MOLECULAR CHARACTERIZATION OF *E. COLI* ISOLATED FROM RAW MILK

A THESIS

BY

MOST. SHAHINUR AKTER

REGISTRATION NO. 1605466

SEMESTER: JANUARY-JUNE, 2018

SESSION: 2016

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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JUNE, 2018

DEDICATED TO MY BELOVED PARENTS

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ABSTRACT

The present study was conducted to isolate and identify *Escherichia coli* from raw milk at Rangpur division of Bangladesh. For this, a total of 60 milk samples were collected from different dairy farms and rural domestic holdings at Rangpur division in Bangladesh. The study was carried out during the period from July, 2017- May, 2018. The milk samples were collected and brought to the Laboratory under Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur and subjected to different bacteriological tests for isolation and identification of bacteria based on morphology, staining, cultural and biochemical examinations. Molecular test using PCR was also studied. The isolated bacteria were *Escherichia coli* 30.00%, *Staphycoccus spp.* 20%, *Salmonella spp* 20%, *Corynebacterium spp*. 16%. On nutrient broth enough whitish turbidity, On MacConkey agar Bright pink colonies were formed and On EMB agar *Escherichia coil* showed greenish black colonies with metallic sheen. In Gram's staining technique the *Escherichia coil* revealed Gram negative, pink color, short rod, cocci, coma, single, pair or in short chain arrangement. The organism in biochemical test

showed MR test positive, VP test negative & Indole positive. In statistical analysis, the prevalence of the isolates in different dairy farm found from this study was non-significant. Present study indicates that the *Escherichia coli* was one of the important bacteria that available in raw milk of the sample collection area at Rangpur district of Bangladesh.

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LIST OF ABBREVIATIONS AND SYMBOLS

BA = Blood agar

BAU = Bangladesh Agricultural University

Dx = Dextrose

e.g. = Example

E.oli = *Escherichia coil*

EMB = Eosin methylene blue

et al. = Associated

Fig. = Figure

gm = Grams

H_2O_2 = Hydrogen peroxide

H_2S = Hydrogen sulfide

hrs = Hours

HSTU = Hajee Mohammad Danesh Science and Technology University

KOH = Potassium hydroxide

L = Lactose

Ltd = Limited

MC = MacConkey

mg = Milligram

MIU = Motility Indole Urease

ML = Maltose

ml = Milliliter

mm = Minutes

mm = Millimeter

MN = Mannitol

MR = Methyl Red

n	= Number
NA	= Nutrient agar
NB	= Nutrient broth
No.	= Number
OIE	= Office International Des Epizooties
PBS	= Phosphate buffered saline
Prof.	= Professor
R	=Resistant
S	= Sucrose
Sec	= Seconds
spp.	= Species
TSI	= Triple sugar Iron
VP	= Voges Proskauer
-	=Negative
%	=Percentage
/	= Per
+	= Positive
<	= Less than
>	= Greater than

CHAPTER-I
INTRODUCTION

Milk is an excellent high quality food providing major nutritional requirement to man at any age and extremely susceptible to spoilage by microbes. Unhygienic production of milk and milk products and improper storage, cause the early spoilage with microorganisms. Bacteria present in the milk cause unpleasant effect on the taste and physical properties and disease. The bacterial contamination of milk not only reduces the nutritional quality but also consumption of such milk threatens health of the society.

Milk is defined to be the lacteal secretion, practically free from colostrums, obtained by the complete milking of one or more healthy cows, 5 days after and 15 days before parturition, which contains not less than 8.5 percent milk solids and not less than 3.5 percent milk fat. Milk constituents include water (87.20%), protein (3.50%), fat (3.70%), milk sugar or lactose (4.90%), ash (0.70%) and dry matter (12.80%). Milk and milk products consist of high moisture, are nearly neutral in pH and are rich in vitamins. Raw milk represents an ideal growth medium for microorganisms (Haridy, 1992).

There is an increasing concern about the presence of zoonotic pathogens in raw milk due to the spread of such pathogens between cows during the milking time by milking equipments and milks' hands (Fox and Gay, 1993). Besides, microorganisms may enter the udder through teat canal, and the bacteria may come out through milk (Smith *et al.,* 2007*). Staphylococcus aureus* and *Escherichia coli* are the two major contaminants of milk. The presence of the pathogen in milk largely depends on fecal contamination, and the presence of pathogen in feces mainly originates from feed contamination (Aycicek *et al.,* 2005).

Hence, milk easily favors the growth and multiplication of many bacteria, even pasteurized or refrigerated. These bacteria may significantly influence the quality of the milk and milk products. Milk contains

relatively few bacteria when it is secreted from the udder of a healthy animal. However, during milking operations, it gets contaminated from the exterior of the udder and the adjacent areas, dairy utensils, milking machines, the hands of the milking man, from the soil and dust.

In these way bacteria, yeasts and molds get entry into the milk and thus constitute the normal flora of milk. Milk might also be contaminated with pathogenic bacteria or bacterial toxins which may serve as vehicle for the transmission of diseases to humans such as salmonellosis, diarrhea, food poisoning, tuberculosis etc. Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder.

Beyond this stage of milk production, microbial contamination can generally occur from three main sources; from the udder, from the exterior of the udder and from the surface of milk handling and storage equipment. Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, feces and grass.

The fresh milk deteriorates easily to become unsuitable for processing and human consumption (FAO, 2001). Moreover, fresh milk may get microbial contamination from utensils, animal skin, environment, or water used for cleaning etc. (FAO, 2008). Foodborne diseases are a common and widespread global problem. Several outbreaks have been reported as a result of consuming contaminated milk that may look, taste and smell perfectly normal but are in fact contaminated with large number of harmful bacteria (Centers for Disease Control and Prevention, CDC, 2009). Milk contaminated by high levels of spoilage bacteria usually becomes unsuitable for further processing since it does not meet the consumer's expectations in terms of health (nutritional value), safety (hygienic quality) and satisfaction (sensory attributes) (Nanu *et al.,* 2007). The presence of Coliforms in food of animal origin indicates environmental and fecal contamination since these micro-organisms are abundant in the environment food (Shojaei and Yadollahi, 2008). However, milk is an excellent medium for growth and transmission of

different bacterial pathogens to humans (Donkor *et al.,* 2007). *Escherichia coli* are a Gram negative, rod-shaped bacterium, a member of the family Enterobacteriaceae and commonly found in the lower intestine of warm-blooded organisms (Singleton, 1999). Raw or processed milk is a well-known good medium that supports the growth of several microbes with resultant spoilage of the product or infections/ intoxications in consumers (Murinda *et al.,* 2004; Oliver *et al.,* 2005). Microbes may gain entry into raw milk directly from dairy cows experiencing sub clinical or clinical mastitis (Rodojcic-Prodaova and Necev, 1991), from the farm environment particularly the water source (Eberhart, 1977) and utensils used for the storage of milk on farm or during transportation (Freedman, 1977). Markets and consumers for raw milk and their products have existed in many parts of the world. Raw unpasteurized milk is consumed directly by a large number of people in rural areas and indirectly by a much larger segment of the population via consumption of several types of cheeses. Among the main reasons that people may believe that the raw milk and their products have advantages or value over the pasteurized one.

E. coli is one of the important bacteria of gut flora (Eckburg *et al.,* 2005). Among the pathogenic *E. coli,* Shiga toxigenic *E. coli* (STEC) strains have been reported mostly in Latin America, India, Bangladesh and many other developing countries (Kaddu-Mulindw *et al.,* 2001; Rehman *et al.,* 2014). Pathogenic *E. coli* have been isolated by several researchers in Bangladesh (Nazir *et al.,* 2005; Khatun *et al.,* 2015; Himi *et al.,* 2015) from fecal samples of healthy cattle (Hassan *et al.,* 2014), raw milk of cattle and buffaloes (Alam, 2006; Islam *et al.,* 2008; Hossain *et al.,* 2011; Jahan *et al.,* 2015). In Bangladesh, about 20% of all diarrheal cases is associated with enterotoxigenic *E. coli* (Qadri *et al.,* 2005).

E. coli frequently contaminates food organism and it is a good indicator of fecal pollution (Diliello, 1982; Soomro *et al.,* 2002; Benkerroum *et al.,* 2004).

Presence of *E. coli* in milk products indicates the presence of enteropathogenic microorganisms, which constitute a public health hazard. Enteropathogenic *E. coli* can cause severe diarrhoea and vomiting in infants, and young children (Anon., 1975). Most strains of *E. coli* live as commensals, many perhaps all are opportunistic pathogens of human and animals (Levine, 1984). Though it is a part of normal enteric microflora, but it is capable of producing serious diarrheal diseases, as well as other systemic diseases, especially infection of the urinary tract. *E. coli* and other facultative anaerobes constituted about 0.1% of gut flora (Eckburg *et al.,* 2005) and oro-fecal transmission is the major route through which pathogenic strains of the bacterium cause disease. Over the last half-century it has become increasingly obvious that there are a number of different pathogenic groups of *E. coli*. Pathogenic *E. coli* strains are categorized into pathotypes on the basis of their virulence genes. At least six known pathotypes associated with gastrointestinal infections have been recognized, apart from those opportunistic "nonpathogenic strains" causing urinary tract infections, diarrhoea, septicemia, and meningitis in humans and a number of similar diseases in animals.

E. coli is important as mastitis pathogens and widely distributed in the farm environment (Hogan and Smith, 2003). Amongst the coliforms, *Escherichia coli* organisms are the most common contaminants of raw and processed milk (Quinn *et al.,* 2002). It is a reliable indicator of fecal contamination of water and food such as milk and dairy products (Todar, 2008). If environmental contamination of the milk supply were important, then farm level factors that reflect general hygiene, such as the degree of cleanliness of the facilities and animals cleanliness of milking equipment, and other factors might be important in limiting access of these agents to the milk (McEwes *et al.,* 1988).

Especially in India, rate of infection is still higher Characteristics because of warm and humid climate (Bhatia, A *et al.,* 2007). On heating at normal cooking temperature, the bacteria may be killed but the toxins

remains active (Presscott, L.M *et al.,* 2002). Most *E. coli* are harmless, but some are known to be pathogenic bacteria, causing severe intestinal and extra intestinal diseases in man (Kaper *et al.,* 2004).

This work describes the presence of *Escherichia coli* in raw milk available at rural farmer comprising the unorganized region in Rangpur district.

And this study investigates the microbiological quality and safety of locally produced raw milk. Several works have been done throughout the world regarding the milk and microorganisms of contaminated milk (Hassan *et al.,* 2014). In Bangladesh, a few works have been done on isolation and molecular characterization of *E. coli* from raw milk of cattle and buffalo (Alaine *et al.,* 2006; 1slam *et al.,* 2008; Hossain *et al.,* 2011, Hossain, 2015). Moreover very few works have been reported in Bangladesh on molecular detection of *E. coli* with other pathogenic organisms in cow's raw milk in a specific time period. So, keeping the above facts in mind, the present study was designed with an objective to molecular detection and characterization of bacteria isolated from raw milk samples using 16s rRNA gene. Thus, the objective of this study was to investigate the occurrence of the opportunistic pathogen *E. coli* in cow's milk in Rangpur district.

Considering the above facts, the research work has been conducted with the following objectives.

- Isolation of *E. coli* in raw milk by cultural and biochemical test.
- Characterization of Isolated bacteria by molecular test.

CHAPTER -II

LITERATURE OF REVIEW

Ahlam A. *et al.,* (2014) studied the quality of raw cow milk at the farm level which considered the first step for production of safe and high quality milk. A total of 150 raw cow milk samples were collected from governmental and private farms at Alexandria Governorate, Egypt. The samples were analyzed for bacteriological properties. The mean values of TBC, coliforms, *Staphylococcus aureus* and *Aeromonas hydrophila* count in positive samples were 3.22 × 104 ± 7.28 × 103, 1.4 × 103 ± 3.9 × 102, 5.26 ×102 ± 8.1 × 10 and 6.4 ×10 ± 1.3 × 10 in examined raw cow milk collected from governmental farms while the respective counts in raw cow milk collected from private farms were 1.51 × 105 ± 2.59 × 104, 3.28 × 102 ± 4.7 × 10, 2.95 × 102 ± 7.7 × 10 and 6.8 ×10 ± 4.1 × 10. Also prevalence of some pathogens as *E. coli*, *Salmonellae* and *Yersinia enterocolitica* organisms were detected. The hygienic as well as the public health significance of raw milk were discussed.

Amal, M. Eid (2014) determined the prevalence of food poisoning pathogens in Bulk tank milk from 3 dairy herds in Gharbia Governorate, Egypt. *Escherichia coli*, *Staphylococcal aureus* and *Listeria monocytogenes* were detected in 20, 60, and 10 % in samples collected from farm I , in farm II with percentage of 40, 40 and 20 % and in farm III 20, 20 and 10 % of examined bulk tank milk samples, respectively. Polymerase chain reaction is a powerful technique for detection of pathogens in foods. It is a rapid procedure with both sensitivity and specificity for quick detection and identification of specific pathogenic bacteria from different sources. The eight *E. coli* isolates were screened for the presence of virulence associated genes (stx1, stx2), heat-stable enterotoxin gene (STa) and only one (50%) isolate from farm I encoded the STa gene. The ability of *Staphylococcus aureus* to produce enterotoxins which is linked to Staphylococci enterotoxins SEs genes was investigated by using multiplex PCR, out of 12 Staph. aureus isolated

from the examined BTM samples, 2 isolates were carrying sea gene, 1(16.6%) from farm I and 1(25%) from farm II. *Listeria monocytogenes* detection methods based on PCR amplification of the hly gene sequences specific for confirmation of *L. monocytogenes* and not any other type of *Listeria* have been used for identification of all four obtained isolates and the results obtained from isolation were in line with that of molecular diagnosis as PCR detected only the presence of *L. monocytogenes*. Since presence of these food poisoning microorganisms constitute a potential risk to public health, these findings underscore the need to control them and to limit bacterial multiplication in bulk tank milk.

Asmahan A. Ali and Warda S. Abdelgadir (2011) Estimated that the incidence of opportunistic pathogen, *E. coli*, in raw cow's milk in Khartoum State (Khartoum, Khartoum North and Omdurman). Hundred raw milk samples were randomly collected from different localities/sources of Khartoum State and were inoculated on the relevant bacteriological media. Confirmation was performed using a series of biochemical tests. The results revealed that 63% of the samples were *E. coli* positive. The highest numbers of milk samples contaminated with *E. coli* were obtained from Khartoum north vending shops and Khartoum milk vendors. From twenty samples 9, 10 and 12 contaminated samples were detected from Khartoum, Khartoum North and Omdurman farms respectively with contamination with Coliforms ranging between 3.86±0.1 and 4.18±0.01 and *E. coli* between 3.53±0.1 and 3.93±0.01. Although, fresh cow milk samples collected from milk vendors and milk vending shops were contaminated with almost the same load of Coliform bacteria 4.11 and 4.01 log10 cfu/mL, but the count of *E. coli* ranged from 3.54-3.90 log10 cfu/mL. The highest mean value of coliform was found in milk from Khartoum farms with15.0 x104 cfu/mL, while the lowest mean count of 3.857±0.02 log10 cfu/mL was detected in milk obtained from the Omdurman farms.

Basil A Abbas *et al*., (2017) studied the investigation of the microbiological quality of milk and milk products. Milk was found to be contaminated with several types of bacteria. Most of these bacteria have been found to show different antibiotic resistance patterns against several known antibiotics. Different characterization methods such as conventional biochemical tests and DNA based methods have been applied. Therefore, the aim of this study was to review the recent studies the microbiological quality of milk and milk products.

Ekici. K. *et al.,* (2004) aimed to isolate some pathogens from raw milk of different milch animals. For this aim, 36 samples of sheep milk, 25 samples of goat milk and 4 samples of cow milk were analyzed. Among the 66 total raw milk samples, *S. aureus* was isolated from 12 samples while *E. coli* was isolated from 6 samples. *Salmonella spp*. could not be isolated in any of the samples.

Hafsa Afroz *et al.,* (2013) reported milk and milk products are ideal foods for all age groups in both rural and urban people all around the world. This study reports microbiological status of powder milk samples and antibiotic susceptibility pattern of *E. coli* and *Staphylococcus aureus* isolated from powder milk samples collected from different area of Dhaka, Bangladesh. Twelve samples were collected and seven of them were found acceptable according to codex Alimentarius and ICMSF in terms of total viable count and total coliform*. E. coli* was isolated from 11 samples and *Staphylococcus aureus* was isolated from 6 samples. *E. coli* isolated were resistant to 5 antibiotics and *Staphylococcus aureus* isolates were resistant to 6 antibiotics. Hygienic conditions during production and post-processing should be improved according to HACCP (Hazard Analysis and Critical Control Points) guidelines to improve the microbiological quality and safety of powder milk products.

Islam, M. A. *et al.,* (2016) performed molecular detection of *E. coli* isolated from raw cow's milk. A total of 20 milk samples were collected from different upazila markets of Jamalpur, Tangail, Kishoreganj and Netrokona districts of Bangladesh. Milk samples were cultured onto

various culture media for the isolation of bacteria. The isolated bacteria were identified by studying staining characteristics, cultural properties on different selective media, biochemical tests, catalase and coagulase test, and finally by PCR. Out of 20 samples, 15 (75%) milk samples were found positive for *E. coli*. 15 *Escherichia coli* isolates were amplified by 16S rRNA gene based PCR. Antimicrobial sensitivity test was carried out to ascertain the susceptibility of the organism to various antibiotics. Its results showed that the *E. coli* isolates were resistant to amoxycillin (86.67%) and erythromycin (73.33%) but sensitive to azithromycin (53.33%), ciprofloxacin (86.67%), gentamicin (86.67%), norfloxacin (80%) and streptomycin (66.67%).

Lubote *et al.,* (2014) Aimed to investigate the prevalence of *Salmonella spp*. and *Escherichia coli* (*E. coli*) in milk value chain in Arusha, Tanzania. A total of 75 raw milk samples were collected from smallholder dairy farmers, street vendors and outlet shops in Arusha and Arumeru districts. A questionnaire survey was also used to assess hygienic practices along the chain. Salmonella and *E. coli* were detected in 28/75 (37.33%) and 68/75 (90.67%) samples, respectively. Bangata ward in Arusha showed relatively high prevalence of *Salmonella spp*. (42.11%) while Akeri ward in Arumeru showed relatively low prevalence (31.58%). In milk value chain, the highest prevalence was observed in street vendors (43.75%) while the lowest prevalence was in dairy farms (33.33%). Mean count for *E. coli* from milk producers, vendors and shops were 3.0 × 103, 8 × 103 and 6.6 ×103 cfu/mL, respectively, indicating a significant ($p < 0.05$) increase in *E. coli* load along the chain. Furthermore, confirmatory test showed that *Salmonella* isolates were predominantly identified as *Salmonella* enterica serovar Arizonae. Besides, *Salmonella* and *E. coli*, other enterobacteria detected were *Enterobacter cloacae*, *Klebsiella pneumonia*, and *Serratia marcescens*. Taken together, qualitative and quantitative findings revealed that poor animal husbandry, poor hygienic practices, lack of refrigeration and less awareness of the zoonotic pathogens had a significant impact on the prevalence of detected bacteria, posing a public health risk.

Mayada M. Gwida and Fatma, A, EL-Gohary (2013) examined that the occurrence of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) in raw milk (Market and Farm milk) in Dakahlia Governorate, Egypt. Therefore, the present study was conducted to clarify the role of raw milk in transmitting some zoonotic bacteria such as *S. aureus* and *E. coli* to man aiming to study the source of infection. Three hundred and twenty five random samples of market milk, bulk farm milk, milker hand swabs and human stool specimen were collected to be cultivated on the selective bacteriological media. Identification was performed using a series of different biochemical tests. The obtained results revealed that out of 150 examined market milk 36.66% (55 out 150) and 56.66% (85 out 150) harboring *E. coli* and *S. aureus* respectively. On the other hand, bacteriological examination of 100 raw milk samples collected from different dairy farms clarified that *S. aureus* and *E. coli* were isolated at a percent of (18 and 20%) respectively. Additionally seventy five samples were collected from man representing 50 stool samples (25 diarrheic cases and 25 apparently healthy dairy handlers) and 25 hand swabs from dairy handlers. *S. aureus* and *E. coli* were found to be positive in 7, 40 stool samples out of the total examined (50) with percentages of 14, 80 respectively. Out of 25 human diarrheic cases, *S. aureus* and *E. coli* were isolated with percentages of 8, 88 respectively. Meanwhile *S. aureus* and *E. coli* were isolated respectively from 5 (20%), 18 (72%) stool samples out of 25 apparently healthy dairy handlers. Out of 25 hand swabs from dairy handlers *S. aureus* was isolated with the percentage of 60. However, *E. coli* was isolated with percentage of 20. It could be concluded that the results of the present study clearly indicated that the quality of the raw milk sold in Dakahlia Governorate is considered unsatisfactory and strict hygienic measures are required to improve the quality of raw milk sold in Dakahlia Governorate.

Melese Abate Retal and Addisu Hailu Addis (2015) Milk has an outstanding nutritional quality but it is also an excellent medium for bacterial growth and an important source of bacterial infection when consumed without pasteurization. Microbial contamination might

generally occur from within the udder, exterior to the udder and from the surface of milk handling and storage equipment. Raw milk collection and its transportation to the processing centers present a number of technical, economical and organizational problems in most developing countries. Hygienic quality control of raw milk and milk products in Ethiopia is not usually conducted on routine basis. Some of the disease causing bacteria in the milk are *Salmonella spp.*, *M. bovis*, *Corynebacterium spp.*, *C. perfringens*, *Yersinia enterocolitica*, *Coxiella burnetii*, *Brucella*, *Staphylococcus spp.*, *Campylobacter jejuni*, *M. avium*, *Listeria spp.*, *E. coli*, and other coliforms. Many bacteria could get an easy access to milk and milk products such as *E. coli*, coliform and they are often used as indicator organisms to confirm the bacterial contamination of milk. The higher total bacterial counts and isolation rates of some public health important pathogens were observed in these literatures conducted in different study areas of the country and the consumption of raw/unpasteurized milk carries an important public health risk.

Msalya, G. (2017) Milk in Tanzania has been reported to be contaminated with large number of bacteria. This is because milk is obtained from animals with unknown health status, good milking and handling practice are to a large extent to served, and marketing distribution are done in informal channels. These factors are potential causes of milk-borne diseases and milk quality loss. The aim of this study was to assess nutritional risks in milk as reported in literature over a period of 20 years and through analyses of samples collected during the present study. The issues highlighted in literature were high bacteria and coliform counts exceeding standard levels in East Africa, prevalence of bacteria and drug residues in milk, and adulteration. Based on performed analyses, total bacterial count 1.0 × 107 colony forming units per milliliter (cfu/ml) and total coliform count 1.1 × 107 cfu/ml, also greater than recommended levels, were found. Ten bacteria types were solated from milk samples (five, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, and *Klebsiella spp*.

are reported in Tanzanian for the first time). Two drugs tetracycline and sulphur were detected. Therefore, it is worth noting that integrated research is needed to evaluate the situation and address these challenges.

Muhamed Mubarack, H. *et al.,* (2010) investigated that the microbiological quality of raw milk samples from some of the villages and the surrounding areas of Coimbatore district. Among the 80 raw milk samples used for the study, bacteriological identification revealed a definite dominance of *Lactobacillus sp*. Besides it, the other genera *Staphylococcus*, *Escherichia*, *Bacillus*, *Salmonella* and *Pseudomonas* were isolated on selective agar and broth.

Priyanka Singh and Alka Prakash (2008) *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* were isolated from milk products i.e. curd and cottage cheese, sold at shops in the unorganized sector of Agra region. Of the 116 bacterial isolates from cottage cheese, 15 were confirmed as *E. coli*, 12 as *S. aureus*, and two as *L. monocytogenes*. Fifty-eight isolates were obtained from curd samples of which five were confirmed as *E. coli*, eleven as *L. monocytogenes*, and no *S. aureus* contamination was found in curd. The result indicates that strict preventive measures should be adopted to ensure contamination free milk products for the good health of all consumers.

Rajeev Kumar and Amit Prasad (2010) isolated *Staphylococcus* and *E. coli* from milk (dairy farm, vendors and house) and milk products (viz; Dahi, Ice cream, Gulabjamun, Burfi, Khoa and Butter). All samples were inoculated on different bacteriological media and various biochemical tests were performed for the confirmation of isolates. The result of the present study revealed that out of 135 samples, 25 samples were found contaminated with *Staphylococcus* and *E. coli*. The highest rate of contamination was recorded in Burfi while the lowest was recorded in Ice cream. These enterpathogenic bacteria may cause problems due to improper handling and processing of milk and milk products. These

organisms are significant from public health point of view as they have been associated with the onset of food poisoning in human beings.

Rajesh Singh Tomar *et al.,* (2017) Showed that Milk is a natural and rich resource of minerals, vitamins, protein etc. Milk contamination is a very serious public issue, because millions of people especially children all over the world consume dairy products daily. An extensive study was carried out in Gwalior region of Madhya Pradesh, India. The study was to determine the various Milk handling practices and microbial contamination in various milk samples collected from dairy, milk parlor, and small shops. A total of 90 milk samples were collected from four different areas in Gwalior for the isolation and identification of microbes, from February 2016 to April 2016. Previously described standard protocols were adapted for the isolation and cultivation of microbes. Different biochemical assays like IMVIC test, Catalase test, Oxidaxe test, and selective media were used to identify microbes in the milk samples. The results of the study showed that the presence of bacterial contamination (gram positive and negative types of microbes) in raw milk and pasteurized milk result from unhygienic practices during the pre-milking and storage stage. 72 samples were contaminated with *Staphylococcus* aureus, Escherichia coli, and *Pseudomonas aeruginosa* However, some more bacterial colonies were also observed, but the results are not stable. Also, drug resistant bacteria against Ampicillin and Tetracyclin were reported. On the basis of the present study, it is suggested that dairy farm, dairy shops, and milk parlors must improve the hygienic and storage condition of milk. In addition, public education must be given to all stakeholders in dairy industry on milking and post harvest handling of milk, to curtail the likely losses due to refusal of spoiled milk and milk-borne pathogens resulting from contamination of milk.

Tahlina Tanzin *et al.,* (2016) The objective of this study was to isolate and identify *Staphylococcus aureus* and *Escherichia coli* from raw milk samples of cattle and buffalo, and to evaluate the antibiotic sensitivity

pattern. Materials and methods: A total of 34 milk samples were collected twice from 17 different healthy cattle (n=14) and buffaloes (n=3) at one-month interval, and analyzed in laboratory by staining, cultural and biochemical characteristics followed by polymerase chain reaction targeting gene of *S. aureus* and 16 S rRNA of *E. coli*. Antibiotic sensitivity pattern of the isolated bacteria was assessed using the disc diffusion method. Confirmation of the isolates as *S. aureus* and *E. coli* were carried out by PCR using nuc gene, 16S rRNA gene specific primers specific for *S. aureus* and *E. coli* respectively. A total of 12 samples (35.29%; 11 from cattle, 1 from buffalo) were found to be positive for *S. aureus*; 5 and 7 during first and second month, respectively. The *E. coli* were found in three samples (2 from cattle, 1 from buffaloe); one in first month and two in the second month. The antibiotic sensitivity test using 4 commonly used antibiotics indicated that the most of the isolates were resistant to Gatifloxacin and one isolate showed intermediate resistance to ofloxacin while sensitive to Ciprofloxacin and Levofloxacin. Two different species of bacteria i.e., *S. aureus* and *E. coli* are contaminating with milk samples. The pathogenic bacteria can be controlled effectively by using Ciprofloxacin and Levofloxacin in the case of mastitis in cattle and buffaloes in Bangladesh.

Uddin M.A. *et al.,* (2010) Investigated that the bacteriological quality of locally available raw milk. A total number of 22 raw milk samples were collected from Dhaka city and its surrounding areas during the period from October 2009 to November 2009. The analysis comprised enumeration of total viable bacterial count (TVBC), total coliform count (TCC) and total staphylococcal count (TSC) for the determination of sanitary quality. The highest TVBC, TCC and TSC were 2.36 ×109 cfu/ml, 2.0 × 108 cfu/ml & 4.7 ×107 cfu/ml, respectively. In order to observe the antibiotic-resistance pattern, the antibiogram assay was carried out. All *Escherichia coli* isolated from raw milk exhibited 100% resistance against Rifampin (R) and Tetracycline (TE) and 50% resistance against Nalidixic Acid (NA) but were 100% sensitive against Imipenem (IPM). *Klebsiella spp*. exhibited 100% sensitivity against Imipenem (IPM). *Staphylococcus*

spp. isolated from raw milk exhibited 100% resistance against Rifampin (R) and Nalidixic Acid (NA), but were 100% sensitive to Gentamicin (CN) and Imipenem (IPM). This survey indicates that most of the raw milk samples were not satisfactory in course of public health standard as some pathogenic bacteria were detected from these samples. Frequent use of antibiotic should be prohibited since antibiotic resistant strain is continuously increasing.

CHAPTER-III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Study area and period

The present research work was carried out on dairy cows at Rangpur district. The laboratory works were conducted in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur, during the period from July 2017 to May, 2018.

Table 3.1. Areas of samples collection

Sl. no	Name of the sample collection area	No. of samples
1.	Rangpur pirganj	20
2.	Rangpur sadar	10
3.	Mimima dairy farm, Dinajpur	10
4.	HSTU dairy farm	6
5.	Noyanpur, Dinajpur	6
6.	Jaigirhat, Rangpur	8

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)
- Simmons Citrate Agar Medium, (HI-MEDIA, India)
- Staphylococcus agar no. 110 media, (HI-MEDIA, India)

- Manitol Salt Agar, (HI-MEDIA, India)

3.1.2.2 Liquid media

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

3.1.2.3 Data Analysis

Data were analyzed using SPSS version 21.

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
- Ethyl alcohol (70% and 95%)
- Sugar Media (Dextrose, Maltose, Lactose, Sucrose and Mannitol)
- Gel loading dye
- PCR mastermix, 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
- Centrifused 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

- 11., buffer
- 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
- Isopropanol
- 70% Ethanol
- 95% Ethanol
- 1.5 ml micro centrifuge tubes

3.1.5 Materials used for Polymerase Chain Reaction Table 3.2: PCR Reaction Mixture for 16s rRNA.

Buffer	2.5 111
dNTP	2.5 p.1
MgC12	2.5 ill
Forward Primer (27F)	1.0 p.1
Reverse Primer (1492R)	1.0 1.11
Nano Pure Water	12.5 ill
DNA	2.0 [11
Taq DNA Polymerase	1.0 ill
Final Volume	25 ttl

- Primers used for PCR:

16S rRNA gene region was amplified with the universal primers.

  - Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3')
  - Reverse primer- 1492R (5' TACCTTGTTACGACTT 3')

- Product size: 1500bp
- Thermal Cycler (Thermo cycler, ASTEC, Japan)
- 2% agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask
- Electrophoresis apparatus (Biometra standard power pack P 2T)
- 100 by DNA size marker
- Bromphenicol blue of loading bufter.

- Ethidium bromide (0.5 µg/ml)
- Distilled water
- UV trans-illuminator

3.1.6 Media for Culture

3.1.6.1 Liquid media

3.1.6.1.1 Nutrient broth

Nutrient broth is used for the general cultivation of non-fastidious microorganisms in water, feces and other materials. A liquid medium, it is produced according to the formula from APHA and AOAC, and supports the growth of a great variety of microorganisms that are not very nutritionally demanding. 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.

3.1.7 Semi solid media

3.1.7.1 Motility indole urease (MIU) media

It is a semi solid media used to determine motility indole formation and of the test organisms.

3.1.8 Solid media

3.1.8.1 Nutrient Agar Medium

Nutrient agar is used for cultivating of non-fastidious microorganisms (Cheesbrough, 1985).

3.1.8.2 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).

3.1.8.3 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 noniactose-fermenting gram-negative and Klebshella spp bacteria appear pink (Cheesbrough, 1985).

3.1.8.4 Staphylococcus agar no. 110

Staphylococcus agar no. 110 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, 984).

3.1.8.5 Salmonella-Shigela Agar

Salmonella Shigella Agar (SS Agar) is a selective and differential medium widely used in sanitary bacteriology to isolate Salmonella and Shigeila from feces, urine, and fresh and canned foods. Shigella and the major part of Salmonella: Clear, colorless and transparent (Cheesbrough, 1985).

3.1.8.6 Blood Agar Medium

For the isolation, cultivation and detection of hemolytic reaction of fastidious microorganisms. Blood Agar Base 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.

3.1.8.7 Simmons Citrate Agar Medium

Simmons citrate agar is used for determination the ability of bacteria to ferment citrate as a sole source of carbon.

3.1.8.8 Triple Sugar Iron Agar

Triple sugar iron agar is used for identification of gram negative bacteria that capable of fermenting sugar. Triple sugar iron agar contains lactose, sucrose and glucose.

3.1.9 Media for Biochemical test

Sugar fermentation broth, indole broth, methyl red broth, voges-proskauer broth, Simmons 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 diswashing detergent solution (‘Trix’ Recket and Colman Bangladesh Ltd) overnight Contaminated glassware was disinfected with 2% sodium hypochloride solution prior to cleaning. The glassware were 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. All the glassware was kept in oven at 50°C for future use.

3.3 Experimental layout

The experimental work was divided into two steps: The first step was performed for the isolation and identification of the organisms of the collected sample using cultural, staining and biochemical characteristics. The second step was conducted for the Molecular characterization by

PCR. The layout of the diagrammatic illustration of the present study is shown in figure.

Experimental Layout

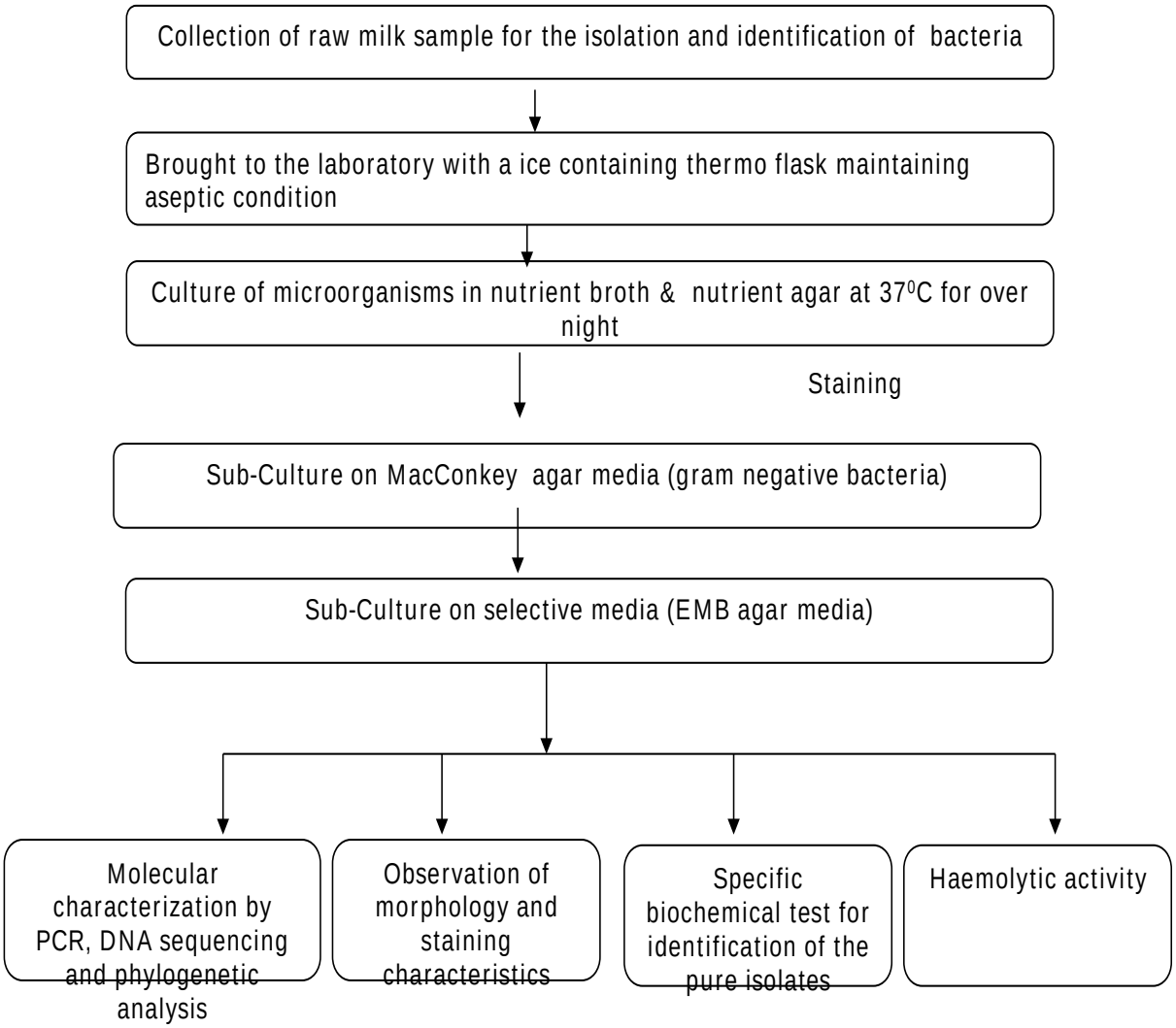


Figure 3.1: The schematic illustration of the experimental layout

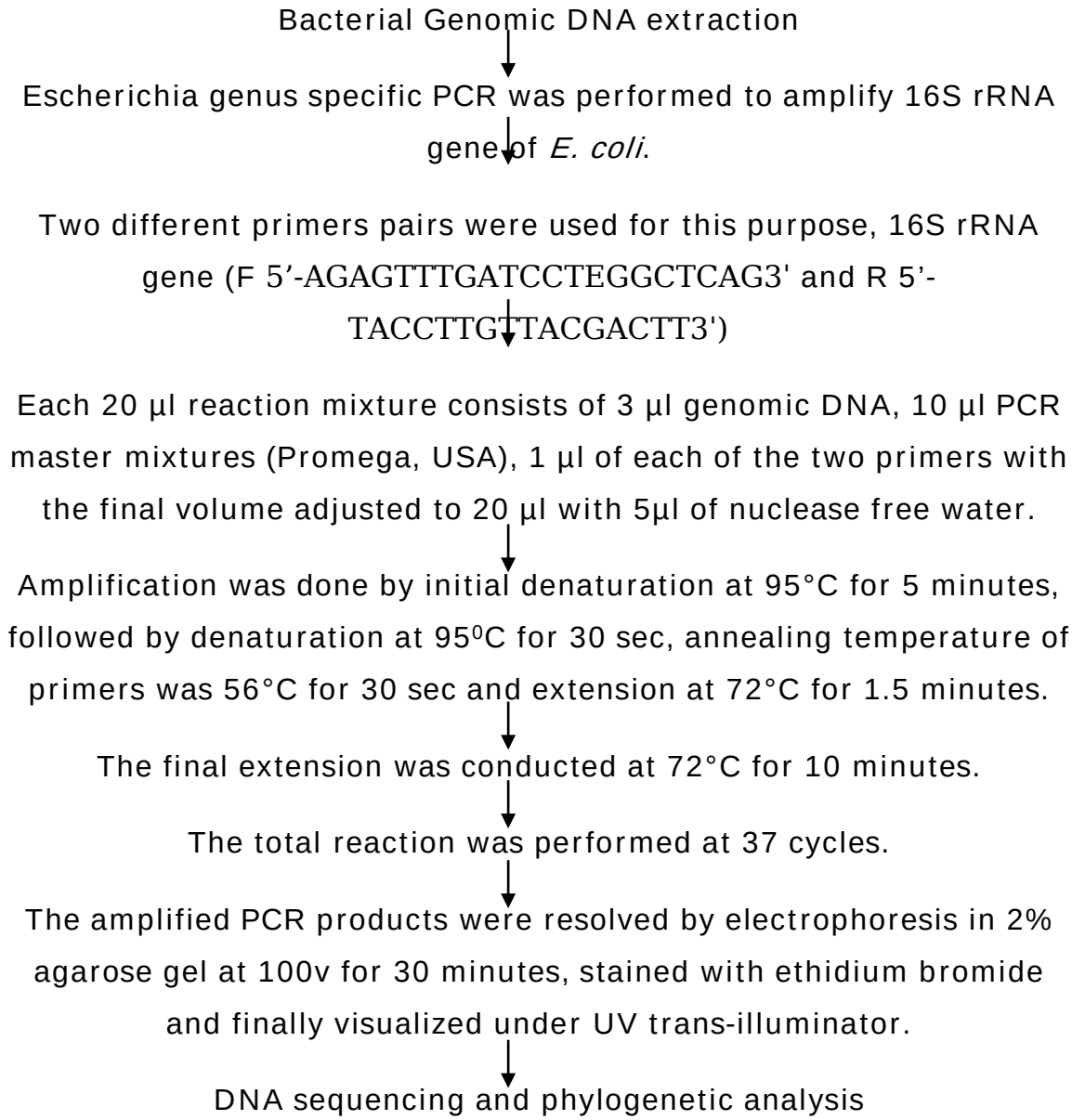


Figure 3.2: Molecular characterization by PCR, DNA sequencing and phylogenetic analysis

3.4 Collection of milk samples

A total of 64 milk samples were randomly collected directly from Rangpur and Dinajpur district by owner and they were instructed to soak the teat 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 each haves into a labeled sterilized test tubes with rubber cap, using routine techniques for aseptic infection. The samples were carried to 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. The remaining samples were stored at 0°C for further use.

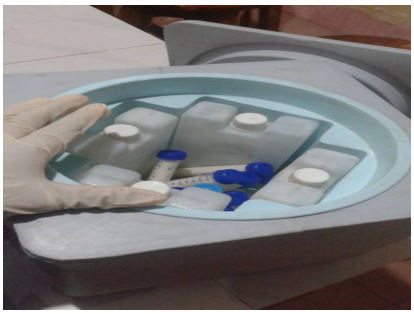



Plate 3.1: Sample collection and preparation

3.5 Preparation of sample

Firstly, prior to taking samples, the screw capped vial in which initially sample of milk was collected was shaken thoroughly for proper mixing of the milk sample. After proper shaking, 1 ml of milk sample was pipetted out from the screw capped vial by sterile pipette. It was transferred to 9 ml diluent kept in a test tube. The test tube was shaken electrically in a whiriling mixturing machine. Decimal dilutions as necessary were prepared in the usual way as per instruction of milk and dairy byproduct, (Harrigan and Mc Cance, 1976).

3.6 Preparation of culture media

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

3.6.1 Nutrient broth media (NB Media)

Thirteen grams of dehydrated nutrient broth (111-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 40^0C in aerator for future use.

3.6.2 Nutrient agar media (NA media)

Twenty eight grams of nutrient agar powder (111-MEDIA) 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 or sred 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 there 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 fur overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use.

3.6.6 Sugar media

The medium consists of peptone water of which fermentable sugar was added to the proportion of 1%. One gram of Bacto peptone (HI-MEDIA) and 0.5 gram of sodium chloride were added in 100 ml of distilled water. The medium was boiled for 5min adjusted to pH 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2% solution was added to peptone water and then in 5 ml into cotton plugged test tubes containing Durham's fermentation and placed inversely. These were then sterilized by autoclaving at 1.2 kg/cm2 121°C for 15 minutes. The sugars used for fermentation were prepared separately 10% solution in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A mild heat was necessary to dissolve the sugar. The sugar

solutions were sterilized in Arnold steam sterilizer at 100°C for 30 minutes for 3 consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at for 24 hours.

3.6.7 Salmonella- Shigella agar

6.3 gram Salmonella- shigella agar base powder was added to 100 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 Petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes 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.

3.6.8 Staphylococcus agar no. 110

149.6 grams of Staphylococcus agar powder (Hi-media, India) 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. Resuspend the precipitate by entle 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).

3.6.9 Simmon's Citrate Agar Media

2.42 gram Simon citrate agar base powder was added to 100 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 there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishespartially 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. A small amount of milk sample was placed on the agar plates with the help of sterilized bacteriological loop on the Petridishes of Simon citrate agar. Then it was placed overnight at 37°C temperature.

3.7 Preparation of reagents

3.7.1 Methyl- Red solution

The indicator MR 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) 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.

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.

3.7.5 Phosphate Buffered Saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate Na_2HPO_4, $12H_{20}$), 0.2 gram of potassium chloride (KC1) and 0.2 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 / cm2 pressure and 121° C for 15 minutes and stored for future use.

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 amyalcohol 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.

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 110 and by streak plate method (Cheesbrough, 1985) to observe the colony characteristic colony morphology of *Escherichia coli, Staphyloccus spp, Streptococcus spp., Corynebacterium spp.* Then was repeatedly sub-

cultured onto Mac-Conkey, EMB agar, SS agar and *Staphylococcus agar* no 110 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.

- 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 were done with saframn for 30 sec.
8. Then gently washed with tap water.
9. Finally examined under microscope with oil immersion. (James G. Cuppuccion Natalie Sherman,1996)

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 eatalase 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 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).

3.10.3 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).

3.10.4 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 (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).

3.10.5 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).

3.10.6 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.

3.10.7 Motility Indole Urease (MIU) test

MIU medium 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 (Cheesbrough, 1985).

3.10.8 Haemolytic activity

To determine the haemolytic property of isolated bacteria, the colonies of bacteria were inoculated on to blood agar media and incubated at 370 for 24 hours. Various types of haemolysis were observed after development of bacteria 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 (1986) and was listed as mentioned below:

3.10.8.1 Alpha **(α)** hemolysis

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

3.10.8.2 Beta **(β)** hemolysis

Complete clear zone of hemolysis around the colony.

3.10.8.3 Gamma **(ɣ)** 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 Poindexter (1971).

- Step 1. A inoculum was picked up with a sterile inoculating ioop and was spread on a 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 coil* 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 coil*.

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 (1979). The organism if *Escherichia coil* 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 organisms 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 PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogénetic Analysis of *E. coli*.

3.14.1 Basic protocol of bacterial genomic DNA isolation

Bacteria from saturated liquid culture are lysed and portions are removed by digestion with protease-k. Cell wall debris, polysaccharides and remaining proteins are removed by Phenol-
Chloroform extraction and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Procedure

- Inoculate a 25 ml of liquid culture with *E. coli.* Grow in conditions appropriate for *E. coli.* until the culture is saturated.
- Spin 1.0 ml of the overnight culture in a micro centrifuge tube for 5 minutes at 10000 rpm.
- Discard the supernatant.
- Repeat this step. Drain well onto a kimwipe.
- Re-suspend the pellet in 467 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ ml Proteinase k to give a

final concentration of 100 Lg/mg Proteinase-k in 0.5% SDS. Mix thoroughly and incubate 30 mm for 1 hr at 37°C.

- Add an approximately equal volume (500 µl) of Phenol/Chloroform/Isoamyl alcohol. Mix thoroughly but veiy gently to avoid shearing the DNA, by inverting the tube until the phase are completely mixed.
- Then centrifuge the tubes at 12000 rpm for 10 minutes,
- Remove aqueous, viscous supematant 400µl) to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of Phenol/Chloroform/isoamyl alcohol extract thoroughly and spin in a microcentrifuge at 10000 rpm for 5 mm.
- Transfer the supernatant to a fresh tube (400 µl).
- Add 1/10th volume of 3 M sodium acetate and mix.
- Add 0.6 volumes of isopropanol to precipitate the nucleic acids, keep on ice for 10 minutes.
- Centrifuge at 13500 rpm for 15 minutes.
- Discard the supernatant.
- Wash the obtained pellet with 1 ml of 95% ethanol for 5 minutes. Then centrifuge at 12000 rpm for 10 minutes.
- Discard the supernatant.
- Dry the pellets as there is no alcohol.
- Resuspend the pellet in 50 iii of TE and then 7.5 i1 of RNase. Store DNA at 4°C for short term and at -20°C for long term.

3.14.2 PCR amplification and sequencing of 16S rRNA

PCR Condition:

Table 3.3: Condition of PCR

Sl. no	Steps	Temperature	Duration	Cycle
1.	Initial denaturation	95°C	5 min	01
2.	Denaturation	95°C	30 Sec	35
3.	Annealing	56°C	30 Sec	
4.	Extension	72°C	1.5 min	

5.	Final extension	72°C	10 min	01
6.	Holding	4°C	hold	

3.14.3 Electrophoresis

Process of Electrophoresis:

- 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.

3.14.4 Nucleotide sequence accession number and BLAST analysis

The nucleotide sequence 16S 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

3.15.5 Chain-termination methods (Sanger sequencing) Steps of Sanger sequencing using ABI 3130 Genetic analyzer

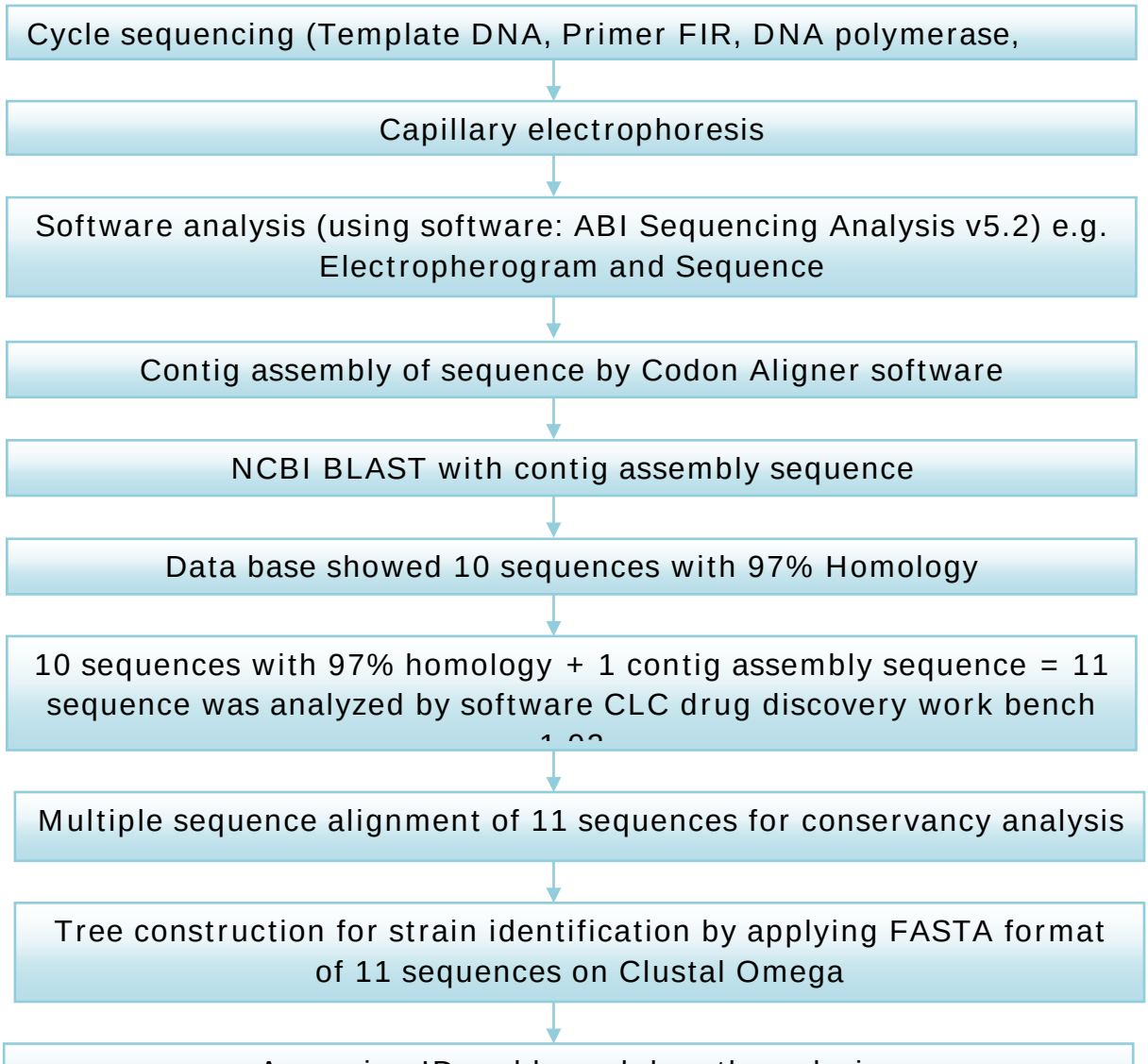


Figure 3.3: Steps of Sanger sequencing using ABI 3130 Genetic analyzer

3.15 Maintenance of stock culture

The stock culture was maintained following the procedures of Choudhury *et al.,* (1987). Nutrient agar slants were used for the maintenance of stock culture for each of the bacterial isolates. One slant was used for an individual isolate. After growth of the 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 organismss 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.

3.16 Statistical Analysis

Data were analyzed using SPSS version 21. The chi-square (χ^2) was used to assess statistical differences between the farms. A p-value more then 0.05 were statistically considered non-significant.

CHAPTER -IV

RESULTS

4.1 Frequency of bacteria isolated from milk from different commercial dairy farms

The results of frequency of different bacterial isolates are presented in Table-4.1. A total of 60 milk samples were examined for the isolation of bacteria.

Table -4.1: Frequency of isolates in milk samples

Name of isolated organisms	No. of isolated organisms	Percentage (%) of isolated organisms
Escherichia coil	18	30
Staphylococcus spp.	12	20
Streptococcus spp.	12	20
Corynebacterium spp	10	14
Unidentified	8	16
Total	60	100

4.2 Results of cultural examination

4.2.1 Nutrient broth

Nutrient broth was inoculated with the milk samples and incubated at 37°C for 24 hours. The growth of bacteria was indicated by the presence of turbidity (plate 4, 5).

4.2.2 Nutrient agar

Agar plates streaked with *E.coli* broth revealed the growth of bacteria after 24hrs of incubation at 3 7°C. The growth of *E.coli* on nutrient agar was characterized by circular, smooth, opaque and colorless colonies (Plate 1, 2, 3).

4.2.3 MacConkey (MC) agar

MacConkey agar plates streaked separately with the organism revealed the growth of after 24 hours of incubation at 37°C aerobically and were indicated by the growth of right- pink colored smooth colonies (Plate 6,7).

4.2.4 Eosin Methylene Blue (EMB) agar

EMB agar plates streaked separately with the organism revealed the growth of bacteria after 4 hours of incubation at 37°C aerobically. The growth was indicated by smooth, circular, Greenish black color colonies with metallic sheen on the agar plate (Plate 8,9).

4.2.5 Blood agar

Blood agar plates streaked separately with the organisms and incubated at 37°C aerobically for 24 hours. Hemolysis on blood agar media was recorded after overnight incubation (Plate 10-14).

4.2.6 Results of Gram's staining

The microscopic examination of Gram's stained smears from MC and EMB agar revealed

Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short

chain (Plate 16-18).

4.2.7 Results of biochemical test

4.2.7.1 Fermentation reaction with five basic sugars

The isolated bacteria fermented the five sugars (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) with the production acid and gas. The change of color from reddish to yellowish indicated acid production. The presence of gas bubble in the inverted Durham's tube indicated gas production.

4.2.7.2 Triple sugar Iron (TSI) agar slant test

The test organisms were culture into TSI agar slant by stab streak method. In TSI agar slant, yellow slant, yellow butt, presence of gas bubbles and absence of black precipitate (plate 39, 40).

4.2.7.3 Motility, Indole, Urease (MIU) test

Suspected colony was inoculated into the tube containing MIU medium. Then the medium was incubated at 37°C for overnight. *E.coli* in MIU medium causing turbidity and urease production with indole positive (Plate 36, 38).

4.2.7.4 Other biochemical tests

All the isolates were indole test positive, catalase test positive, methyl-red positive and VP test negative. Results of these tests are shown in Table 4.4 and Plate 21-35 and 41.43.

Table 4.2: Morphology and staining characteristics of isolated bacterial pathogens

Name of sample	No. of positive isolates	Test performed	Observation	Response				Indication
				Positive isolates	% of positive isolates	Negative isolates	% of Negative isolates	
Milk	60	Microscopic examination by Gram's staining technique	Gram negative (pink colour) small rod-shape, arranged in single or pair	18	30	42	70	*Escherichia coli*
			Gram negative (pink colour) small rod-shape, arranged in single or pair	12	20	48	80	*Staphylococcus spp*
			Gram negative (pink colour) small rod-shape, arranged in single or pair	10	10	50	90	*Corymebacterium spp*
			Gram positive, cocci shape, arranged in chain from	12	20	48	80	*Streptoccus spp*
			No growth	8	16	52	84	*Unidentified*

Table 4.3: Cultural properties of isolated bacterial pathogens

Name of sample	No. of positive isolates	Test performed	Observation	Response				Indication
				Positive isolates	% of positive isolates	Negative isolates	% of Negative isolates	
Milk	60	Nutrient agar	Circular, small, smooth, convex and gray-white cononies were produced	12	20	48	80	*Staphylococcus spp.*
			Small, smooth, circular and grayish colonies	10	10	50	90	*Corynebacterium sp*
			Circular, smooth, opaque and colorless colonies	18	30	42	70	*Escherichia coli*
			Circular, small smooth, convex, and golden, yellowish colonies	12	20	48	80	*Streptococcus spp*
		Blood agar	Hemolysis	12	20	48	80	*Staphylococcus spp*
			Grayish in color about 1mm in diameter and surrounded by a small zone of hemolysis	10	10	50	90	Corynebacterium sp.
			Hemolysis	18	30	42	70	*Escherichia coli*

			β-Type of hemolysis were produced	12	20	48	80	*Streptococcus sp*
		Staphylococcus agar no 110	Golden yellowish colonies	12	20	48	80	*Streptococcus sp*
		MacConkey agar	Bright- pink colored smooth colonies	18	30	42	70	*Escherichia coli*
		Eosin Methylene Blue agar	Black color colonies with metallic sheen	18	30	42	70	*Escherichia coli*

Table 4.4: Characterization of bacterial pathogens by biochemical properties

Name of sample	No. of positive sample	Name of the biochemical tests	Results	Response				Indication
				Positive isolates	% of positive isolates	Negative isolates	% of Negative isolates	
Milk	60	TSI agar slant reaction	Yellow slant, yellow butt & absence of gas bubbles.	12	20	48	80	*Staphylococcus spp*
			Yellow slant, yellow butt & absence of gas bubbles.	10	10	50	90	*Corynebacterium sp*
			Not done	18	30	42	70	*Escherichia coli*
		MIU test	-	12	20	48	80	*Staphylococcus*
			-	10	10	50	90	*Corynebacterium*
			+	18	30	42	70	*Escherichia coli*
		Indole test	-	12	20	48	80	*Staphylococcus*
			-	10	10	50	90	*Corynebacterium*
			+	18	30	42	70	*Escherichia coli*
			-	12	20	48	80	*Streaptcoccus*
		MR test	+	12	20	48	80	*Staphylococcus*
			-	10	10	50	90	*Corynebacterium*

			+	18	30	42	70	*Escherichia coli*
			+	12	20	48	80	*Streaptcoccus*
		VP test	-	12	20	48	80	*Staphylococcus*
			-	10	10	50	90	*Corynebacterium*
			-	18	30	42	70	*Escherichia coli*
		Catalase test	+	12	20	48	80	*Staphylococcus*
			-	10	10	50	90	*Corynebacterium*
			+	18	30	42	70	*Escherichia coli*
			+	12	20	48	80	*Streaptcoccus*

TSI = Triple Sugar Iron; MIU = Motility Indole Urease; MR = Methyl red; VP Voges-Proskauer; + = positive reaction; - = negative reactio

Plate 1. Culture of *E. coli* on Nutrient agar showing circular, smooth colourless

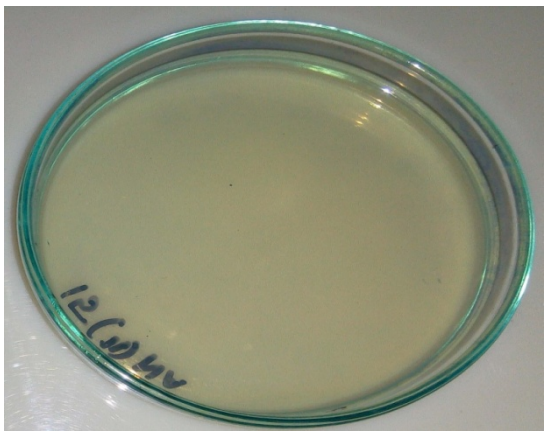


Plate 2. Nutrient agar (Control)

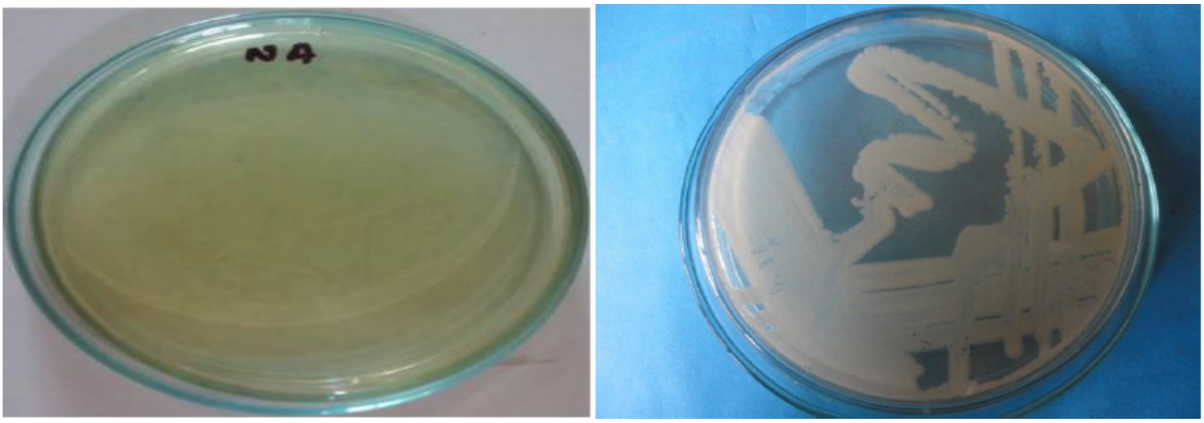


Plate 3: *Staphylococcus spp* produce small yellow colonies on Nutrient Agar (Right) and uninoculated control (Left)

Control

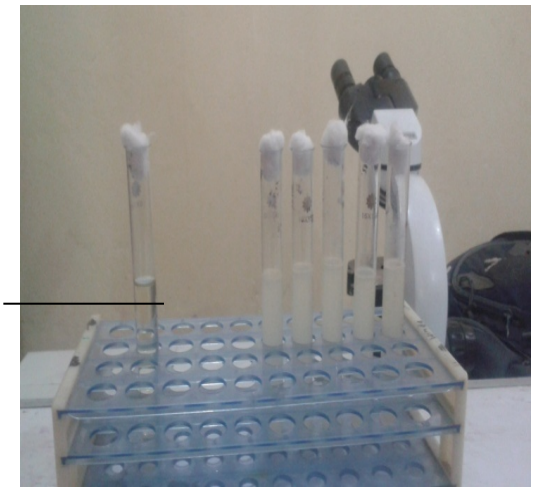


Plate-4: Culture of *E. coli* in nutrient broth showing turbidity (right) fresh

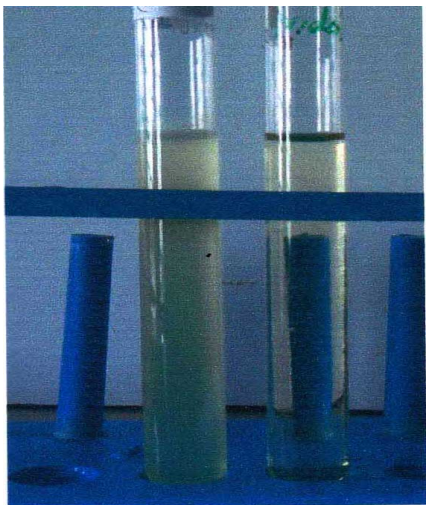


Plate-5: Culture of *Corynebacteriam sp.* on in nutrient broth

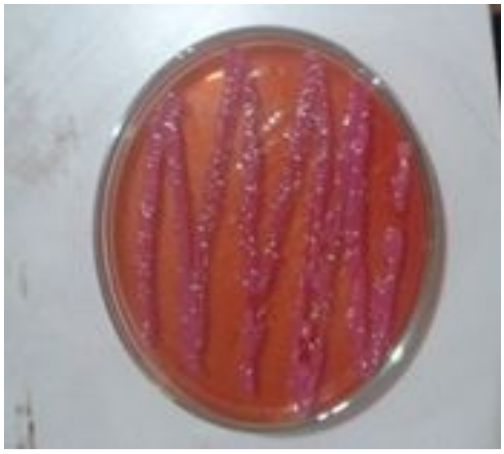


Plate-6: Lactose fermenting organisms produce bright pink

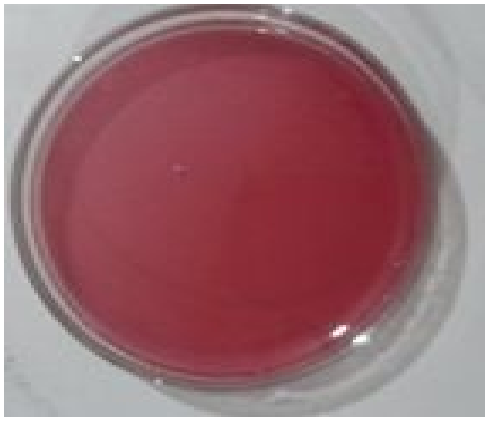


Plate-7: MacConkey agar (control)

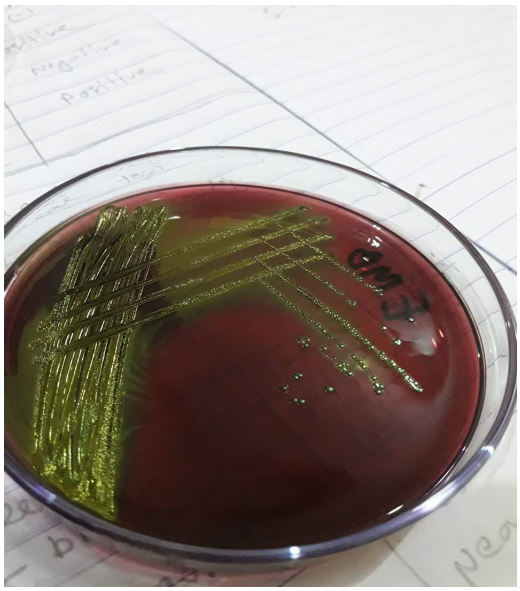


Plate-8: Growth of *E. coli* on EMB agar showing greenish black coloniless with metallic sheen

Plate-9: EMB agar (control)

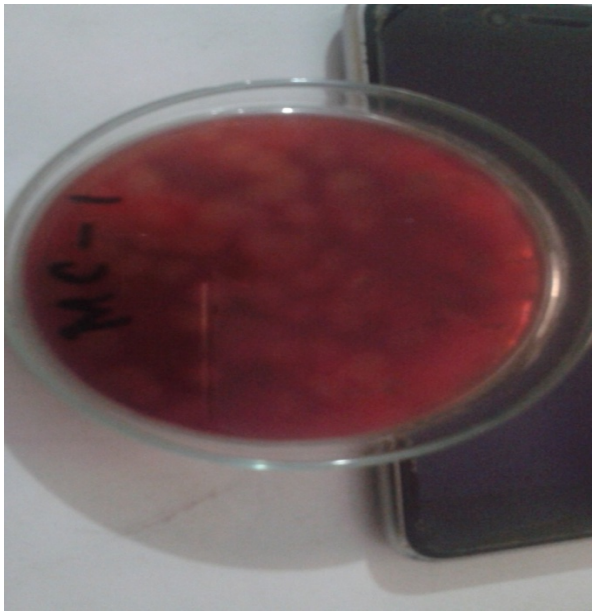


Plate-10: Culture of *E. coli* on Blood agar observed characteristic hemolysis

Plate-11: Blood agar (control)

Plate 12: Blood agar media showing hemolytic reaction for *staphylococcus spp*

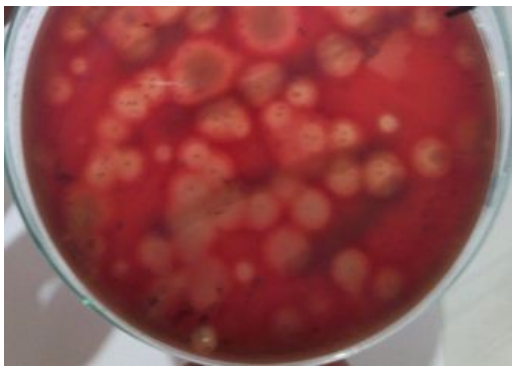


Plate No 13: β-hemolytic colonies of *Streptococcus spp* on Blood

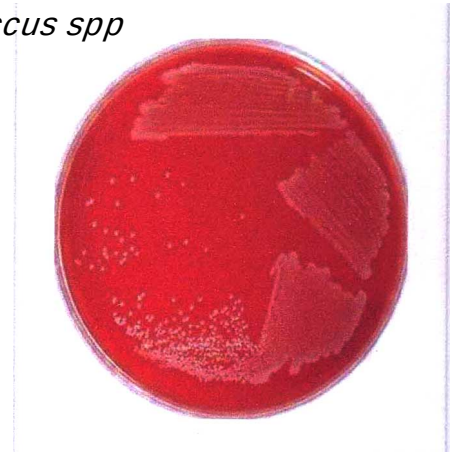


Plate 14: Culture of *Corynebacterium sp.* on blood agar showing grayish colony about 1 mm in diameter and surrounded by a zone of hemolysis (left) and nuinoculated control

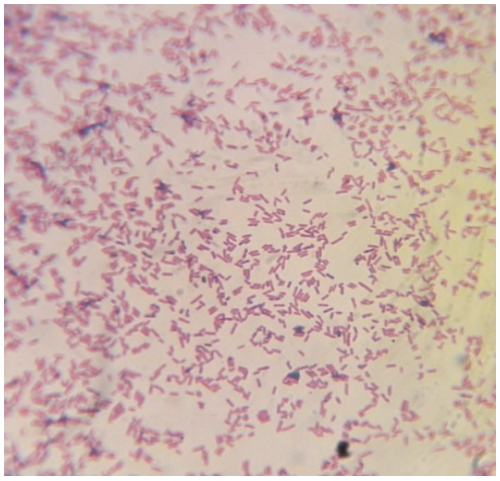


Plate-15: *E. coli showing* Gram-negative (Pink colour) small rod shape, arranged in single or paired at 100x

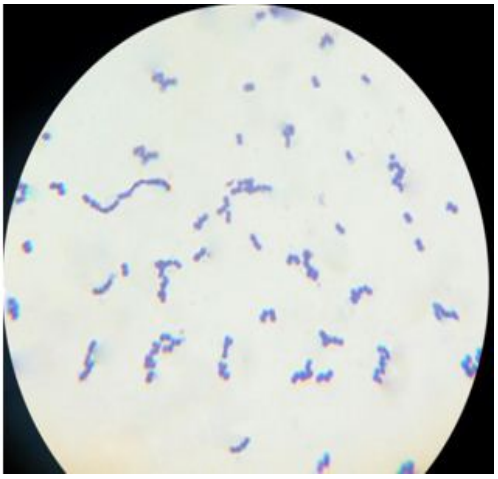


Plate no 16: Gram positive short chain shaped violet colour *streptococcus spp* under 100xmicroscopy.

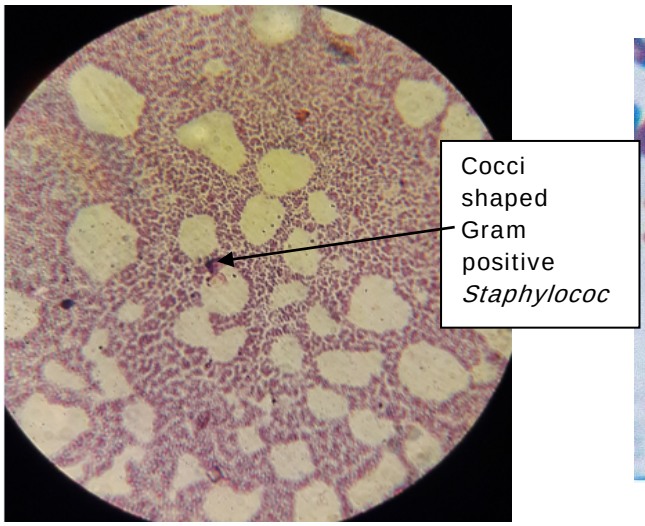


Plate 17: Gram positive cocci *Staphylococ*

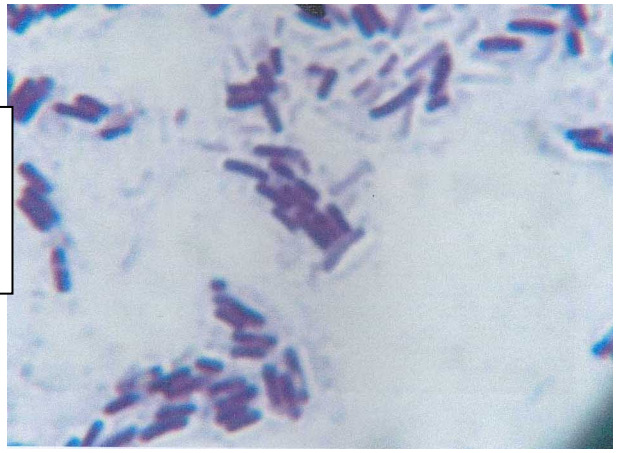


Plate 18: *Carynebacterium sp* showing gram positive (Violet color) short cocco bacilli or rods arranged in bundles and singly also at 100x (Gram's staining)

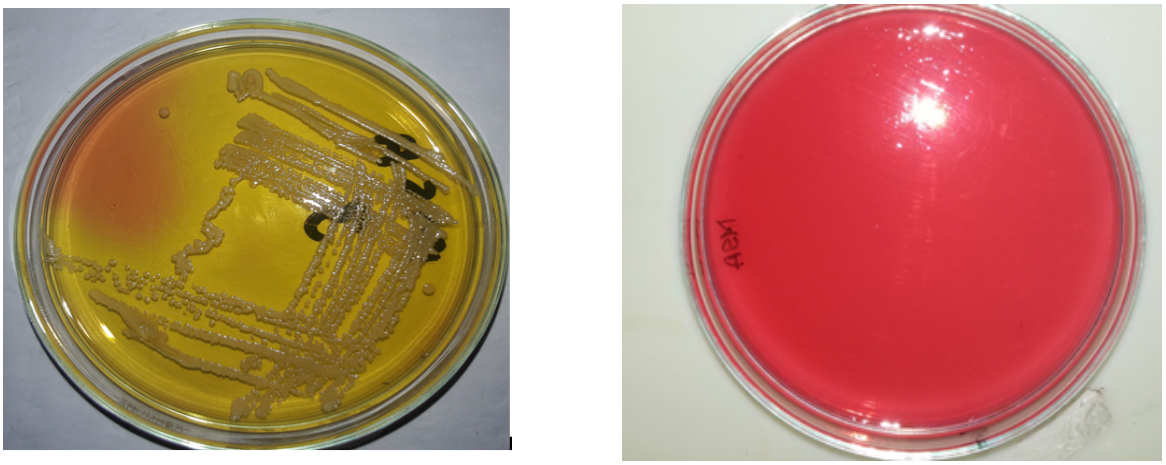


Plate 19: *Staphylococcus spp* produce medium yellowish colony on Manitol salt agar MSA (left) and uninoculated control (right)

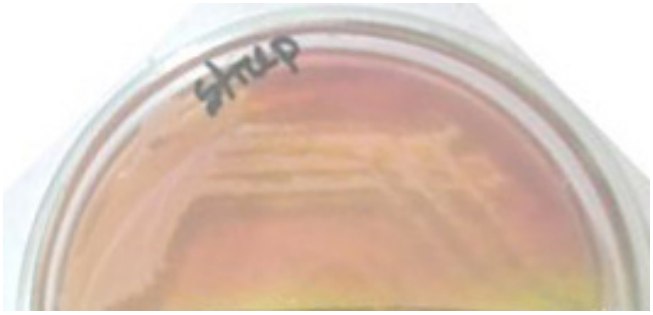


Plate No 20: *Streptococcus spp* on Staphylococcus Agar No. 110

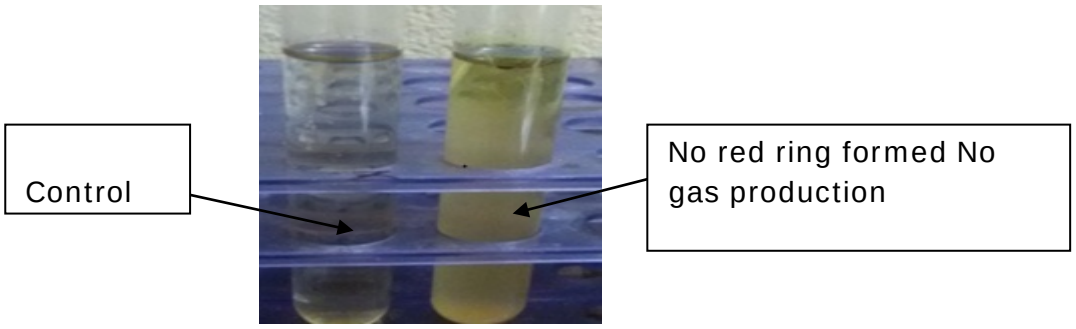


Plate 21: *Staphylococcus* spp showing negative result (right) on Indole with control (left)

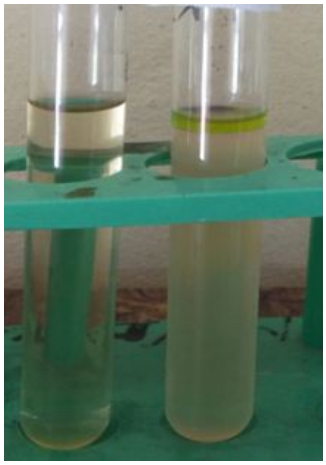


Plate no 22. Indole test were negative on *Streptococcus spp*

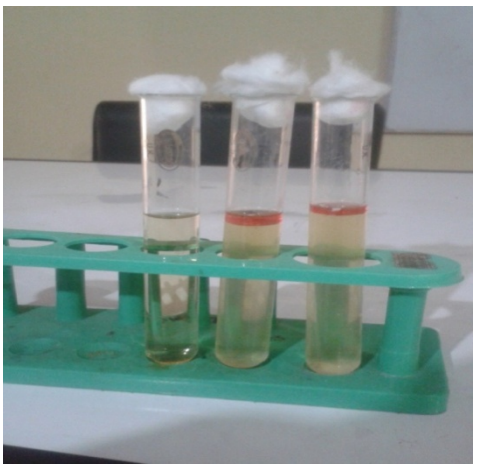


Plate-23: Indole test showing positive results with a red colour in the reagent layer indication indole production with reaction of *E. coli* (Right) and

Plate 24: Indole test showing no change of the medium with the reaction of *Coryebacterium sp*. (righ) and uninoculated controle (Left)

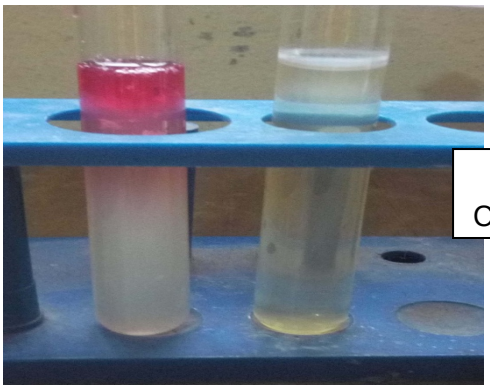


Plate 25. Methyl –Red test for *E. coli* showing the medium was change to bright red colour (left) and

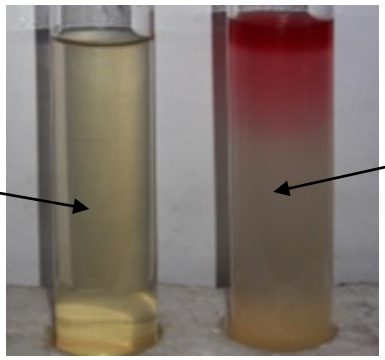


Plate 26: *Staphylococcus spp* showing positive result (right) on MR with control (Left)

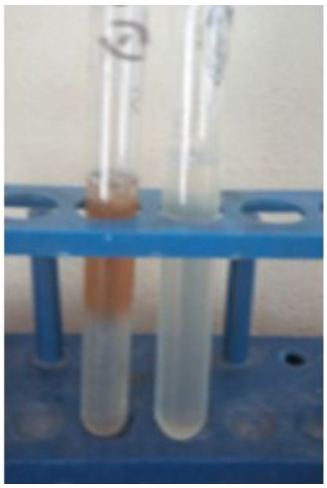


Plate no 27. MR test negative for *Streptococcus spp*

Plate 28: Methyl- red test for *corymebacterium sp.* showing the medium was changed to yellow color (left) and uninoculated control (right)

Plate 29. Voges-Proskauer test for *E. coli* showing no change of the medium (Right) and uninoculated control (Left)

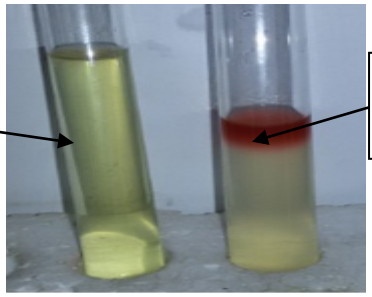


Plate 30: *Staphylococcus spp* showing positive result (right) on VP control (left)

Plate no 31. *Streptococcus spp* showing positive result (left) on VP control (right)

Plate 32. Simmon's citrate test for *E. coli* showing no change of the medium (Right) and

Plate 33: *Staphylococcus spp* showing negative result (right) on Citrate with control (left)

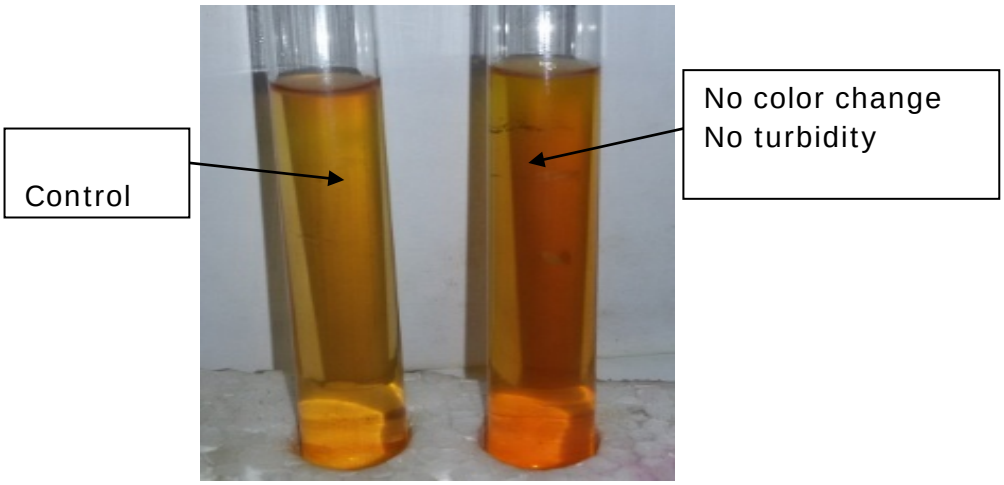


Plate 34: *Staphylococcus spp* showing negative result (night) on Citrate utilzation with control (left)

Plate no 35: *Streptococcus spp* were negative results by no changed medium green to blue

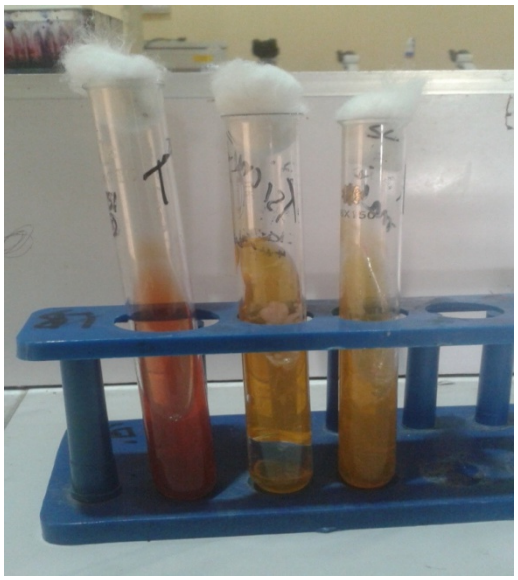


Plate 36. Motility Indole Urease test causing turbidity and Urease production with indole positive by *E. coli* (Right) and uninoculated control

Plate 37: MIU *test* showing *Staphylococcus spp* were non motile result

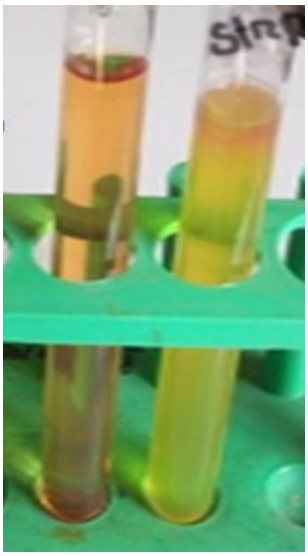


Plate 38: MIU test showing positive results on *Streptococcus spp.*

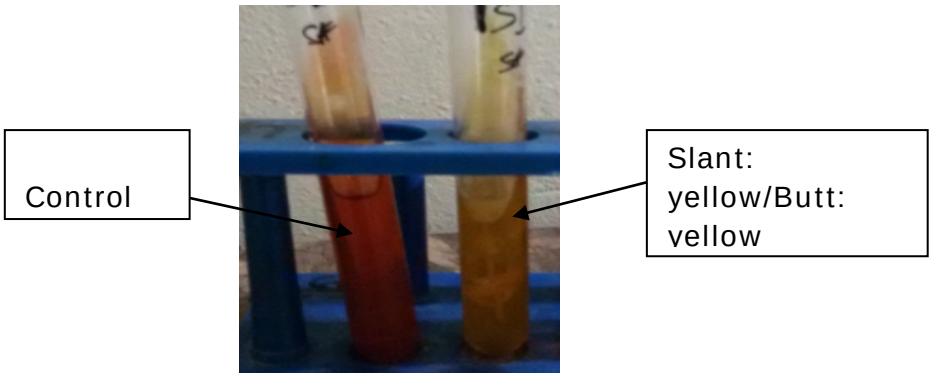


Plate 39: *Staphylococcus spp* showing positive result (right) on TSI with control (left)

Plate 40: *Streptococcus spp* showing positive result (left) on TSI with control (right)

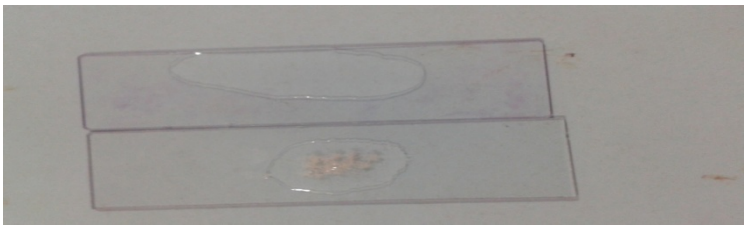


Plate 41. Catalase test (positive) for *E. coli* indicating by formation of gas bubbles

Plate No 42: Catalase negative on *streptococcus spp*

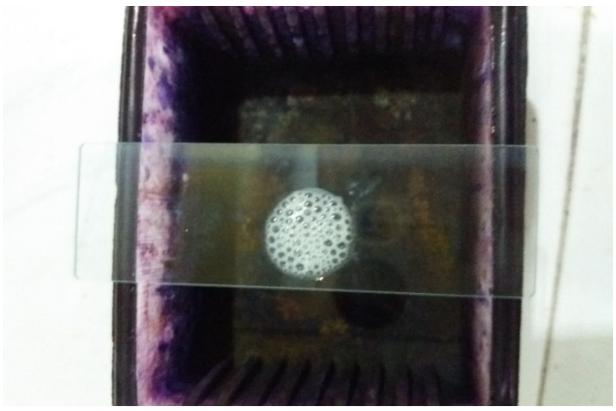
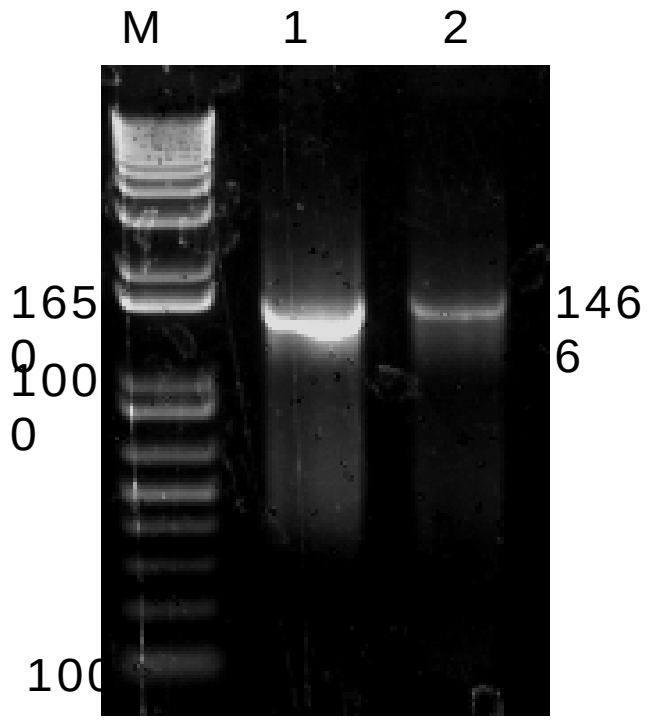


Plate 43: Catalase test of *Staphylococcus spp* showing bubble formation indicating positive reaction.

4.2.8 Result of PCR Amplification Sequencing of 16s rRNA Genes with universal Primers and phylogenetic analysis of *E. coli*

Out of 60 sample of E. coli was resent in 18 cases and the percentage was 30%, 16S rRNA gene region was amplified with the universal primers, Forward primer
27F (5′-AGAGTTTGATCCTEGGCTCAG3') and Reverse primer
1492 R (5'- TACCTTGTTACGACTT3'). PCR Amplification band was found at 1466 bp.



M: Marker, 1kb plus DNA ladder

Figure 4.1: Results of Amplification of 16s rRNA Gene region of *E. coli*

Note: M: Marker 1kb Plus DNA ladder

PCR: Polymerase Chain Reaction

kb: Kilo base

4.2.9 Contig sequence of *Escherichia coli*

Contig_ E2:1466 bp, Blast: 97%

Identified Strain: Escherichia marmotae

Contig E2

TGCAGTCGACGCCGACAGGAAACAGCTTGCCGCTTCGCTGACGAGTGGCG
GACGGGGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCT
TCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGG
GGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACC
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTA
TGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGT
AAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAA
TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCT
CGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCT
GGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTC
AGGTGCGAAAGCGTgGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCGAYTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCC
GGAGCTAACGCGTTAARTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAA
AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTTGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTT
TCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCG

CAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGAC
TGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGC
CCTTACGACCAGGGCTACACACGTGGTACAATGGCATATACAAAGAGAAG
CGACCTCGCGAGAGCAAGCGGACTTCATAAAGTATGTCGTAGTCCGGATT
GGAGTGTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGAT
CAGAATGATACGGTGAATACGTTCCCGGGCTTTGTACACCCCGCCCGTCA
CACCATGGGAGTGGGTTGCGTAAAGAAGTAGGTAGGTTAACATTCGGGAG
G

4.2.10 Electropherogram

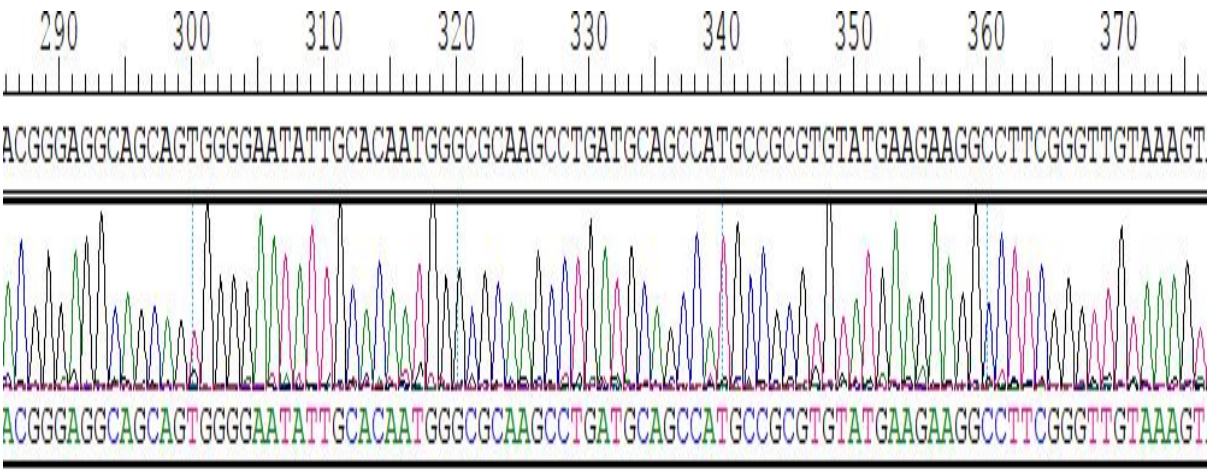


Figure 4.2: Electropherogram of 16s rRNA Gene Sequence of *Escherichia coli*

4.2.10 Phylogenic tree analysis of *Escherichia coli*

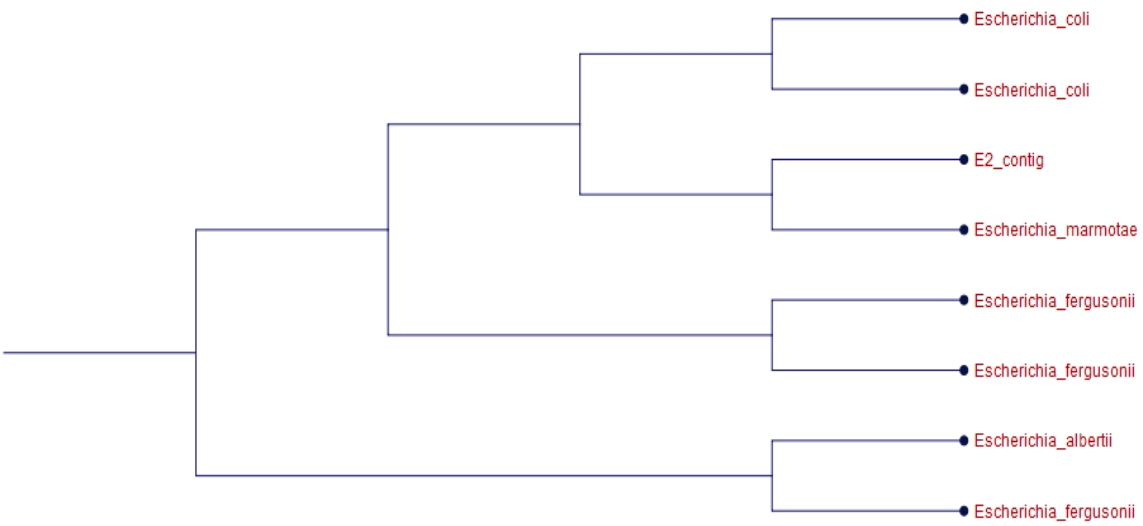


Figure 4.3: Phylogenic tree analysis of *Escherichia coli*

4.2.12 Statistical Analysis

Table 4.5: Statistical Analysis

Sample collection Area	Number of sample	Positive number of sample (%)	Chi Square	P Value
Rangpur, Pirgonj	20	5	2.460	0.782
Rangpur Sadar	10	3		
Mimima Dairy farm Dinajpur	10	4		
HSTU Dairy farm Dinajpur	6	1		
Nayanpur, Dinajpur	6	3		
Jaigirhat, Rangpur	8	2		
Total	60	18		

Table number shows the statistical analysis of different dairy farms, A p-value was more than 0.05 were statistically considered non significant.

CHAPTER -V

DISCUSSION

60 raw milk samples were analyzed in the laboratory by different cultural, biochemical, staining and molecular methods to detect *Escherichia coli* in raw milk. Out of 60 samples, 30% (n=18) were contaminated with *E. coli*. 20% (n=12) were contaminated with *Staphylococcus spp*. 20% (n=12) were contaminated with *salmonella spp.*14% (n=10) were contaminated with *Corynebacterium spp* and 16% (n=8) were unidentified. Out of 60 samples, 18 samples were revealed the positive result for *E. coli*. *E. coli* was identified and confirmed by cultural examination, morphological studies, staining characters and biochemical tests and finally PCR were performed for the amplification of specific gene (16s rRNA gene) of isolated bacteria. In statistical analysis, the prevalence of the isolates in different dairy farm found from this study was non-significant.

In MC agar plates, the samples showed bright pink or red colonies in MC agar were identified as *E. coli* (Plate 1). In EMB agar colonies produced by *E. coli* are smooth, circular, black color colonies with metallic sheen (Plate 8). The microscopic examination of Gram's stained smears from MC agar and EMB showed that the isolated bacteria were Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain (Plate 15). *E. coli* showed negative result to catalase. DNA extracted from *E. coli* isolates was used in the PCR assay. PCR primers targeting 16S rRNA gene of *E. coli* amplified 1466 bp fragments of DNA confirmed the identity of *E. coli*. Result of PCR for *E. coli* is shown in (Plate 4). Milk is the best media for the growth of many bacteria in which some of them are pathogenic. As we know fresh milk is enriched with pathogenic and non pathogenic bacteria which can be transmitted to human by milking and consumption of milk.

Coliform bacteria present in the fresh raw milk might be hazardous if proper boiling of milk is not done during consumption. It causes disease if proper hygienic procedure is not maintained during milking. In most

cases, milk containing *E. coli* was obtained from animals with subclinical mastitis. Zafolon *et al.* (2008) studied at Nova Odesa, São Paulo and showed that the prevalence of *E. coli* was 57.3%. The results obtained in my study are likewise higher when compared to those formerly documented (Soomro *et al.,* 2002; Kumer and Prasad, 2010; Hossain, 2015).

Based on observations made throughout the collection of samples, we concluded that the improper hygiene practice and poor management before and during milking may have contributed to the contamination of milk with *E. coli*, and the commercial farms are more vulnerable in this case. The incidence at a considerable high percentage indicates the alarming situation both for dairy farming and for public health. The presence of *E. coli* in the milk sample is an appealing as well as an important finding of this study.

CHAPTER-VI

SUMMARY AND CONCLUSION

The results from this study indicate that the farming sites from where the samples were collected are pretty much well maintained. Because, the prevalence of the isolates found from this study was non-significant. But it concluded that raw milk available for consumers have a high *E. coli* contamination. Thus the results of the present study warn the need for more precaution. The present research was undertaken with a view to isolating and characterizing *Escherichia coli* present in cow's raw milk sample collected from different region of Bangladesh. Out of 60 samples, 18 (30%) milk samples were found positive for *E. coli*. 18 *Escherichia coli* isolates were amplified by 16S rRNA gene based PCR.

Concluding remarks

- A concise, informative prevalence of bacteria prevalent in cow's raw milk were considered on the basis of number of collected sample from different dairy farm.
- Molecular characterization by using PCR techniques confirm presence *Escherichia coli* in collected raw milk sample.
- Proper udder hygiene and sanitation should maintain during milking to decrease number of these bacteria and that will increase the national economy as a high protein source.

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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/in2 presssure 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/in2 pressure for 15 minutes.

Eosine methylene blue(EME) 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^2pressure 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^2pressure for 15 minutes.

Salmonella Shigella Agar	Gram/Liter
Lactose	10.0gm
Bile salt No.3	8.5gm
Sodium citrate	8.5gm
Sodium Thiosulfate	8.5gm
Beef extract	5.0gm
Proteose peptone	5.0gm
Ferric citrate	1.0gm
Brilliant Green	0.33gm
Nuetral Red	0.025gm
Agar	13.5 gm
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

APPENDIX-II

Composition of the media used in biochemical test

MR-VP broth	Gram/Liter
Peptone	7.0
Dextrose	50
Dipotassiuni 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.

Indoletryptopon broth medium	Gram/Liter
Tryptone	10.0
Distilled water	1000ml

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

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	l.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