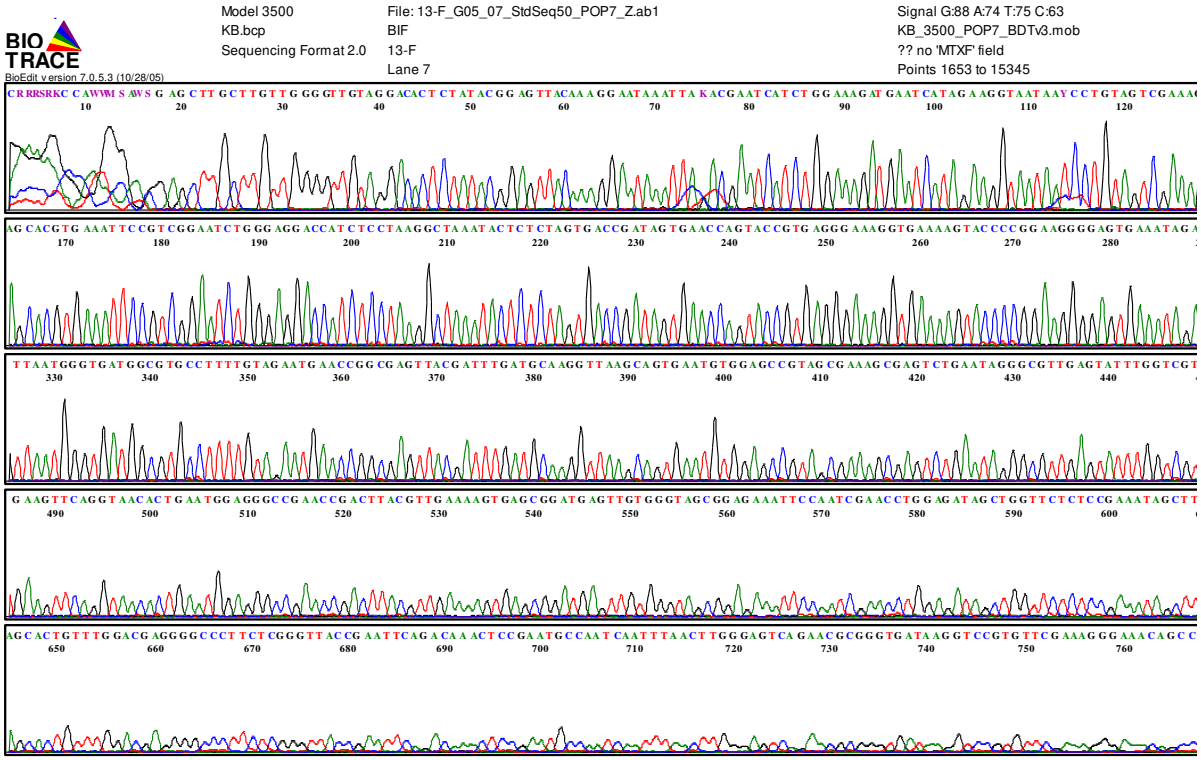
GTAGGACAATGGTAGGAGAGCGTTMTAAGGGCGTTGAAGCTTGATCGCAG

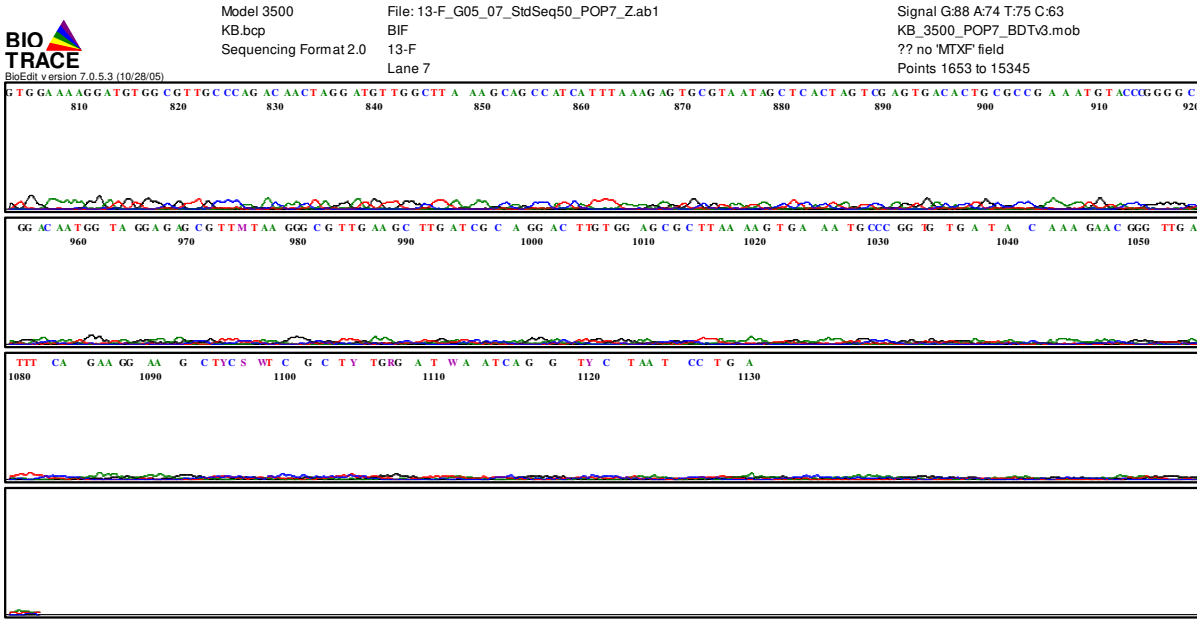
GACTTGTGGAGCGCTTAAAAGTGAAATGCCCGGTGTGATACAAAGAACGG

GTTGAAATCCCGTCCACCGAATGAYTAAGTTTCAGAAGGAAGCTYCSWTC

GCTYTGRGATWAATCAGGTYCTAATCCTGA

4.7.1.2 Chromatogram of Forward (F) Primer





4.7.1.3 Reverse Primer Sequence

>13_R

CAWYGACGAWCGMATKAAACGAWTATGGTGGTACAGGAATATCAACCTGT

TATCCATCGCCTACGCCTGTCGGCCYCARCTTAGGACCCGACTAACCCAG

AGCGGACGAGCCTTCCTYTGRAAACCTTAGTCAATCGGTGGACGGGATTC

TCACCCGTCTTTCGCTACTCACACCGGCATTCTCACTTCTAAGCGCTCCA

CATGTCCTTGCGATCATGCTTCAACGCCCTTAGAACGCTCTCCTACCATT

GTCCTACGGACAATCCACAGCTTCGGTAATATGTTTAGCCCCGGTACATT

TTCGGCGCAGTGTCACTCGACTAGTGAGCTATTACGCACTCTTTAAATGA

TGGCTGCTTCTAAGCCAACATCCTAGTTGTCTGGGCAACGCCACATCCTT

TTCCACTTAACATATATTTTGGGACCTTAGCTGGTGGTCTGGGCTGTTTC

CCTTTCGAACACGGACCTTATCACCCGCGTTCTGACTCCCAAGTTAAATT

GATTGGCATTCGGAGTTTGTCTGAATTCGGTAACCCGAGAAGGGCCCCTC

GTCCAAACAGTGCTCTACCTCCAATAATCATCACTTGAGGCTAGCCCTAA

AGCTATTTCGGAGAGAACCAGCTATCTCCAGGTTCGATTGGAATTTCTCC

GCTACCCACAACTCATCCGCTCACTTTTCAACGTAAGTCGGTTCGGCCCT

CCATTCAGTGTTACCTGAACTTCAGCCTGGTCATGGGTAGATCACCTGGT

TTCGGGTCTACGACCAAATACTCAACGCCCTATTCAGACTCGCTTTCGCT

ACGGCTCCACATTCACTGCTTAACCTTGCATCAAATCGTAACTCGCCGGT

TCATTCTACAAAAGGCACGCCATCACCCATTAACGGGCTCTGACTACTTG

TAAGCACACGGGTTTCAAGTTCTATTTCACTCCCCYTTCCGGGGTACTTT

TCACCTTTCCCTCACGGTACTGGGTTCACTATCGGTCACTAGARAGTATT

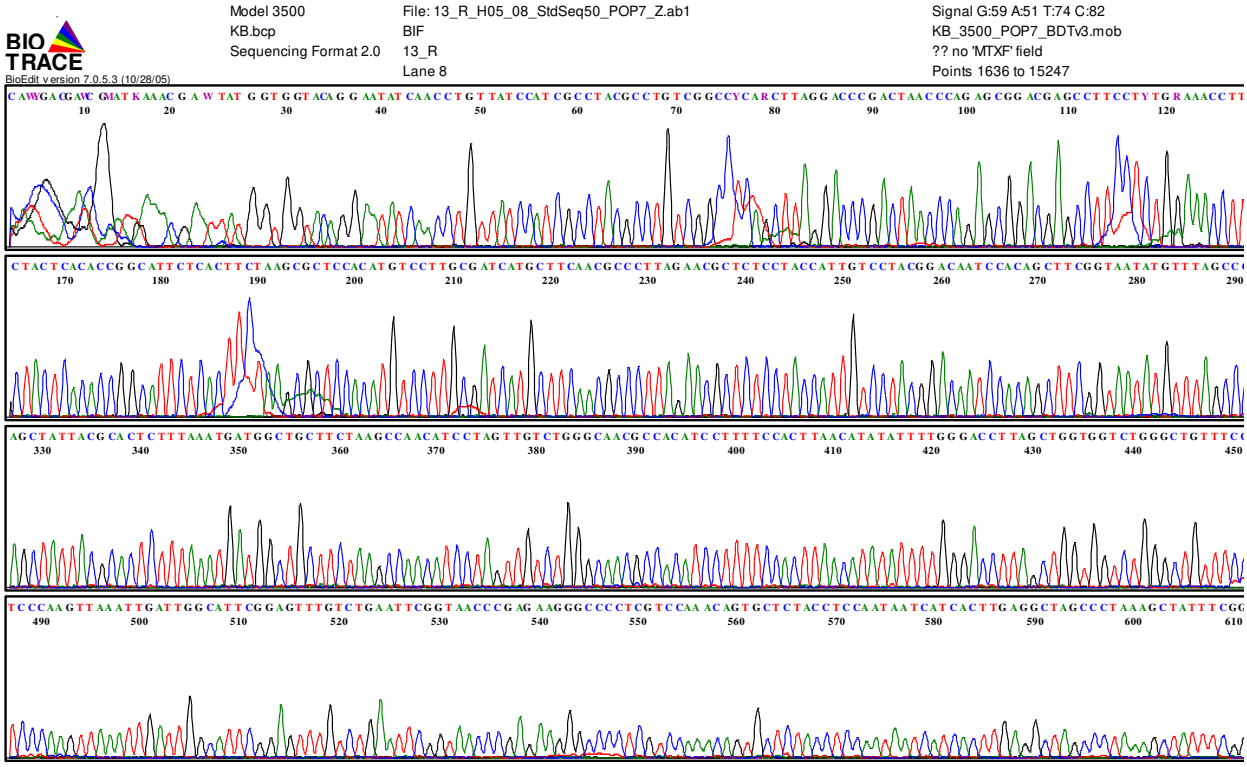
TWAGCCTTACGARAATGGTTCCTCCCARATTCCGACGGAATTTCACGTGC

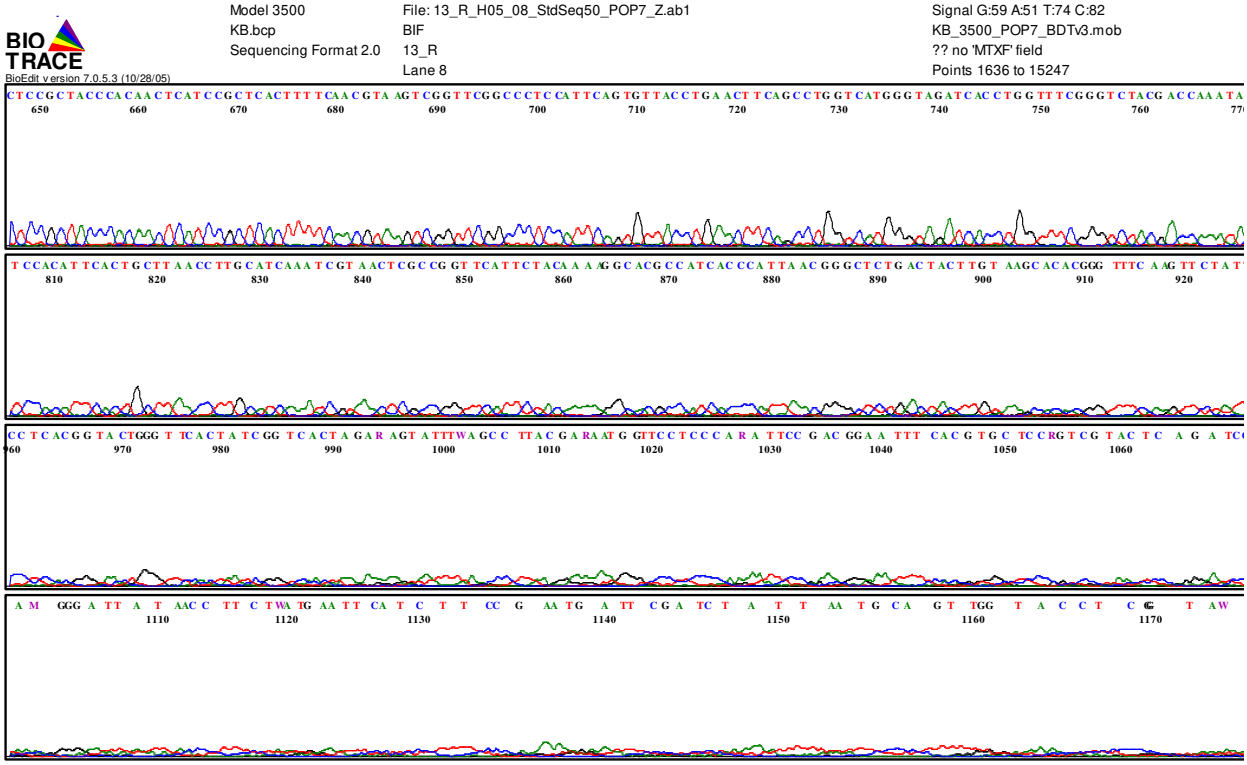
TCCRGTCGTACTCAGATCCMCTCAGAAGAGAAKTASASSSKTKTSSRACT

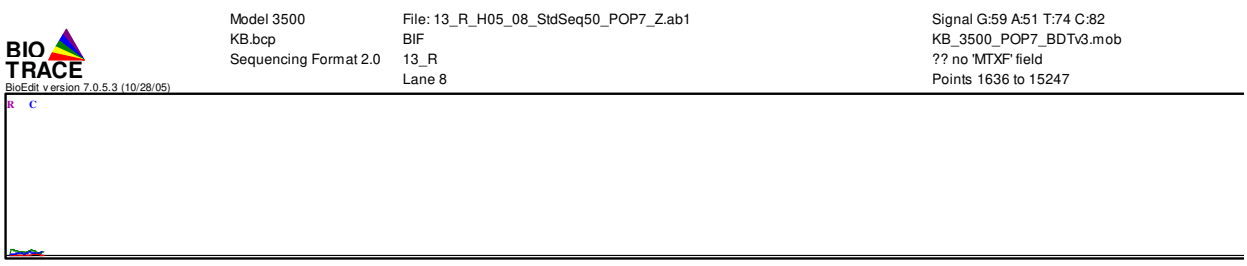
AMGGGATTATAACCTTCTWATGAATTCATCTTCCGAATGATTCGATCTAT

TAATGCAGTTGGTACCTCGGTAWGAGAGTGTYCCCTTACACRC

4.7.1.4 Chromatogram of Reverse (R) Primer







4.8 Characterization of *Streptococcus agalactiae* by Molecular Technique

4.8.1 Characterization by DNA Amplification with PCR

Out of 48 samples of *Streptococcus* sp. was present in 28 cases and the percentage was 58%, was amplified with the specific primers Sag 40 (F); Sag 445 (R) that revealed *Streptococcus agalactiae.*

PCR Amplification band was found at 405 bp.

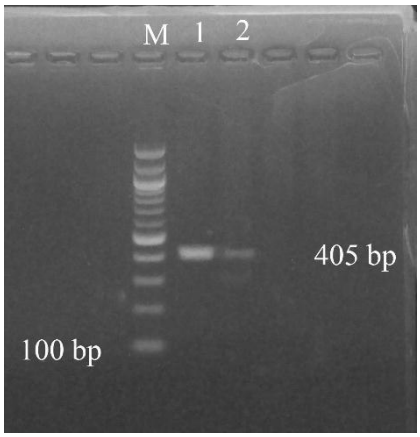


Figure 4.4: Results of PCR of *Streptococcus agalactiae*

Note: M: Marker 100 bp Plus DNA ladder

PCR: Polymerase Chain Reaction

4.9 Characterization of *Streptococcus* sp. by DNA Amplification, Sequencing by Following Primers

4.9.1 Characterization by PCR, 23S rRNA Gene Region Amplification

Out of 48 samples of *Streptococcus* sp. was present in 28 cases and the percentage was 58%, 23S rRNA gene region was amplified with the universal primer Uni 1870 (F); Uni 2308 (R)

PCR Amplification band was found at 438 bp.

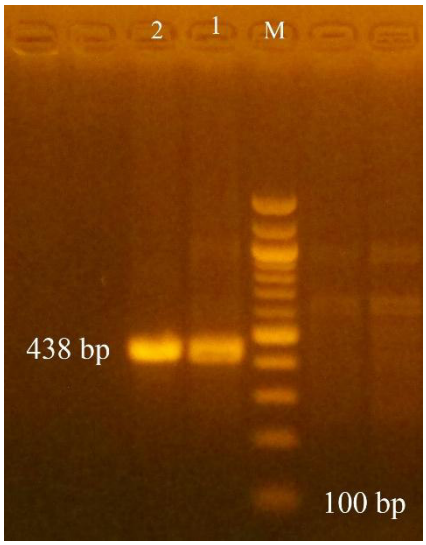


Figure 4.5: Results of PCR of *Streptococcus* sp.

Note: M: Marker 100 bp Plus DNA ladder

PCR: Polymerase Chain Reaction

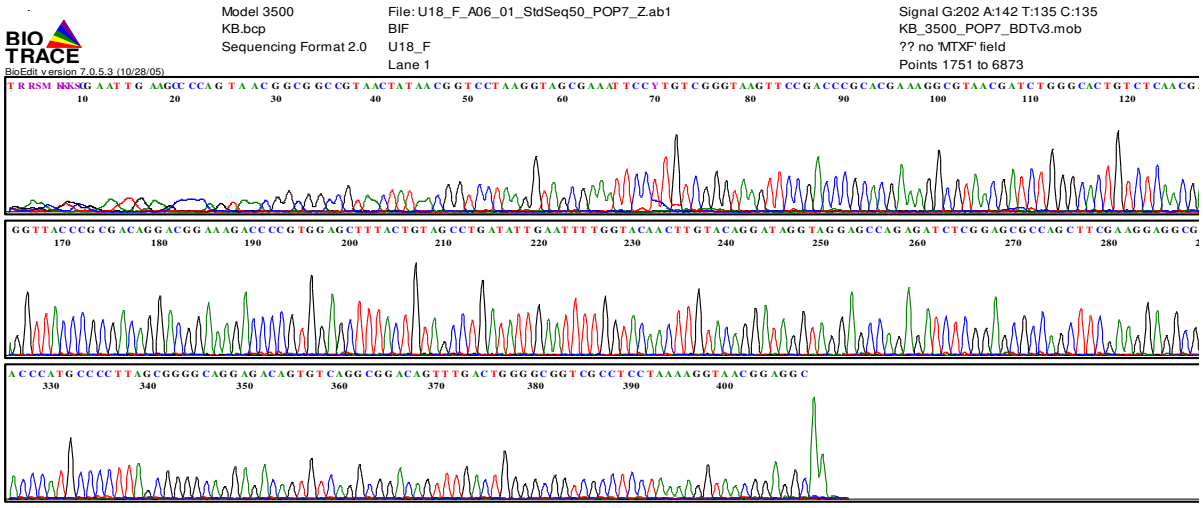
4.9.2 Sequence of *Streptococcus* sp. Forward and Reverse Primer

4.9.2.1 Forward Primer Sequence

>U18_F

TRRSMKKKSCGAATTGAAGCCCCAGTAACGGCGGCCGTAACTATAACGGT
CCTAAGGTAGCGAAATTCCYTGTCGGGTAAGTTCCGACCCGCACGAAAGG
CGTAACGATCTGGGCACTGTCTCAACGAGAGACTCGGTGAAATTATAGTA
CCTGTGAAGATGCAGGTTACCCGCGACAGGACGGAAAGACCCCGTGGAGC
TTTACTGTAGCCTGATATTGAATTTTGGTACAACTTGTACAGGATAGGTA
GGAGCCAGAGATCTCGGAGCGCCAGCTTCGAAGGAGGCGTCGGTGGGATA
CTACCCTGGTTGTATTGAAATTCTAACCCATGCCCCTTAGCGGGGCAGGA
GACAGTGTCAGGCGGACAGTTTGACTGGGGCGGTCGCCTCCTAAAAGGTA
ACGGAGGC

4.9.2.2 Chromatogram of Forward (F) Primer



4.9.2.3 Reverse Primer Sequence

>U18_R

CMTYYAMTGTCCGCCTGAMCTGTCTCCTGCCCCGCTAAGGGGCATGGGTT

AGAATTTCAATACAACCMSGKTAGTATCCCACCGACGCCTCCTTCGAAGC

TGGCGCTCCGAGATCTCTGGCTCCTACCTATCCTGTACAAGTTGTACCAA

AATTCAATATCAGGCTACAGTAAAGCTCCACGGGGTCTTTCCGTCCTGTC

GCGGGTAACCTGCATCTTCACAGGTACTATAATTTCACCGAGTCTCTCGT

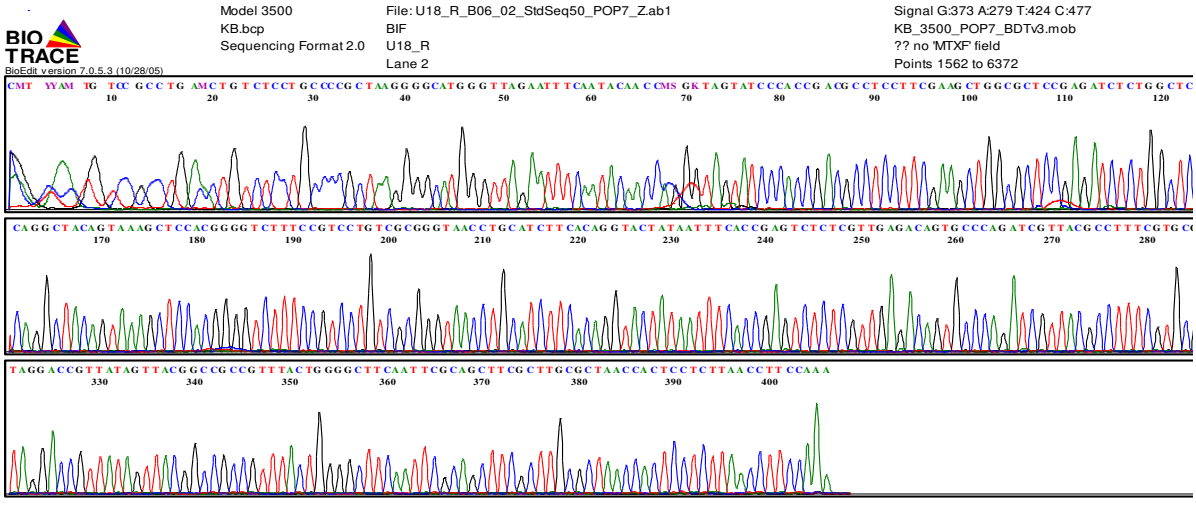
TGAGACAGTGCCCAGATCGTTACGCCTTTCGTGCGGGTCGGAACTTACCC

GACAAGGAATTTCGCTACCTTAGGACCGTTATAGTTACGGCCGCCGTTTA

CTGGGGCTTCAATTCGCAGCTTCGCTTGCGCTAACCACTCCTCTTAACCT

TCCAAA

4.9.2.4 Chromatogram of Reverse (R) Primer



4.10 Characterization of *Klebsiella* sp. by Molecular Technique

4.10.1 Characterization by DNA Amplification with PCR

Out of 48 samples of *Klebsiella* sp. was present in 30 cases and the percentage was 62.5 %, was amplified with the Universal Primers (16S rRNA); Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') Reverse primer- 1492R (5' TACCTT GTTACGACTT 3'). During PCR band was found at 1492 bp. Generally, band visualized in between 1466 bp to 1500 bp according to Lane *et al.* (1991); Devereux and Wilkinson (2004) and Anna *et al*., (2010).

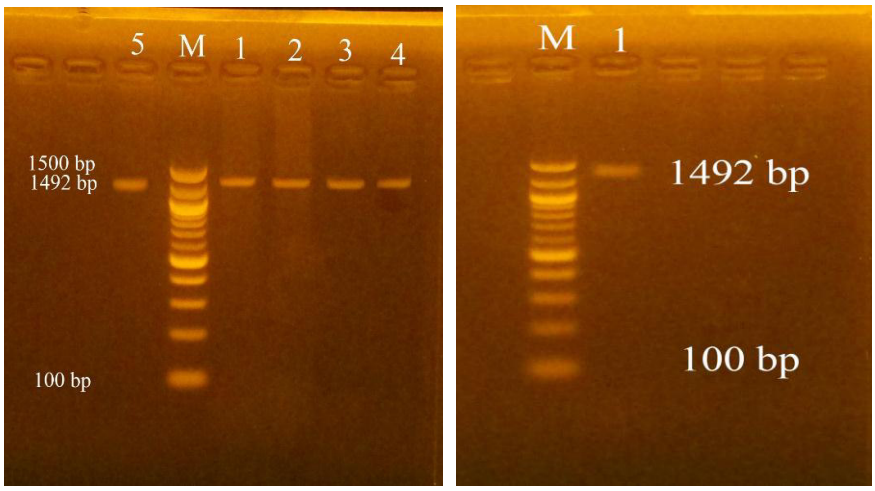


Figure 4.6: Results of PCR of *Klebsiella* sp.

Note: M: Marker 100 bp Plus DNA ladder

PCR: Polymerase Chain Reaction

4.11 Molecular Detection of *Streptococcus agalactiae* by RT-PCR (Real Time Polymerase Chain Reaction)

Extracted DNA run with Go taq prob and reference primer. PCR max (Eco 48) showed the following graph. That indicates suspected samples contain *Streptococcus agalactiae.* Samples are arranged 1, 2, 3,4 and NTC up to down. The florescence vs cycle (40) given in APPENDIX-IV. Gradually upward graph indicates that samples are contain suspected nucleic acid. DNA are detected by florescence. For RT-PCR primer needed with 200 bp or below 200 bp. Here we performed RT-PCR to observe the pattern of graph and what type of graph produced by *Streptococcus agalactiae* which were positive and confirmed by PCR.

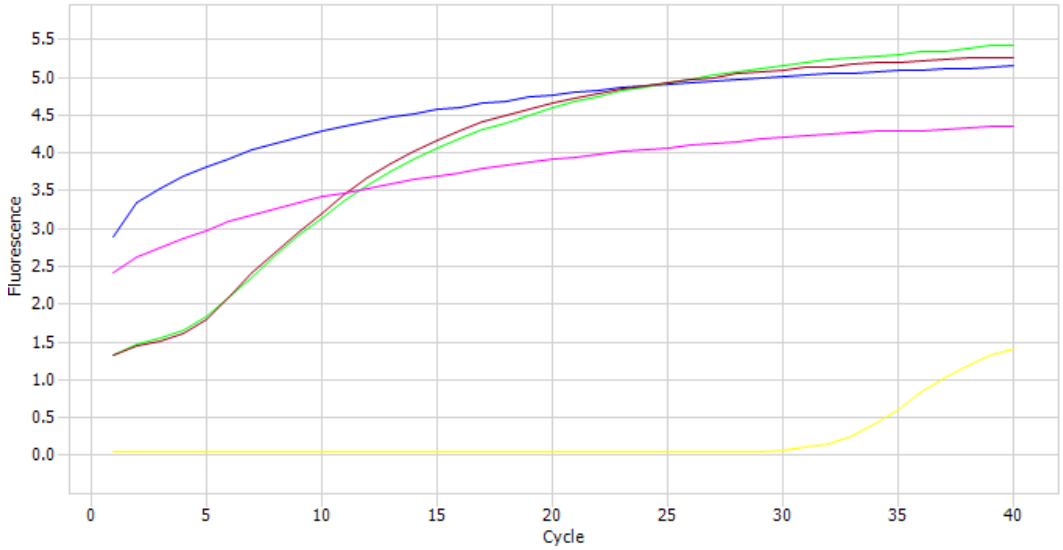


Figure 4.7: Results of RT- PCR of *Streptococcus agalactiae*

Legends- Yellow line is Negative control (NTC), Green, Purple, Pink and Blue lines are sample 1, 2, 3 and 4.

4.12 Phylogenetic Tree Result

4.12.1 Phylogenetic tree of *Staphylococcus aureus* (23S rRNA)

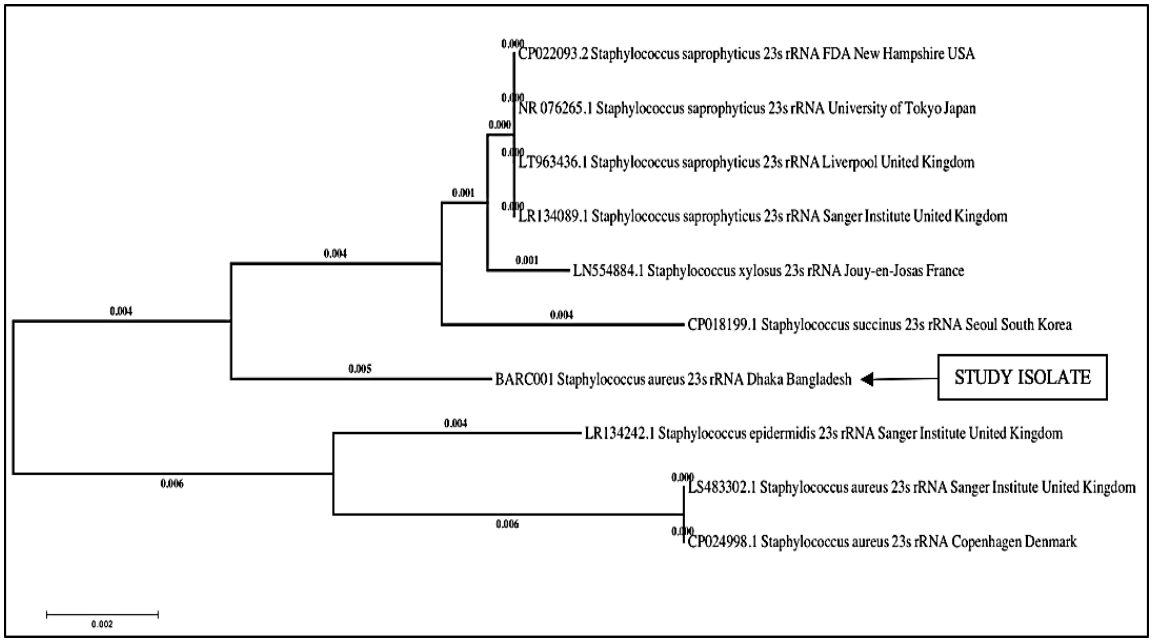


Figure 4.8: Phylogenetic tree of *Staphylococcus aureus* (23S rRNA)

Phylogenetic tree of *Staphylococcus aureus* 23S rRNA gene sequence of present study isolates and related Gene bank sequence. The tree was inferred using the

clastalWmultiple sequence alignment and Neighbor-Joining method Saitou and Nei (1987) using MEGA software (Kumar *et al.,* 2016). Bootstrap value was used as 1000 for tree clustering. The evolutionary history, the optimal tree with the sum of branch length = 0.03591732 is shown. Number at the nodes represents the level of bootstrap support based on the neighbor-joining analysis Saitou N. and Nei M. (1987). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method Tamura K. *et al.,* (2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 1034 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar S. *et al.,* 2016).

4.12.2 Phylogenetic tree of *E. coli*

23S rRNA gene region was amplified with the Primer Eco 223 (F); Eco 445 (R)

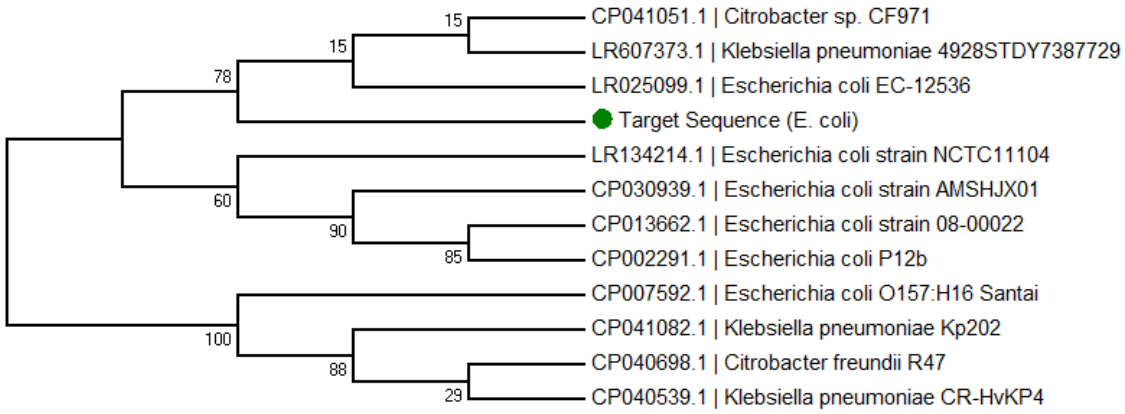


Figure 4.9: Construction of Phylogenetic tree of Target Sequence (*E. coli*) and its Phylogenetic Neighbours

Methods used in the study

Bootstrap: 1000 replicates (It construct the phylogenetic tree for 1000 times and show the average maximum number of repeats for best result)

Method: Neighbor joining

4.12.3 Phylogenetic tree of *Streptococcus* sp. (suspected species-*S. agalactiae*)

23S rRNA gene region was amplified with the universal primer Uni 1870 (F); Uni 2308 (R)

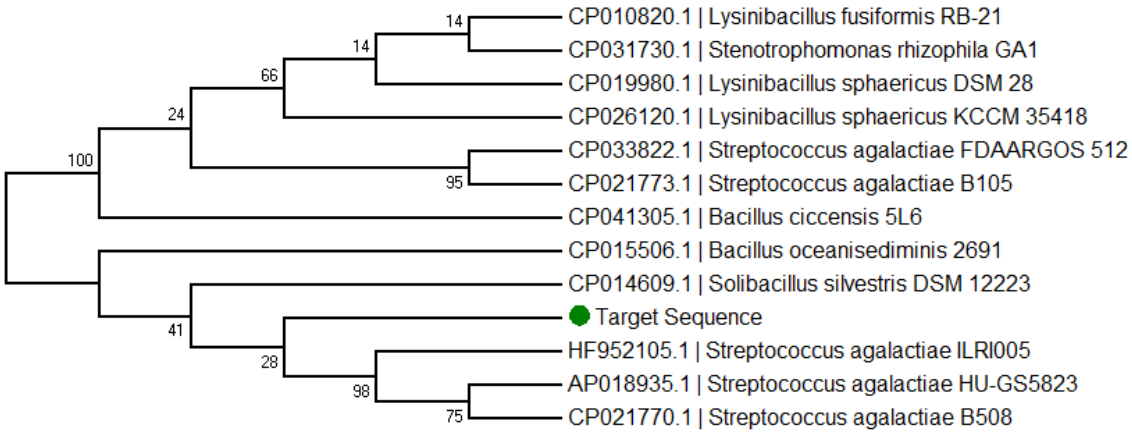


Figure 4.10: Construction of Phylogenetic tree of Target Sequence (*S. agalactiae*) and its Phylogenetic Neighbors

Methods used in the study

Bootstrap: 1000 replicates (It construct the phylogenetic tree for 1000 times and show the average maximum number of repeats for best result)

Method: Neighbor joining

4.13 Antimicrobial Susceptibility Pattern for *Escherichia coli*, *Klebsiella* sp. and *Staphylococcus aureus* and *Streptococcus* sp.

Table 4.5: Antibiogram Study of all Isolates

		Antibacterial agents and Disc concentration (µg/disc)															
Isolates	Diameter of zone of inhibition(mm)	AMX (30)	C (30)	NX (10)	TE (30)	GEN (10)	B (10)	AZM (30)	E (15)	NV (30)	MET (5)	VA (30)	LE (5)	CIP (5)	CH (25)	NA (30)	Fly
E.coli	Sensitive	20 mm	23 mm	30 mm	13 mm	23 mm		20 mm					33 mm	36 mm		23 mm	
	Intermediate																22 mm
	Resistant						0		0	0	0	0			0		
Streptococcus sp. Including *Streptococcus agalactiae*	Sensitive			1 mm	21 mm	22 mm		15 mm	12 mm				1 mm			1 mm	12 mm
	Intermediate																
	Resistant	0	0				0			0	0	0		0	0		
Staphylococcus aureus	Sensitive	15 mm		19 mm		21 mm		23 mm					26 mm	29 mm			
	Intermediate		20 mm				11 mm		17 mm							24 mm	
	Resistant				0					0	0	0			0		0
Klebsiella sp.	Sensitive		24 mm	25 mm		19 mm		25 mm					22 mm	30 mm		17 mm	
	Intermediate																
	Resistant	0			0		0		0	0	0	0			0		0

(**Legends:** GEN = Gentamicin, AMX = Amoxicillin, C = Chloramphenicol, CIP = Ciprofloxacin, AZM = Azithromycin, E = Erythromycin, VA = Vancomycin, NX = Norfloxacin, TE = Tetracycline, B = Bacitracin, NV = Novobiocin, MET = Methicillin, LE = Levofloxacin, CH = Cephradine, NA = Nalidixic acid, 0 = No zone of inhibition.)

From the table 4.5 most of the isolates were MDR. CIP (5 µg/disk) produced highest (36 mm) zone in the case of *E. coli* and *Klebsiella* sp. showed 30 mm zone. All the isolates were completely resistant to Methicillin, Novobiocin and Vancomycin. Gentamicin and Azithromycin showed good antimicrobial response against all the isolates. This two and Ciprofloxacin, Levofloxacin, Norfloxacin and Chloramphenicol are very much effective in the case of mastitis in dairy cows. House fly showed highest zone (22 mm) in case of *E. coli* which is notoriously appeared in current study. Extracted antibiotic from the house fly might be the alternative choice to treat BM.

4.14 Development of Vaccine Seed

In tenfold dilution desire CFUs were found in tube 6,7, 8. Pure bacterial culture which were formalin killed taken in a tube. After required centrifuge and resuspension with normal saline. Suspended solution and adjuvant were taken equally (Both were taken @5ml) in a tube to formulate bulk volume. All processes were maintained according to previously mentioned references. Bulk 40 ml PFIV candidate stored in 4°C until further use.

Figure 4.11: Montanide@ISA 206 Adjuvant Mixed with the Formalin Killed Bacterial Samples and Making Seed

4.14.1 Result of Vaccine Seed Management and Commercialization

Quality control like sterility and safety of vaccine candidate was good. There was no other organism except *E. coli, Staphylococcus aureus, Streptococcus* sp. and *Klebsiella* sp. Only above mentioned isolates were available in vaccine candidate observed by culturing on different selective media. The formalin inactivated vaccine candidate containing Montanide adjuvants are reported to have no toxic effect even after booster dose (Barnett *et al.,* 1996). Field trial and immune response/antibody titer of inactivated vaccine candidate was not performed yet due to time limitation. The vaccine seed were stored at -80°C until further recommendation.

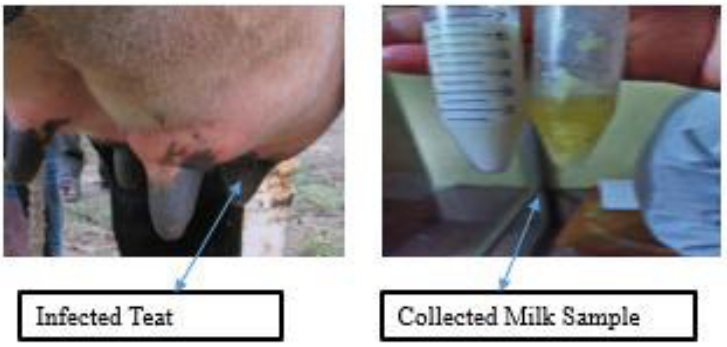


Figure 4.12: Infected Teat and Collected Milk Sample

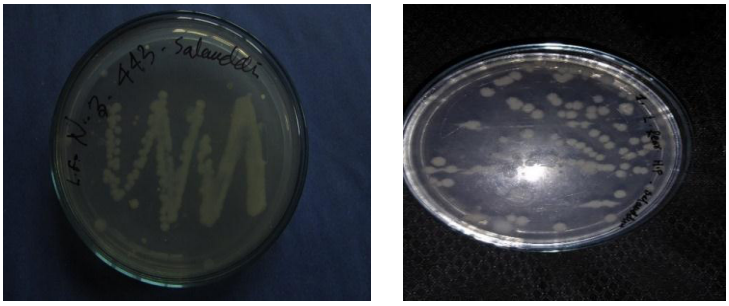


Plate 1: Nutrient Agar Media

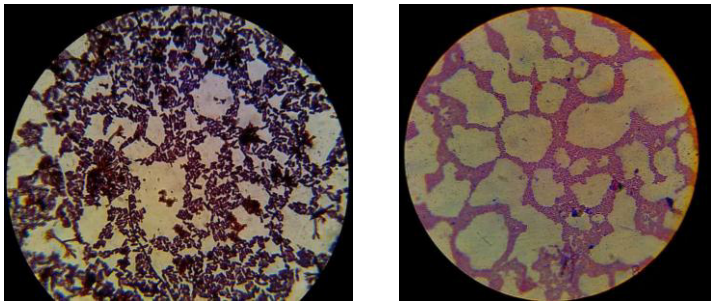


Plate 2: Microscopic view of Nutrient Agar Media

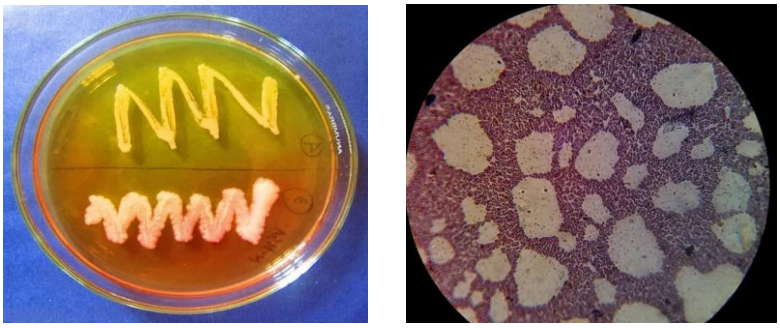


Plate 3: *Staphylococcus* sp. in MSA and Microscopic View

Plate 4: Pink Colony of *Klebsiella* sp. in EMB (Left), Blakish Pink Colony of *E. coli* (Middle) in EMB and Mixed Colony of *Klebsiella* sp. and *E. coli* (Right) in EMB Agar Media

Plate 5: *Klebsiella* sp. in MacConkey Agar Media

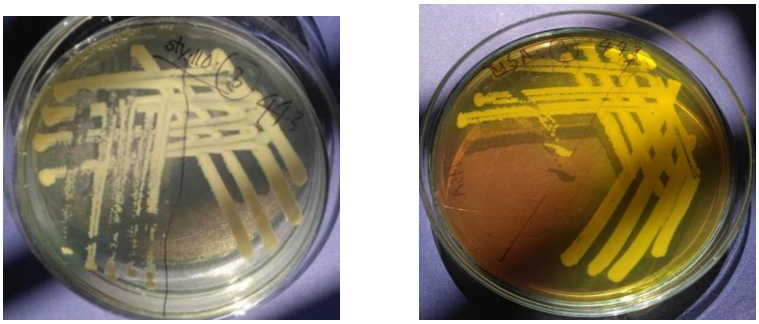


Plate 6: *Staphylococcus* sp.in Staphylococcus Agar No. 110 (Left) and *Staphylococcus* sp. in MSA (Right)

Plate 7: Pure Culture of *Streptococcus agalactiae* in Streptococcus Agalactiae Selective Agar Base (Anaerobic)

Plate 8: *E coli* in EMB (Metallic Sheen Color Colony)

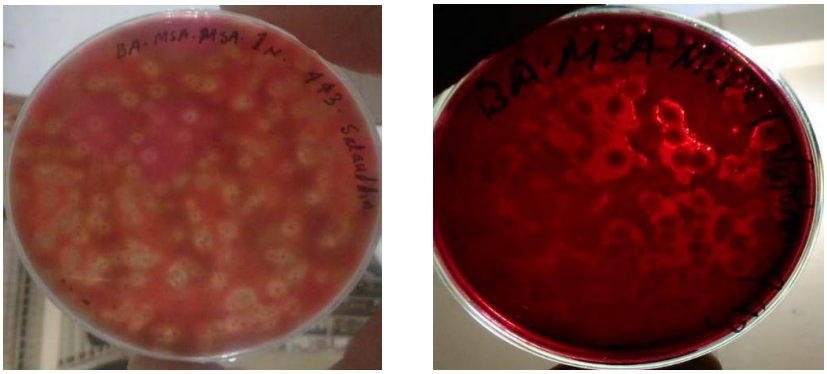


Plate 9: Sheep Blood Agar (Left) Complete Hemolysis *Streptococcus* sp. (*Streptococcus agalactiae*); Ox Blood Agar *Staphylococcus* sp. (Right)

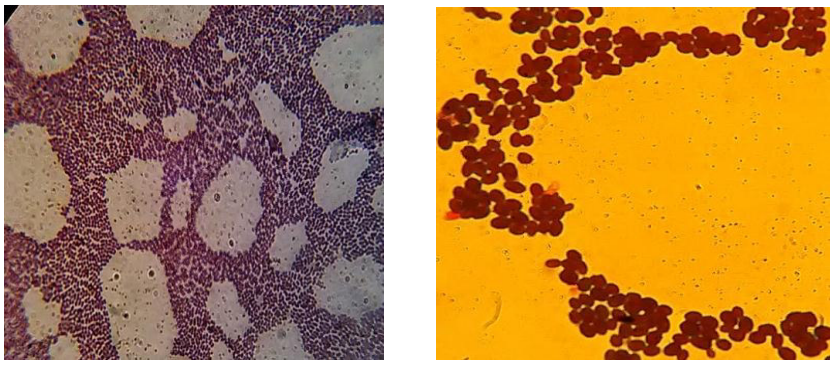


Plate 10: Microscopic View, Cluster gram positive *Staphylococcus* sp. from MSA Agar Media (Left); From Staphylococcus Agar No. 110 (Right)

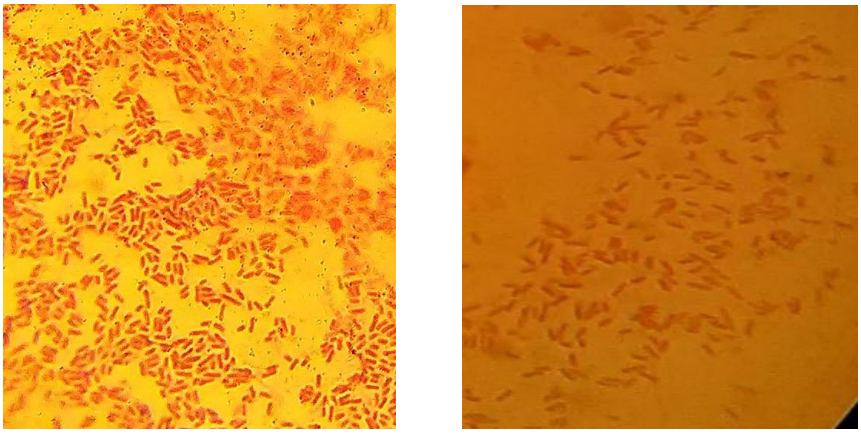


Plate 11: Microscopic view, Gram negative, short rod with capsule *Klebsiella* sp. from MacConkey Agar Media (Left); From EMB, rod shape, Gram negative *E. coli* (Right)

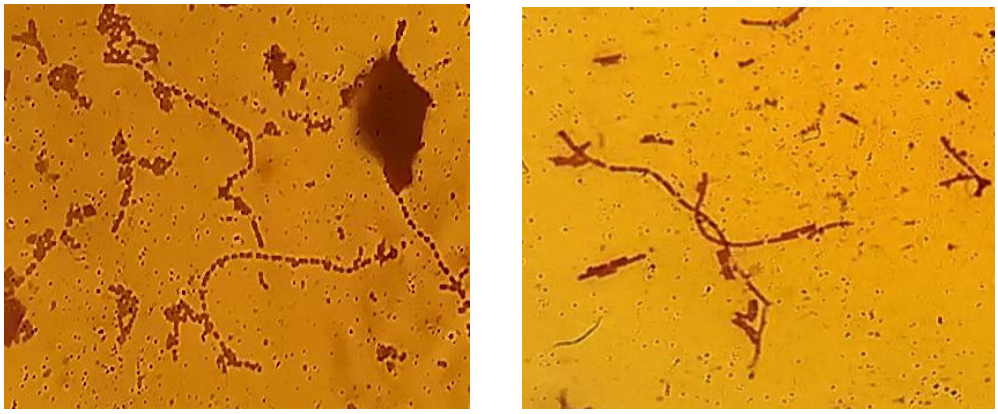


Plate 12: Microscopic View, *Streptococcus* sp. and *Streptococcus agalactiae* in Streptococcus Agalactiae Selective Agar Base (Anaerobic)

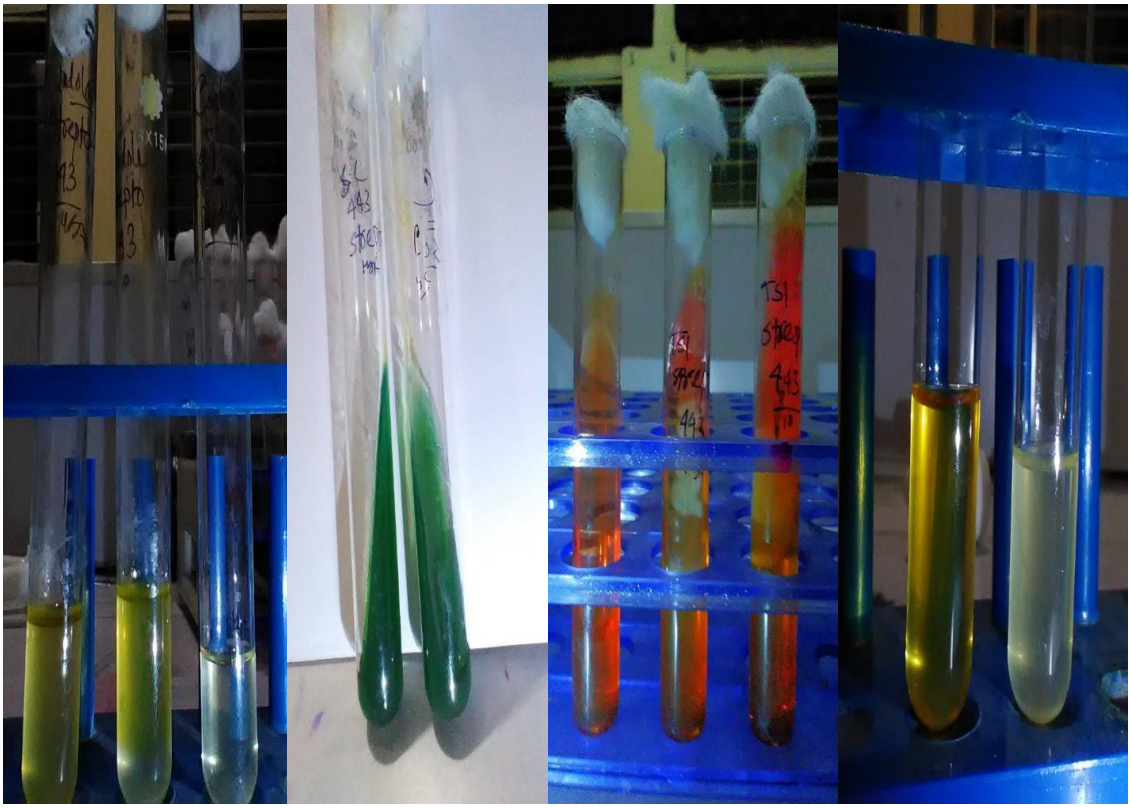


Plate 13: Biochemical test of *Streptococcus* sp. (Left to Right) Indole-, Simmon's citrate-, TSI and MR-

Plate 14: Catalase test, left side *Streptococcus* sp. show no bubble

Plate 15: Voges-Proskauer reaction, left side *Streptococcus* sp. show negative reaction

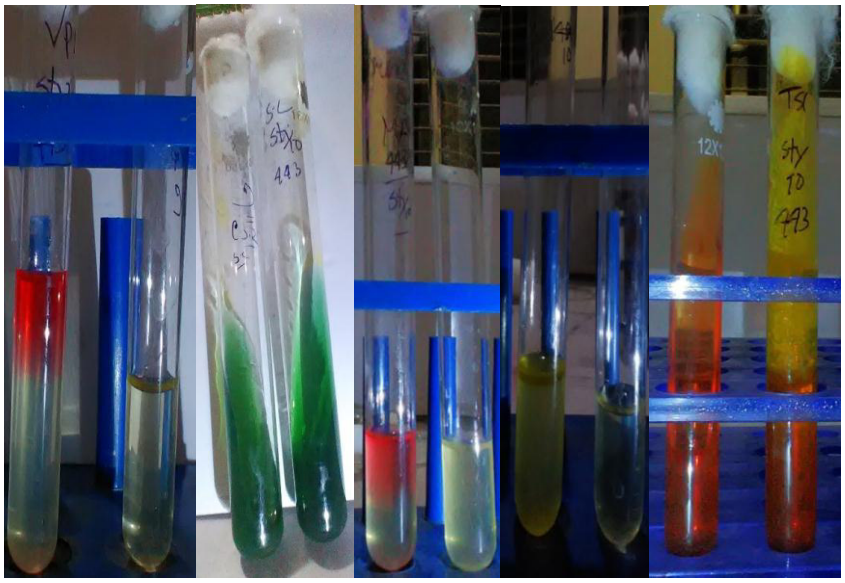


Plate 16: Biochemical test of *Staphylococcus* sp. (Left to Right) VP +, Simmon's citrate-, MR+, Indole-, TSI

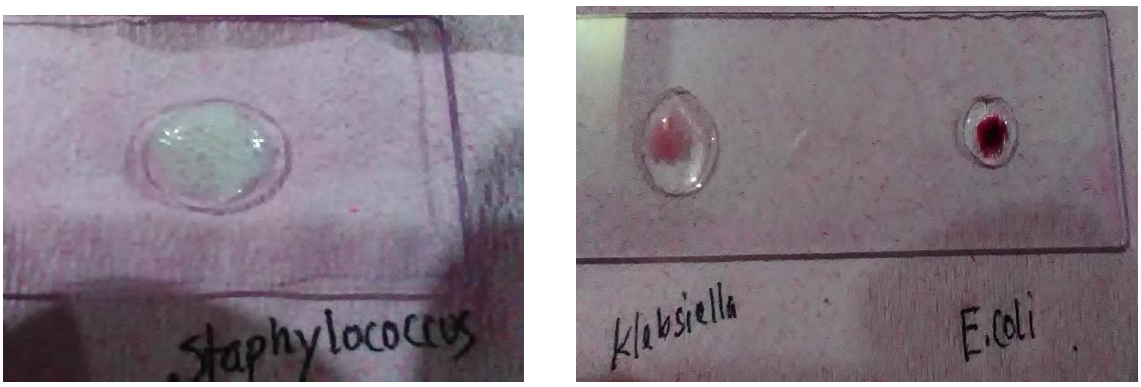


Plate 17: Catalase test, *Staphylococcus* sp. show bubble (Left); *Klebsiella* sp. give positive result and *E. coli* show no bubble (Right)

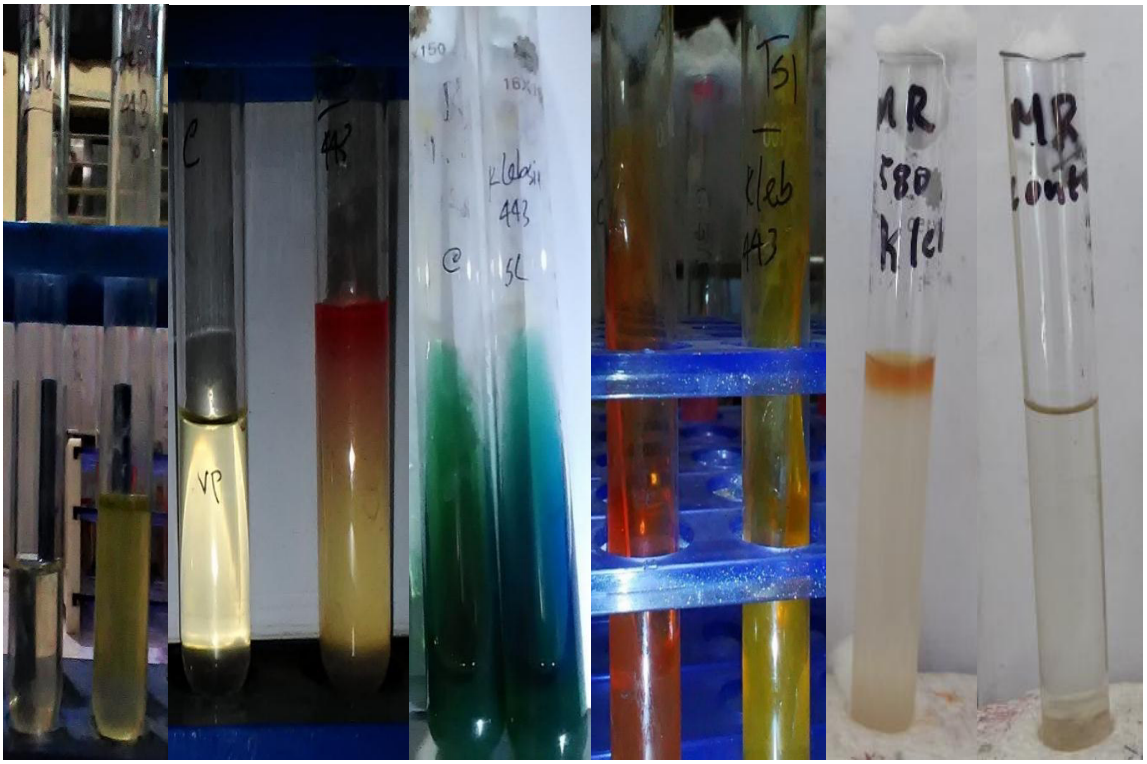


Plate 18: Biochemical test of *Klebsiella* sp. (Left to Right) Indole-, VP +, Simmon's citrate+, TSI, MR-

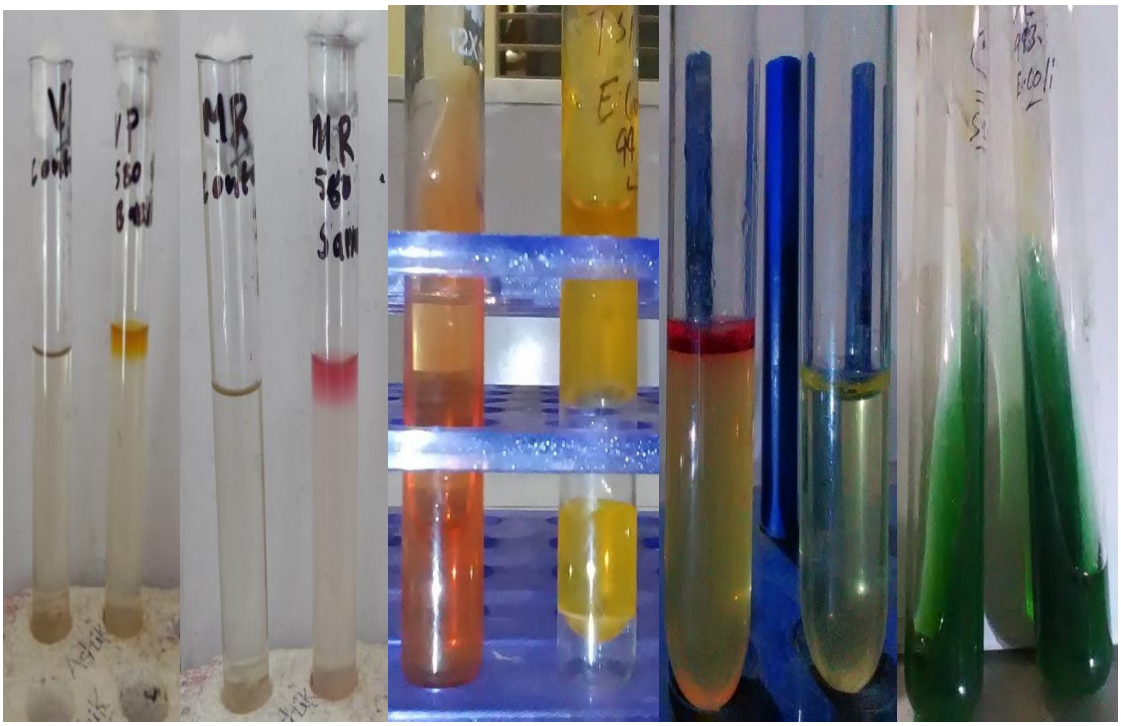


Plate 19: Biochemical test of *E. coli* (Left to Right) VP -, MR+, TSI, Indole +, Simmon's citrate-

Plate 20: DNA extraction and PCR steps at a glance Like Z alphabet

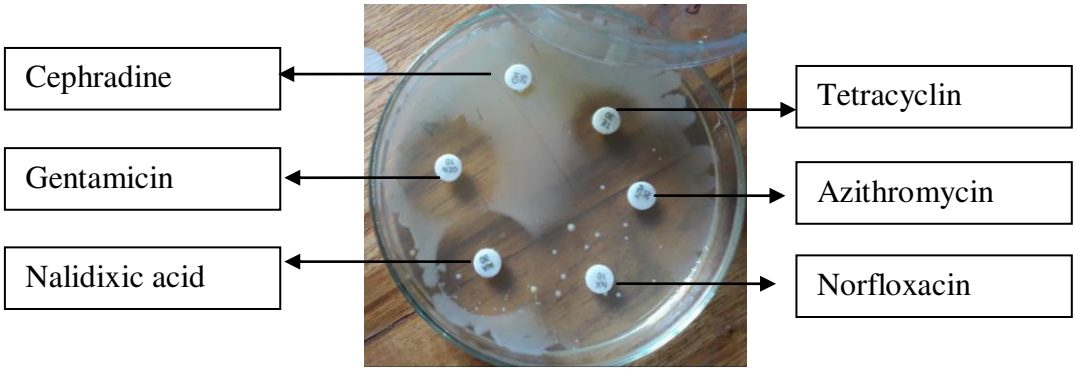


Plate 21: Antibiogram of *E. coli* in MHA

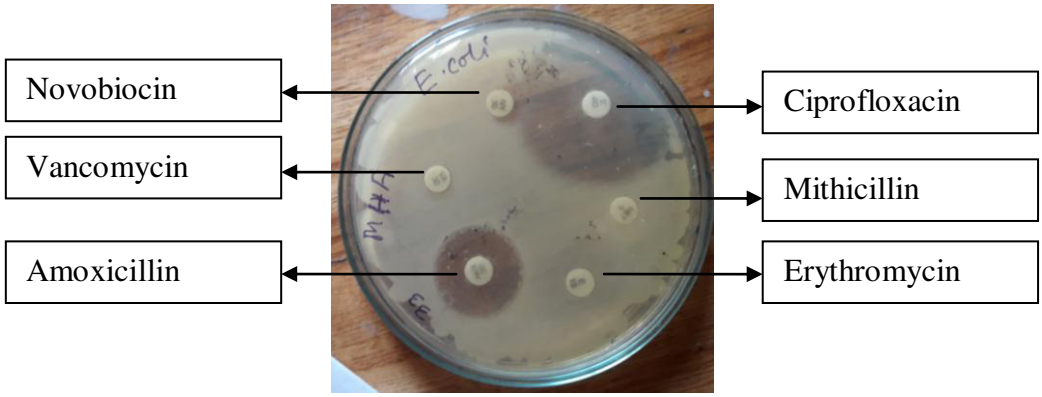


Plate 22: Antibiogram of *E. coli* in MHA

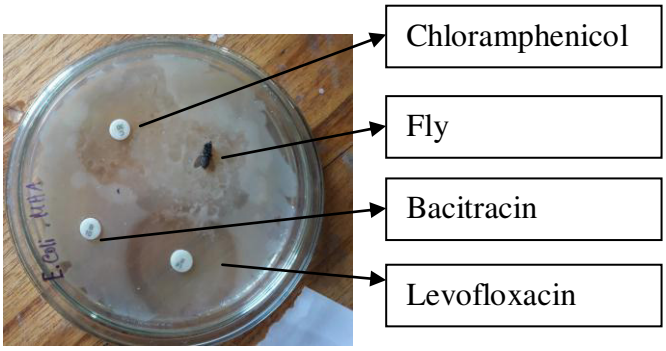


Plate 23: Antibiogram of *E. coli* in MHA

Plate 24: Antibiogram Measurement by Scale

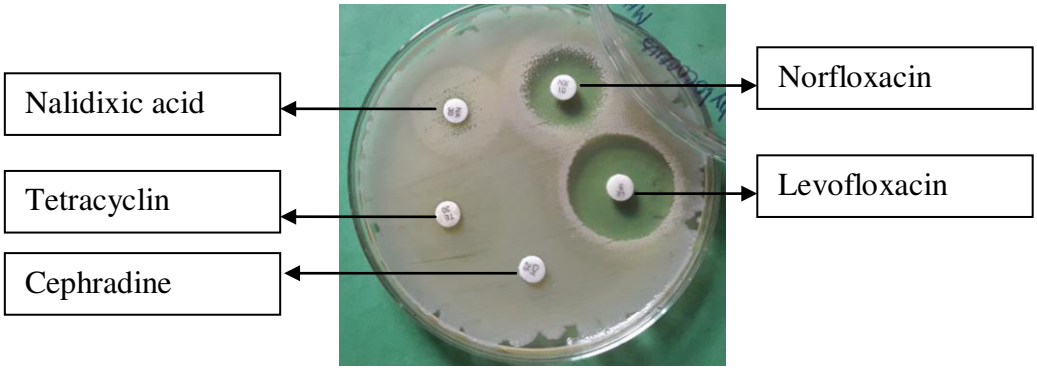


Plate 25: Antibiogram of *Staphylococcus aureus* in MHA

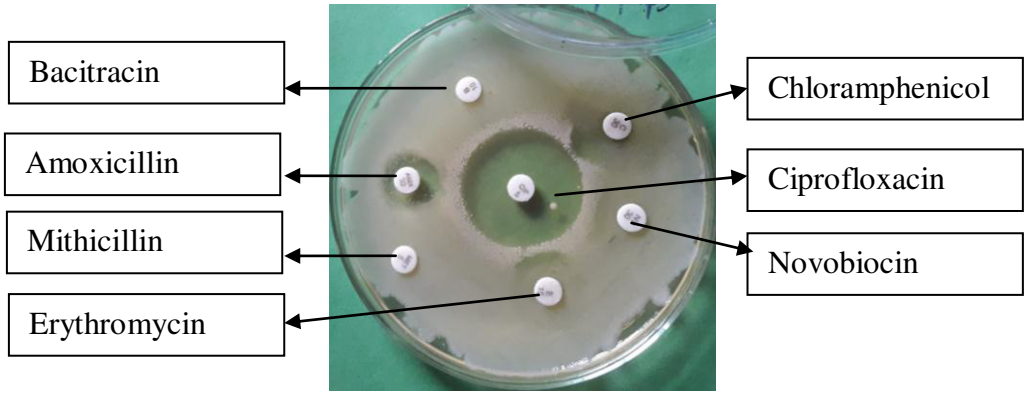


Plate 26: Antibiogram of *Staphylococcus aureus* in MHA

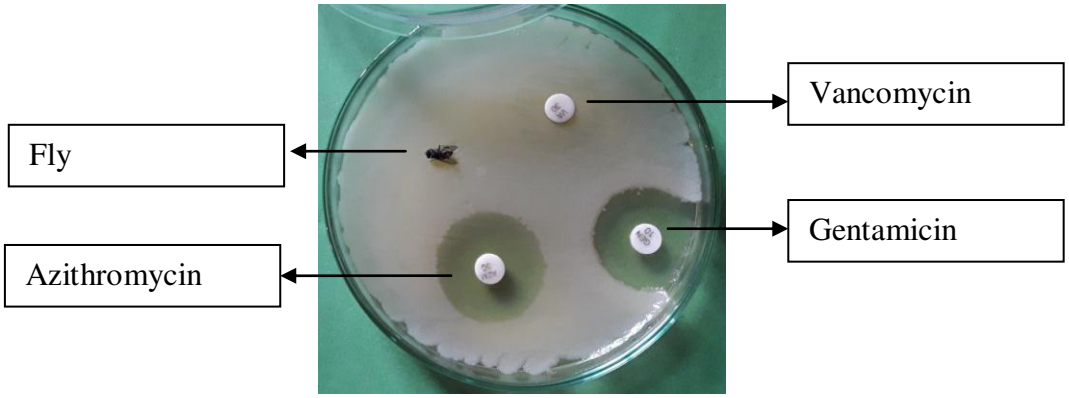


Plate 27: Antibiogram of *Staphylococcus aureus* in MHA

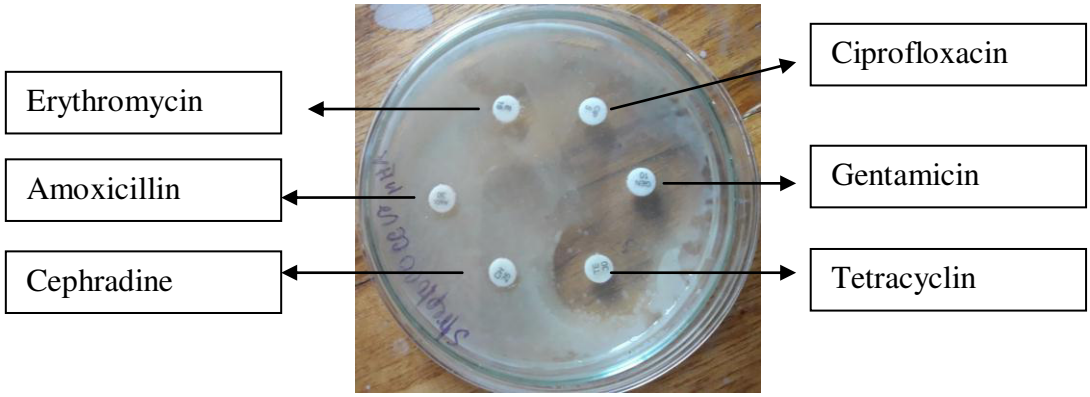


Plate 28: Antibiogram of *Streptococcus* sp. (*S. agalactiae*) in MHA

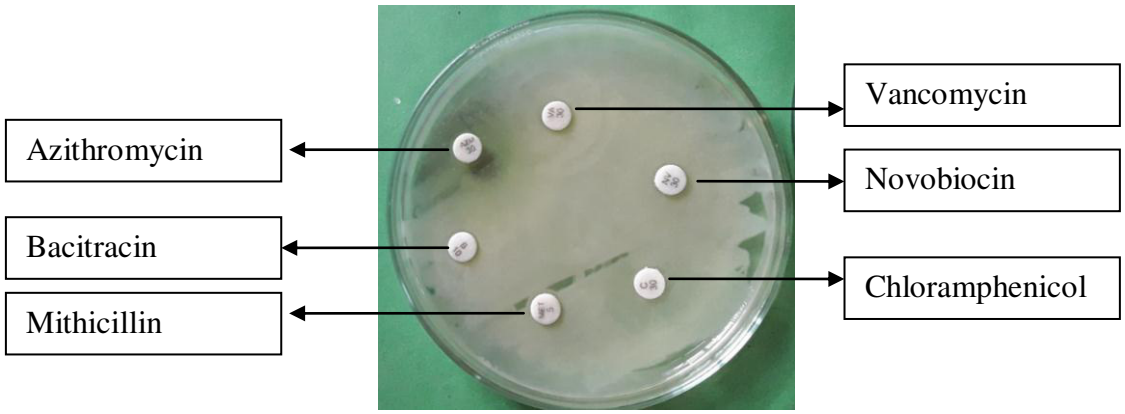


Plate 29: Antibiogram of *Streptococcus* sp. (*S. agalactiae*) in MHA

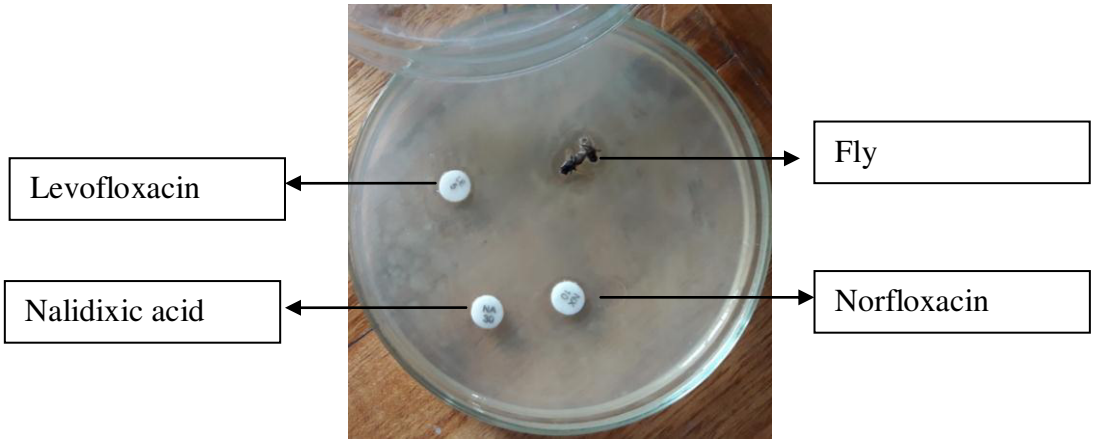


Plate 30: Antibiogram of *Streptococcus* sp. (*S. agalactiae*) in MHA

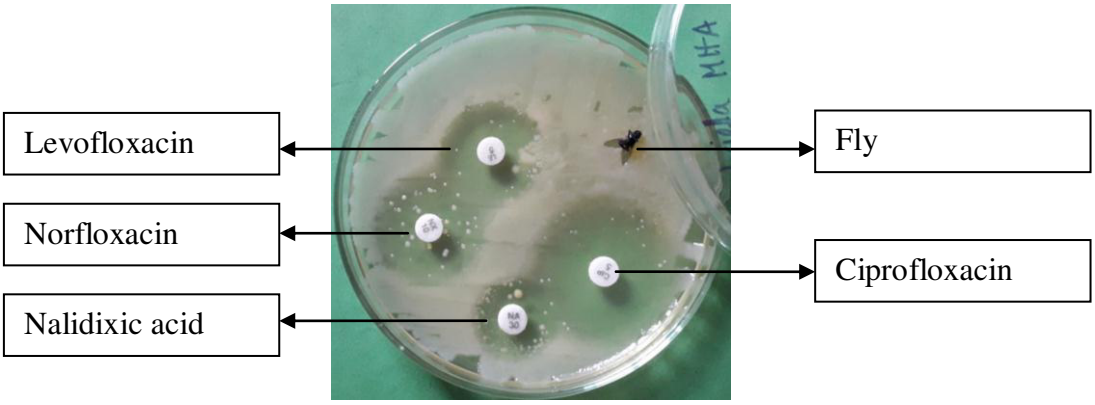


Plate 31: Antibiogram of *Klebsiella* sp. in MHA

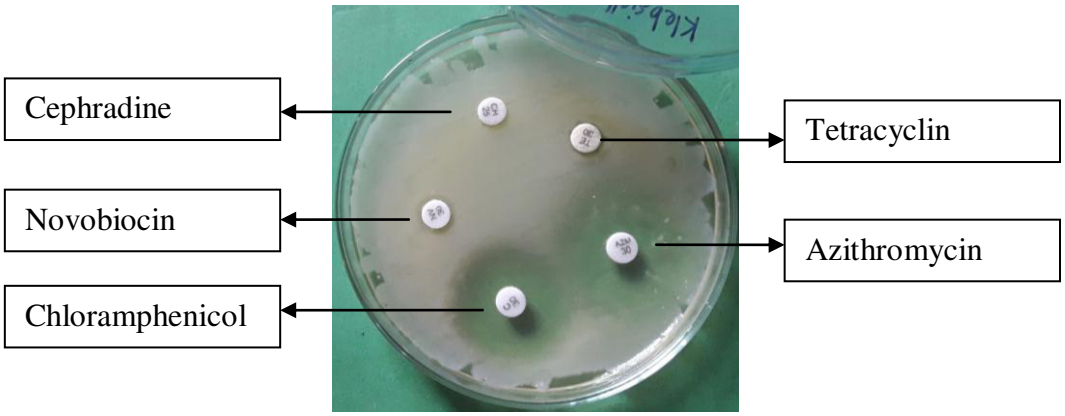


Plate 32: Antibiogram of *Klebsiella* sp. in MHA

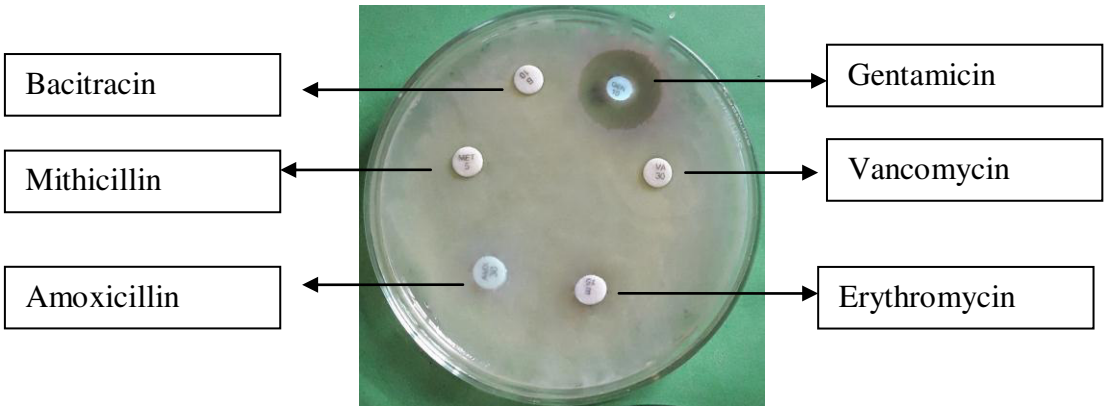
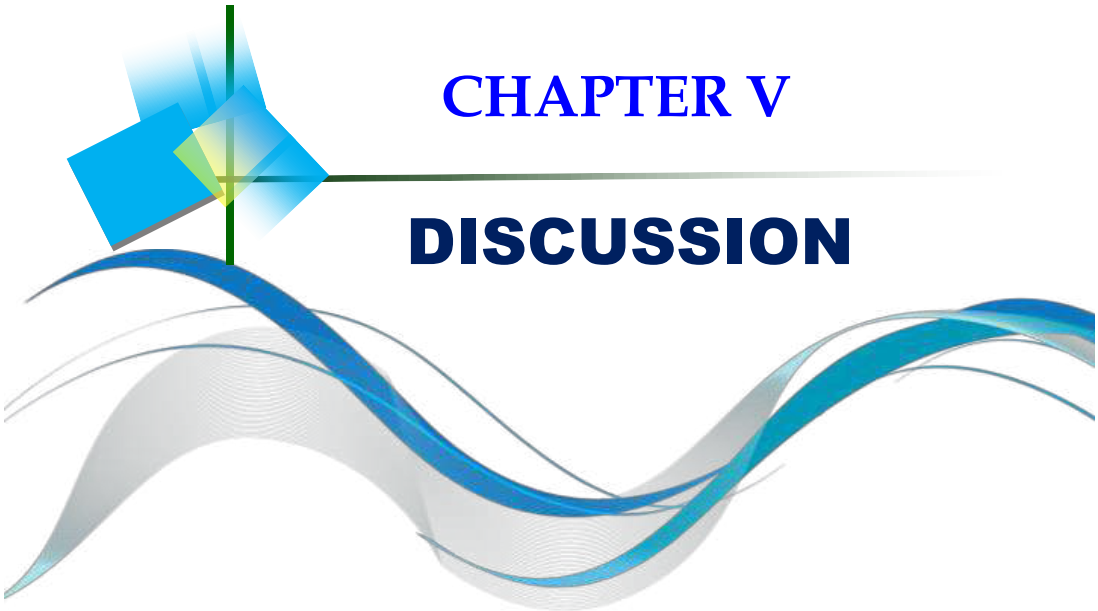


Plate 33: Antibiogram of *Klebsiella* sp. in MHA



CHAPTER V

DISCUSSION

CHAPTER-V

DISCUSSION

The research topic entitled as a step towards development of vaccine against bovine mastitis was selected with main objectives covering proper isolation, characterization and development of a vaccine against bovine mastitis. Bovine mastitis (BM) is an ultimate threat due to its high economic losses. There are a lot of pathogenic organisms responsible for BM. Some are Gram-positive, some are Gram-negative bacteria and also a few fungal infections behind it.

In the present study 48 mastitis milk samples were analyzed in laboratory, in which *E. coli*, *Staphylococcus* sp., *Streptococcus* sp. and *Klebsiella* sp. were isolated and identified by using cultural, morphological and biochemical techniques. This research work only focused on four major bacterial isolates. 100% *E. coli* appeared in the collected mastitis milk samples which have the similarity with the work Zafolon *et al.,* (2008). *E. coli* identified by Eco 223 and Eco 455 primer and showed band at 232 bp. *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus* sp. and *Klebsiella* sp. give band in gel electrophoresis at 1267 bp, 405 bp, 438 bp and 1492 bp respectively by using primers mentioned in table 3.3, 3.4. All primers and PCR condition followed by Riffon *et al.,* (2001) with little modification. Sau 234 and Sau 1501 forward and reverse primer with annealing temperature 58°C, 60.5°C, 60.9°C and 61.3°C they were given band in gel electrophoresis very deeply. In the case of *Streptococcus agalactiae* given band at 405 bp with Sag 40, Sag 445 specific primer where annealing temperature was 60°C, 61°C, 62.5°C, 63.2°C which is similar to Riffon *et al.,* (2001). These findings supported by Riffon *et al.,* (2001) with good remarks. In this study the reference annealing temperature was also followed but at 70°C did not found the reference band. However, PCR for *Streptococcus agalactiae* was performed with above-mentioned gradient annealing temperatures. RT-PCR also performed to detect *Streptococcus agalactiae* and observed the graphs with the above-mentioned primer where used Go Taq probe (Promega Co. Ltd.).

All the identified organisms are amplified with 16S rRNA and 23S rRNA gene and also reference gene. Here primers (Eco 223 plus Eco 455; Sau 234 plus Sau 1501: Uni 1870

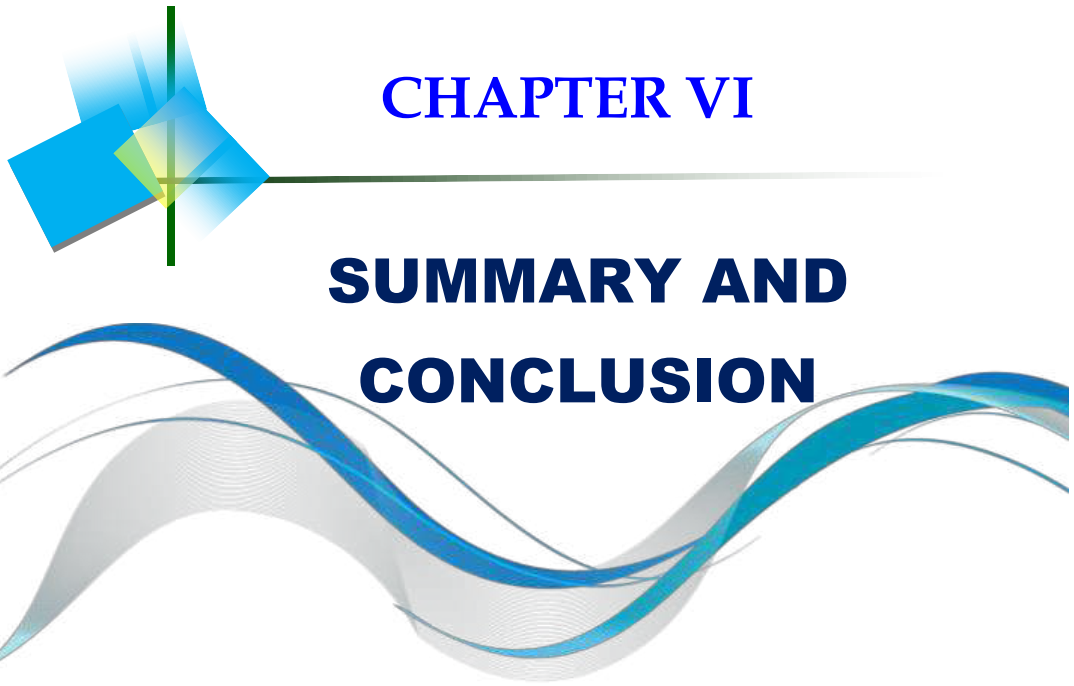
plus Uni 2308) were designed based on a DNA sequence coding for 23S rRNA. Phylogenetic analysis revealed their molecular identification. In the present study, we had done the sequencing of forward and reverse primer of *E. coli, Staphylococcus aureus* and *Streptococcus* sp. The present findings were similar with NCBI database genome sequences found through BLAST at 99% (*S. aureus)* identity, 90% or above homology found in the case of *E. coli* and *Streptococcus* sp. (*S. agalactiae).* Phylogenetic study helps to identify study isolates more specifically at molecular level.

Antibiogram study showed that most of the isolated organisms are Multi-Drug Resistance (MDR). Wang *et al.,* (2015) studied Methicillin Resistance *Staphylococcus aureus* (MRSA), that is similar with our study even here *Staphylococcus aureus* coagulase positive. In the current research all the isolates showed about 5 to 8 drug resistant. Gentamycin showed 22 mm zone in the case of *Streptococcus agalactiae*. Ciprofloxacin 5 micro gram showed 29 mm, 30 mm and 36 mm in the case of *Staphylococcus aureus, Klebsiella* sp. *and E. coli* respectively. In this study also observed the antimicrobial activity of house fly (*Musca domestica*). House flies were just caught and stored in a tube filled with PBS. After the dead, flies were just placed on MHA media along with other antibiotic discs. Most of the cases house fly showed very little complete 12 mm zone (*Streptococcus agalactiae*) but in the case of *E. coli* showed an intermediate zone (22 mm) that was quite big. If the antibacterial agent extracted from fly (*Musca domestica*) that might be helpful to protect MDR bacterial infection like BM. Bovine Mastitis is both costly and frequently appeared disease throughout the world (Seegers *et al.,* 2003). Conventional used of antibiotics for the treatment of BM led to the emergences of MDR organisms (Tenhagen *et al.,* 2006).

To make inactivated seed isolates were inoculated on selective media from the stock culture and also were inoculated in broth medium. To calculate the CFU/ml were made 10-fold dilution for each isolate. Diluted broth culture inoculated on PCA at the rate of 0.1 ml for *Staphylococcus aureus* (10^{-7} tube), *E. coli* and *Klebsiella* sp. (10^{-6} tube) except *Streptococcus* sp. where used 10^{-7} tube with the volume of 0.2 ml. Mainly on PCA cultured the isolates from 5^{th}, 6^{th}, 7^{th} and 8^{th} tube from 10-fold dilution. All the cases we got our desire CFU at 6^{th} and 7^{th} tube. For inactivation of isolates were taken equal volume (10 ml of each) of broth and formalin (0.4% V/V) in a tube and performed centrifuge @ 7000 rpm for 20 sec. After that suspended the sediment with normal saline. To make the bulk volume of formalin-inactivated bacteria had taken 5 ml of suspended

solution and where added 5 ml of reference adjuvant. Mixed gently and store at 4°C. After that prepared the pure culture media for all the isolates which were incorporated in vaccine seed for sterility test. Sterility as satisfactory confirmed by observing no foreign contamination on the pure culture as well as observed the colony morphology and staining of bacteria that were cultured. For safety test inject double dose intramuscularly according to OIE (2013) the vaccine candidate there were no adverse or hazardous conditions appeared after 14 days of observation.

The newly developed formalin killed vaccine candidate contains four major BM causing bacteria. The aim of current study is to formulate BM vaccine which will be commercially available. But there is no newly formulated efficient vaccine available in local area Riffon *et al.,* (2001). Current research work helps to commercialized BM vaccine. Also helps to choose an effective drug to treat BM cows. This polyvalent formalin-inactivated vaccine (PFIV) seed will be commercialized if we get recommendation and patent of this research protocol. It is predicted that; further work will help to commercialize a new PFIV which will be an effective vaccine against BM. That makes a new way in the field of veterinary practice and may increase milk production.



CHAPTER VI

SUMMARY AND CONCLUSION

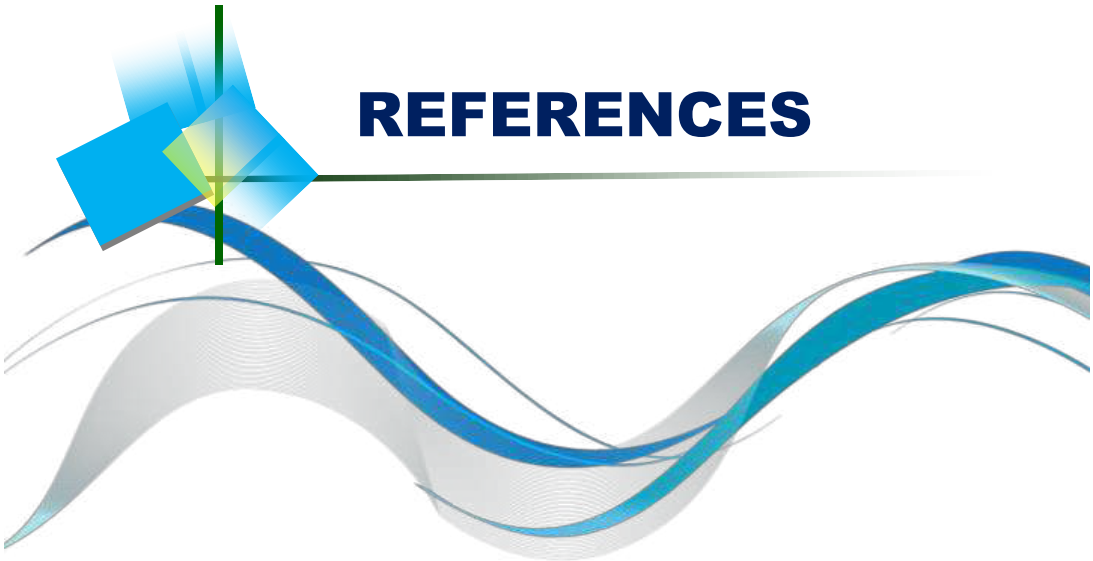
CHAPTER-VI

SUMMARY AND CONCLUSION

Current research work reveled that both small scale and large dairy farms are not safe from bovine mastitis in Bangladesh. Mastitis confirmed by CMT test kit. Isolation, identification and biochemical test of mastitis milk samples indicates *E. coli* and *Staphylococcus aureus* which is methicillin resistant. Both are notoriously appeared in every sample within study area. Besides *Streptococcus* sp. including *Streptococcus agalactiae* and *Klebsiella* sp. suspected *Klebsiella pneumoniae* observed as the same with lower rate and identified by PCR. The entire isolated organism confirmed by molecular technique PCR, RT-PCR. They were given specific band in gel electrophoresis with specific primer. Antibiogram study showed their antibiotic sensitivity and susceptibility. That helps to choose specific drugs. 16S rRNA and 23S rRNA gene based phylogenetic analysis helps to reveal molecular specification of isolates. This study helps the practitioners and farmers to choose the right antibiotics in term of treatment. On the other hand, newly formulated polyvalent formalin inactivated vaccine (PFIV) seed helps to commercially available BM vaccine in Bangladesh. That helps to prevent bovine mastitis in Bangladesh.

Recommendation for further study

- **Field trial design and animal experiment**
  - Field trial and animal experiment will be followed according to Sayed *et al.,* (2015)
- **Determination of immune response of vaccine seed**
  - According to Leitner *et al.,* (2000) immune response should be performed based on ELISA and the result will be observed based on Tong *et al.,* (2014).



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REFERENCES

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APPENDICES

APPENDICES

APPENDIX-I

Composition of the media used:

Nutrient Agar	**Grams/Liter**
Peptone	5.0gm
Bacto beef extract	3.0gm
NaC1	5.0gm
Agar	15.0gm
Distilled water	100 ml
PH	7.2

Sterilized at 121°C under 15 lb/in^2 pressure for 15 minutes.

MacConkey agar	**Grams/Liter**
Bctoeptone	17.0gm
Proteas Peptone	3.0gm
Lactose	10.0gm
Bile Salt	1.5gm
Agar	15.0gm
Neutral red	0.03gm
Crystal violet	0.100gm
Distilled water	1000 ml
PH	71

Sterilized at 121°C under 15 lb/in^2 pressure for 15 minutes.

Eosine Methylene Blue (EMB) agar Gram/Liter

Peptone	10.0gm
Lactose	10.0gm
K_2HpO_4	2.0gm
Eosin	0.4gm
Methylene blue	0.065gm
Agar	20.0gm
Distilled water	1000 ml
PH	6.8

Sterilized at 121°C under 1 Sib/in^2 pressure for 15 minutes.

Mueller Hinton Agar	**Gram/Liter**
Beef infusion	20.0gm
Bactocasamino acid (technical)	17.5gm
Starch	1.5gm
Bacto agar	17.5gm
Distilled water	1000 ml
pH	73

Sterilized at 121°C under 151 b/in^2 pressure for 15 minutes.

Mannitol Salt Agar	**Gram/Liter**
Proteas peptone	10.0gm
Beef extract	1.0gm
D-Mannitol	10.0gm
NaCl	75.0gm
Phenol red	0.025gm
Agar	20gm
Distilled water	1000ml

Sterilized at 121°C under 15 lb/in^2 pressure for 15 minutes.

Blood Agar Gram/Liter

Blood Agar	60 gm
Distilled Water	1000 ml
Bovine blood	5.00 ml
Or Nutrient agar	500 ml

Sterile Defibrinated blood 25 ml

Normal Saline	**Gram/Liter**
NaCl	0.85gm
Distilled water	1000ml

Autoclaved at 121°C for 15 minutes

Plate Count Agar (PCA)

Ingredients	Gms / Litre
Tryptone	5.000
Yeast extract	2.500
Dextrose (Glucose)	1.000
Agar	15.000

Final pH (at 25°C) 7.0±0.2

Formula adjusted, standardized to suit performance parameters

APPENDIX-II

Composition of the media used in biochemical test

MR-VP broth	Gram/Liter
Peptone	7.0
Dextrose	50
Dipotassium phosphate	5.0
Distilled water	1000ml
PH	6.9

Sterilized at 121°C under 151 b/in^2 pressure for 15 minutes.

Triple Sugar Iron TSI Agar	Gram Liter
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Lactose	10.0
Saccharose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosuiphate	0.3
Phenol Red	0.024
Agar	12.0
PH	7.4

Sterilized at 121°C under 151 b/in^2 pressure for 15 minutes.

Simmons citrate Agar	**Gram/Liter**
Magnesium sulphate	0.2
Manoammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Brom-Thymol Blue	0.08
PH	6.8

Sterilized at 121°C under 15 lb/in^2 pressure for 15 minutes.

Indole tryptopon broth medium	**Gram/Liter**
Tryptone	10.0
Distilled water	1000ml

Sterilized at 121°C under 15 lb/in^2 pressure for 15 minutes.

Streptococcus Agalactiae Selective Agar Base

Streptococcus Agalactiae Selective Agar is recommended for selective isolation of **Streptococcus agalactiae** from dairy products.

Composition**

Ingredients	Gms / Litre
Peptic digest of animal tissue	10.000
Meat extract	5.000
Sodium chloride	5.000
Esculin	1.000
Thallous sulphate	0.333
Crystal violet	0.0013
Agar	13.000
Final pH (at 25°C)	7.4±0.2

**Formula adjusted, standardized to suit performance parameters

APPENDIX III

Composition of chemicals and reagents

Crystal violet

Solution-A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

Solution-B

Ammonium oxalate	0.8
Distilled water	80.0 ml

Note-Mix the solution A and B

Gram's iodine

Iodine	1.0g
Potassium iodide	2.0 g
Distilled water	300.0 ml
Ethyl alcohol	(95%)
Ethyl alcohol (100%)	95.5 ml
Distilled water	5.0 ml

Safranin

Safranin O	0.25 ml
Ethyl alcohol (95%)	10.0 ml
Distilled water	100. 0 ml

APPENDIX-IV

RT-PCR Thermal Condition and Cycle

Application	Quantification/DNA Binding Dye/DNA/Standard Curve				
Instrument	Eco™ Real-Time PCR system				
Software	Eco™ Software v5.2.13.0				
Last Date Saved	10/13/2018 7:44 PM				
Stage	**Step**	**Temperat**	**Duration**	**Cycles**	
UDG Incubation	Step 1	95	00:02:00	1	
PCR Cycling	Step 1	95	00:00:15	40	
PCR Cycling	Step 2	60	00:01:00	40	
Total Program Length	1 Hour 11 Minutes 55 Seconds				
Total Cycle Count	40				

Dye	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Cycle 7	Cycle 8	Cycle 9	Cycle 10	Cycle 11	Cycle 12	Cycle 13	Cycle 14	Cycle 15	Cycle 16	Cycle 17	Cycle 18	Cycle 19
Green	2.880007	3.34231	3.519588	3.689879	3.819573	3.929488	4.038529	4.118423	4.207088	4.281491	4.351535	4.411277	4.473271	4.517032	4.573188	4.608299	4.660885	4.687731	4.73625
Green	1.328606	1.479678	1.542883	1.651734	1.831785	2.079789	2.359604	2.633048	2.901466	3.140895	3.369546	3.568356	3.745846	3.913674	4.056305	4.191749	4.304792	4.40215	4.504251
Green	1.321765	1.456359	1.514239	1.618237	1.794311	2.096821	2.413888	2.688327	2.946685	3.207025	3.454114	3.665895	3.856071	4.02076	4.165494	4.28481	4.404061	4.505564	4.584437
Green	2.42033	2.614328	2.737939	2.872685	2.979505	3.089204	3.182007	3.261251	3.336213	3.418867	3.475622	3.538046	3.596641	3.647828	3.698021	3.743554	3.7999	3.841995	3.873472
Green	0.045431	0.044777	0.045986	0.044537	0.044953	0.044443	0.044267	0.044297	0.043697	0.04389	0.043967	0.043573	0.04371	0.043354	0.043645	0.043127	0.043418	0.043195	0.043304

Cycle 20	Cycle 21	Cycle 22	Cycle 23	Cycle 24	Cycle 25	Cycle 26	Cycle 27	Cycle 28	Cycle 29	Cycle 30	Cycle 31	Cycle 32	Cycle 33	Cycle 34	Cycle 35	Cycle 36	Cycle 37	Cycle 38	Cycle 39	Cycle 40
4.765111	4.796526	4.828856	4.85931	4.876697	4.907131	4.9337	4.945876	4.975598	4.998267	5.008364	5.027895	5.045528	5.06064	5.078469	5.089412	5.097538	5.109424	5.122261	5.138839	5.146072
4.598144	4.680151	4.747854	4.818747	4.864054	4.935836	4.969417	5.025741	5.06603	5.107551	5.162041	5.194561	5.227653	5.256272	5.28572	5.3076	5.338617	5.348086	5.386701	5.413895	5.42886
4.665592	4.726608	4.78091	4.852996	4.886083	4.920947	4.964331	4.995809	5.045824	5.076105	5.096025	5.125663	5.140997	5.166789	5.185843	5.197444	5.224754	5.245921	5.250039	5.249306	5.263665
3.917473	3.948448	3.977475	4.016627	4.043467	4.072989	4.10063	4.130407	4.15079	4.177388	4.204919	4.237251	4.248535	4.26207	4.28739	4.290951	4.295487	4.30793	4.326595	4.358084	4.354987
0.043449	0.043263	0.043101	0.043422	0.0429	0.043873	0.045032	0.046838	0.051519	0.058977	0.074891	0.10488	0.161376	0.26166	0.412606	0.60839	0.822851	1.025135	1.189092	1.317157	1.414816

APPENDIX V

Sl. No. **Questionnaire for the surveillance/investigation of Bovine Mastitis**

1. Particulars of the owner: Date: ……

- Name: ……..…… Vill: ……Post: ……District: ……..
- Mobile: ……

2. Particulars of Farm & House:

Small	Medium	Large

- Type of floor: Concrete ☐ Other …….
- Hygienic condition: Poor ☐ Good ☐ Very Good ☐
- Feeding: Ready feed Other……
- Time of Feeding: …… Time /day
- Watering: …….. Time /day
- Floor Cleaning: …… Time /day
- Washing house by Antiseptic powder ☐ Disinfectant ☐ Water ☐
- Washing of Animal daily ☐ weakly ☐ monthly ☐

3. Disease History:

- Deworming: ☐
- History:
- Clinical sign:

- **Milk Color:**
- **Test:**
- Types of Disease: Bacterial ☐ Viral ☐ Fungal ☐ Parasitic ☐ Others ☐
- Name of Disease: ……

4. Vaccination:

Name of vaccine	Age (day)	Remarks

5. Treatment given: Yes ☐ No ☐

Name of medicine used	For	Remarks

Opinion of farmer about disease:

Signature of Investigator