Assessment of microbial load in fecal sample of fat-tailed sheep with molecular characterization and antibiogram study

A THESIS

BY

NABIL MAHMUD

REGISTRATION NO.: 1605464

SEMESTER: JULY-DECEMBER, 2017

SESSION: 2016-2017

MASTER OF SCIENCE
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY

UNIVERSITY, DINAJPUR-5200

DECEMBER, 2017

Assessment of microbial load in fecal sample of fat-tailed sheep with molecular characterization and antibiogram study

A THESIS

BY

NABIL MAHMUD

REGISTRATION NO.: 1605464

SEMESTER: JULY-DECEMBER, 2017

SESSION: 2016-2017

Submitted to the
Department of Microbiology
Hajee Mohammad Danesh Science and Technology University, Dinajpur
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2017

Assessment of microbial load in fecal sample of fat-tailed sheep with molecular characterization and antibiogram study

A THESIS

BY

NABIL MAHMUD

REGISTRATION NO.: 1605464

SEMESTER: JULY-DECEMBER, 2017

SESSION: 2016-2017

Approved as to style and content by

_____	_____
Research Supervisor	**Research Co- Supervisor**
Dr. Mir Rowshan Akter	Dr. Farzana Afroz
Associate Professor	Assistant Professor
Department of Microbiology	Department of Microbiology

_____

Dr. Md. Khaled Hossain
Chairman
Examination committee and
Chairman
Department of Microbiology

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2017

DEDICATED
TO MY
BELOVED PARENTS

ACKNOWLEDGEMENT

The author is ever grateful and indebted to the Almighty Allah without whose grace he would have ever been able to pursue his higher studies in this field of science and to complete his research work successfully for the degree of Master of Science (MS) in Microbiology.

The author sincerely desires to express his heart felt respect, deepest sense of gratitude, sincere appreciation and profound indebtedness to his respected teacher and Research Supervisor, ***Dr. Mir Rowshan Akter****, Associate Professor, Department of Microbiology, Faculty of Veterinary and Animal Science (FVAS), Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur for her constant supervision, scholastic guidance, uncompromising principles, helpful advice, constant inspiration, affectionate feeling, radical investigation and constructive criticism in all phases of this study and preparing the manuscript.*

The author finds it a great pleasure in expressing his heartfelt gratitude and immense indebtedness to his honorable teacher and respected Research Co-Supervisor, ***Dr. Farzana Afroz****, Assistant Professor, Department of Microbiology, Faculty of Veterinary and Animal Science (FVAS), Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, for her sympathetic supervision, kind co-operation, inspiration and valuable suggestions, constructive criticism and valuable advice for the completion of the research work and preparation of the thesis.*
The author wishes to express his cordial respect and profound gratitude to his teachers ***Dr. MD. Khaled Hossain,*** *Associate Professor and Chairman,* ***Dr. Md. Fakhruzzaman****, Associate Professor,* ***Dr. Atiqul Haque****,* ***Dr.Most.Deloara Begum****, Department of Microbiology, FVAS, HSTU, Dinajpur, for their valuable advice and constant inspiration throughout the entire period of study.*

The author expresses his cordial appreciation to ***MD. Ayub****,* ***MD. Saiful****,* ***MD.Ansar*** *and all other office staff of the Department of Microbiology FVAS, HSTU, Dinajpur.*

The author expresses heartfelt gratitude and gratefulness to his beloved parents, for their blessing, inspiration, sacrifice and moral support which opened the gate and paved the way to his higher studies and also provided his with the best of everything in his life.
The author is sincerely thankful and grateful to his friend ***Samina Akter*** *for her dedication, helping hand and cordial co-operation.*

The author is also cordially grateful to the persons who helped him in sample collection from the farms, and equally thankful to ***National Institute of Biotechnology (NIB)*** *for their co-operation.*

Finally, the author is extending his cordial thanks to all his friends, relatives and well-wishers.

The Author

December, 2017

ABSTRACT

This study was conducted to observe the microbial load in the rectal swab and in fecal samples of lamb and adult fat-tailed sheep with molecular characterization and antibiogram study of the identified isolates. The study was performed during the period from July, 2017 to December, 2017. A total of 32 samples were collected, of which 16 were from Dinajpur (8 rectal swab and 8 fecal samples) and 16 were from Nilphamari (8 rectal swab and 8 fecal samples). The organisms were isolated by using standard microbiological method. The result revealed that in case of total aerobic count, the microbial load was higher in Dinajpur ($1.53\times10^7\pm0.05$ CFU) than that of in Nilphamari ($1.46\times10^7\pm0.06$ CFU) and in case of total anaerobic count, it was also higher in Dinajpur ($0.78\times10^7\pm0.012$CFU) than that of in Nilphamari ($0.62\times10^7\pm0.05$).

Among the thirty two samples, the prevalence of the isolates were, *Escherichia coli* 11 (34.37%), *Salmonella spp* 10 (31.25%), *Klebsiella spp* 7 (21.87%). and *Clostridium spp* 4 (12.5%).

The bacteria were isolated by using different bacteriological culture media (MacConkey agar, EMB agar, SS agar, BGA agar, brain heart infusion agar, blood agar and Robertson's cooked meat medium) and identified by different biochemical tests (catalase test, indole test, MR reaction, VP reaction, TSI test, citrate utilization test and MIU test). The bacteria were identified by using Gram's staining technique and out of four isolates 3 isolates were found to be gram negative rods (*Escherichia coli, Salmonella spp, Klebsiella spp.*), while one was gram positive, small rod (*Clostridium spp.*). Among four (4) identified isolates, *Klebsiella spp.* was characterized by using molecular technique like PCR and electropherogram and it was characterized as *Klebsiella pneumoniae.*

From the antibiogram study it was observed that *Escherichia coli, Salmonella spp.* and *Klebsiella pneumoniae* were highly susceptibile to Ofloxacin, while these isolates were individually susceptible to Flumequine, Cefixime and Vancomycin respectively. Colistin was susceptible to *Escherichia coli* and *Klebsiella pneumoniae. Escherichia coli, Salmonella spp.* and *Klebsiella pneumoniae* were resistant to Cefepime, Cefaclor, Tobramycin, Aztreonam and Vancomycin. *Clostridium spp.* exhibited high susceptibility to Colistin, Ciprofloxacin and Levofloxacin. Gentamycin and showed resistance to Nalidixic Acid, Co-Trimoxazole, Amoxycillin, Erythromycin, Cefalexin, Vancomycin, Cloxacillin and Penicillin. The study suggests that the proper dosing of the antibiotics and maintenance of hygiene should be established in all the farms of fat-tailed sheep in Bangladesh to inhibit the multi drug resistant bacteria to keep the sheep and the individuals directly involved with the rearing sheep safe from all the possible zoonotic diseases.

CONTENT

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

-	Negative
#	Identifying number
%	Percentage
@	At the rate of
+	Positive
µg	Microgram
µl	Microlitre
^{0}C	Degree of celcius
Assist	Assistant
BA	Blood Agar
BD	Bangladesh
EMB	Eosin Methylene Blue
ER	Erythromycin
et al.	Associated
etc	Etcetera
Gm	Gram
H.S	Hemorrhagic septicemia
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
HSTU	Hajee Mohammad Danesh Science and Technology University
MARMC	M. Abdur Rahim Medical College
SHD	Sadar hospital of Dinajpur
IBCH.	Islami bank community hospital, Dinajpur

LIST OF ABBREVIATIONS AND SYMBOLS (Cont.)

HSTU	Hajee Mohammad Danesh science and Technology University, Dinajpur
M.S	Master of Science
MC	Mac-Conkey Agar
MSA	Mannitol Salt Agar
MI	Milliliter
MIU	Motility Indole Urease
MR	Methyl Red
NA	Nutrient Agar
NB	Nutrient Broth
No.	Number
PBS	Phosphate Buffer Saline
Prof.	Professor
PSS	Physiological Saline Solution
RPM	Rotation Per Minute
SC	Subcutaneous
SE	Standard Error
SL	Serial number
spp	Species
SSA	Salmonella- Shigella Agar
v/v	Volume by volume
vp	Voges-Proskauer
w/v	Weight by volume

CHAPTER 1

INTRODUCTION

The **fat-tailed sheep** is a general type of domestic sheep known for their distinctive large tails and hindquarters. Fat-tailed sheep breeds comprise approximately 25% of the world sheep population and are commonly found in northern parts of Africa, the Middle East, Pakistan, Afghanistan, Iran, North India, Western China, Somalia and Central Asia. Bangladesh is not common for fat-tailed sheep but some place of here found this animal.

Two general varieties of fat-tails exist, the broad fat-tails and the long fat-tails. The majority of fat-tailed sheep breeds have broad fat-tails, where the fat is accumulated in baggy deposits in the hind parts of a sheep on both sides of its tail and on the first 3–5 vertebrae of the tail. In the long-tailed sheep the fat is accumulated in the tail itself, which may grow so large that it drags on the ground and hinders copulation (Benham 1987).

The earliest record of fat-tailed sheep is found in ancient Uruk (3000 BC) and Ur (2400 BC) on stone vessels and mosaics. Sheep were specifically bred for the unique quality of the fat stored in the tail area and the fat (called *Elyah*, Arabic: أليــة) was used extensively in medieval Arab and Persian cookery.

In Bangladesh, sheep is an important livestock sector, which contributes significantly to the food security and welfare of rural households. The sheep population of Bangladesh in 2015 was about 1.9 million head. About 83.9% of total households own livestock.

They are adapted to breeding in desert and semi desert regions; they are not particular about fodders and are capable of using poor, sparse pastures and tolerating long migrations. The majority of breeds in this group have characteristically high meat productivity. The rams weigh 110-130 kg, sometimes 180 kg, the ewes 60-85 kg, sometimes 130 kg. The kurdiuk weighs 5-7 kg, in some breeds (Gissar) reaching 20-30 kg. Dressed yield after pasturing is 53-56 percent (maximum 60 %). The wool productivity is low, the average clipping in most breeds is 2.0-2.2 kg. The fleece is not homogenous and is used to manufacture coarse fabrics, carpets and felt foot wear. In order to improve the wool production, the fat-tailed sheep are crossbred with fine-wooled and semifine-wooled breeds. The milk production of fat-tailed ewes reaches 120 kg (35-55 kg of the commercial milk). Fertility is 105-120 lambs per ewes.

The sciatica might be treated by dissolving the fat sheep tail and then on its division on 3 equal parts, and then drinking on an empty stomach in three equal parts, per one each morning.

Although, this animal is not originated from Bangladesh, but due to their high adoptability and naturally strong immune system, they are being introduced and being farmed in different areas of Bangladesh.

The native breeds (*Ovis aries*) include the Awassi, Arabi, Na'aimi, Karadi and Hamdani. These are all fat-tailed, carpet-wool producers with some potential to produce milk. Most of the income of sheep producers is derived from the sale of lambs. Milk is partially consumed by the farmers as an important source of protein and the remainder is sold as ghee, yogurt and cheese. People of Bangladesh are not habituated with consuming milk from fat-tailed sheep.

Domestication of livestock by man introduced a major cultural revolution. Hominoids and early men were hunters and gatherers for thousands of years. The climatic fluctuations, which followed the end of glacial period some 14000 years ago, may have been instrumental in forcing man to domesticate animals. Records of the domestication of sheep date back as early as 7000 BC in the Near East.

The conservation of domestic animal diversity is essential to meet future needs in our country. In order to cope with an unpredictable future, genetic reserves capable of readily responding to directional forces imposed by a broad spectrum of environments must be maintained. Maintaining genetic diversity is an insurance package against future adverse conditions. Due to diversity among environments, nutritional standards and challenges from infectious agents, a variety of breeds and populations are required. These act as storehouses of genetic variation which forms the basis for selection and may be drawn upon in times of biological stress such as famine, drought or disease epidemic. The wide range of breeds and species are each specifically adapted to a different set of conditions.

Bacterial infections are an important issue in intensive sheep breeding. The most important thing is a high level of sheep infection with *Escherichia coli, Salmonella spp, Klebshilla spp, Micrococcus spp, Staphylococcus spp, Streptococcus spp, Corynebacterium spp, Lactobacillus spp, Fusobacterium spp* (Mueller 1984).

Escherichia coli is the common enteric commensal in animal species and part of strains of this microorganism is an established diarrhoeal pathogen and associated with human disease (Duffy, 2003).

Due to the global expansion of fat-tailed sheep farming and lack of information about the prevalence of common microbes in fat-tailed sheep, the present study was designed with following objectives:

I. To determine the microbial load in fat-tailed sheep by using total viable count.
II. To isolate and identify the bacteria by using morphological, cultural and biochemical techniques.
III. To characterize the identified bacteria by using PCR and electropherogram.
IV. To detect the susceptibility pattern of commercially available antibiotic against identified isolates.

CHAPTER 2
REVIEW OF LITERATURES

2.1 Objective I: To observe the microbial load in fat-tailed sheep by using total viable count.

Hailu T *et al.* (2016) isolated non-typhoidal *Salmonella* from Sheep feces. They collected a total of 113 sheep fecal samples and processed bacteriologically according to the protocol recommended by the International Standardization Organization designed for isolation of Salmonella from food and animal feces (ISO-6579, 2002) with some modifications. Among a collected 113 fecal samples, 7(6.19%) were positive for Salmonella. However, the difference was not statistically significant (p-value>0.05). Based on ages groups the highest prevalence of Salmonella isolate was obtained from old sheep 2(12.5%) and the adult sheep yields the least. But, the difference was not statistically significant (p-value>0.05). In this study the higher prevalence of Salmonella isolate was pointed out from male with 7.69% than female sheep with 5.41%, even though the association between sexes was not statistically significant (P-value>0.05). The disease was found more prevalent in the animals living together with human.

Knight DR *et al.* (2013) studied the Prevalence of gastrointestinal Clostridium difficile carriage in Australian sheep and lambs. A total of 371 sheep and lamb fecal samples were received in seven batches from three different geographic areas in eastern Australia and two in Western Australia. The overall rate of detection in sheep and lambs was low (4.0%); however, carriage/colonization in lambs (6.5%) was statistically significantly higher than that in sheep (0.6%) (P = 0.005). Seven distinct PCR ribotype patterns were observed, three of which were known international ribotypes (UK 056 [n = 1], UK 101 [n = 6], and UK 137 [n = 2]), while the remainder were unable to be matched with our available reference library. This low rate of carriage/colonization in Australian ovines suggests they are unlikely to be a major source/reservoir of human infections.

Uzal *et al.* (2004) stated that *Clostridium perfringens* produces disease in sheep, lambs and other animal species, most of which are generically called enterotoxemias. This microrganism can be a normal inhabitant of the intestine of most animal species including humans but when the intestinal environment is altered by sudden changes in diet or other

factors, C. perfringens proliferates in large numbers and produces several potent toxins that are absorbed into the general circulation or act locally with usually devastating effects on the host. History, clinical signs and gross post-mortem findings are useful tools for establishing a presumptive diagnosis of enterotoxaemia by C. *perfringens* in sheep and lambs although no definitive diagnosis of these diseases can be made without laboratory confirmation. Because all types of C. *perfringens* can be normal inhabitants of the intestine of most animals, culture of this micro-organism from intestinal contents of animals has no diagnostic value unless a colony count is performed and large numbers (usually more than 104-107 CFU/g) of C. *perfringens* were isolated.

Mueller RE *et al*. (1984) determined successive changes in aerobic and anaerobic bacterial counts and changes in the generic composition of the epimural community in lambs from 1 to 10 weeks.

2.2 Objective II: To isolate and identify the bacteria by using morphological, cultural and biochemical techniques.

Saeed Alamian *et al.* (2015) discovered that full doses of *Brucella melitensis* strain can be used conjunctively to vaccinate pregnant Iranian sheep during late pregnancy without abortifacient effects, prolonged antibody responses and vaccine strain excretion in milk and vaginal discharges.

Aslani MR *et al*. (2015) diagnosed infective vegetative endocarditis of the right heart of a 3-year-old fat-tailed ram. Tachycardia, marked brisket edema, jugular veins distention and pulsation and pale mucous membranes were the clinical findings. Hematology revealed changes consistent with moderate dehydration (PCV 42%). There was a moderate leucocytosis (WBC count 16.4 × 109/L) with 82% neutrophils, 14% lymphocytes, and 4% monocytes. Bacterial culture of the heart valve lesions on blood agar resulted in the isolation of α-hemolytic Gram-positive and non-acid-fast rods. The microorganism was identified as *Erysipelothrix rhusiopathiae* using biochemical tests.

Reza Kheirandish *et al*. (2013)reported the first case of pulmonary nocardiosis in sheeps in Iran. Nocardia spp. are known as causes of bovine mastitis, pneumonia, cutaneous or subcutaneous abscesses and disseminated disease. Pulmonary infection by Nocardia spp. was

diagnosed in a sheep. Grossly, the animal had multiple white, firm, and small nodules in the lung. Microscopically, the nodules consisted of foci of pyogranulomatous inflammation with the presence of macrophages, multinucleated (langhans-type) giant cells and eosinophilic amorphous material with rosette-shape configurations (Splendore-Hoeppli reaction). Tissue samples of the lung were streaked onto bovine blood agar, incubated at 37°C for 48-72 hours under aerobic condition, and stained with Gram and modified Zeihl-Neelsen staining. Bacteriological investigations confirmed numerous thin, gram-positive, beaded, branching, filamentous and non- acid fast organisms. Finally, on the basis of characteristic gross, histopathological and bacteriological findings, pulmonary nocardiosis were diagnosed.

Garcia JP *et al.* (2013) evaluate d the contribution of epsilon toxins (ETX) to *C. perfringens* type D pathogenicity in an intraduodenal challenge model in sheep, goats, and mice using a virulent *C. perfringens* type D wild-type strain (WT), an isogenic ETX null mutant (*etx* mutant), and a strain where the *etx* mutation has been reversed (*etx* complemented). All sheep and goats, and most mice, challenged with the WT isolate developed acute clinical disease followed by death in most cases. Sheep developed various gross and/or histological changes that included edema of brain, lungs, and heart as well as hydropericardium. Goats developed various effects, including necrotizing colitis, pulmonary edema, and hydropericardium. No significant gross or histological abnormalities were observed in any mice infected with the WT strain. All sheep, goats, and mice challenged with the isogenic *etx* mutant remained clinically healthy for ≥24 h, and no gross or histological abnormalities were observed in those animals. Complementation of *etx* knockout restored virulence; most goats, sheep, and mice receiving this complemented mutant developed clinical and pathological changes similar to those observed in WT-infected animals. These results indicate that ETX is necessary for type D isolates to induce disease, supporting a key role for this toxin in type D disease pathogenesis.

Samad MA (2011) found that all types of emerging, reemerging and neglected zoonotic diseases are widely prevalent and pose a great threat to human health in Bangladesh. Neglected Veterinary medical profession and its extension services, poor people without any knowledge of zoonotic diseases who are in close contact with livestock and their products, and unhygienic processing, maintaining and marketing the livestock and livestock products have made the situation graver in Bangladesh.

Miah *et al.* (2011) observed that the present study was conducted to isolate the causal agent of enteritis (E) from Sheep. A total of 50 jejunal samples from affected lamb were collected for isolation of *Clostridium perfringens,* the causal agent of enteritis. The novel method for isolation of *Clostridium perfringens* was stab culture in association with pouring of olive oil on the surface of the stab culture. The collected samples in nutrient broth were kept in a candle jar and incubated for 24 hrs at 37°C. For maintenance of anaerobic condition olive oil (2-3 ern) were poured on surface of culture broth in test tube. In Gram's staining, the morphology of the isolated bacteria was Gram-positive, rod-shaped, anaerobic, spore-forming single or paired in arrangement. The addition of olive oil on the surface of stab culture was effective for the growth of *Clostridium perfringens.* The characterization of *Clostridium perfringens* was then performed by other routine cultural study, staining procedure and different types of biochemical tests. The occurrence of enteritis was 8%.

EI-Khodery and Osman (2010) reported that faeces sample bacteriologically from 56 lambs with acute lamb dysentery revealed that *clostridium perfringens* was the most common pathogen (45 cases) followed by *Staphylococcus aureus* (seven cases) then *Streptococcus uberis* (three cases).

Vettorato MP *et al.* (2009) demonstrated that healthy sheep in Sao Paulo, Brazil, can be carriers of potential human pathogenic shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *Escherichia coli*. About 86 *E. coli* strains previously isolated from 172 healthy sheep from different farms were studied. Significance and impact of the study was as some of the STEC serotypes presently found have been involved with haemolyticuraemic syndrome (HUS) in other countries, the important role of sheep as sources of STEC infection in our settings should not be disregarded.

Evans J *et al*. (2008) compared CT-RMac and TBX agars as isolation medium for VTEC (Vero toxin-producing *Escherichia coli*) O26 from Scottish animal faeces and highlights that VTEC O26 may be missed if only CT-RMac agar is used.

Lahti (2008) reported that *Clostridium perfringens* type A was the predominant type isolated in our study and most of the isolates carried. No attempts, however, were made to differentiate between the consensus and atypical. Distribution frequency of the ~2-toxin was dependent on the animal host species. It was most frequent among avian isolates.

Hariharan H *et al.* (2007) took a fecal sample from a 42-year-old goat with a 2-month history of poor weight gain and diarrhea yielded a moderate growth of an organism resembling *Salmonella spp*. on MacConkey agar. The organism was identified as *Escherichia fergusonii*. The animal was euthanized. Samples of intestine, lung, liver, and kidney yielded the same organism, *E. fergusonii*.

Steven Pao *et al.* (2005) detected *Salmonella* strains and *Escherichia coli* O157:H7in 17 and 5 small ruminants in Virginia, respectively, out of 287 tested small ruminants.Background microflora interfered with the fecal analysis. The combination of Salmonella enzyme immunoassay (EIA) detection and xylose-lysine-deoxycholate agar isolation was satisfactory. Modifying enrichment to a 1:100 dilution enabled effective E. coli O157:H7 detection by EIA and isolation by sorbitol-MacConkey agar with cefixime-tellurite.

Collie (2002) evaluated that *Clostridium perfringens as* a Gram positive, anaerobic spore forming rod. There are five strains of C. *perjringens,* designated A through E. Each strain produces a unique spectrum of toxins. The epsilon toxin is made by types Band D. This toxin is a pore forming protein; it causes potassium and fluid leakage from cells. In addition to the epsilon toxin, *Clostridium perfringens* type D strains produce alpha toxin and type B strains produce alpha and beta toxins. C. *perfringens* type B causes severe enteritis in young lambs and piglets. Type D causes enterotoxemia in sheep and lambs and on rare occasions, in cattle. All five strains can infect wounds in any species.

Tillotson *et al* (2002) detected that C. *perfringens* was detected in 332 of 731 fecal samples of sheep farm. Therefore, it can be concluded that type A C. *perfringens* is probably a normal inhabitant of lambs intestinal tracts. Subsequently, all C. *perfringens* isolates were subjected to genotyping. Results showed that type A C. *perfringens* was the most prevalent genotype, whereas type C C. *perfringens* was identified only in 0.9% of samples. Type A C. *perfringens* genes may be found in the feces of healthy lambs, whereas type C is seldom found in healthy others animal.

Vatn *et al* .(2000) reported that abomasal disease in lambs aged 2-5 weeks, made during the period 1993-1998, included 67 cases and 45 non-affected controls. Gross pathological findings included various combinations of bloat, haemorrhage and ulcers in the abomasum. *Sarcina*-like bacteria were found in sections and smears from the abomasum of 79% *(53/67)*

of the cases. From one case, a lamb with abomasal bloat, the anaerobic "packet" forming *Sarcina ventriculi* was cultivated from the abomasal contents and identified by biochemical reactions. *Sarcina* like bacteria were observed microscopically in specimens from 94% (44/47) of the lambs with abomasal gas and in 45% (9/20) of those with ulcers or haemorrhage or both but little gas. On culture, abomasal contents from 41 cases yielded *Clostridium fallax*from 16 (39%) and *Clostridium sordellii* from eight (20%); abomasal cultures from 30 control lambs were negative for the three bacterial species. Quantitative cultivation, carried out on abomasal contents from live lambs and lambs dead ;3 h, showed that *Clostridium perfringens, Escherichia coli* and *Lactobacillus* spp. were present in the majority of both cases and controls, with no significant differences in the mean numbers.

Lewis CJ *et al.* (1998) studied the Sudden death in sheep associated with *Clostridium sordellii.* For this purpose, *Clostridium sordellii* was isolated from 37 sheep, in 29 flocks, which died suddenly between 1993 and 1995. The sheep were of all ages, but the most severe gross lesions affected lambs four to 10 weeks of age. In older weaned lambs and ewes the gross changes were less marked and more variable. Thirty sheep suffering from a variety of other conditions were examined and *C sordellii* was not isolated. The isolation of *C sordellii* has been reported only twice before from sheep in Britain, and on both occasions no detailed investigations were described. The evidence from this study indicates that *C sordellii* should be considered when investigating the cause of sudden death in sheep of all ages in Britain.

Netherwood *et al.* (1998) stated that C. *perfringens* was significantly associated with sheep diarrhea, being isolated from 57% (240/421) of diarrheic lambs, versus 27%.

Gilmour NJL (1980) found *Pasteurella haemolytica* causes two distinct disease syndromes in sheep. *P. haemolytica* biotype A causes septicemia in young lambs and pneumonia in all ages of sheep. Biotype T produces an acute systemic disease affecting principally the upper alimentary tract and lungs in young adult sheep.

2.3 Objective III: To characterize the *Klebsiella pneumoniae* by using PCR and electropherogram.

Mourad Ben Said *et al.* (2016) first observed first molecular evidence of *Borrelia burgdorferi* sensulato in the ruminants in Tunisia, and also demonstrated that host species such as goats and sheep may play an important role in natural Lyme disease cycles in this

country. They investigated 1,021 ruminants (303 goats, 260 sheep, 232 cattle and 226 camels) from different bioclimatic areas in Tunisia for the presence of *B. burgdorferi* s.l. DNA in blood by real time PCR. Prevalence rates were 30.4% (92/303) in goats, 6.2% (16/260) in sheep, 1.3% (3/232) in cattle, and 1.8% (4/226) in camels.

Hajializadeh R Valilo *et al.* (2016) designed a study to identify genetically resistant animals to gastrointestinal nematode (GIN) infections using microsatellite polymorphisms of Ovar-DRB1 gene in Iranian Ghezel sheep breed lambs. They randomly selected 120 male Ghezel lambs, which were at 4 to 6 months of age from six different sheep flocks in East Azerbaijan province (n=20 per flock). These lambs were naturally infected with GINs, and individual fecal samples were collected twice with a week interval to evaluate fecal egg counts (FEC). Blood samples were also collected for DNA isolation and PCR was performed to amplify the second exon and microsatellites within the second intron of the Ovar-DRB1 gene. this study showed a strong association between microsatellite polymorphism of Ovar-DRB1 gene and resistance to GIN infections in Ghezel sheep lambs.

Ahmed M.A. Mansour *et al.* (2014) did the trials for isolation of Klebsiella pneumoniae from diseased and apparently healthy farm animals (cows, sheep, goats and camels) for recognition of Klebsiella pneumoniae subspecies.They noticed that there was a marked variation between incidences of *Klebsiella pneumoniae* subspecies in examined animals as regards to health condition. The frequency was greater among samples collected from diseased animals 25.2% as compared with apparently healthy one 5.5%. They found that there was great difference between the prevalence of *Klebsiella* isolated from various animal origins. On biochemical identification *Klebsiella pneumoniae* subsp. pneumoniae was the most prevalent followed by *Klebsiella pneumoniae* subsp. ozaenae and *Klebsiella pneumoniae* subsp. Rhinoscleromatis. *Klebsiella pneumoniae* subsp. rhinoscleromatis was not isolated from apparently healthy animals. The in vitro sensitivity of isolates of *Klebsiella pneumoniae* subspecies recovered from different animal species to 23 antimicrobial agents was tested.SDS-PAGE analysis showed that CPSs of *Klebsiella pneumoniae* subspecies contained wide variety of different molecular weights which ranged from 15.52 kDa to106.29 kDa and gave 10-13 bands.

Edismauro G *et al.* (2014) observed the occurrence and molecular characterization of enterohemorrhagic *Escherichia coli* serotype O157:H7 isolates from rectal swabs from 52

healthy dairy cattle belonging to 21 farms in Mid-West of Brazil. The isolates were further characterized by serotyping and two hundred and sixty *E.coli* isolates were obtained of which one hundred and twenty six were characterized as shiga toxin-producing *E. coli*. The prevalence of O157:H7 was found to be 1.92% (1/52 animals), demonstrating that healthy dairy cattle from farms in the Mid-West of Brazil are an important reservoir for highly pathogenic *E. coli* O157:H7.

Arash Javanmard *et al.* (2011) aimed to study the association between GDF9 and BMP15 gene polymorphism and litter size in fat-tailed sheep. A total of 97 mature ewes from four breeds (Afshari=19; Baluchi=18; Makui=30 and Mehraban=30) were genotyped for the BMP15 HinfI and GDF9 HhaI polymorphisms by PCR-RFLP technique. The highest and lowest mutant allele frequencies were found in Makui (0.27) and Afshari (0.10) sheep for the BMP15 gene and in Afshari (0.24) and Mehraban (0.18) sheep for the GDF9 gene, respectively. Litter size was significantly influenced by genotype of the ewe for two genes ($P < 0.01$). Heterozygous genotypes for both loci showed higher litter size than homozygous genotypes ($P < 0.01$). None of the individuals carried homozygous genotype for both of the GDF9 and BMP15 variants in these breeds. The individuals carrying the mutant allele for one of the investigated candidate gene still showed fertile phenotype. Thus, existence of homozygosity at one of the BMP15 and GDF9 variant is not probably able to block normal hormonal pathway of reproduction in fat-tailed sheep.

Blanco M *et al.* (2003) obtained fecal swabs from 1,300 healthy lambs in 93 flocks in Spain in 1997 and examined for Shiga toxin-producing *Escherichia coli* (STEC). STEC O157:H7 strains were isolated from 5 (0.4%) animals in 4 flocks, and non-O157 STEC strains were isolated from 462 (36%) lambs in 63 flocks. A total of 384 ovine STEC strains were characterized in this study. PCR showed that 213 (55%) strains carried the stx1 gene, 10 (3%) possessed the stx2 gene, and 161 (42%) carried both the stx1 and the stx2 genes. Enterohemolysin (ehx A) and intimin (eae) virulence genes were detected in 106 (28%) and 23 (6%) of the STEC strains, respectively. The STEC strains belonged to 35 O serogroups and 64 O:H serotypes (including 18 new serotypes The majority (82%) of ovine STEC strains belonged to serotypes previously found to be associated with human STEC strains, and 51% belonged to serotypes associated with STEC strains isolated from patients with hemolytic-uremic syndrome. Thus, this study confirms that healthy sheep are a major reservoir of STEC strains pathogenic for humans.

2.4 Objective IV: To detect the susceptibility pattern of commercially available antibiotic against identified isolates.

López-Martín JI *et al.* (2016) observed the prevention of human salmonellosis requires prior monitoring of *Salmonella* in animals. They determined Salmonella enterica serovars from different animal origins were isolated and their antimicrobial susceptibility. The constant release of these serovars to the environment, reaching also animal food, is a permanent potential risk for public health, turning into a first priority the establishment of control and antibiotic therapy strategies.

CHAPTER 3

MATERIALS AND METHODS

The present study was conducted during the period from July, 2017 to December, 2017 Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh. The detailed outline of the materials and methods are given below.

3.1 Materials

3.1.1 Study area

The samples were collected from Dinajpur (Nizam & Brothers sheep farm, Setabganj) and Nilphamari (Mini zoo, Nilsagor agro industries limited), Bangladesh and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for laboratory analysis.

3.1.2 Experimental animals

The experimental animals were categorized according to their age groups as follows; group A; lamb (≤ than 1 year), group B; adult sheep (≥ than1 year).

3.1.3 Sample collection

A total 32 samples comprising; 16 rectal swabs and 16 fecal samples were collected from fat-tailed sheep of different aged groups. The rectal swabs were collected with the help of pre-sterilized cotton bud and immediately transferred into pre-sterilized screw-cap test tube containing PBS (phosphate buffer solution) under aseptic condition. On the other hand, the fecal samples were collected by using pre-sterilized plastic bags separately and transferred to the ice box containing plenty of ices then brought to the department of microbiology for laboratory analysis.

Plate 1: Sample collection (left) and transferring of sample in ice box (right).

Table 1: Collection of samples from fat-tailed sheep based on their age groups.

Location of Fat-tailed sheep farm	Age of fat-tailed sheep				Total number of collected samples
	Group A (lamb; ≤ than 1 year)		Group B (adult; ≥ than1 year)		
	Rectal swab	Fecal sample	Rectal swab	Fecal sample	
Dinajpur	4	4	4	4	16
Nilphamari	4	4	4	4	16
Total	16		16		32

3.1.4 Preparation of samples

3.1.4.1 Fecal samples:

After collecting the fecal samples under aseptic condition, the samples were transferred to the laboratory in the ice box and were mixed with adequate amount of PBS. Then the mixture of feces and PBS were mixed very well using a shaker to convert the solid fecal samples into liquid form for serial dilution.

3.1.4.2 Rectal swabs:

Rectal swabs were collected by using the pre-sterilized cotton buds from the rectum of the animals into the screw-cap test tubes containing PBS and transferred to the microbiology laboratory maintaining aseptic conditions. Then each screw-cap test tubes were subjected to serial dilution.

3.1.5 Determination of total viable count

For each dilution, the number of colony forming units (CFU) should be counted on the plates. Typically, numbers between 30 and 300 are considered to be in the range where one's data is statistically accurate. If the number of CFUs on the plate are greater than 300, this might be recorded in the table as TNTC (too numerous to count). Alternatively, if the CFU numbers are greater than 300 and diluted bacterial suspension was distributed on the surface of the plate uniformlyandthe CFUs can be discerned as individual colonies; the plate should be divided into 4 sectors, the number of bacteria in one sector should be counted and multiplied by four.If the number of CFUs on the plate is below 10, the number of CFUs should be recorded, but using this data in the calculations is not needed.

Dilution factor	**Number of bacterial colonies (CFUs)**									**Avg # CFU**	**Avg # bacteria/ml**
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**		
$1:10^{-1}$											
$1:10^{-2}$											
$1:10^{-3}$											
$1:10^{-4}$											
$1:10^{-5}$											
$1:10^{-6}$											
$1:10^{-7}$											
$1:10^{-8}$											
$1:10^{-9}$											
$1:10^{-10}$											

*T=Trial.

Calculating the number of bacteria per ml of serially diluted samples:

To calculate the number of bacteria per mL of diluted sample one should use the following equation:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \rightarrow \frac{\text{Number of CFU}}{\text{ml}}$$

For example, if for the 1x 10^{-8} dilution plate we plated **0.1 ml** of the diluted cell suspension and counted 200 bacteria, then the calculation would be:

200/0.1 mL x 10^{-8} or 200/10^{-9} or 2.0 x 10^{11} bacteria per ml.

3.1.6 Data analysis:

Data were analyzed using SPSS version 21.

3.1.7 Glassware and appliances:

The glassware's and appliances were used during the whole process of the experiment are as follows : scalpel, forceps, scissor, petridishes, tray, test tubes, conical flask, micro pipette, slides, test tube racks, bacteriological incubator, autoclave, refrigerator, biosafety cabinet, shaker, colony counter, electronic balance, ice boxes, microscope, spirit lamp, inoculating loop, gel documentation, gel electrophoresis, thermal cycler, microcentrifuge, gel casting tray, microwave oven, PCR tube etc.

3.1.8 Chemicals and reagents:

The chemicals and reagents used for the study were; Gram's iodine, crystal violet, acetone alcohol, safranin, methyl red solution, Kovac's reagent, alpha napthol, 10% potassium hydroxide, PBS, glycerol, 3% H_2O_2, TAE buffer, agarose, gel-loading dye, PCR mastermix, DNA ladder (100bp), Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3'), Reverse primer- 1492R (5' TACCTTGTTACGACTT 3') and other common laboratory reagents and chemicals.

3.1.9 Media for Culture

3.1.10 Liquid media

3.1.10.1 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.

3.1.10.2 Selenite broth

Selenite Broth is used as a selective enrichment for the cultivation of Salmonella spp. that may be present in small numbers and competing with intestinal flora. This medium must not be autoclaved. Once prepared it is steamed at 100°C for 30 minutes. There should be a very slight red precipitate. To minimize the risk of teratogenicity to workers, sodium selenite must be added separately to the medium. It has a pH of approximately 7.1. Selenite broth gives pale or colorless colonies. (Leifson, E. 1939)

3.1.10.3 Tetrathionate broth

The use of Tetrathionate Broth for tile cultivation of Salmonella species was first described by Mueller. Mueller demonstrated that lactose fermenting members of the Enterobacteriaceae were inhibited or killed in the medium while the salmonellae grew almost unrestricted. Schaeffe was one of the first investigators to demonstrate the enrichment properties of the medium. He reported obtaining four times as many isolates of salmonellae after specimen enrichment in Tetrathionate Broth than found on direct platings. Kauffman modified the formula and reported marked increase in recovery of *Salmonella.* Tetrathionate Broth Base, A.P.H.A. is enriched with iodine solution for the selective cultivation of Salmonella species. Bile salts are incorporated in the medium as the selective agent to retard

the growth of Gram-negative bacilli other than salmonellae, and inhibit Gram-positive organisms.

3.1.11 Semi solid media

3.1.11.1 Motility indole urease (MIU) media

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

3.1.12 Solid media

3.1.12.1 Nutrient Agar Medium

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

3.1.12.2 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. Escherichia coli colonies grow with a metallic sheen with a dark center, Aerobacter aerogenes colonies have a brown center, and nonlactose-fermenting gram-negative and Klebshella spp bacteria appear pink (Cheesbrough, 1985).

3.1.12.3 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).

3.1.12.4 Brilliant Agar Medium

Brilliant Green Agar is highly selective medium for the isolation of Salmonella spp. Escherichia coli: Inhibited-moderate growth and Yellow-green colour of colony. Salmonella: good growth and pink-white color of colony (Cheesbrough, 1985).

3.1.12.5 Salmonella-Shigela Agar

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

3.1.12.6 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.

3.1.12.7 Simmons Citrate Agar Medium

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

3.1.12.8 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.

3.1.13 Media for Biochemical test

Sugar fermentation broth, indole broth, methyl red broth, voges-proskauer broth, Simmon's citrate agar, triple sugar iron agar and motility indole urease (MIU).

3.2 Methods

The experimental layout is schematically presented in figure 1. The entire study is divided into four steps. The first step includes the total viable counts of the collected samples. The second steps includes isolation and identification of the bacteria from the sample by cultural, morphological and biochemical test. Third step includes evaluation of antibiotics sensitivity against the isolated bacteria and fourth step is molecular characterization by using PCR.

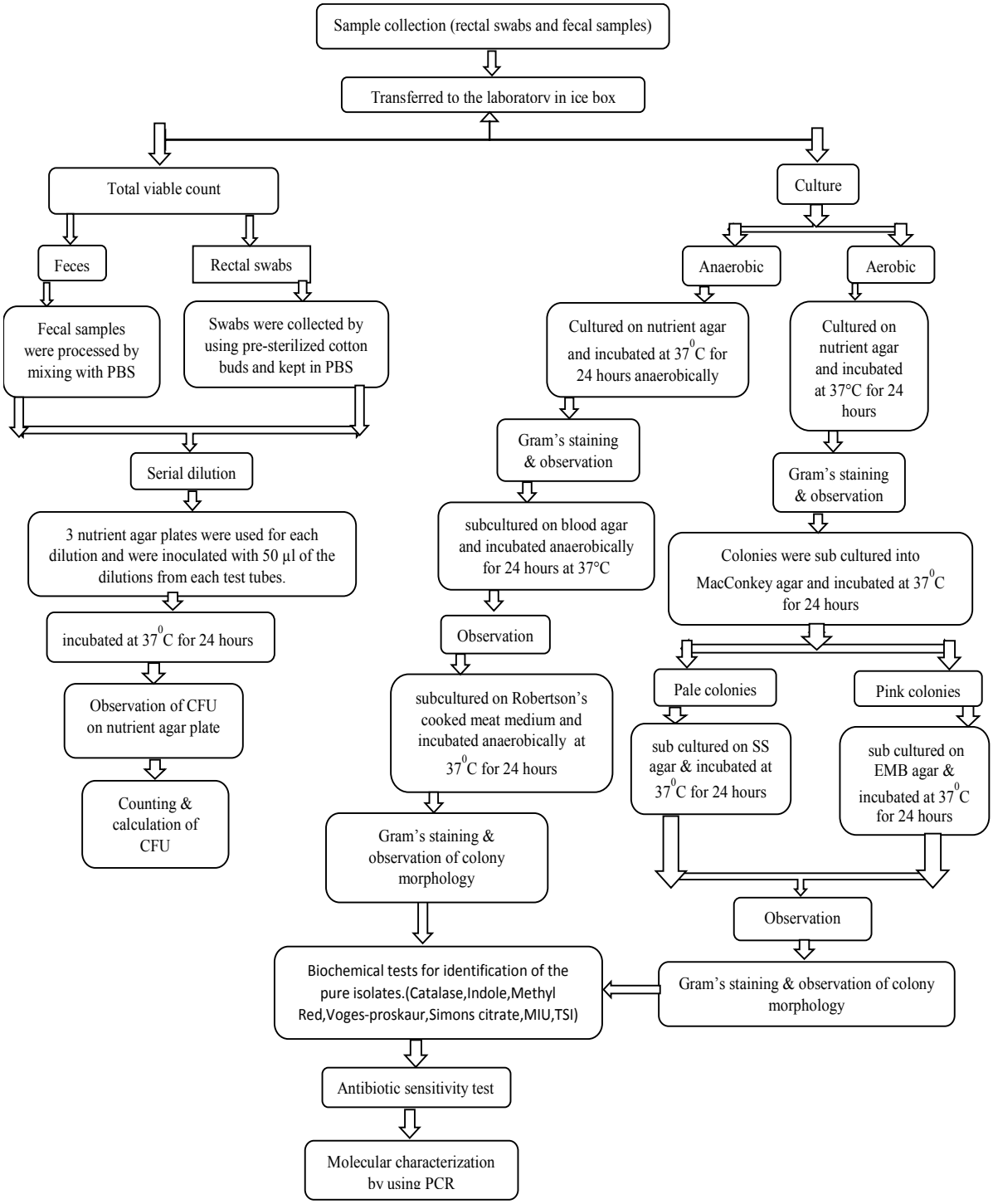

Figure 1: The schematic illustration of the experimental layout.

3.2.1 Preparation of Culture Media:

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

3.2.1.1 Nutrient agar(NA) media

28 grams of nutrient agar powder 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 petri dish and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use.

3.2.1.2 Eosin Methylene Blue (EMB) agar

Thirty six grams of EMB agar base 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 and I to 50° C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petri dish sized and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37° C for overnight to check their sterility and petri dishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use.

3.2.1.3 Mac Conkey agar

51.5 grams Mac Conkey agar base 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 45^0- 50^0C 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. Petri dishes, these were incubated at 37° C for overnight to

check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use.

3.2.1.4 Salmonella Shigela (SS) Agar:

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by boiling for 5 minutes. After boiling the medium was put into water bath at 45^0- 50^0C 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. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use.

3.2.2 Preparation of reagents

3.2.2.1 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.

3.2.2.2 Methyl Red

A quantity of 17 gms of MR-VP medium 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^oC for overnight o check their sterility and then in refrigerator for future use.

3.2.2.3 Alpha- naphthol solution

Alpha- naphthol solution was prepared by dissolving 5 gm of Alpha- naphthol in 100 ml of 95% ethyl alcohol.

3.2.2.4 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in100 ml of cooled water.

3.2.2.5 Phosphate Buffered Saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate Na_2HPO_4, $12H_2O$), 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 / cm^2 pressure and 121° C for 15 minutes and stored for future use.

3.2.2.6 Indole reagent (Kovac's reagent)

This solution was prepared by dissolving 25 ml of concentrated Hydrochloride acid in 75 ml of amyl alcohol 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.

3.3 Viable count of the collected samples:

Samples were collected and each samples were diluted with PBS as 10^{-1}, 10^{-2},10^{-3},10^{-4},10^{-5}. Then 50 µl samples were taken and spread in nutrient agar plate following the spread-plate method and incubate at 37ºC for 24 hours. The number of organisms per ml of original culture is calculated by multiplying the number of colonies counted by the dilution factor:

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

3.3.1 Cultivation and isolation of organisms:

Samples were collected and each of the samples were 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 Mac Conkey, SS agar and EMB agar by streak plate method (Cheesbrough, 1985) to observe the colony characteristic colony morphology of *Escherichia coli, Salmonella spp.* and *Klebsiella pneumoniae.* Then was repeatedly sub-cultured onto Mac-Conkey, SS agar and EMB agar and until the pure culture morphology (shape, size, surface texture, edge and elevation, color, opacity etc) were obtained.

3.3.2 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 decolorization step, and a counterstain is used to impart a pink color to the decolorized gram- negative organisms.

3.3.3 Preparation of Gram's staining solution:

The four solutions needed for the Gram staining procedure.

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

3.3.4 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 organisms 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 1 min and gently washed with tap water.
5. Smears were gently flooded with Grams iodine mordant and let stood for 1 min 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 safranin for 30 sec.
8. Then gently washed with tap water.

9. Finally examined under microscope with oil immersion. (James G. Cuppuccion , Natalie Sherman ,1996*)*

3.3.5 Biochemical examination:

Isolated organism with supporting growth characteristics of suspected identified by biochemical test are performed Sugar fermentation test, Oxidase test, Catalase test, Indole test , MR Test, Voges-proskauer test, Simmon's citrate, Triple sugar iron agar(TSI), Motility Indole Urease **(**MIU) test, Selenite broth.

3.3.5.3 Catalase test

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

3.3.5.4 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 (0.5ml) 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).

3.3.5.5 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).

3.3.5.6 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 acetone 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).

3.3.5.7 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).

3.3.5.8 Triple Sugar Iron Agar (TSI)

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.

3.3.5.9 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).

3.4 PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Klebsiella pneumoniae.*

3.4.1 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 *Klebsiella spp.* Grow in conditions appropriate for *Klebsiella spp.* 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 µg/mg Proteinase k in 0.5% SDS. Mix thoroughly and incubate 30 min for 1 hr at 37^0C.
- Add an approximately equal volume (500 µl) of Phenol/Chloroform/Isoamyl alcohol. Mix thoroughly but very 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 supernatant (~ 400 µl) 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 min.
- Transfer the supernatant to a fresh tube (~ 400 µl).
- Add $1/10^{th}$ 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 µl of TE and then 7.5 µl of RNase. Store DNA at 4^0C for short term and at -20^0C for long term.

3.4.2 PCR amplification and sequencing of 16S rRNA

PCR Condition:

Table 2: Condition of PCR.

Step	Temperature	Duration	Cycles
1. Initial denaturation	95ºC	5 min	01
2. Denaturation	95ºC	30 Sec	35
3. Annealing	56ºC	30 Sec	
4. Extension	72ºC	1.5 min	
5. Final extension	72ºC	10 min	01
6. Holding	4ºC	hold	-

3.4.3 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 (0.5µg/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.

3.4.4 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

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

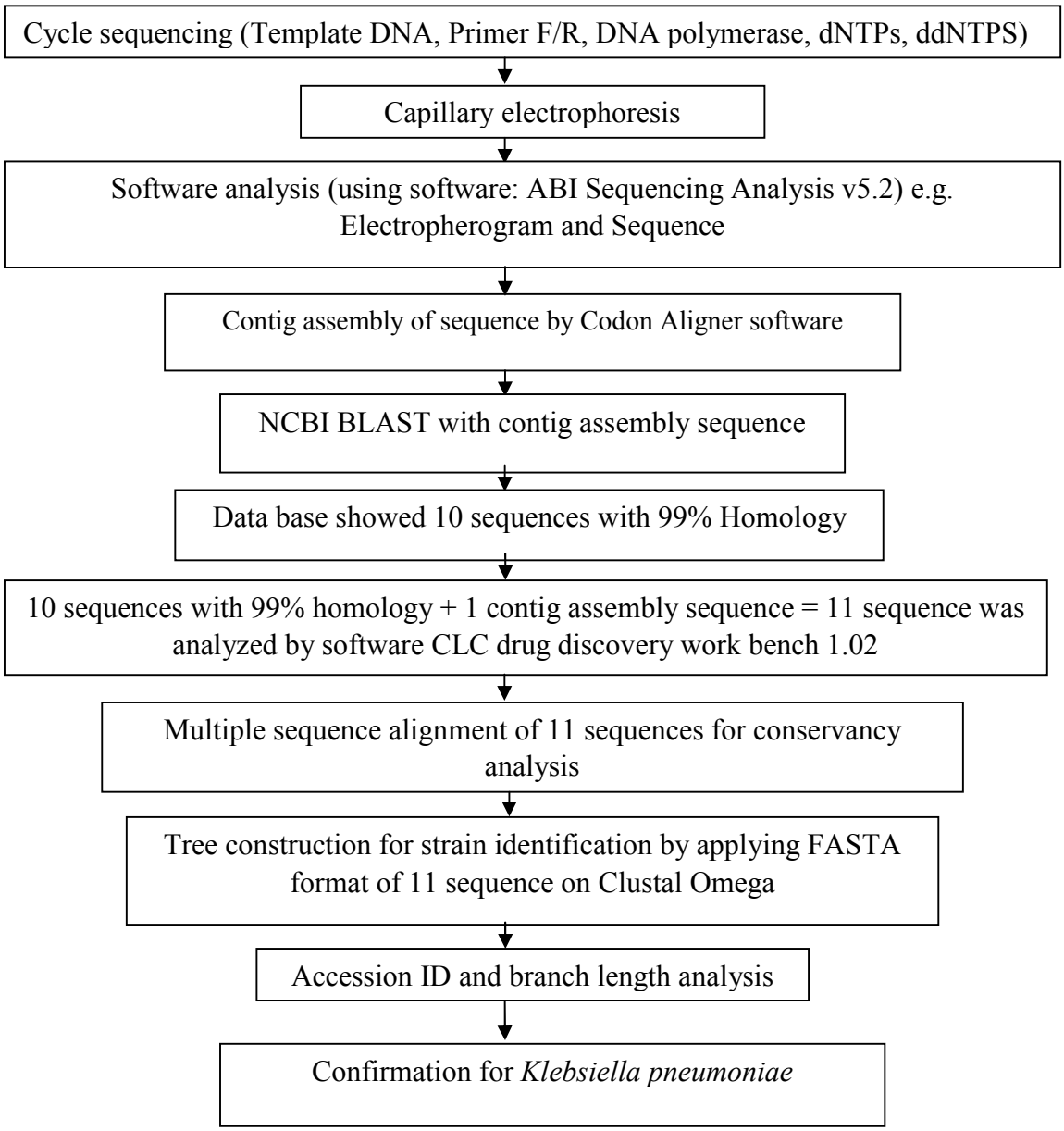

Figure 2: Steps of Sanger sequencing using ABI 3130 Genetic analyzer.

3.5 Antibiotic sensitivity test against isolated microbes:

To determine the drug Sensitivity and resistance patterns of isolated organisms used different types of commercially available antimicrobial discs, (Mast diagnostics Mersey side, UK.) Which were showed in (Table 1). The antibiotic resistance was determined by Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (Difco), according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI 2011). After overnight incubation at 37 °C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant or sensitive in accordance with company recommendations.(Cappuccino 2005*). E.coli, Salmonella spp.,* and *Klebsiella spp.* isolates were tested for sensitivity to (12 of routine and practical antibiotics) Ofloxacin (5 µg), Colistin (10 µg), Cefaclor (30 µg), Aztreonam (30 µg), Cefxime (5 µg), Tobramycin (10 µg), Cefepime (3 µg), Flumequine (30 µg), Cefalexin (30 µg), Vancomycin (30 µg). The disks were purchased from national company. The results were interpreted by special manufacturer's tables.

Table No 3: Antimicrobial agents with their disc concentration

Sl. No	Antimicrobial agents	Symbol	Disc code (ug/disc)	Diameter of zone of inhibition (ZOI)		
				Resistant	Intermediate	Susceptible
1	Aztreonam	ATM	30	≤17	18-20	≥21
2	Cefixime	CFM	5	≤15	16-18	≥19
3	Colistin	CL	10	≤10	-	≥11
4	Cephradine	CH	25	≤13	14-20	≥21
5	Cephalexin	CN	30	≤14	-	≥14
6	Cefepime	FEP	30	≤31	32-36	≥37
7	Cefaclor	CEC	30	≤23	24-26	≥27
8	Ofloxacin	OFX	5	≤12	13-15	≥16
9	Flumequine	UB	30	≤2	-	≥2
10	Tobramycin	TOB	10	≤18	19-25	≥26
11	Vancomycin	VA	30	≤9	10-11	≥12

Sl. No	Antimicrobial agents	Symbol	Disc code (ug/disc)	Diameter of zone of inhibition (ZOI)		
				Resistant	Intermediate	Susceptible
1	Nalidixic Acid	NA	30	≤22	23-27	≥28
2	Ciprofloxacin	CIP	5	≤15	16-20	≥21
3	Colistin	CL	10	≤10	-	≥11
4	Azithromycin	AZM	30	≤13	14-17	≥18
5	Cephalexin	CN	30	≤14	-	≥14
6	Amoxicillin	AMX	30	≤13	14-17	≥18
7	Levofloxacin	LE	5	≤13	14-16	≥17
8	Erythromycin	E	15	≤13	14-17	≥18
9	Gentamycin	GEN	10	≤12	13-14	≥15
10	penicillin	P	10	≤11	12-21	≥21
11	Vancomycin	VA	30	≤9	10-11	≥12
12	Cloxacillin	COX	1	≤10	-	≥11
13	Co-Trimoxazole	COT	25	≤21	-	≥75

These antibiotic discs were used for the antibiogram study of *Clostridium spp.*

3.6 Maintenance of stock culture

During the experiment it was necessary to preserve the isolated organisms for longer periods. Sterile buffered glycerin (20%) was prepared by mixing of 20 parts pure glycerin and 80 parts PBS. Then a loop full of thick bacterial culture of each isolate was mixed with 20% sterile buffered glycerin in small vial and preserved at -20°C / -80°C. This method was more appropriate for preserving bacteria with no deviations of their original characters for long time even for several years.

Plate 2: Fat-tailed sheep; from Dinajpur (A) and Nilphamari (B and C).

Plate 3: A portion of the collected samples in screw capped tubes (A); Culturing of anaerobic organisms in anaerobic condition in anaerobic jar with anaerogen (B); Counting of colony forming unit (CFU) by using a colony counter machine (C).

HAPTER 4

RESULT

Result of morphological, staining, cultural, biochemical, antibiotic sensitivity including percentage of incidence and molecular detection of isolated bacteria are presented in the table and described below under the following headings:

4.1 Total viable count

A total of 32 samples comprising rectal swabs (16) and fecal samples (16) were collected from Dinajpur and Nilphamari. The experimental animals were categorized into; lambs (≤ than 1 year) and adult sheep (≥ than1 year). In every case the samples were collected at 2 weeks interval.

4.1.1Total aerobic viable count

A total of 32 samples comprising rectal swabs (16) and fecal samples (16) were collected from Dinajpur and Nilphamari from lambs (≤ than 1 year) and adult sheep (≥ than1 year). In every case the samples were collected at 2 weeks interval.

Table 4: Total aerobic viable count of both location.

Location	Aerobic (CFU/µl of rectal &fecal samples) X $10^{-5}/10^{-6}/10^{-7}$																Total	P value
	Lamb								Adult									
	Fecal samples (n=8)				Rectal swabs (n=8)				Fecal samples (n=8)				Rectal swabs (n=8)					
	1st wk	3rd wk	5th wk	7th wk	1st wk	3rd wk	5th wk	7th wk	1st wk	3rd wk	5th wk	7th wk	1st wk	3rd wk	5th wk	7th wk		
Dinajpur	1.98 ×10^7 ± 0.01	1.69 ×10^7 ± 0.05	1.53 ×10^7 ± 0.02	1.31 ×10^7 ± 0.1	1.76 ×10^7 ± 0.06	1.59×10^7 ± 0.08	1.39×10^7 ± 0.06	1.08 ×10^7 ± 0.01	1.76×10^7 ±0.13	1.74×10^7 ±0.16	1.58× 10^7 ±0.04	1.50×10^7 ±0.17	1.44×10^7 ±0.15	1.44×10^7 ±0.15	1.39 ×10^7 ±0.06	1.33 ×10^7 ±0.14	1.53×10^7 ±0.05	0.000
Nilphamari	1.97 ×10^7 ± 1.60	1.61 ×10^7 ± 1.30	1.58 ×10^7 ± 1.31	1.90 ×10^7 ± 1.60	1.63 ×10^7 ±1.38	1.00×10^7 ±0.77	1.35×10^7 ±1.13	1.25 ×10^7 ±1.03	1.23×10^7 ±0.93	1.27×10^7 ±0.97	1.26×10^7 ±0.96	1.32 ×10^7 ±1.02	1.39×10^7 ±1.75	1.65×10^7 ±1.38	1.57 ×10^7 ±1.28	1.38 ×10^7 ±1.12	1.46×10^7 ±0.06	0.000

From the table it has found that, the aerobic microbial load was the highest during the 1st week of collection and gradually decreased as the animal ages. The microbial load were higher in Dinajpur than that of in Nilphamari. P value was statistically highly significant.

Table 5: Total anerobic viable count of both location.

Location	Anaerobic (CFU/µl of rectal & fecal samples) X $10^{-5}/10^{-6}/10^{-7}$																Total	P value
	Lamb								Adult									
	Fecal samples				Rectal swabs				Fecal samples				Rectal swabs					
	1st wk	3rd wk	5th wk	7th wk	1st wk	3rd wk	5th wk	7th wk	1st wk	3rd wk	5th wk	7th wk	1st wk	3rd wk	5th wk	7th wk		
Dinajpur	1.01 ×10^7 ± 0.01	1.46 ×10^7 ± 0.02	1.62 ×10^7 ± 0.02	1.83 ×10^7 ± 0.01	0.64 ×10^7 ± 0.01	0.75×10^7 ± 0.01	0.85×10^7 ± 0.01	0.94 ×10^7 ± 0.01	0.46×10^7 ±0.004	0.49×10^7 ±0.02	0.55×10^7 ±0.02	0.59×10^7 ±0.004	0.32×10^7 ±0.01	0.35×10^7 ±0.01	0.38 ×10^7 ±0.01	0.41 ×10^7 ±0.01	0.78×10^7 ±0.12	0.000
Nilphamari	0.86 ×10^7 ± 0.004	0.85 ×10^7 ± 0.01	0.92 ×10^7 ± 0.01	0.97 ×10^7 ± 0.004	0.62 ×10^7 ± 0.01	0.67×10^7 ± 0.004	0.73×10^7 ± 0.004	0.76 ×10^7 ± 0.01	0.42×10^7 ± 0.004	0.48×10^7 ± 0.01	0.48×10^7 ± 0.04	0.57 ×10^7 ± 0.004	0.34×10^7 ± 0.01	0.35×10^7 ± 0.01	0.36 ×10^7 ± 0.02	0.50 ×10^7 ± 0.02	0.62×10^7 ±0.05	0.000

This table showing that, the anaerobic count was lowest during the first week of collection and it was increasing as the animal was aging. The total anaerobic count was higher in Dinajpur than that of in Nilphamari. P value was statistically highly significant.

4.2 Prevalence of the isolates:

Table 6: Prevalence of the isolates in both location.

Isolated bacteria	Dinajpur		Nilphamari		Percentage %
	Rectal	Fecal	Rectal	Fecal	
Escherichia coli	**50% (4)**	**25% (2)**	**50% (4)**	**12.5% (1)**	**34.37%(11)**
Salmonella spp.	**25% (2)**	**37.5% (3)**	**37.5% (3)**	**25% (2)**	**31.25%(10)**
Klebsiella pneumoniae	**25% (2)**	**12.5% (1)**	**25% (2)**	**25% (2)**	**21.87%(7)**
Clostridium spp.	**12.5% (1)**	**25% (2)**	**0%**	**25%(1)**	**15.63%(5)**
Total number of isolates identified	**9**	**8**	**9**	**6**	**100%**

Among all the isolates, it seems that, presence of *Escherichia coli* has been more frequent, *Salmonella spp.* and *Klebsiella pneumoniae* were moderate and the prevalence of *Clostridium spp.* was more frequent in Dinajpur than Nilphamari.

4.3 Isolation and identification of organisms by cultural characteristics

The cultural characteristics of *Escherichia coli, Salmonella spp, Klebsiella pneumoniae and Clostridium spp*., on various selective media are presented in Table-7.

Table 7: Isolation of bacteria from different samples by using cultural characterisics.

Name of the culture media	Name of the organisms			
	Escherichia coli	***Salmonella spp.***	***Klebsiella pneumoniae***	***Clostridium spp.***
Nutrient agar	Large, circular, low convex, grayish, white, moist colonies were found.	Smooth. Opaque, translucent colonies were found,	Large, circular, smooth, convex, colonies.were found	Fuzzy, off-white colony
MacConkey agar	Bright, pink colored, smooth colonies were found.	Small, white or pale, translucent dewdrop like colonies were formed.	Round, pink, slightly raised, translucent and mucoid colonies were found.	—
EMB agar	Smooth and green Metallic sheen colonies were formed.	—	Round, pink, slightly raised, translucent and mucoid colonies were found.	—
SS agar	—	Opaque, smooth, round with black centered colonies were found.	—	—
Brilliant green agar	—	Red to pink-white colonies	—	—
Robertson cooked meat medium	—	—	—	Turbid growth
Brain heart infusion agar	—	—	—	Yellowish white colony
Blood agar				Beta-hemolytic colony

All the isolates produced whitish, opaque, smooth or fuzzy colony on the nutrient agar (plate-4, table-7). On the other hand, *Escherichia coli* and *Klebsiella pneumoniae* produced pink

colonies on MacConkey agar due to lactose fermentation (plate-9, table-7) whereas, *Salmonella spp.* gave pale colonies for not fermenting lactose (plate-9, table-7). *Escherichia coli* and *Klebsiella pneumoniae* produced metallic green sheen and dark pink colonies on eosin methylene blue agar respectively (plate-11, table-7). *Salmonella spp.* gave black centered or black colonies on Salmonella shigella agar (plate-10, table-7) and red to pink-white colonies on brilliant green agar (plate-12, table-7). The anaerobic *Clostridium spp.* produced beta-hemolytic colonies on blood agar (plate-14, table-7), yellowish white colony on brain heart infusion agar (plate-13, table-7) and turbid growth on Robertson cooked meat medium (plate-15, table-7).

4.4 Identification of isolated organisms by using morphological characteristics:

Table 8: Identification by using morphological characteristics.

Staining and morphological characteristics	Name of the organisms
Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain.	***Escherichia coli***
Gram negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain.	***Klebsiella pneumoniae***
Gram negative, pink colored and very small plump rods.	***Salmonella spp.***
Gram positive, purple/violate colored and small rod shaped organisms arranged in single, pairs or short chain.	***Clostridium spp.***

All the aerobic isolates turned out to be gram negative rods and arranged as single or in pairs or chains (Table-7, Plate 5-7) whereas, the only anaerobic isolate *Clostridium spp.* was observed as gram positive rod, arranged in pairs or chains or single (Table-7, Plate-8).

4.5 Characterization of field isolates by using different biochemical techniques.

Table 9: Biochemical properties of the isolated organisms.

Name of the tests performed	Observation		Remarks
Catalase test	Presence of bubble.		*Klebsiella spp, Salmonella spp.*
	Absence of bubble.		*Escherichia coli, Clostridium spp.*
Indole test	Presence of a cherry red colored ring on the surface of the media.		*Klebsiella spp, Salmonella spp, Escherichia coli.*
	Absence of a cherry red colored ring on the surface of the media.		*Clostridium spp.*
MR reaction	Presence of a red colored ring on the surface of the media.		*Salmonella spp, Escherichia coli*
	Absence of a red colored ring on the surface of the media.		*Klebsiella spp, Clostridium spp.*
VP reaction	Presence of a red colored ring on the surface of the media.		*Klebsiella spp, Salmonella spp.*
	Absence of a red colored ring on the surface of the media.		*Escherichia coli, Clostridium spp.*
Simon's citrate	Formation of Prussian blue color on the slant		*Klebsiella spp, Salmonella spp.*
	No color change of the medium		*Escherichia coli, Clostridium spp.*
MIU test	Diffuse, hazy growth, slightly opaque media		*Klebsiella spp, Escherichia coli.*
	No color change		*Salmonella spp, Clostridium spp.*
TSI test	Slant	Yellow	*Klebsiella spp, Escherichia coli.*
		Red	*Salmonella spp, Clostridium spp.*
	Butt	Yellow	*Klebsiella spp, Escherichia coli.*
		Black	*Salmonella spp.*
		Red	*Clostridium spp.*
	H_2S	Presence	*Salmonella spp, Clostridium spp.*
		Absence	*Klebsiella spp, Escherichia coli.*

Catalase test was performed by placing a drop of hydrogen peroxide on slides and mixing the colony of the bacteria to be tested thoroughly. Presence of bubbles indicates the positive result (Table-9, Plate-16). Indole test was performed by inoculating the buffered peptone water broth with the bacteria to be tested, followed by incubation for 24 hours at 37°C. The next day tubes were observed after adding Kovac's reagent. Formation of cherry red colored ring on the surface of the broth medium indicates positive result (Table-9, Plate-17). Methyl red test was performed by inoculating the target organisms into MR medium and incubating for 48 hours at 37°C. Formation of red colored ring on the surface of the broth medium indicates positive result (Table-9, Plate-18). Voges-Proskauer test was performed by inoculating the target organisms into VP medium and incubating for 72 hours at 37°C. Formation of red colored ring on the surface of the broth medium indicates positive result (Table-9, Plate-19). The organisms to be tested were inoculated onto Simmon's citrate agar medium slant and incubated for 24 hours at 37°CThe turning of the media slant into Prussian blue color indicates the positive result (Table-9, Plate-20). MIU medium was inoculated by the organisms to be tested and incubated for 24 hours at 37°C. Positive result will change the color of the medium. The red ring formation on the surface of the media, diffuse, hazy growth and slightly opaque media indicates positive result (Table-9, Plate-21). TSI agar slant was inoculated by the organisms to be tested and incubated for 24 hours at 37°C. Glucose fermentation is indicated by yellow butt, lactose fermentation by yellow slant and H_2S production by blackening of the medium and gas production by presence of bubble or gas space (Table-9, Plate-22).

4.6 Characterization of *Klebsiella pneumoniae* by DNA amplification, sequencing and phylogenetic analysis

4.6.1 Characterization by DNA amplification with PCR

Molecular characterization confirmed that this isolate is *Klebsiella pneumoniae*. 16S rRNA gene region was amplified with the universal primers, Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') Reverse primer- 1492R (5' TACCTTGTTACGACTT 3'). (Plate-23).

4.6.2 Contig Sequence of *Klebsiella pneumoniae*

>464

GGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGT
GACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGAT
AACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGG
ACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGG
GTAACGGCTCACCTAGGCGCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGG
AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCATTCGGGTT
GTAAAGCACTTTCAGCGGGGAGGAAGGCGATGAGGTTAATAACCTTNTCGATTG
ACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACT
GGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG
CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTG
ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGTCGANTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAG
CTAACGCGTTAANTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
TGATTTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGG
TGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTAGCGGTTAGGC
CGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATA
CAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTC
CGGATTGGAGTCTCCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGA
TCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
ATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTAC
CACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAAC
CTGCGGCTGGATCA

Base pair : 1492; BLAST: 99% similar.

Identified strain: *Klebsiella pneumoniae* strain DSM 30104

4.7 Antibiogram study:

The total four isolates *Escherichia coli*, *Salmonella spp, Klebsiella pneumoniae* and *Clostridium spp*. obtained from rectal swab and fecal samples of fat-tailed sheep were subjected to Antibiotic Sensitivity test shown in table and along with the pictures.

Table 10: Antimicrobial Susceptibility Pattern for *Escherichia coli, Klebsiella pneumoniae* and *Salmonella spp.*

Sl. No	Antimicrobial agents	Symbol	Disc code (ug/ disc)	*Escherichia coli*			*Klebsiella pneumoniae*			*Salmonella spp.*		
				S	I	R	S	I	R	S	I	R
1	Aztreonam	ATM	30	-	-	100	-	-	100	-	-	100
2	Cefixime	CFM	5	-	-	100	-	33.3	66.7	100	-	-
3	Colistin	CL	10	100	-	-	100	-	-	-	-	100
4	Cephradine	CH	25	-	100	-	-	33.3	66.7	-	-	100
5	Cephalexin	CN	30	-	-	100	-	-	100	-	-	100
6	Cefepime	FEP	30	-	-	100	-	-	100	-	-	100
7	Cefaclor	CEC	30	-	-	100	-	-	100	-	-	100
8	Ofloxacin	OFX	5	100	-	-	100	-	-	100	-	-
9	Flumequine	UB	30	100	-	-	-	33.3	66.7	-	-	100
10	Tobramycin	TOB	10	-	-	100	-	-	100	-	-	100
11	Vancomycin	VA	30	-	-	100	100	-	-	-	33.3	66.7

[Legends: S= Susceptible; I= Intermediate, R= Resistant]

Escherichia coli, Klebsiella pneumoniae and *Salmonella spp.* shown sensitivity to Ofloxacin while, Colistin was susceptible to *Escherichia coli* and *Klebsiella pneumoniae* Flumequine, Cefixime and Vancomycin were individually susceptible to *Escherichia coli, Salmonella spp.* and *Klebsiella pneumoniae* The organisms were resistant to rest of the antibiotics.

Table 11: Antimicrobial Susceptibility Pattern for *Clostridium spp.*

Sl. No	Antimicrobial agents	Symbol	Disc code (ug/disc)	*Clostridium spp.*		
				S	I	R
1	Nalidixic Acid	NA	30	-	-	100
2	Co-Trimoxazole	COT	25	-	-	100
3	Colistin	CL	10	100	-	-
4	Ciprofloxacin	CIP	5	100	-	-
5	Azithromycin	AZM	30	66.7	33.3	-
6	Amoxycillin	AMX	30	-	-	100
7	Levofloxacin	LE	5	100	-	-
8	Erythromycin	E	15	-	-	100
9	Gentamycin	GEN	10	66.7	33.3	-
10	Cefalexin	CN	10	-	-	100
11	Vancomycin	VA	30	-	-	100
12	Cloxacillin	COX	1	-	-	100
13	Penicillin	P	10	-	-	100

[Legends: S= Susceptible; I= Intermediate, R= Resistant]

Clostridium spp. exhibited high sensitivity to Colistin, Ciprofloxacin and Levofloxacin while, intermediate sensitivity to Azithromycin and Gentamycin and showed resistance to Nalidixic Acid, Co-Trimoxazole, Amoxycillin, Erythromycin, Cefalexin, Vancomycin, Cloxacillin and Penicillin.

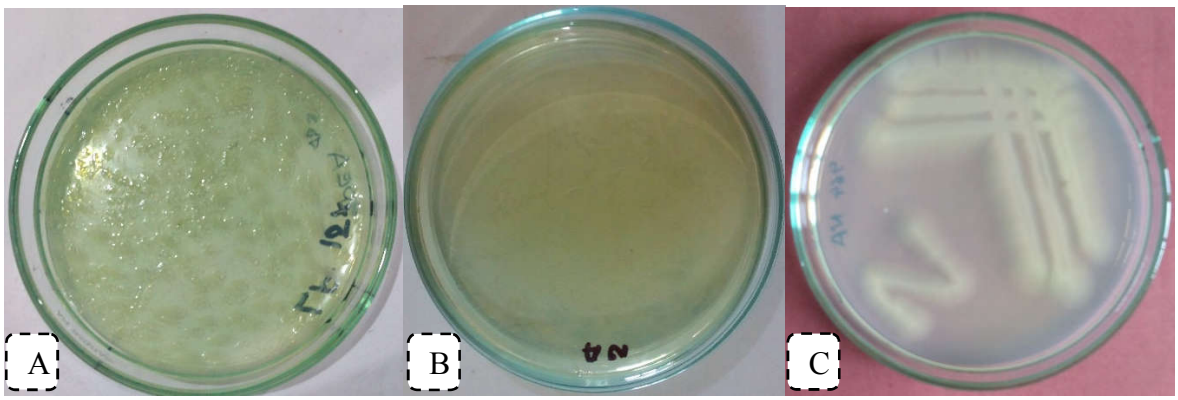

Plate 4: Growth of microorganisms on nutrient agar under aerobic condition (A); Control plate (B); Anaerobic condition (C).

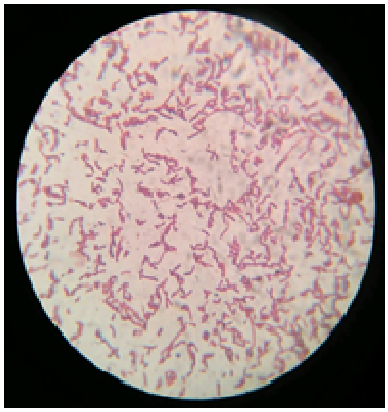

Plate 5: Gram negative large rod, pink colored *Escherichia coli* under microscope

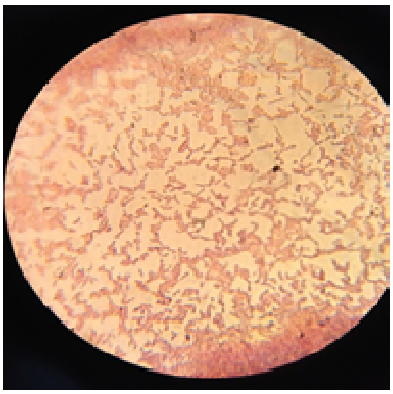

Plate 6: Gram negative large rod, pink colored *Klebsiella pneumoniae* under microscope

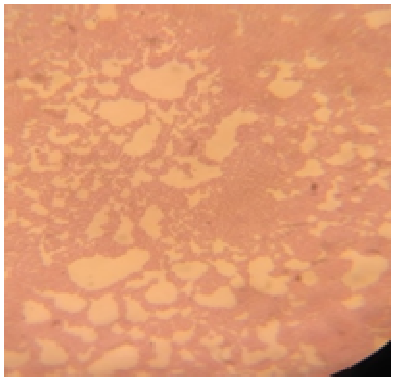

Plate 7: Gram negative small rod, pink colored *Salmonella spp.* under microscope

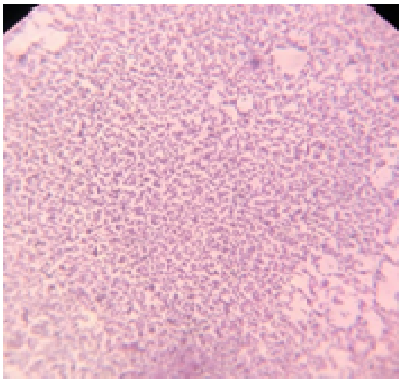

Plate 8: Gram positive small rod, violet colored *Clostridium spp.* under microscope

Plate 9: Growth on MacConkey agar; *Escherichia coli* (A), *Klebsiella pneumoniae* (B), *Salmonella spp.* (C) and Control plate (D).

Plate 10: Growth of *Salmonella spp.* on Salmonella-Shigella agar (A); Control (B)

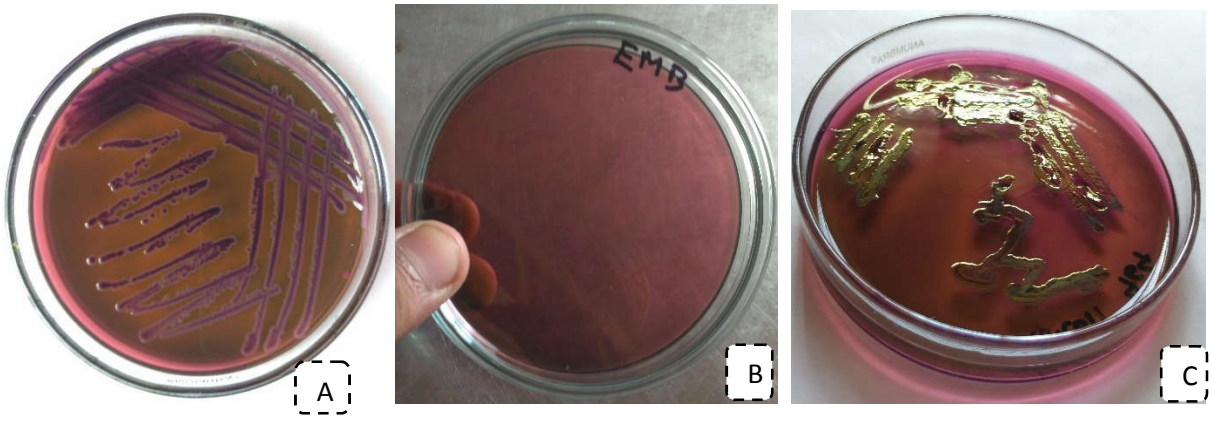

Plate 11: Growth of *Klebsiella pneumoniae* on EMB agar (A); Control (B); *Escherichia coli.* (C).

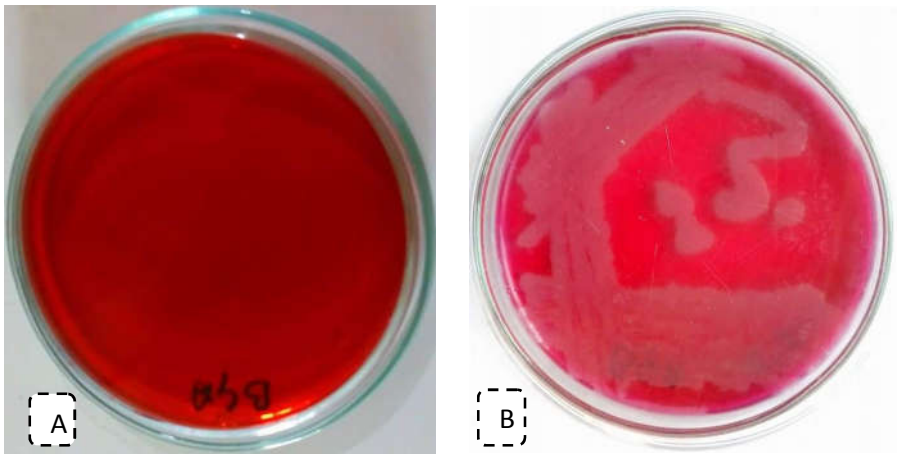

Plate 12: Growth of *Salmonella spp.* On brilliant green agar (B); and control (A).

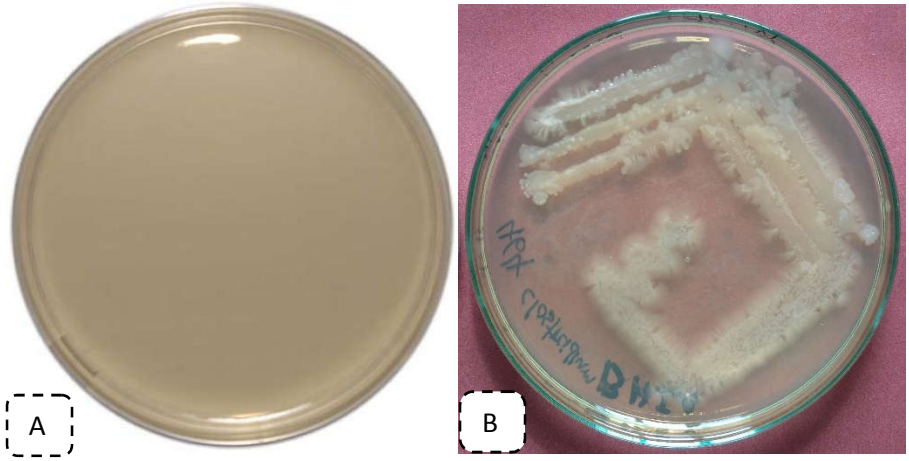

Plate 13: Growth of *Clostridium spp.* on brain heart infusion agar (B); and control (A).

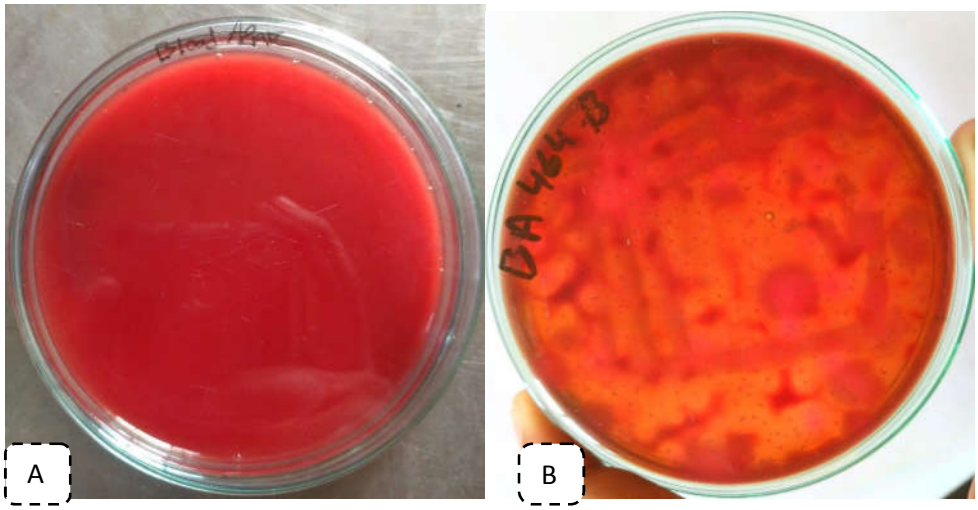

Plate 14: Growth of *Clostridium spp.* on the blood agar (B); and control (A)

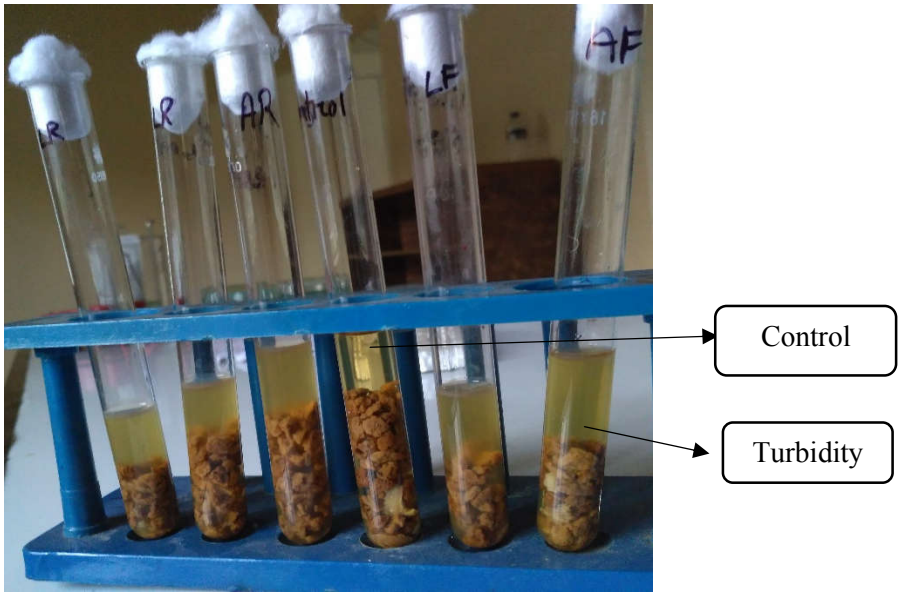

Plate 15: *Clostridium spp.* showing turbid growth in Robertson's cooked meat broth medium (left and right); and control (middle).

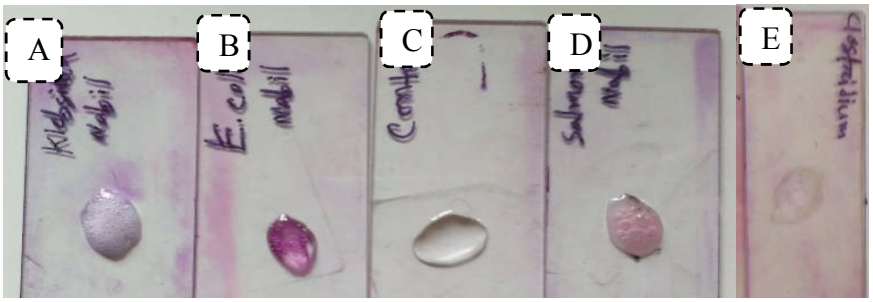

Plate 16: Catalase test; *Klebsiella pneumoniae* (A), *Escherichia coli* (B), *Salmonella spp.* (D), *Clostridium spp.* (E) and control (C).

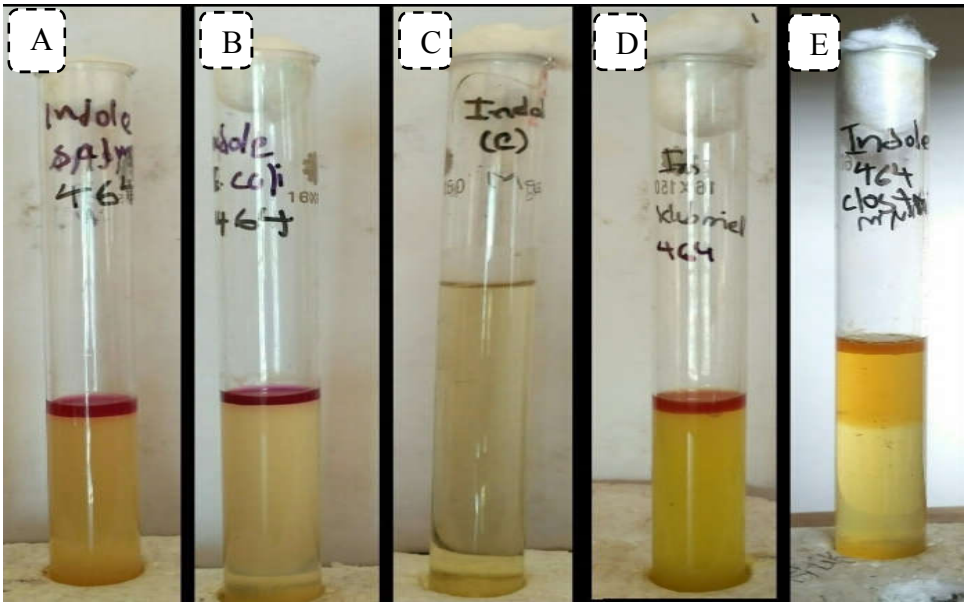

Plate 17: Indole test; A= Indole positive, B= Indole positive, C=Control, D= Indole positive, E= Indole negative.

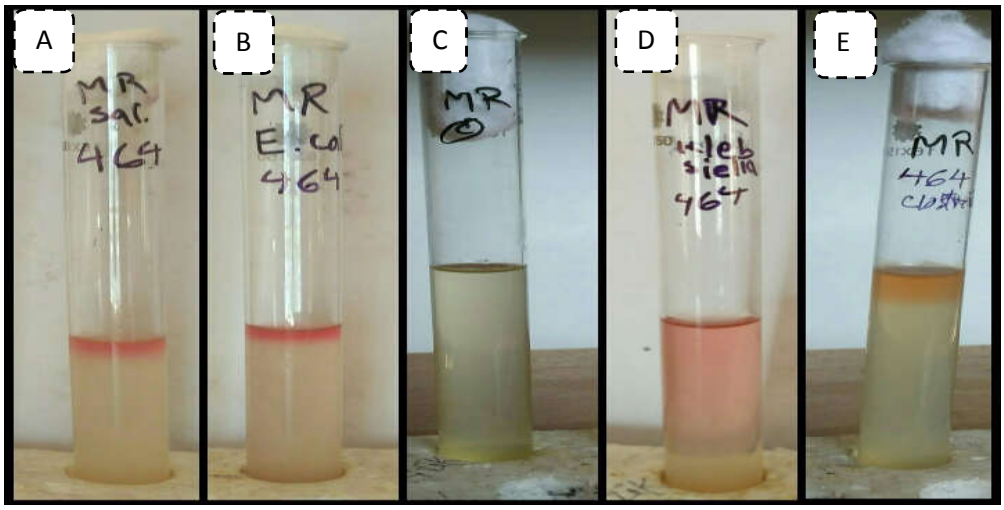

Plate 18: Methyl red reaction; A= MR positive, B= MR positive, C=Control, D= MR negative, E= MR negative

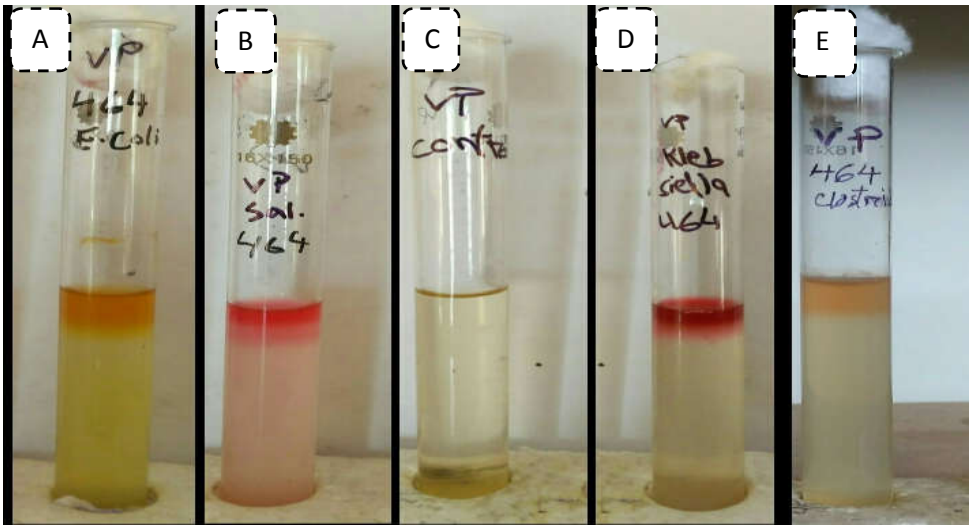

Plate 19: Voges-Proskauer reaction; A= VP negative, B= VP positive, C=Control, D= VP positive, E= VP negative

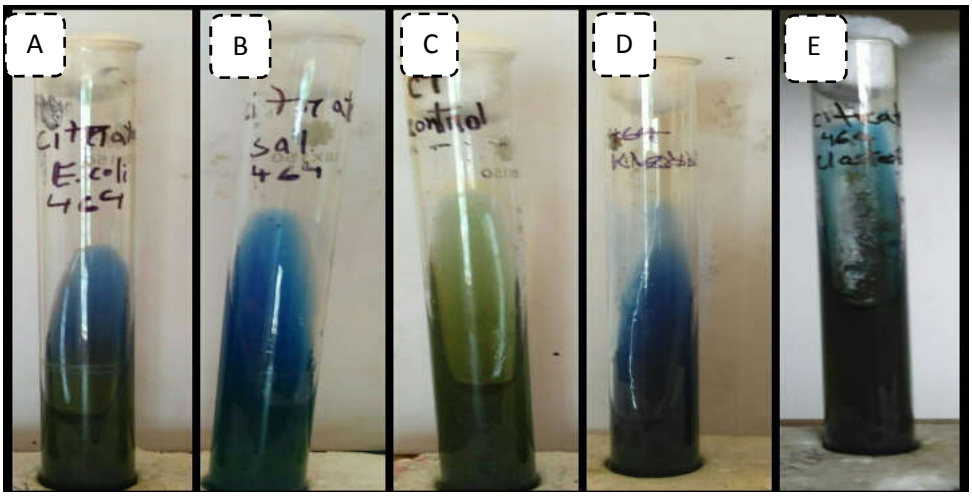

Plate 20: Simmon's citrate test; A= negative, B= positive, C=Control, D= positive, E= negative

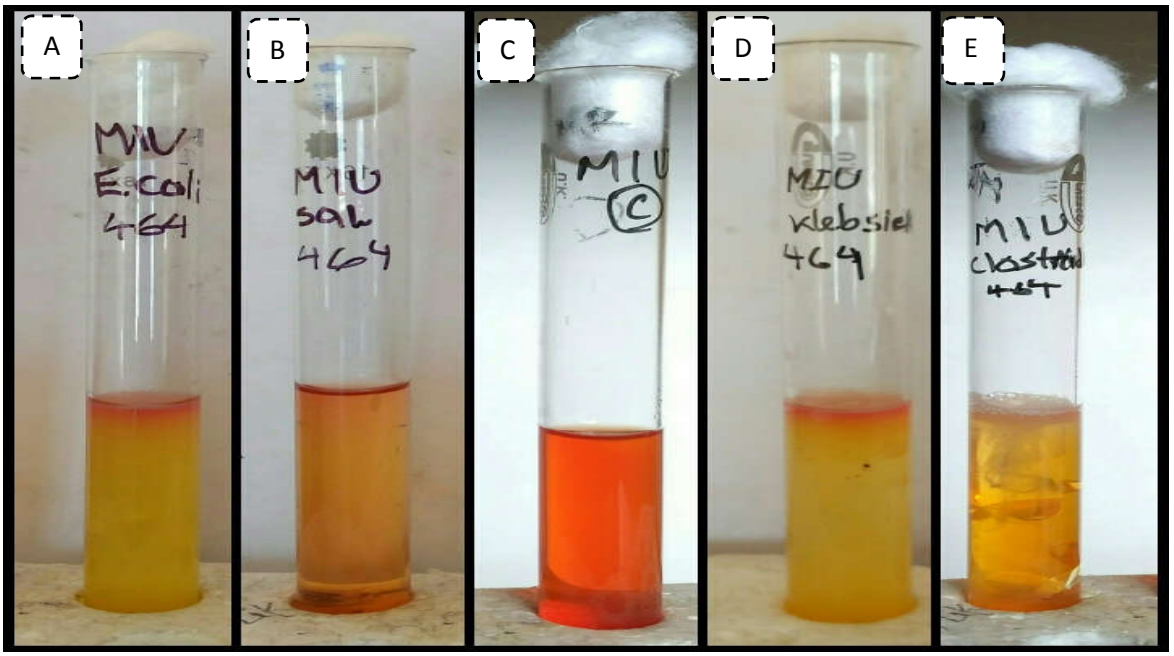

Plate 21: Motility indole urease (MIU) test; A= positive, B= negative, C=Control, D= positive, E= negative

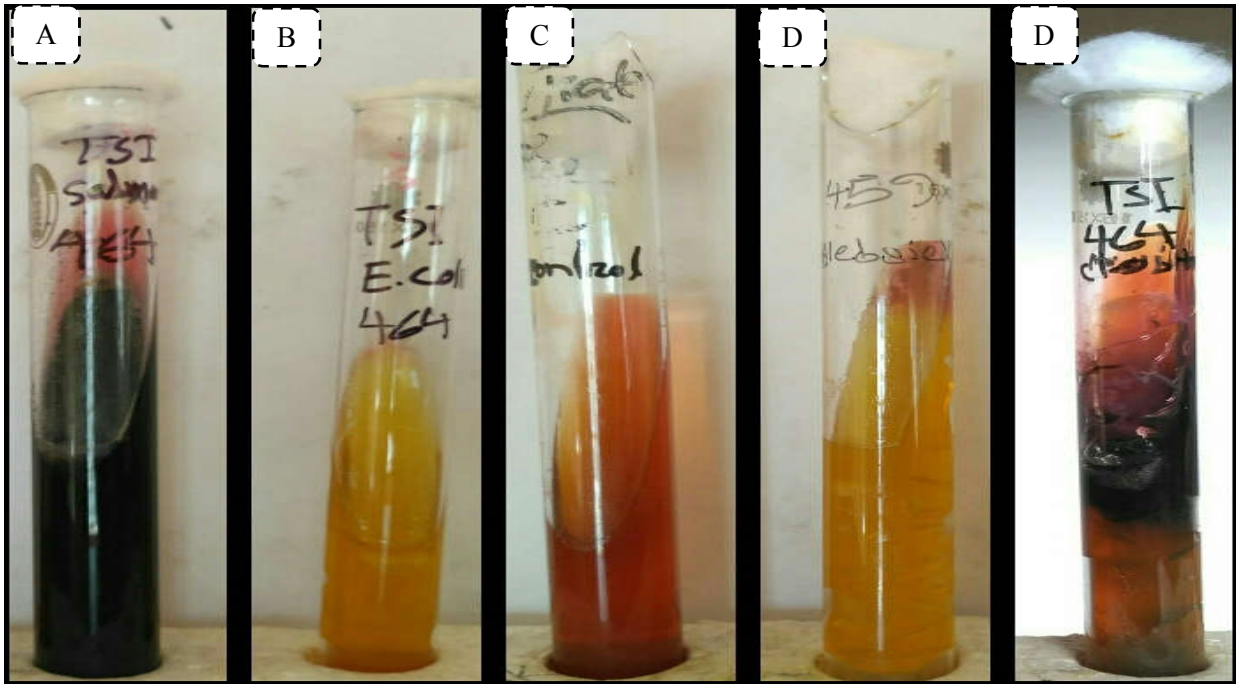

Plate 22: TSI test; *Salmonella spp.* showing red slant, black butt (A), *Escherichia coli.* Showing yellow butt and slant with gas production (B), Control of TSI (C), *Klebsiella pneumoniae* showing yellow butt and slant with gas production (D), *Clostridium spp.* showing red slant and butt with H_2S production (E).

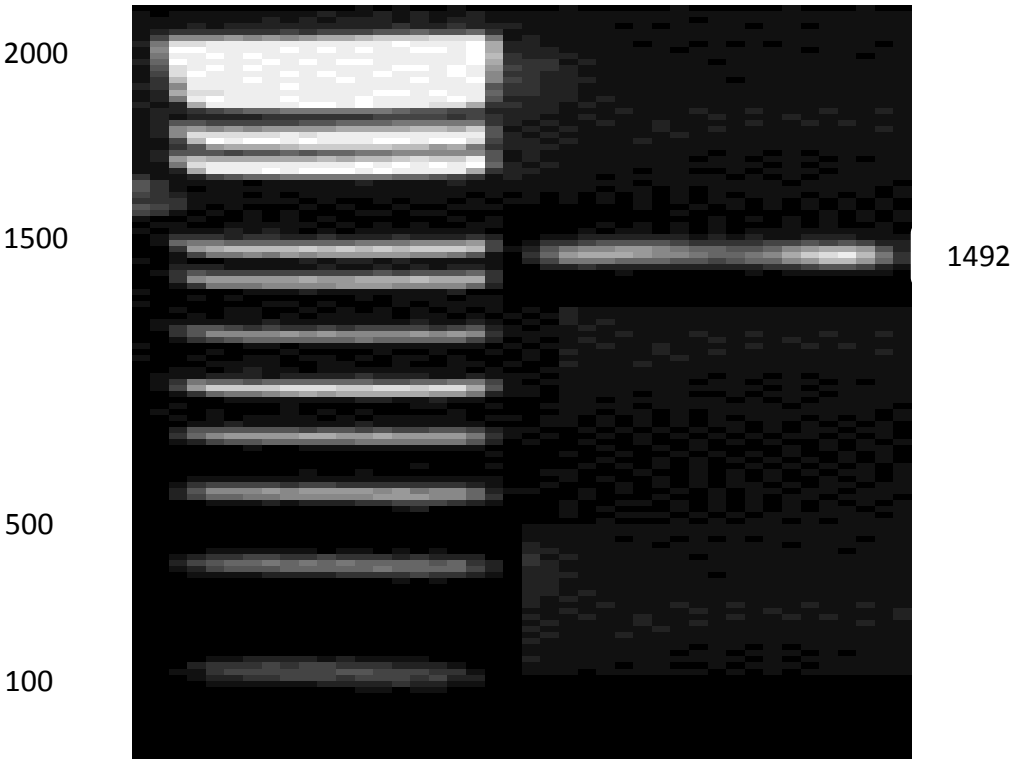

Plate 23: Result of amplification of 16S rRNA gene region of *Klebsiella pneumoniae* by using PCR.

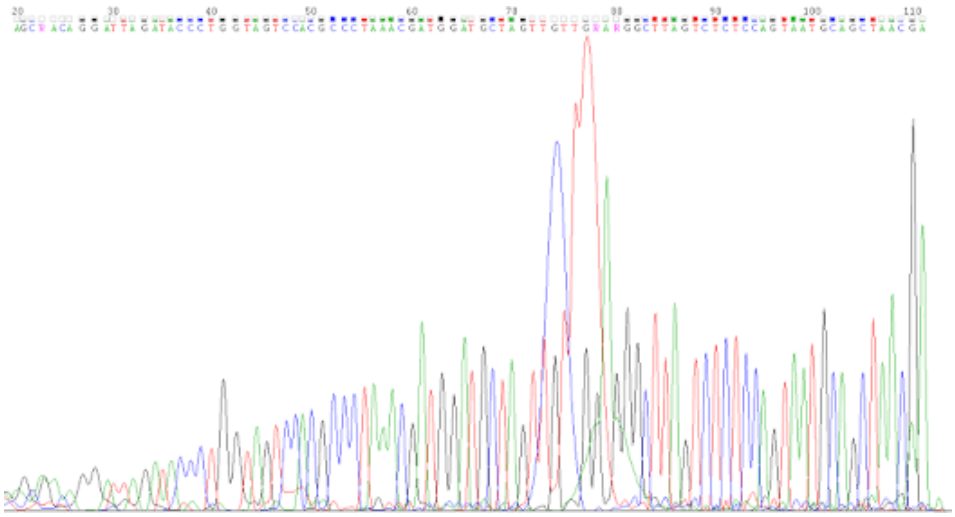

Plate 24: Electropherogram

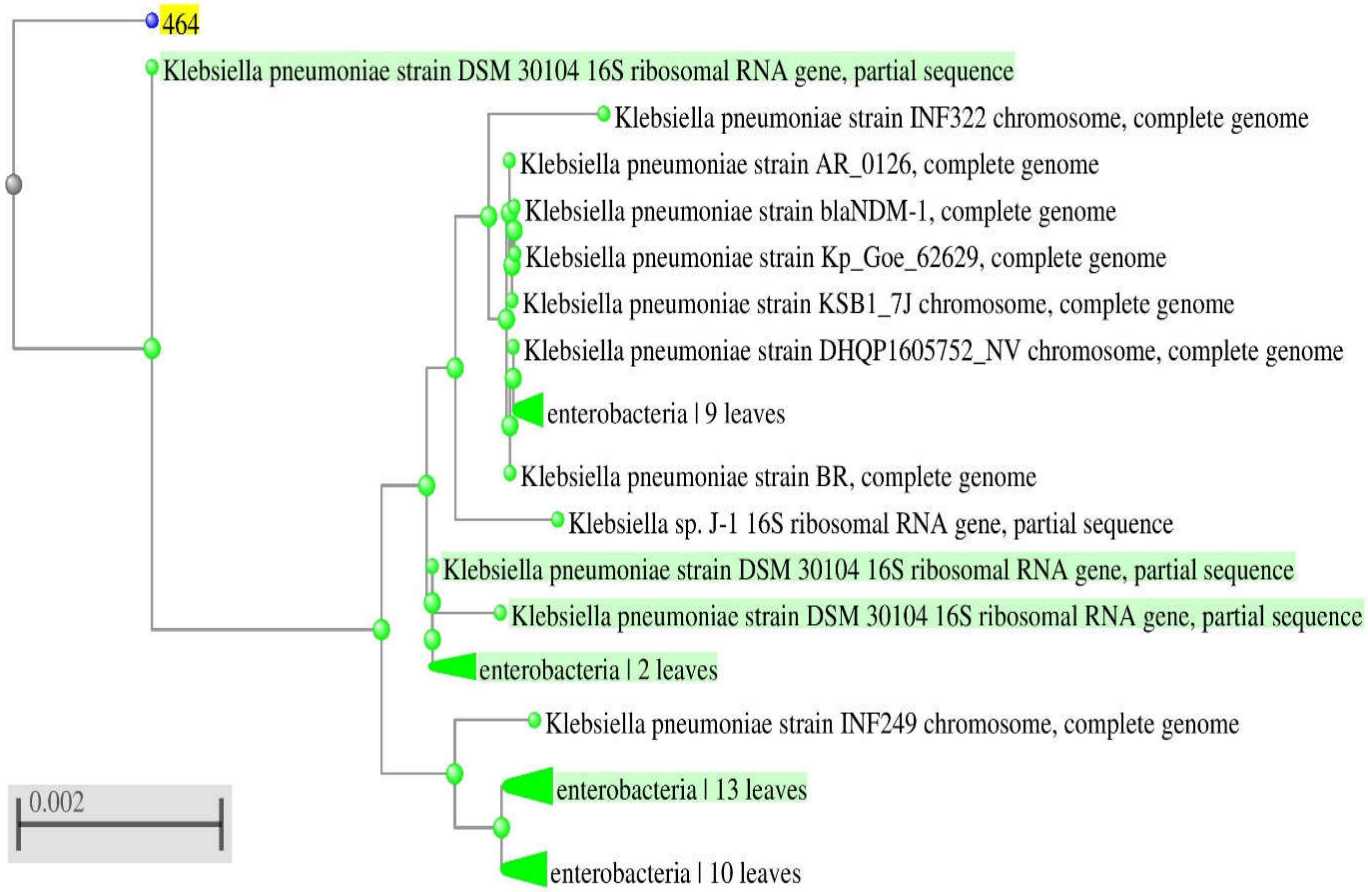

Plate 25: Phylogenic tree analysis of *Klebsiella pneumoniae.*

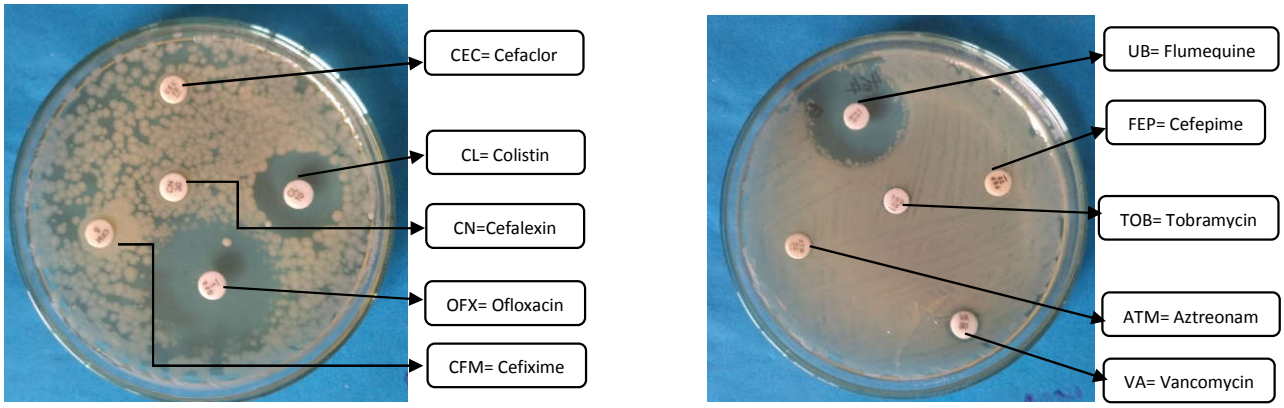

Plate 26: Antibiogram study of *Escherichia coli.*

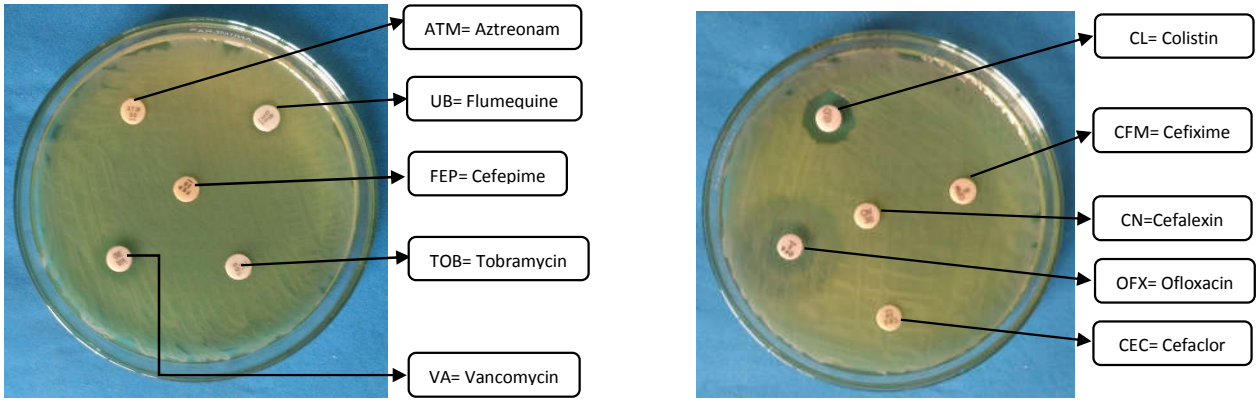

Plate 27: Antibiogram study of *Klebsiella pneumoniae.*

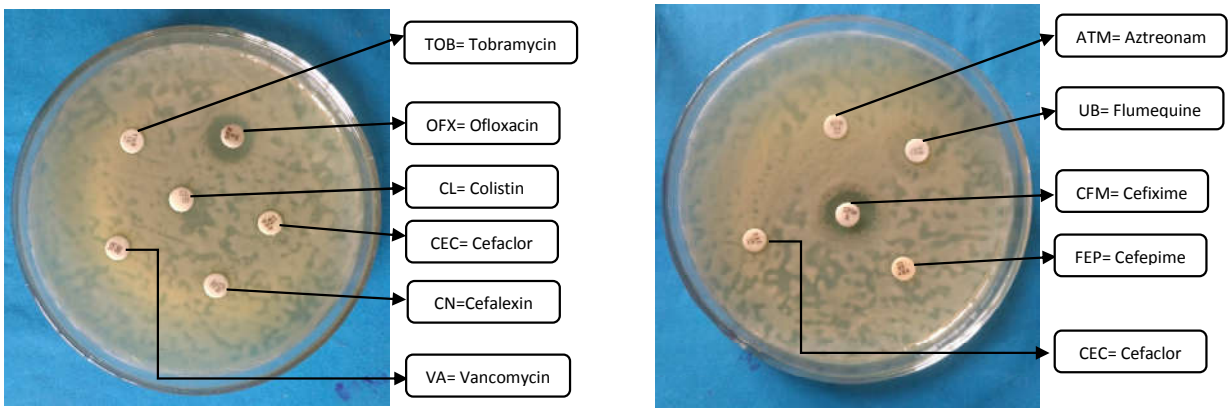

Plate 28: Antibiogram study of *Salmonella spp.*

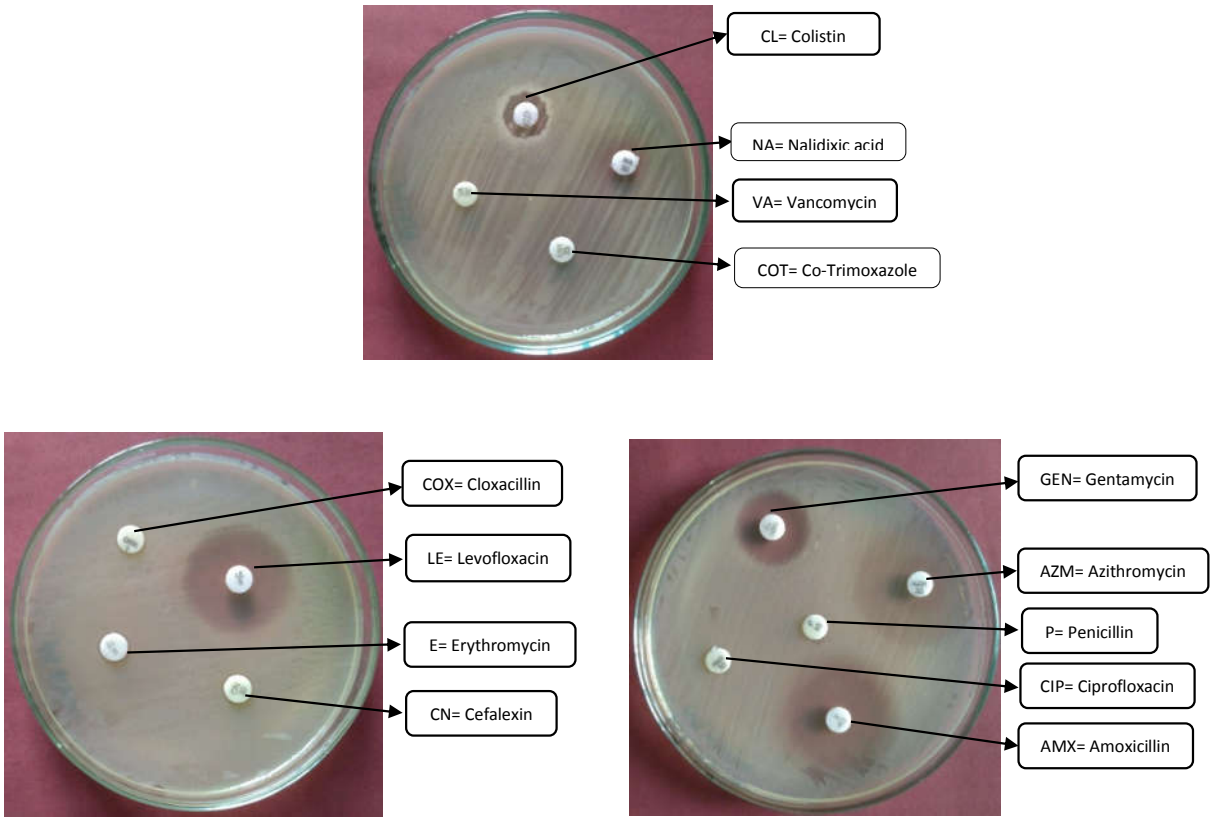

Plate 29: Antibiogram study of *Clostridium spp.*

CHAPTER 5

DISCUSSION

The history of rearing fat-tailed sheep in Bangladesh is not very primitive. Since, the animal is very expensive, it's not yet being reared at the farming level all over the country, but many ritzy people are practicing the rearing of fat-tailed sheep and looking forward to take this practice to the farming level from just luxury purpose. This study was aimed to observe the microbial load (both aerobic and anaerobic) with molecular characterization and antibiogram study of the isolated organisms from the feces and rectal swabs of different aged fat-tailed sheep in Dinajpur and Nilphamari. A total of 32 samples comprising fecal samples (16) and rectal swabs (16) were collected from different aged fat-tailed sheep from different farms in Dinajpur and Nilphamari and were submitted to the microbiology laboratory for bacteriological analysis.

The current study is probably the first study to be conducted in Dinajpur on the fat-tailed sheep to observe the microbial load, their antibiogram study and molecular characterization. As the animal is not very popular at the farming level yet, this study was conducted in order to represent some information on fat-tailed sheep. The fat-tailed sheep is widely known for it's large tail and hindquarters. This animal is used to cope up with extreme environments such as, heat and desiccation and also naturally resistant to many diseases which is usually found in other small ruminants. Fat-tailed sheep has health benefits too, the sciatica might be treated by dissolving the fat sheep tail and then on its division on 3 equal parts, and then drinking on an empty stomach in three equal parts, per one each morning (www.healthwantcare.com).

The present study was reflected on the molecular characterization of bacteria isolated from fat-tailed sheep with microbial assessment and antibiogram study. The collected samples were subjected to total viable count (aerobic and anaerobic), morphological, cultural, biochemical and antibiogram study. In addition, the identified isolate was subjected to the molecular characterization by PCR with sequencing of 16S rRNA gene and phylogenetic study.

In this study the result of total viable counts were expressed by CFU (colony forming unit). In each case the average CFU was counted at 2 weeks interval. The result revealed that in case of total aerobic count, the microbial load was higher in Dinajpur ($1.53\times10^{7}\pm0.05$ CFU)

than that of in Nilphamari ($1.46\times10^7\pm0.06$ CFU) and in case of total anaerobic count, it was also higher in Dinajpur ($0.78\times10^7\pm0.012$CFU) than that of in Nilphamari ($0.62\times10^7\pm0.05$). It was found that, the total aerobic count was the highest during the first week of collection and were gradually decreasing as the animals were aging. On the other hand, unlike the total aerobic count, the total anaerobic count was increasing as the animals were aging. This finding is supported by (Mueller RE *et al*. 1984).

In our present study among thirty two (32) samples, sixteen (16) samples were collected from Dinajpur which comprises eight fecal samples and eight rectal swabs which contain 2 (25%) and 4 (50%) *Escherichia coli,* 3 (37.5%) and 2 (25%) *Salmonella spp,* 1 (12.5%) and 2 (25%) *Klebsiella pneumoniae,* and 2 (25%) and 1 (12.5%) *Clostridium spp.* The rest of the sixteen (16) samples were collected from Nilphamari, which were also consisted of eight fecal samples and eight rectal swabs and revealed 1 (12.5%) and 4 (50%) *Escherichia coli,* 2 (25%) and 3 (37.5%) *Salmonella spp,* 2 (25%) and 2 (25%) *Klebsiella pneumoniae* and 1 (25%) *Clostridium spp.* was found in the fecal samples whereas, rectal swab from Nilphamari revealed no *Clostridium spp.* The differences among the prevalence's of the isolates from two different places involves several factors including the extent of hygienic practicing.

In this study the prevalence of different organisms were studied on the basis of their age and spatial differences. In our observation it was observed that the highest prevalence found in lambs (≤ than 1 year) in comparing with the adults (≥ than1 year). Whereas, the prevalence was higher in Dinajpur than Nilphamari.

In this study the prevalence of *Escherichia coli* in fecal samples collected from Dinajpur was 4 (50%), which is higher than the findings of Evans j.*et al.* (2008).

The total isolation of *Salmonella spp.* was 31.25%(10) in this study, which is higher than the isolation of López-Martín JI *et al.* (2016), and Hailu T *et al.* (2016).

In a previous study, the incidence of *Klebsiella pneumoniae* subspecies were recovered from fecal samples and the percentage of the prevalence was 40.9%, from sheep (Ahmed M.A. Mansour *et al.* 2014), which was much higher than the current study (21.87%). Ahmed M.A. Mansour *et al.* (2014) found that, the prevalence of *Klebsiella* was higher in sheep, followed by cow, goats and lastly camels with percentages of 8.5, 5.5, 4.1 and 3.9%, respectively.

In a previous study, the prevalence of *Clostridium spp.* was 1(0.6%) in sheep and lambs (Daniel R. Knight*et al.* 2013) whereas, the prevalence of *Clostridium spp.* in the

current study was higher 5 (15.63%). Gram's staining from blood agar revealed gram-positive, violet colored, small rod shaped appearance, arranged in single or paired under the microscope. The organism was catalase and indole negative, did not utilized citrate, was motile in motility indole urease test, produced hydrogen sulfide gas in triple sugar iron agar. In the MR test, the absence of red color in the media after addition of methyl red, indicated the negative result. In the voges-proskauer test, there was no change of color of the media after adding alpha napthol and 10% potassium hydroxide, which indicated the negative result. These results are similar with the results of Blocker *et al.* (2001), Galizzi *et al.* (2001), Miyata *et al.* (2001) and Gilbert *et al.* (2001).

In the present study , the bacteria were isolated from fecal samples and rectal swabs on different bacteriological culture media like, nutrient agar, MacConkey agar, eosin mythelene blue agar, salmonella-shigella agar, brilliant green agar, Robertson's cooked meat medium, brain heart infusion agar and blood agar. In our study, it was observed that the isolated organisms might be *Escherichia coli, Klebsiella pneumoniae, Salmonella spp* and *Clostridium spp* which were later confirmed by morphological characters (Gram's staining), different biochemical tests.

In this study, the identified isolate (*Klebsiella pneumoniae*) was subjected to molecular characterization by using PCR and phylogenetic analysis. This finding is supported by Ahmed M.A. Mansour *et al.* (2014).

In our study, the antibiogram study revealed that all of the isolated organisms were susceptible to various antibiotics used in this study at varying levels. All the tested isolates (*Escherichia coli, Klebsiella pneumoniae, Salmonella spp.*) were highly susceptible to Ofloxacin, suggesting that this antibiotic would be the first choice of drug for the treatment purpose. On the other hand *Escherichia coli* were also highly susceptible to Colistin and Flumequine. In this study it was also observed that *Klebsiella pneumoniae* were highly susceptible to Colistin and Vancomycin and *Salmonella spp.* were also susceptible to Cefixime suggesting that these antibiotics could be the first choice of drug in treatment purpose. These results are supported by López-Martín JI *et al.* (2016) and Ahmed M.A. Mansour *et al.* (2014).

The individuals who involved in handling fat-tailed sheep, the veterinarian, persons with specific medical conditions such as a chronic illness, immunodeficiency and pregnancy may be get infected by any zoonotic bacteria if proper care not taken.

In this study, *Clostridium spp.* were highly susceptible to Colistin, Ciprofloxacin and Levofloxacin, suggesting that these antibiotics could be the first choice of drug for the treatment purpose. The susceptibility pattern of *Clostridium spp.* was partially similar to the findings of Singh *et al.* (2010), Bona *et al.* (2007), and Schotte (2004).

The antimicrobial agents are of great value for devising curative measures against bacterial infections. It also emphasize to have judicious selection of antibiotic prior to treatment.

Out of four isolates (*Escherichia coli, Salmonella spp, Klebsiella spp.* and *Clostridium spp.*) only one isolate like *Klebsiella* was characterized by using PCR techniques. In this case the field isolates were amplified by DNA amplification, sequenced and analysed by phylogenetic tree analysis. These findings are supported by Ahmed M.A. Mansour *et al.* (2014).

The result of isolation, identification and antibiogram study of the isolated organisms in the rectal and fecal sample from the lambs and adult sheep belonging to Dinajpur and Nilphamari indicated that if the rearing of fat-tailed sheep is practiced in a unhygienic way, these isolated organisms can be a threat to human and the sheep itself, as the feces of sheep contains shiga toxin producing *Escherichia coli, Salmonella spp, Klebsiella pneumoniae* and *Clostridium spp*. From this study, the further study can also help in following ways-

- Molecular characterization of *Escherichia coli, Salmonella spp* and *Clostridium spp.*
- Lethal effect of toxins extract of *Clostridium spp.* and development of toxoid from local isolates.
- Development of vaccines against local isolates.

CHAPTER 6

SUMMARY AND CONCLUSION

The results from this study indicates 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 (the organisms are present at a non significant level to cause diseases). But, it has found that, the lamb feces contain *Clostridium spp.* which is alarming. The degree of prevalence of these isolates is mainly dependent on the hygienic condition of the places, where the sheep are being reared. That is why this study reveled two different level of prevalence's from two different places.

It has found that the microbial load in feces remains the highest in the lamb and deceases gradually as the lamb ages to adult. The individuals, who directly involved in rearing fat-tailed sheep, are at a high risk of catching any zoonotic disease if the proper hygienic and sanitation measures are not maintained. Moreover, these animals are from a different climate, and has adopted well in our country environment. It's necessary to maintain a routine veterinarian check up and vaccination if needs.

The isolates were subjected to antibiogram study and from the result of the antibiogram study, it has found that all the isolates were resistant against most of the antibiotic, which is not good at all. To those antibiotics, whom the isolates were susceptible, should be applied in recommended dose for treatment purpose. The proper dosing of the proper antibiotics should be established in all the farms of fat-tailed sheep in Bangladesh to inhibit the multi drug resistant bacteria, as the animal have already adopted to the country environment, its gaining popularity. Finally, the proper hygiene should be maintained to keep the sheep and the individuals directly involved with the rearing sheep safe from all the possible zoonotic diseases.

- **Concluding remarks:**
  - A concise, informative prevalence of bacteria prevalent in fat-tailed sheep were considered on the basis of their age and spatial differences.
  - Antibiogram study emphasize judicious selection of antibiotics prior to treatment may inhibit the multidrug resistance.
  - Proper hygiene should be maintained to increase the fat-tailed population in context of Bangladesh that will increase the national economy as a high protein source.

CHAPTER 7
REFERENCES

Ahmed Mansour MA, Hoda Zaki M, Nibal Hassan A and Abdulrahman Al-Humiany A (2014). Molecular characterization and immunoprotective activity of capsular polysaccharide of *klebsiella pneumoniae* isolated from farm animals at taif governorate. *American Journal of Infectious Diseases* 10 (1): 1-14.

Arash Javanmard, Azadzadeh N, Ali Esmailizadeh K (2011). Mutations in bone morphogenetic protein 15 and growth differentiation factor 9 genes are associated with increased litter size in fat-tailed sheep breeds. *Veterinary Research Communication*, 35(3): 157-167.

Aslani MR, Ebrahimi Kahrisangi A, Baghban F, Kazemi A, Heidari M and Salehi N (2015). Endocarditis associated with *Erysipelothrix rhusiopathiae* in a fat-tailed ram. *International Journal of Veterinary Research,* 16(3): 310-312.

Benham JT (1987). Sheep with Two Tails: Sheep's Tail-Fat as Cooking Medium in the Middle East, *The Cooking Medium,* pp 47-48.

Blanco M, Blanco JE, Mora A, Rey J, Alonso JM, Hermoso M, Hermoso J, Alonso MP, Dahbi G, González EA, Bernárdez MI and Blanco J (2003). Serotypes, Virulence Genes, and Intimin Types of Shiga Toxin (Verotoxin)-Producing *Escherichia coli* Isolates from Healthy Sheep in Spain. *Journal of Clinical Microbiology*, 41(4): 1351-1356.

Carter GR (1986). Essentials of Veterinary Bacteriology and Mycology. 3rd edn., pp. 312-330.

Cheesbrough M (1985). Medical laboratory manual for tropical countries. Vol. II. Microbiology. pp. 400-480.

Collie RE and McClane BA (2002). Evidence that the enterotoxin gene episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology*. 36: 30-36.

Duffy G (2003). Vero Toxigenic *Escherichia coli* in animal feces, manures and slurries, *Journal of Applied Microbiology*, 94: 945-1035.

Edismauro Freitas GF, Marcos RAF, Jefferson FNP, Fabrício Conceição R and Cecilia Moreria N (2014). Enterohemorrhagic *Escherichia coli* O157:H7 from healthy dairy

cattle in Mid-West Brazil: occurrence and molecular characterization. *Pesquisa Veternaria Brasileira,* 34(1):24-28.

El-Khodery SA and Osman SA (2010). Acute Lamb Dysentery in lamb clinical findings and treatment outcomes. *Tropical Animal Health and Production.* 50: 30-32.

Evans J, Knight HI, Smith AW, Pearce MC, Hall M, Foster G, Low JC and Gunn GJ (2008). Cefixime–tellurite rhamnose MacConkey agar for isolation of Vero cytotoxin-producing *Escherichia coli* serogroup O26 from Scottish cattle and sheep faeces. *Letters in Applied Microbiology* 47 (3): 148–152.

Garciaa JP, Adamsb V, Beingessera J, Hughesb ML, Poonb R, Lyrasb D, Hillc A, McClaneb BA, Roodb DJI and Uzala FA (2013). Epsilon Toxin Is Essential for the Virulence of *Clostridium perfringens* Type D Infection in Sheep, Goats, and Mice. *Infection and Immunity,* 81(7): 2405-2414.

Gilmour NJL (1980). *Pasteurella haemolytica* infections in sheep. *The Veterinary Quarterly*, 2(4): 191-198.

Hailu T, Kebede B (2016). Isolation of Non-Typhoidal *Salmonella* from Sheep faeces in Eastern Hararghe, Ethiopia. *Applied Microbiology: Open Access* 2(3): 2471-9315.

Hajializadeh Valilou R, Rafat SA, Firouzamandi M and Ebrahimi M (2016). Use of Microsatellite Polymorphisms in Ovar-DRB1 Gene for Identifying Genetic Resistance in Fat-Tailed Ghezel Sheep to Gastrointestinal Nematodes. *Iranian Journal of Applied Animal Science,* 6(4): 879-886.

Harry Hariharan, Alfonso López, Gary Conboy, Mada Coles, Tammy Muirhead (2007). Isolation of *Escherichia fergusonii* from the feces and internal organs of a goat with diarrhea. *Canadian Veterinary Journal,* 48:630–631.

Knight DR, Riley TV (2013). Prevalence of gastrointestinal Clostridium difficile carriage in Australian sheep and lambs. *Applied and Environmental Microbiology*. 79(18): 5689-5692.

Lahti P, Heikinheimo A, Johansson T, Korkeala H, (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS *l151-cpe* or IS *1470-like-cpe)* as a common cause of food poisoning. *Journal of Clinical Microbiology.* 46:371-373.

Leifson, E. (1939). "New selenite selective enrichment medium for the isolation of typhoid and paratyphoid bacilli". *American Journal of Hygiene*, 24: 423–432.

Lewis CJ, Naylor RD (1998). Sudden death in sheep associated with *Clostridium sordellii. The veterinary record,* 142(16):417-421.

López-Martín JI, González-Acuña D, García CA, and Carrasco LO (2016). Isolation and Antimicrobial Susceptibility of *Salmonella Typhimurium* and *Salmonella Enteritidis* in Fecal Samples from Animals. *Journal of Antimicrobial Chemotherapy*, 2:1, 1-6.

M. A. Samad (2011). Public health threat caused by zoonotic diseases in bangladesh. *Bangladesh Journal of Veterinary Medicine*, 9 (2): 95 – 120.

Miah MS, Asaduzzaman M, Sufian MA, and Hossain MM (2011). Isolation of Clostridium perfringens, Causal agents of enteritis in lamb. *Journal Bangladesh Agricultural University*. 9(1): 97-102.

Mourad Ben Said, Hanène Belkahia, Alberto Alberti, Khaoula Abdi, Manel Zhioua, Monia Daaloul-Jedidi, Lilia Messadi (2016). First molecular evidence of *Borrelia burgdorferi* sensu lato in goats, sheep, cattle and camels in Tunisia. *Annuals of Agricultural and Environmental Medicine*, 23(3), 442–447.

Mueller RE, Iannotti EL, and Asplund JM (1984). Isolation and Identification of Adherent Epimural Bacteria During Succession in Young Lambs, *Applied And Environmental Microbiology*, 47(4): 724-730.

Plug I, and Badenhorst S (2001). The Distribution of Macro mammals in South Africa over the past 30000 years. Transvaal Museum, Pretoria.

Reza Kheirandish, Masoud Sami, Reza Ghanbarpour, Soodeh Alidadi (2013). Pulmonary nocardiosis in a fat-tailed sheep, *Eurasian Journal Veterinary Science,* 29,(4): 220-224.

Saeed Alamian, Ramin Bagheri Nejad, Hamid Reza Jalali, Armin Kalantari, Afshar Etemadi (2015). Innocuousness of conjunctival vaccination with *Brucella melitensis* strain Rev.1 in pregnant Iranian fat-tailed ewes. *Veterinary Science Development*, 5(2): 112-114.

Songer JG (1996). Clostridial enteric diseases of domestic animals. *Clinical Microbiology*. 9: 216-234.

Steven Pao, Dhartika Patel, Aref Kalantari, Joseph P. Tritschler, Stephan Wildeus and Brian L. Sayre (2005). Detection of *Salmonella* Strains and *Escherichia coli* O157:H7 in Feces of Small Ruminants and Their Isolation with Various Media. *Applied and Environmental Microbiology,* 71(4): 2158-2161.

Uzal FA, Songer JG (2004). Diagnosis of *Clostridium perfringens* intestinal infections in sheep. *Journal of Veterinary Disease Investigation.* 20 : 253-265.

Vettorato MP, de Castro AFP, Cergole-Novella MC, Camargo FLL, Irino K and Guth BEC (2009). Shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *Escherichia coli* strains isolated from healthy sheep of different populations in Sao Paulo, Brazil. *Letters in Applied Microbiology* 49 (1): 53–59.

APPENDIX

APPENDIX 1

	Composition of Media	
1.	**Nutrient broth**	
	Ingredients per litter of deionized water	**g/L**
	Peptone	5.0
	Sodium chloride	5.0
	Beef extract	1.5
	Yeast extract	1.5
	Final pH(at25°C)	7.4±0.2
2.	**Nutrient agar**	
	Ingredients per litter of deionized water	**g/L**
	Beef extract	3.0
	Peptone	5.0
	Sodium chloride	5.0
	Agar	20.0
	Final pH	7.1±0.1
3.	**Salmonella Shigella agar**	
	Ingredients per litter of deionized water	5.00 gm
	Peptic digest of animal tissue	
	Beef extract	5.00 gm
	lactose	10.00 gm
	Bile salts mixture	8.50 gm
	Sodium citrate	10.00 gm
	Sodium thiosulphate	8.50 gm
	Ferric citrate	1.00 gm
	Brilliant green	0.00033gm
	Neutral red	0.025 gm
	Agar	15.00 gm
	Distilled water	1000 ml
	Final pH(at25°C)	7.0±0.2 gm

5. Thiosulfate-Citrate-Bie Salts-Sucrose (TCBS) agar

Ingredients per litter of deionized water	gtL
Sucrose	20.0 gm
Dipepton	10.0 gm
Sodium Citrate	10.0 gm
Sodium Thiosaulfate	10.0 gm
Sodium Chloride	10.0 gm
Yeast extract	5.0 gm
Sodium cholate	3.0 gm
Ferric citrate	1 gm
Bromothymol blue	0.04 gm
Thymol blue	0.04 gm
Agar	15.gm

6. MacConkey Agar

Ingredients	**g/L**
peptone	17.0
Protease peptone	3.0
Lactose	10
Bile salt	1.5
Sodium cholride	5.0
Agar	13.5
Neutral Red	0.03
Crystal violet	0.001
Final pH	7.1±0.2

7. Eosine methylene blue agar

Ingredients	**g/L**
Peptone	100
Lactose	10.0
K2HP04	2.0
Eosin	0.4

	Methylene blue	0.065
	Agar	20.0
	Final pH	6.8±0.2

8. Blood agar

Ingredients	**g/L**
Agar	15.0
Beef extract	10.0
Peptone	10.5
Sodium chloride	5.0
Final pH	7.3±0.2

9. MR VP medium (Himedium, India)

Composition	7.0
Buffered peptone	
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH(at 25°CO	6.9±0.2

11. Simmon's citrare agar

Ingredients	**g/L**
Magnessium sulphate	0.20
Ammunium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothvmol blue	0.08
Agar	15.0

12. TSI Agar slant

Ingredients	3.00 gm
Lab Lamco Powder	
Yeast extract	3.00 gm
Peptone	20.00gm

Sodium chloride	5.00 gm
Lactose	10.00gm
Sucrose	10.00gm
Glucose	1.00 gm
Ferric citrate	0.3 gm
Sodium thiosulphate	0.3 gm
Phenol red	0.3 gm
Agar	12.00gm
Distilled water	1000

APPENDIX 2

Preparation of reagents

1. Peptone water

peptone	1 gm
Distilled water	1000 ml

2. Kovacs reagent for indole preparation 5 gm

P- dimethyl aminobenzal dehyde

Amyl alcohol	75 gm
Conc. HCL	25 ml

3. V-P reagent-1

5% alpha- naphthanol in absolute ethyl alcohol

4. V-P reagent-2

40% potassium hydroxide containing 0.3% creatine. The ingredient was dissolved by heating gently over a steam bath. When in solution, added 0.052 gm of cotton blue dye.

5. Methyl red Solution

Methyl red	0.05 gm
Ethanol(absolute)	28 ml
Distilled water	22 ml

6. Phenol red solution

0.2^{0}o aqueous solution of phenol red

7. Gram stain solutions

a. **Stock crystal violet**

crystal violet	10 gm
Ethyl alcohol	1000m1

b. Stock oxalate

Ammonium oxalate	1 gm
Distilled water	1000 ml

Crystal violet working solution: 20 ml of solution no. I mixed with 80 ml of solution no. 2. Additional dilution was made when desired.

c. Lugol's Iodine solution

Iodine crystal	1 gm
Potassium iodide	2gm

Dissolved completely in 10 ml of distilled water, then added to distilled water to make 300 ml. stored in ambar bottle.

d. Ethyl alcohol 250 ml

e.	**Acetone**	250 ml
f.	**Counterstain**	2.5 ml
	Safranine	
	Ethyl alcohol (95%)	100 ml

Safranine working solution:

The stock safranine is usually diluted as 1:4 with distilled water.