BACTERIAL LOAD IN FECAL SAMPLE AND GROWTH PERFORMANCE OF SHEEP AT GOBINDAGONJ UPAZILLA OF GAIBANDHA DISTRICT OF BANGLADESH

A THESIS

BY

SYEDA SALMUN RIFAT

Registration No.: 1305067

Semester: January-June/2015

MASTER OF SCIENCE (M.S.)

IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY
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ABSTRACT

The present study was conducted to identify the organisms in feces of sheep and their effects on growth performance of sheep at Gobindogonj Upazilla, Gaibandha. The samples were analyzed to determine the total viable count, prevalence of gram negative bacteria, *Eschrechia coli* and *Salmonella spp.* The sheep were categorized into three (3) groups namely group A, group B and group C according to their body weight gain at same age. For the determination of bacterial load a total of 45 samples were tested where *E.coli* present in 20 sample and prevalence was 44.44%, (group A- 60%, Group B -40% and group- C 33.33%), and *Salmonella spp* was present in 11 samples and prevalence was 24.44% (group A- 33.33%, Group B- 26.67% and group C-13.33%). From this study the result represented that the total viable counts/g feces in group A, group B and group C were 9.8×10^6, 4.9×10^4 and 3.4×10^3 CFU/g sample respectively, the *E. coli* counts/g feces in group A, group B and group C were 4.24×10^6, 2.4×10^4 and 5.5×10^2 CFU/g of sample respectively and *Salmonella spp.* counts/g sample on group A, group B and group C were 3.2×10^3, 2.1×10^2 and 1×10^2 CFU/g feces respectively. The study showed that group A had highest bacterial load (6.99 Log 10/g) and group C had lowest bacterial load (3.53Log10/g).In conclusion it is evident that *E. coli* and *Salmonella spp* was successfully detected through different bacteriological agar

media and biochemical reaction indicating the isolated organisms were responsible for possible faecal pollution and also a negative effect on growth performance of sheep in reared in research area.

CONTENTS

CONTENTS (Contd.)

CHAPTER	TOPIC	PAGE NO

CONTENTS (Contd.)

CONTENTS (Contd.)

CONTENTS (Contd.)

LIST OF TABLES

LIST OF PLATES

LIST OF PLATES (Contd.)

List of abbreviation and symbols

TVC	:	Total viable count
TEC	:	Total *E. coli* count
CFU	:	Colony forming units
cm^2	:	Centimeter square
E. Coli	:	*Escherichia coli*
e.g.	:	Example
ELISA	:	Enzyme-linked immunosorbent assay
EMB	:	Eosin Methylene Blue agar
et al.	:	Associated
TSC	:	Total Salmonella spp count
EU	:	European Union
FAO	:	Food and Agriculture organization
Fig.	:	Figure
GDP	:	Gross Domestic Product
GNP	:	Gross National Product
Gm	:	Grams
hrs	:	Hours
HSTU	:	Hajee Mohammad Danesh Science and Technology University
H2S	:	Hydrogen sulphide
HWS	:	Hot Water Supply
ISO	:	International Organization for Standardization
Ib	:	Pound
Kg	:	Kilogram
log	:	Logarithms
MCA	:	MacConkey Agar
mg	:	Milligram
min	:	Minutes
MIU	:	Motility Indole Urease
ml	:	Milliliter
mm	:	Millimeter
NA	:	Nutrient agar

No.	:	Number
PCR	:	Polymerase Chain Reaction
PH	:	Numerically it is the negative logarithm of that concentration of hydrogens ions (= H+) (= protons) in a solution.
Prof.	:	Professor
SL.	:	Serial
SPC	:	standard plate count
spp.	:	Species
Sq	:	Square
SSA	:	Salmonella-Shigella agar
TBC	:	Total bacterial counts
TCC	:	Total coliform count
TSI	:	Triple Sugar Iron agar
TVC	:	Total viable counts
WB	:	Warner-Bratzler
US	:	United State
WHO	:	World Health organization
-	:	Negative
%	:	Percentage
/	:	Per
+	:	Positive
<	:	Less than
>	:	Greater than
µg	:	Microgram
^{0}C	:	Degree Celsius

CHAPTER I

INTRODUCTION

Bangladesh is an agro-based country where 80 percent of the population lives on agriculture. Since livestock plays a vital role in the economy of Bangladesh, therefore its contribution bears significant strain on maintaining domestic resources essential for promotion of lifestyle and livelihood of landless and marginal farmers. It is evident that livestock wealth provides about 9% of the Gross National Product (GNP), which is 6.5% of Gross Domestic Product (GDP) (Ahmed, 1992; Rahman and Rahman, 1998). Among the livestock species, sheep is considered as an important and promising animal resource in Bangladesh for contributing meat and wool. During the last twelve years sheep population increased 2.5 times, with annual growth rate of 5% and there are 2.7 million sheep (BBS, 2008), of which 32% are reared in three ecological zones, Barind, Jamuna basin and Coastal areas. Most of the sheep are indigenous, with few crossbreds (Bhuiyan, 2006) and are capable of bi-annual lambing and multiple births. In our country, goats and sheep are mainly kept by the poor farmers and distressed women in extensive system under ranged condition without any supplementation. This system of production causes reduced growth rate and poor reproductive performance, which in turn results in severe economic losses. (Sultana *et al* , 2011).The unemployed persons, landless farmers and rural women of our country

ubiquitously rear sheep, capable of helping in poverty alleviation all over the world including Bangladesh, Currently the contribution of sheep in Bangladesh can be summarized as a source of meat, skin and wool. Bangladesh possesses 3.156 million sheep at present (BER, 2014). Sheep rearing is increasing in this country to fulfill the growing demand of animal protein. But bacteria retard it's growth. Sheep is susceptible to certain pathogenic (disease-causing) bacteria, such as *Salmonella*, *E.coli, Corynebacterium spp, Listeria monocytogenes, Clostridium spp, Campylobacter spp, Fusobacterium spp* etc. Many times, a combination of viral and bacterial agents infect the lungs as a result of stress such as weaning, transport, change of weather, poor air quality. Tetanus infection is caused by *Clostridium tetani,* when the bacteria gains entry to the body through contamination. Most cases of tetanus in sheep are secondary to tail docking and castration, especially when rubber bands are used in the process.

Escherichia coli is considered as the normal bowel flora of different species of mammals and birds but some strains of *E. coli* possess pathogenic character due to the acquisition of virulent factors. Microbial characteristics associated with virulent *E. coli* include production of enterotoxin, verotoxin, colicins and siderophores, type-1 pili and motility, resistance to the lytic action of the host complement and antibiotics (Dho *et al*., 1984; Chulasiri *et al*., 1989).

Salmonellosis has been recognised in all countries, but appears to be most prevalent in areas of intensive animal husbandry, especially in pigs and calves and some types of poultry reared in confinement. Reptiles are commonly asymptomatic carriers of *Salmonella*. Several serovars are host specific (e.g. *S. abortusovis* in sheep or *S. Typhi* in humans) or host adapted (e.g. S. *Choleraesuis, S. Dublin*). (OIE Terrestrial Manual, 2008)

The present studies was conducted with the following objectives-

- To determine the bacterial load in feces of sheep.
- To isolate the bacteria from sheep feces.
- To know the effects of microorganisms on growth performance of sheep.

CHAPTER II

REVIEW OF LITERATURE

N.R. Atieno *et al.* (2013) mentioned that goat and sheep sludge had the highest fecal *coliform* (FC) contamination with a mean density of 3.9×10^5 ($\pm3.5\times10^5$) MPN/100 ml and *Vibrio* and *Salmonella* species were more frequently detected in samples which also showed high incidence of fecal bacteria indicator.

W. Bruha (2013) stated the incidence of *Salmonella spp*. In fecal and hide samples before transportation and the incidence in hide samples after transportation. Microbroth

dilution plates were used to established antibiotic resistant profile on the samples. Twenty-seven percent of all samples (n=600) tested positive for *Salmonella* using several selective and enrichment media. Of those, 40% were classified as serogroup B and 48% were from serogroup C. Other serogroup values were10% or less of the total

A. Dabassa *et al*. (2012), evaluated the existing safety status of foods, studied 180 animal samples composed from cattle, goat,and sheep, meat and feces were analyzed for microbial load determination using conventional culture method. The aerobic mesophilic counts varied from 3.0 to 9.0 log10 CFU/g. *Coliforms* were encountered in mostsamples. *Enterobacteriacea* were present at 2-4 log10 CFU/g in 19 %(45) meat, and 21% (22) faeces samples. *Salmonella* species were present in 4.4 % (8.0) of all the samples. out of 60 cattle, 60 goats and 60 sheep part 4(13.3%) beef, 1(3.3%) cattle feces,1(3.3%) chevon, 1(3.3%) mutton, 1(3.3%) sheep feces were contaminated with *Salmonella.*

M.N. Munsi *et al. (*2012) reported that *Escherichia coli* alone was found in 6 samples (30%), *Escherichia coli.* Combined with *Proteus mirabilis* was found in 12 (60%) and no bacteria could be detected in 2 of the 20 fecal samples of sheep of BLRI goat and sheep research farm.

M. Wessam *et al.* (2012), detected *Salmonella* isolates from 309 different apparently healthy samples (97 fecal, 71 bile, 102 intestinal content, 18 livers and 21 spleen)

collected from slaughtered sheep in a slaughterhouse. The results revealed that the incidence rate of *salmonella* isolates in fecal samples was 7.2 %, in bile samples 8.5 % and in intestinal content 9.8%. While no isolation was obtained from the liver and spleen samples. The total positive sample of *salmonella* isolates was 23 (7.4%)

EM Moriarty *et al.* (2011) studied on the survival of enteric bacteria 10 freshly collected sheep fecal samples on pastures was meseared in each of four seasons. Ten freshly collected feces were placed on pasture, and concentrations of *Escherichia coli, Enterrococci,* and *Campylobacter spp*. were monitored until exhaustion of the fecal samoles. In all four seasons, there was an increase in Enterrococcal concentrations by upto 3 orders of magnitude, with peak concentrations recorded between 11 and 28 days after deposition. *E.coli* concentrations increased in 3 out of four seasons by upto 1.5 orders of magnitude, with peak concentrations recorded between 8 and 14 days after deposition.

N. Sultana *et al.* (2011) reported that there were no significant differences ($p > 0.05$) in service per conception, litter size, lambing interval, gestation length, service period, birth weight and weaning weight between intensive and semi-intensive feeding system.

CJ Lewis *et al.* (2007) stated that enterotoxemia in sheep is caused by the various types of *C. perfringens*. In the UK, they are the commonest clostridial diseases to affect sheep.

The five distinct types of *C. perfringens* are distinguished by their toxicological properties. Types B,C and D are of major importance to sheep, while type A is occationally pathogenic.

M. Gourmelon *et al.* (2007) identified the origin of the fecal contamination observed in French estuaries, two library-independent microbial source tracking (MST) methods were selected: i) Bacteroidales host-specific 16S rRNA gene markers and ii) F-specific RNA Bacteriophage genotyping. The specificity of the Bacteroidales makers was evaluated on human and animal (bovine, pig, sheep and bird) feces.

P. Cox *et al.* (2005) reported that *Clostridium perfringens* spores in pooled animal fecal samples from domestic animals were highly variable, with the majority of horse, calf, sheep and adult cattle samples being either equal to or less than the detection limit of 100 CFU/g. He found 1.0 $x10^5$ to 1.9 $x10^8$CFU/g.

R.W. Weaver *et al*. (2005) reported the presence of indicator bacteria in dry manure were often as high as that in fresh manure from horse and sheep. They found 4.96log10·g–1 and 6.25log10·g–1 *Enterococcus spp*. in fresh and dry manure of sheep respectively. And *E. coli* found 6.05 log10·g–1 and 5.63 log10·g–1 in fresh and dry manure respectively.

S. Pao *et al.*(2005) reported aerobic organisms at 7.2 ± 0.7 $\log_{10}$ CFU/g, yeasts and molds at 3.4 ± 0.7 $\log_{10}$ CFU/g, and generic *E. coli* organisms at 5.5 ± 1.0 $\log_{10}$ (most probable number)/g.

Alvseike *et al*. (2002) http://aem.asm.org/content/71/4/2158.full - aff-1 reported that small ruminants, such as sheep and goats, are potential carriers of *Salmonella* and *E. coli* O157:H7.

Graves *et al.* (2002) reported the general methods of isolates from known sources is to collect fecal samples directly from the animals or from fresh droppings.

Hindson *et al.* (2002) stated that the lambs from birth to first few weeks of age, the bacterial species mainly involved are *E. coli*, *Salmonella spp, Clostridium perfringens* type B and *campylobacter*. The bacterial causal agents of scouring in case of growing lambs include *salmonella spp, Campylobacter* and *Listeria.*

Radostits *et al*. (2000) stated that *E. coli* is a major cause of diarrhoea in calves, piglets, lambs, and the term "*collibacillosis*" is commonly used. It causes loss in this age group of animals

P.J. Hadley *et al.* (1997), stated that the condition of the fleece significantly affected the microbial load on these parts of the dressed carcass, with the carcases derived from sheep with increasingly dirty fleeces carring upto 1000

times more microorganisms, and a higher proportion of the carcases being contaminated with *Enterobacteriaceae.*

Rahman (1995) stated that bacterial agents causing diarrhoea in sheep are *Escherichia coli, Salmonella spp.* and *clostridium perfringens*, where *proteus spp.,* and some other species of bacteria may be associated in some cases.

CHAPTER III

MATERIALS AND METHODS

The present research work was conducted during January to June, 2015 at the Microbiology laboratory in the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The detailed outline of Materials and Methods are given below.

3.1 Materials

3.1.1 Study area

The research work is carried out at 3 unions of Gobindagonj upazilla at Gaibandha district of Bangladesh.

3.1.2 Study population

A total 165 sheep were classified into three different groups namely group A, group B and group C according to their body weight gain and different pasture land. These animals were fed same type of concentrate feed and practiced same management system like they reared in semi-intensive system, but they grazed about 8 hours in a day in different pasture land..

3.1.2.1 Group A

Contained 50 animals of 6 months aged having 7 kg body weight, grazed on low land near the water. From 50 aminals 15 animals were selected randomly for this study.

3.1.2.2 Group B

Contained 55 animals of 6 months aged having 8 kg body weight, grazed on both low land and fallow land. From 55 aminals 15 animals were selected randomly for this study.

3.1.2.3 Group C

Contained 65 animals of 6 months aged having 9 kg body weight, grazed on fallow land which were clean and contained green grasses. From 65 aminals 15 animals were selected randomly for this study.

3.1.3 Field sample

Fecal samples of sheep were collected aseptically directly from the rectum or just after deposition and taken into small polybags using disposable hand gloves and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University for laboratory analysis maintaining proper cool chain.

3.1.4 Instrument and appliances

Phase contrast microscope, test tubes, cotton, hand gloves, plastic syringe (5 ml), micropipette (1 ml, 500 µl, 10-20 µl), glass slides, eppendorf tubes, magnifying glass, marker pen, ice-box, spirit lamp, cover slips, inoculating loop and rack, autoclave, refrigerator, conical flask, colony count machine, digital weight balance and stirring machine

3.1.5 Laboratory preparation

All items of glasswares including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides, vials and mesearing cylinder soaked in a household dishwashing detergent solution (‘Trix, Recket and Colman Bangladesh Ltd.) for overnight, contaminated glasswares were disinfected in 2% sodium hypo chloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly and finally sterilized by autoclaving for 15 minutes at 121^0 C under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven over at 50^0 C. Disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50^0 C for future use.

3.1.5 Media for culture

3.1.5.1 Liquid media

2% buffered peptone water (Hi-media, India).

3.1.5.2Solid media

4 Nutrient agar base (Hi-media, India).
5 Plate count agar media (Hi-media, India).
6 Salmonenella-Shigella agar (Hi-media, India).
7 Brilliant green agar (Oxoid, England).
8 Eosin methylene blue (EMB) agar (Hi-media, India).
9 MacConkey agar medium (Hi-media, India).
10 Triple sugar iron (TSI) agar slant (Hi-media, India).
11 Motility, Indole, Urease (MIU) medium (Hi-media, India).

3.1.6 Reagents

- Gram’s staining reagent: Crystal violet, Gram’s iodine, Acetone and Safranine.
- Alpha-naphthol solution.
- Kovac’s reagent.
- Ethyl alcohol (70% and 95%).

3.1.6.1 Liquid Media

3.1.6.1.1 Nutrient broth

Nutrient broth (NB) was used to grow the organisms from the samples collected from the study areas before performing biochemical test (Cheesebrough, 1985).

3.1.6.2 Solid media

3.1.6.2.1 Plate count agar

Plate count agar was used to determine of plate count of microorganisms by pour plate method (Cheesebrough, 1985).

3.1.6.2.2 Nutrient agar

Nutrient agar (NA) medium was used to grow the organisms from the collected samples (Cheesebrough, 1985).

3.1.6.2.3 Salmonella-Shigella agar

Salmonella-Shigella (SS) agar medium was used as a selective medium for salmonella organism which causes enhancement of the grouth of salmonella while inhibiting the growth of other contaminating organisms shows typical colony character (Cheesebrough , 1985).

3.1.6.2.4 MacConkey agar

MacConkey agar (MC) medium was used to culture the organisms under the family Enterobacteriaceae (Cheesebrough, 1985).

3.1.6.2.5 Eosin methylene blue agar

Eosin methylene blue (EMB) agar medium was used to observe the growth of *Escherichia coli* (Cheesebrough, 1985).

3.1.6.2.6 Brilliant green agar

Brilliant green agar (BGA) was used as a selective madium for the isolation and identification of *Salmonella* organism (Cheesebrough , 1985).

3.2 Methods

3.2.1 Experimental layout

The samples were collected directly from sheep from Gobindagonj upazilla for determination of bacterial load, isolation and identification of bacterial pathogens by morphology, staining and cultural characteristics. Characterization of bacteria were done by cultural and biochemical reactions.

EXPERIMENTAL LAYOUT

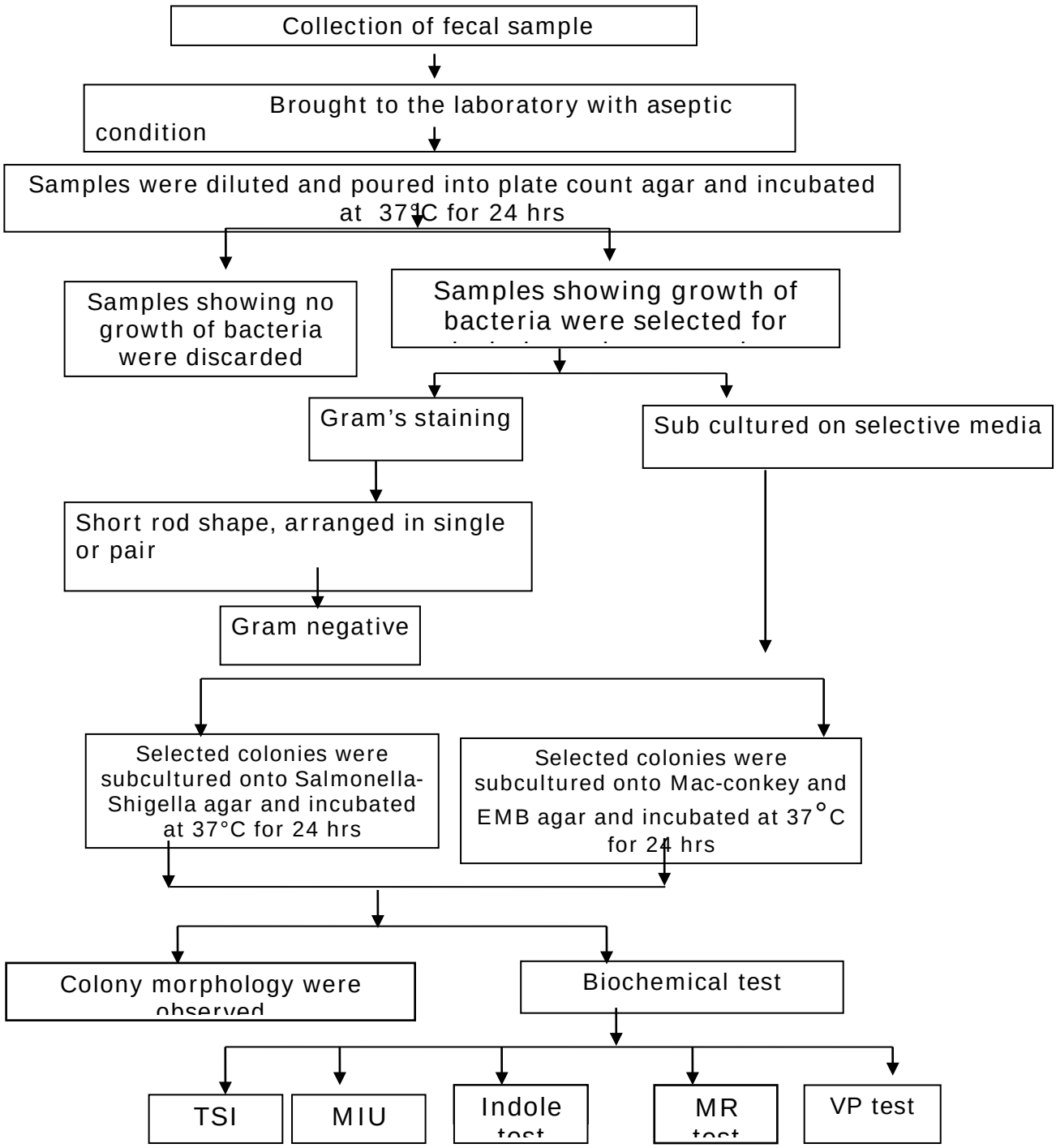


Fig. 1 The schematically illustration of layout of the experiment

3.2.2 Collection of fecal sample

Fecal samples from three Unions of Gobindoganj upazilla were collected aseptically into small polybags which placed on ice box and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur with necessary precautions for bacteriological examination.

3.2.3 Sample preparation

1gm of each fecal sample mixed with 100 ml of 2% buffered peptone water by stirring done by stirring machine in a small conical flask.

3.2.4 Serial dilution of the sample

In serial dilution the original inoculum was diluted in a series of dilution tubes. At first 10 sterile test tubes were placed on a test tube holder rack containing 9 ml of 2% buffered peptone water. Then 1ml sample from 1^{st} test tube mixed with 2^{nd} test, then from 2^{nd} test tube to 3^{rd} test tube and finally 9^{th} to 10^{th} test tube and 1ml discard from 10^{th} test tube by the help of pippet and in every steps mixing was done properly.

3.2.5 Enumeration of total viable count (TVC)

For the determination of total bacterial count, 1 ml of each ten-fold dilution was transferred and spread on dupli-cate plate count agar using a fresh pipette for each dilution. The diluted samples were spread as quickly as possible on the

surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate. The plates were then kept in an incubator at 37^0 C for 24-48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the total viable count. The total viable count was calculated according to ISO (1995). The result of the total bacterial count was expressed as the number of organism or colony forming unit per ml (CFU/ml) of sample and multiplied with 100 to calculate the colony in 1gm of feces.

3.2.6 Enumeration of total *E. coli* count (TEC)

For the determination of TEC count 1ml of each tenfold dilution was transferred to EMB agar. For each dilution five test plates containing EMB agar were used. All the agar plates were incubated at 37 ^{o}c temperature for 48 hours. The total *E. coli* count was calculated according to ISO (1995). The result of the TEC was expressed as the number of organism or colony forming units per gm (CFU/g) of sample.

3.2.7 Enumeration of total *Salmonella* Count (TSC)

For the determination of TSC, 1ml of each tenfold dilution was transferred to Salmonella-Shigella agar. For each dilution five test plate containing Salmonella-Shigella agar were used. All the agar plates were incubated at 37 ^{o}C temperature for 48 hours. The total salmonella count was

calculated according to ISO (1995). The results of the total salmonella count was expressed as the number of organism or colony forming units per ml (CFU/ml) of sample and multiplied with 100 to calculate the colony in 1gm feces.

3.2.8 Isolation and identification of pathogens

The entire samples were selected for bacteriological culture.

3.2.8.1 Preparation of culture media and reagents

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

3.2.8.1.1 Liquid Media

3.2.8.1.1.1 Nutrient broth

13 gram of Bactonutrient broth (Difco) was dissolved in 1000 ml of cold distilled water and heated upto boiling to dissolve it completely. The solution was then distributed in tubes, stoppered with cotton plugs and sterilized in the autoclave machine at l21°C and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was judged by incubating overnight at 37°C and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.8.1.2 Solid media

3.2.8.1.2.1 Plate count agar

17.6 gram of plate count agar powder was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, 20 ml 0f the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^0C for overnight to

check their sterility and used for culture characterization (Carter, 1979).

3.2.8.1.2.2 Nutrient agar

28 gram of nutrient agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37^0C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.2.8.1.2.3 Salmonella-Shigella agar

6.3 gram of dehydrated salmonella-shigella agar (High Media, india) was suspended in 100 ml of cold distilled water taken in a conical flask and heated upto boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass ptridishes (medium sized) and 15 ml quanties in sterile glass petrdishes (large size) to form a thick layer therein. To accomplished the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged by keepig the petridishes in the incubator at 37^0C for overnight and then used for cultural

characterization or stored at 4^0C in refrigerator for future used (Carter, 1979).

3.2.8.1.2.4 Eosin methylene blue agar

36 gram of EMB agar base (Hi-media, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed.

The sterility of the medium was judged and used or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.8.1.2.5 Mac conkey agar

51.50 gram of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000 ml of cold distilled water taken in a conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium

was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.8.1.2.6 MIU medium

18 grams of MIU agar (Difco) was suspended in 950 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. 95 ml of it was despensed into flasks and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then it was cooled to about 50°C and 5 ml sterile 40% basal medium was added aseptically. After mixing were dispensed into sterile test tubes. Then allowed to cool in an upright position. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.2.8.1.2.7 Brilliant Green Agar (BGA)

BGA medium was used as a selective medium for the isolation and identification of salmonella organisms (Cheesebrough, 1985). According to the direction of manufacturer 52 gm of BGA powder (Oxoid, England) was suspended in 1000 ml of distilled water in a conical flask. It was then gently heated with gentle agitation and brought just to the boil to dissolve the medium completely. After strelization by autoclaving the medium was cooled to 50°C, mixed properly and poured into sterile petridishes (10ml in each petridish) and allowed to solidify. Then the petridishes were incubated at 37°C for over night to check their sterility and used to culture the organism or stored at 4ºC in refrigerator for future use (Carter, 1979).

3.2.8.1.3 Reagents preparation

3.2.8.1.3.1 Methyl Red-Voges Proskaure test

3.2.8.1.3.1.1 Methyl Red-Voges Proskaure broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the test tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and used for biochemical characterization or stored at 4°C in refrigerator for future use (Cheesbrough, 1985).

3.2.8.1.3.1.2 Methyl red solution

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300 ml of 95% alcohol and diluting this to 500 ml with the addition of 200 ml of distilled water.

3.2.8.1.3.1.3 Voges-Proskauer solution

3.2.8.1.3.1.3.1 Alpha-naphthol solution

Alpha-naphthol solution was prepared by dissolving 5 grams of 1-naphthol in 100 ml of 95% ethyl alcohol.

3.2.8.1.3.1.3.2 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 gram of potassium hydroxide crystals in 100 ml of cold distilled water.

3.2.8.1.3.2 Indole
test

3.2.8.1.3.2.1 Kovac's reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of amyl alcohol and to this mixture 5 grams of paradimethyl-aminohenzyldehide crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1969).

3.2.8.1.3.3 Phosphate buffered saline solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of sodium chloride (NaCl), 2.89 gram of disodium hydrogen phosphate ($Na_2HPO_{4,}$ $12H_2O$), 0.2 gram of potassium chloride (KCl) and 0.2 gram of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121°C maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1985).

3.2.8.2 Culture of fecal sample

Each fecal sample earlier put into transport media was divided and inoculated separately in nutrient agar (NA) and plate count agar to promote growth of bacteria. Each group of these media was incubated at 37^0C for overnight. The colonies on primary cultures were repeatedly sub-cultured by streak plate method (Cheesbrough, 1985

3.2.8.3 Morphological characterization of organisms's Gram's staining method

- A loopful of sterile distilled water was placed in the center of a clean sterile slide.
- A small colony was picked up with a bacteriological loop and was mixed with distilled water on the slide.
- The colony was made to thin smear on a slide.
- The smears were fixed by air drying.

- 0.5% crystal violet solution was then applied on the smear for one minute.
- After washing Gram's iodine was then added to act as mordant for one minute and then wash again.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was washed with water.
- Safranine was added as counter stain and allowed for one minute.
- The slide was then washed with water.
- Then the slide was blotted with blot paper and was allowed to air dry. The slide was examined under microscope with high power objective (100X) using immersion oil.

3.2.8.4 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into nutrient agar media by streak plate technique to obtain isolated colonies as per:

Step-1: An inoculum was picked up with a sterile loop and spread on a area of the medium in the petridish.

Step-2: The loop was sterilized by being heated as red hot in a flame.

Step-3: The inoculum was spread over the reminder of the plate by drawing the cooled parallel line.

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

3.2.9 Techniques for the Isolation and Identification of bacteria

3.3.9.1 Culture in ordinary media

Culture of fecal samples (n=45) as soon as earlier and inoculated separately with plate count agar media and were incubated at 37^{O}C for overnight.

Observation: bacteria grown in 45 no. of samples.

3.2.9.2 Staining methods

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

Then samples stained by grams staining technique and observed under microscope into 100x objectives.

Observation: Mixed culture of gram positive and negative bacteria.

3.2.9.3 Culture on different selective Media

3.2.9.3.1 Mac-Conkey agar

Samples were sub-culture on Mac-conkey agar media and inoculated at 37^{O}C for overnight. After that rose pink color colony is formed.

Observation: Gram negative lactose fermenter bacteria were present.

3.2.9.3.2 EMB agar

Samples of positive lactose fermenter were taken and sub-culture on EMB agar media and incubated at 37^{O}C for overnight. Then it's shown slightly circular colonies with dark center and slightly metallic sheen.

Observation: Suspected cases of *E. Coli*.

3.2.9.3.3 Salmonella -Shigella agar

Sample of positive lactose fermenter were taken and sub-culture on SS agar media and incubated at 37^{O}C for overnight, which after inoculation, raised, black centered, smooth round colony was present.

Observation: suspected sample of *Salmonella.*

3.2.9.4 Brilliant green agar:

Sample of positive lactose fermenter were taken and sub-culture on Brilliant green agar media and incubated at 37^{O}C for overnight, which after inoculation, showing pale pink colony against a pinkish background.

Observation:

- Colony with EMB agar reveal gram negative, pink color, large rod shaped appearance, arranged in single or paired indicated as suspected *E. Coli* spp.
- The colony from SS agar were pink colored, small, rod shaped, single, or paired arranged indicated as *Salmonella* spp.

CHAPTER IV

RESULTS

4.1 Bacterial load

4.1.1 Enumeration of total viable bacteria

The result represented on the table 1 showed that total viable bacterial load on three different samples under the three groups of sheep. The result in table 6 reveals that average values of TVC/g of sample of group A, group B and group C were 9.8×10^6, 4.9×10^4 and 3.4×10^3 respectively.

Table 1. Density of average total viable bacteria

Bacterial density of fecal sample obtained from different groups

SL no.	Group	No. of animal analyzed	Total viable count CFU/g sample	Total viable count log10/g
1	A	15	9.8×10^6	6.99
2	B	15	4.9×10^4	4.69
3	C	15	3.4×10^3	3.53

4.1.2 Enumeration of *E. coli* bacteria

The result represented on the table 2 showed that *E.coli* load on three different samples of 3 groups of sheep were not uniform but varried quite considerably. The *E.coli* counts/g sample on group A, group B and group C were 4.24×10^6, 2.4×10^4 and 5.5×10^2 CFU/g of sample respectively.

Table 2. Density of average *E. coli* bacteria count

Sl. No.	Group	No. of animal analyzed	Total *E.coli* CFU/g sample	Total *E. coli* count log10/g
1	A	15	4.24×10^6	6.63

2	B	15	2.4×10^4	4.38
3	C	15	5.5×10^2	2.75

4.1.3 Enumeration of *Salmonella spp*

The result represented on the table 3 showed that *Salmonella spp.* load on three different samples of 3 groups of sheep were not uniform but varried quite considerably. The *Salmonella spp.* counts/g sample on group A, group B and group C were 3.2×10^3, 2.1×10^2 and 1×10^2, respectively.

Table 3. Density of average *Salmonella spp* count

Sl. No.	Group	No. of animal analyzed	Total *E.coli* CFU/g sample	Total *E. coli* count log10/g
1	A	15	3.2×10^3	3.51
2	B	15	2.1×10^2	2.38
3	C	15	1×10^2	2.00

4.2. Identification of *Escherichia coli* by different bacteriological methods

4.2.1 Results of cultural examination

4.2.1.1 MacConkey (MC) agar

E. coli produced bright pink colored colonies on Mac conkey agar (Plate 10).

4.2.1.2 Eosin methylene blue (EMB) agar

EMB agar plates streaked separately with the organism revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically. The growth was indicated by smooth, circular, black color colonies with metallic sheen on the agar plate (Plate 8).

4.2.2. Results of Gram's staining

The microscopic examination of Gram's stained smears from MC and EMB agar revealed Gram-negative, pink colored, small rod shaped organisms arranged in single, pairs or short chain (Plate 9).

4.2.3 Results of biochemical test

4.2.3.1 Triple sugar iron (TSI) agar slant test

Triple sugar iron (TSI) agar slant reaction showing yellow color butt, gas positive, no H_2S production by *E. coli* (Plate 18).

4.2.3.2 Motility, Indole, Urease (MIU) test

Escherichia coli in MIU medium causing turbidity and urease production with indole positive (Plate 14).

4.2.3.3 Other biochemical tests

All the isolates were indole test positive, methyl-red positive and VP test negative. Results of these tests are shown in Table 4 and Plate 15, 16, and 17.

Table 4. Result of biochemical test for *E. coli*

Different biochemical test	Result
Indole	≠
MR	+
VP	-

4.3 Identification of *Salmonella* spp. by different bacteriological methods

4.3.1 Results of cultural examination

4.3.1.1 Salmonella-shigella agar

The organisms were showing black centered, smooth, small round colony (Plate 11)

4.3.1.2 Brilliant Green Agar

Growth of salmonella in Brilliant Green Agar showing pale pink colour colonies against a pinkish background (Plate 12)

4.3.2 Results of Gram's staining

The organisms were observed as Gram-negetive small rod shaped organism arranged singly or pairs (Plate 13)

4.3.3 Results of biochemical test

4.3.3.1 Triple sugar iron (TSI) agar slant test

In TSI agar slant, red slant, yellow butt, gas posiyive, and presence of black precipitate indicate H_2S positive (Plate 19).

4.3.3.2 Other biochemical tests

All the isolates were methyl-red positive, Voges-Proskauer test negative, Motility, Indole, Urease (MIU) test and indole test negetive. Results of these tests are shown in Table 5.

Table 5. Result of biochemical test for *Salmonella spp*

Different biochemical test	Result
Indole	-
MR	+
VP	-

Table 6: Cultural characteristics of organisms

SL No.	Media used	Colony characteristics	Organisms
1	Mac-conkey agar	Bright pink colored colonies	*E. coli*
2	EMB agar	Smooth, circular, black color colonies with metallic sheen	*E.coli*
3	Salmonella-shigella agar	Black centered, smooth, small round colony	*Salmonella spp.*
4	Brilliant green agar	Pale pink colour colonies agaist a pinkish background	*Salmonella spp.*

4.4 Prevalence of *E. coli* bacteria

Total 45 samples were tested where *E. coli* found in 20 sample prevalence was 44.44%, where prevalence of *E. coli* on group A was 60%, Group B was 40% and group was C 33.33% (Table 7)

Table 7: Prevalence of *E. coli* bacteria

Sl no.	Group	Pasture land	Age (months)	Management system	Total no. of sample analyzed	No. of posive for *E. coli*	Prevalence (%)
1	A	Low land near water	6	Semi-intensive	15	9	60
2	B	Fallow land and near the water	6	Semi-intensive	15	6	40
3	C	Clean fallow land full of green grass	6	Semi-intensive	15	5	33.33

4.5 Prevalence of *Salmonella spp.*

Total 45 samples were tested and 11 samples showed positive characteristics for *Salmonella spp,* prevalence was 24.44% where prevalence on group A was 33.33%, Group B was 26.67% and group C was 13.33% (Table 8)

Table 8: Prevalence of *Salmonella spp.*

Sl no.	Group	Pasture land	Age (month)	Management system	Total no. of sample analyzed	No. of posive for *E. coli*	Prevalence (%)
1	A	Low land near water	6	Semi-intensive	15	5	33.33
2	B	Fallow land and near the water	6	Semi-intensive	15	4	26.67
3	C	Clean fallow land full of green grass	6	Semi-intensive	15	2	13.33

4.6 Effects bacteria on the basis of growth performance (body weight)

All sheep were same aged, but had different body weight and had different bacterial load on feces. The body weight of group A, group B and group C were 7, 8, 9 kg respectively. Where group A had highest bacterial load (6.99 Log 10/g) and group C had lowest bacterial load (3.53Log10/g) (Table 9)

Table 9: Effects bacteria on the basis of growth performance (body weight)

SL No.	Group	Body weight	Total value count Log10/g	Total *E. coli* count Log10/g	Total *Salmonella* count Log10/g
1	A	7	6.99	6.63	3.51
2	B	8	4.69	4.38	2.38
3	C	9	3.53	2.75	2.00

Plate 1: Fecal sample

Plate 2: Diluted fecal sample.

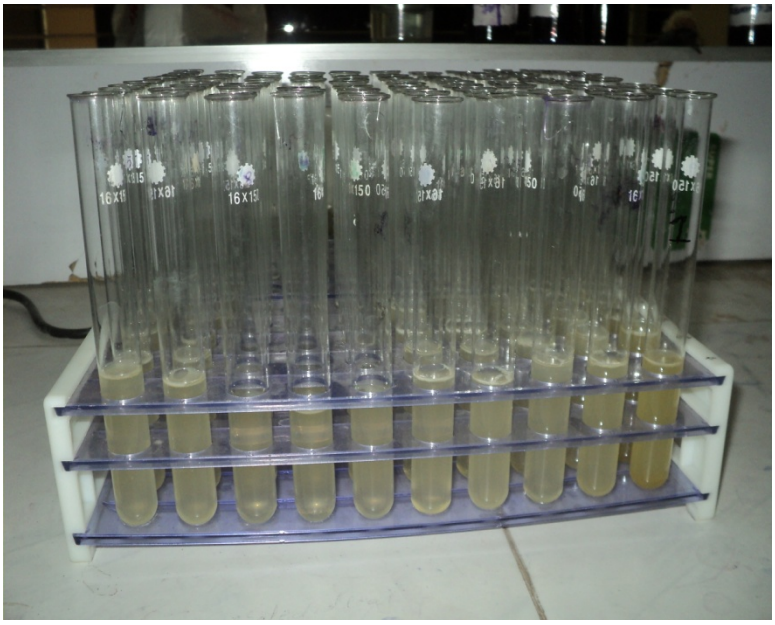


Plate 3: Ten fold dilution of fecal sample.

Plate 4: Colony count in counter machine.

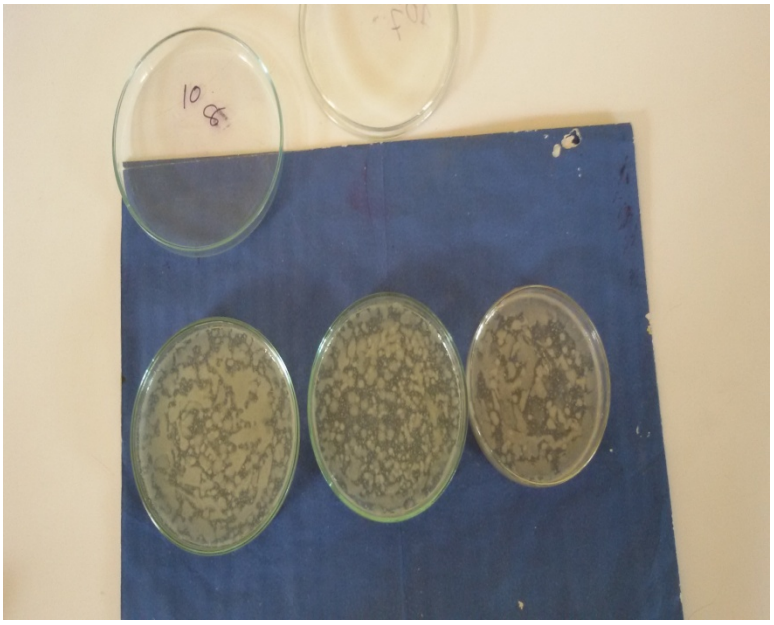


Plate 5: Colony of bacteria in plate count agar for TVC

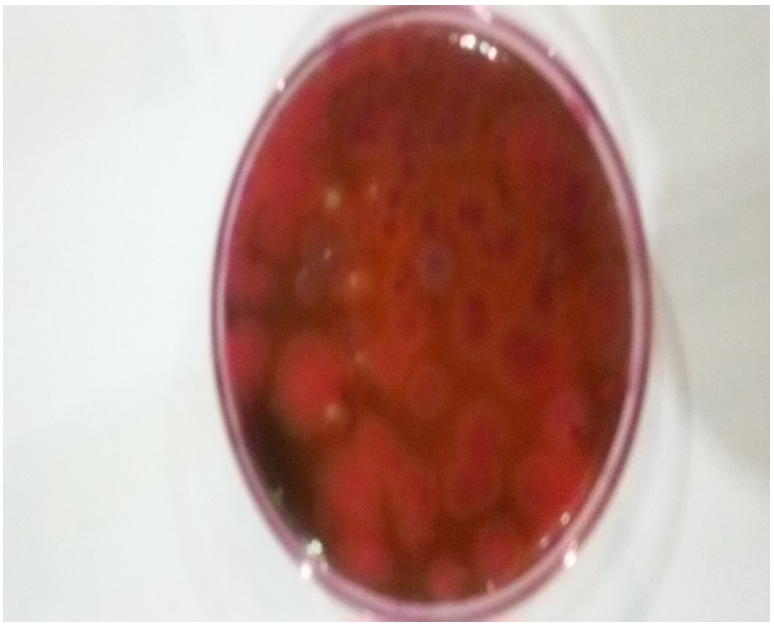


Plate 6: Colony of bacteria in EMB agar

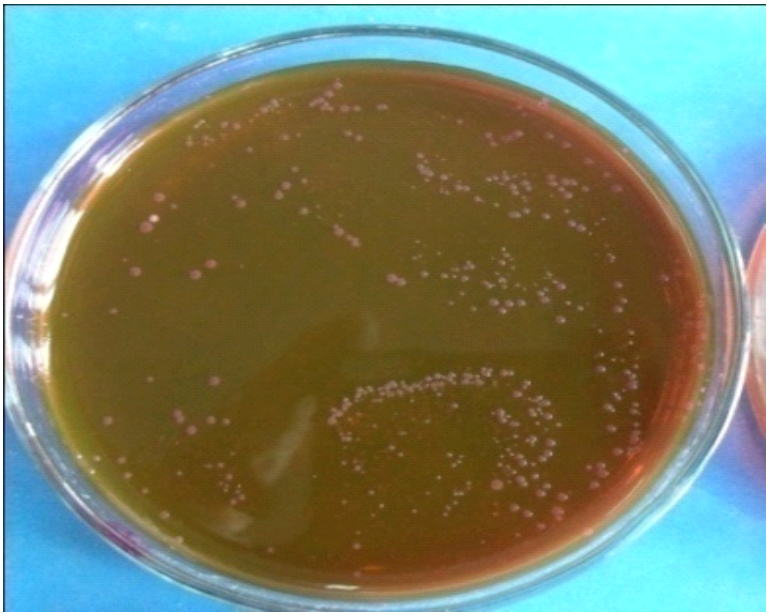


Plate 7: Colony of bacteria in SS agar

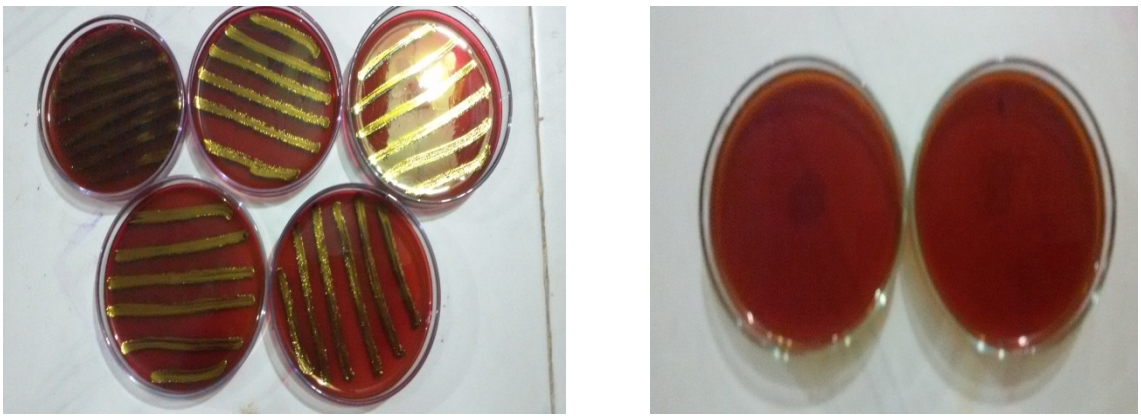


Plate 8: Metallic sheen produced by *E. coli* on EMB agar (left) and control (right).

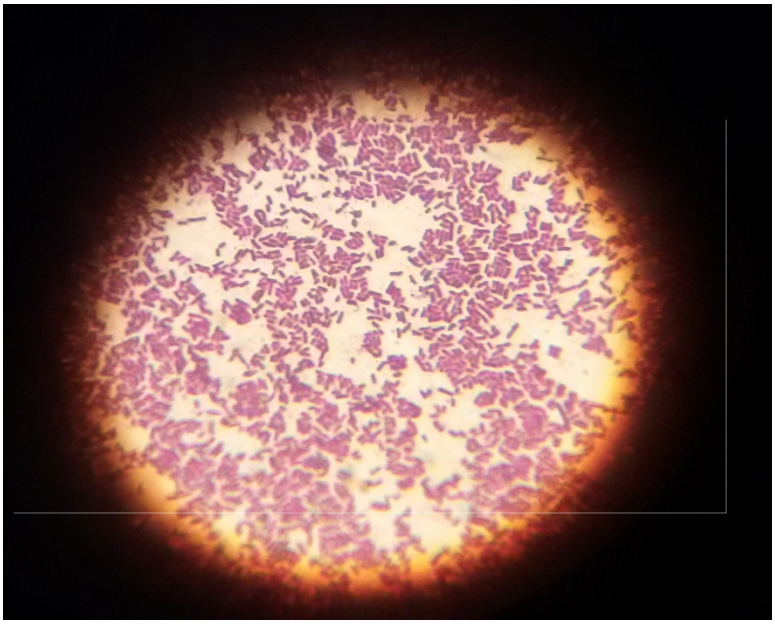


Plate 9: *E. coli* showing pink colour small rod-shape, arranged in single or pair at 100x magnification (Gram's staining).

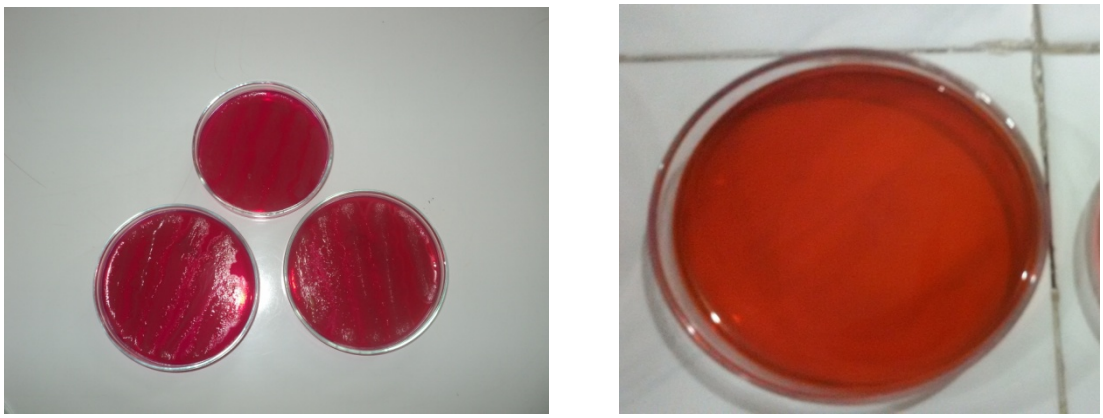


Plate 10: *E. coli* produced bright pink colored colonies on MacConkey agar (left) and control (right).

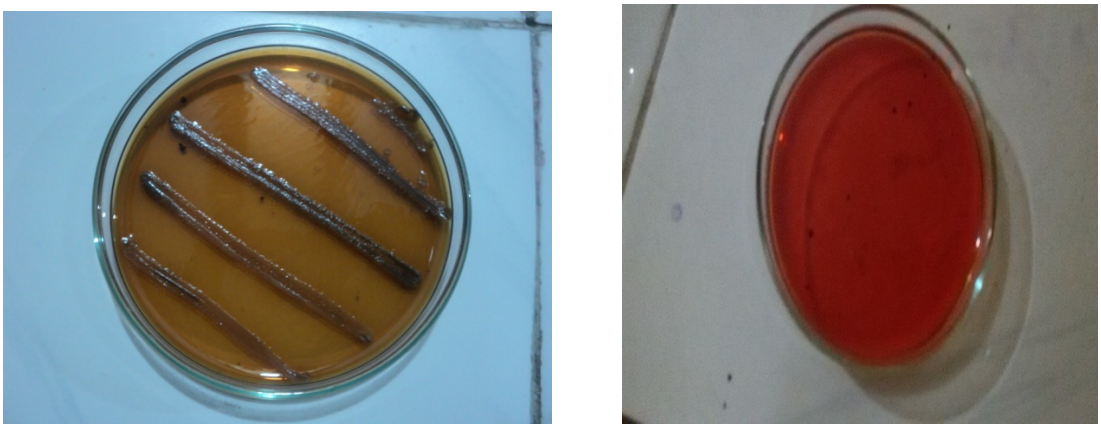


Plate 11: Growth of microorganism in Salmonella-Shigela (SS) agar as black centered, smooth, small round colony (left) which compare with control petridis (right)

Plate 12: Growth of *Salmonella spp* in Brilliant Green Agar showing pale pink colour colonies agaist a pinkish background (left) and control (right)

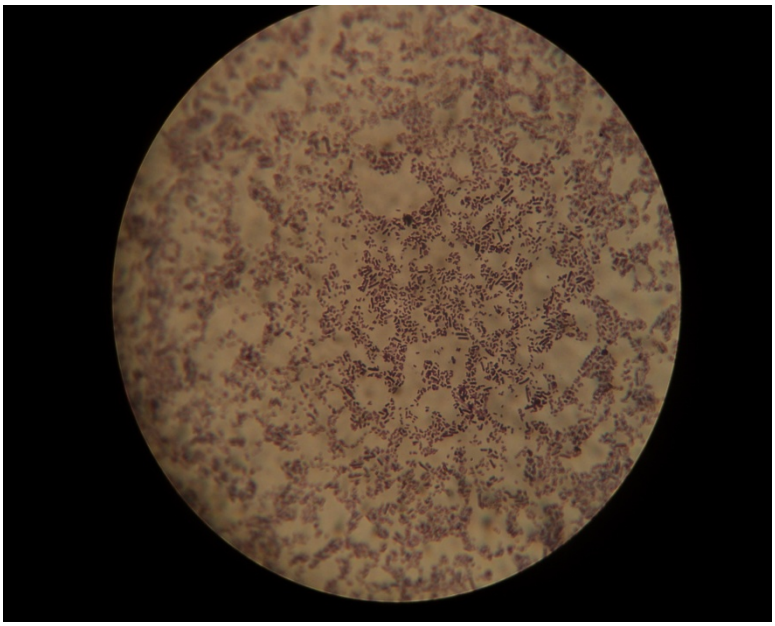
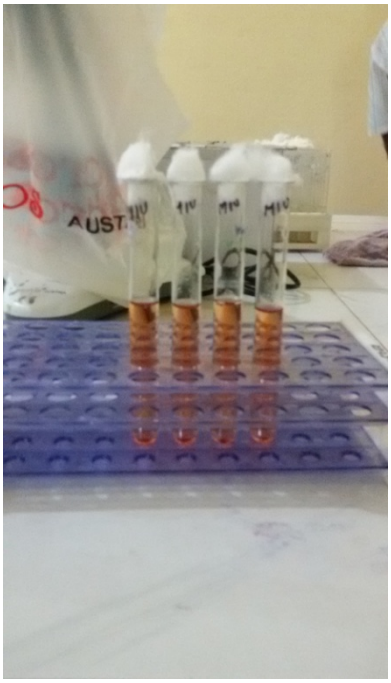


Plate 13: *Salmonella spp* showing pink colour, small rod-shape, arranged in single or pair at 100x magnification (Gram's staining).



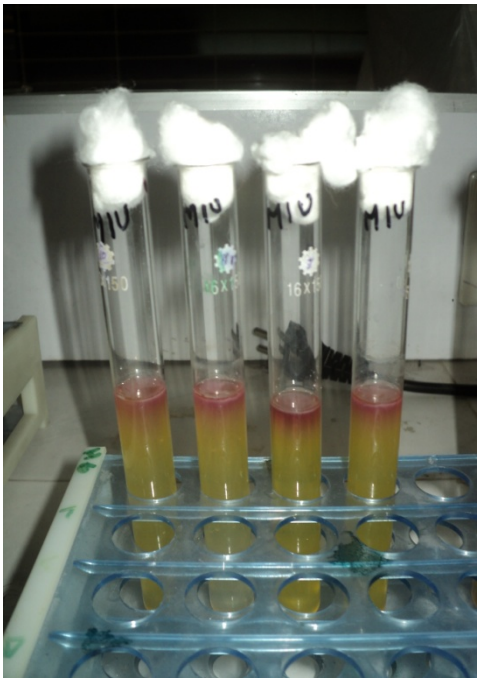


Plate 14: Motility Indole Urease (MIU) test causing turbidity and urease production with indole positive by *E. coli* (right) and control (left)

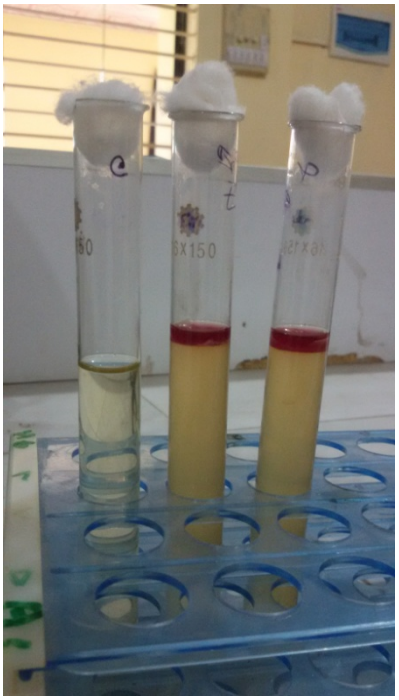


Plate 15: Indole test showing positive results with a red color in the reagent layer indicating indole production with reaction of *E. coli* (right) and control (left).

Plate 16: Methyl-Red test for *E. coli* showing the medium was changed to bright red color (right) and control (left).

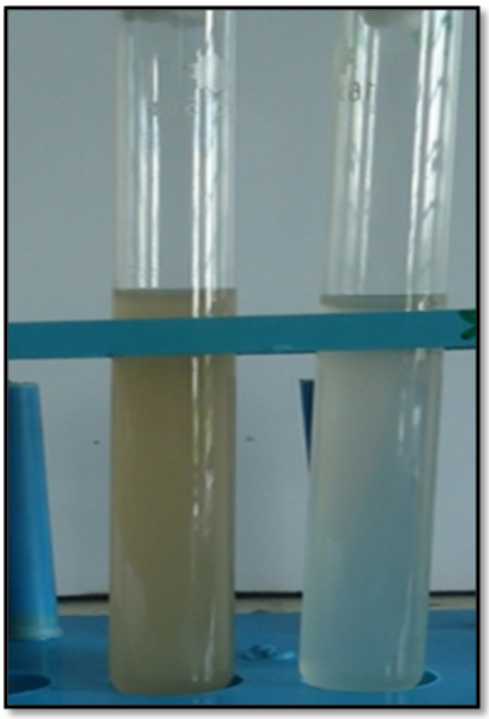


Plate 17: Voges-Proskauer test for *E. coli* showing no change of the medium (left) and control (right)

Plate 18: Culture on triple sugar iron (TSI) agar slant reaction showing yellow color butt and slant (left) indicate,gas produce but no H_2S production by *E. coli* and control (right)

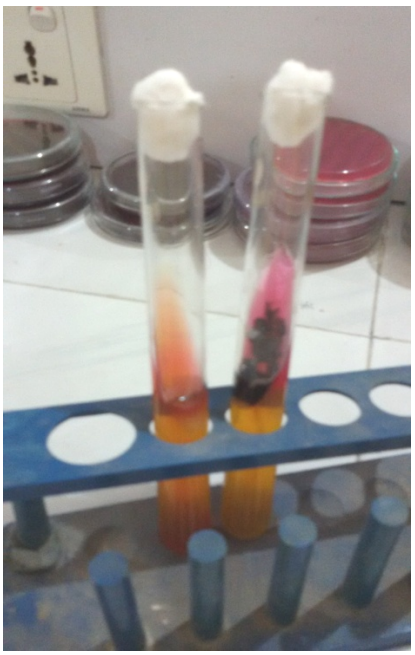


Plate 19: Triple Sugar Iron (TSI) slant test showing yellow butt, red slant and black colour in the slant due to hydrogen sulfied production (right), and control (left)

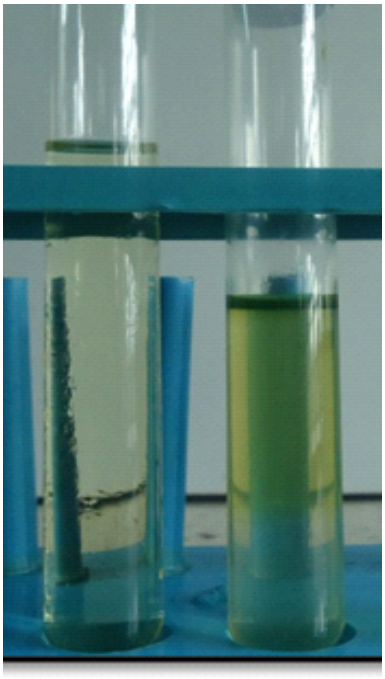


Plate 20: Indole test showing no change of the medium with the reaction of *Salmonella spp* (left) and control (right).

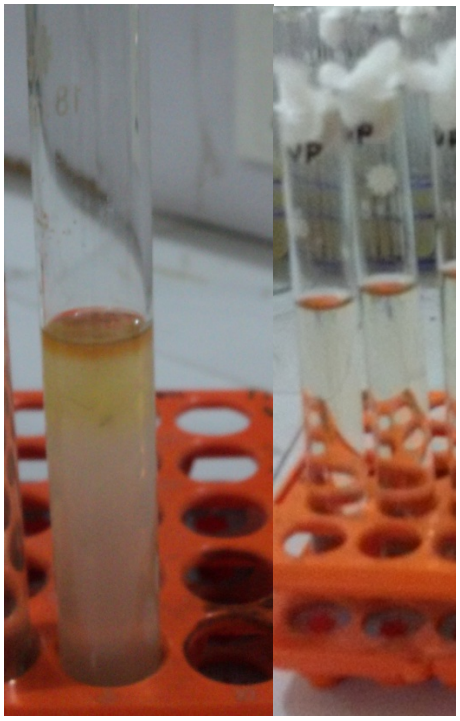


Plate 21: Salmonella spp show no colour change on indole test (left), compared with control (right)

CHAPTER V

DISCUSSION

The present study showed that among 45 tested samples, *E. coli* found in 20 sample, was 44.44%, where percentage of *E. coli* on group A was 60%, Group B was 40% and group C was 33.33%. Alternatively the isolation of *E. coli* from fecal samples of diarrhoeic sheep was found 60% (combined with *Proteus mirabilis*) and *E. coli* alone found 30% (MN Munsi *et al.* 2012).

From total 45 samples, 11 samples showed positive characteristics for *Salmonella spp,* percentage was 24.44% where percentage on group A was 33.33%, Group B was 26.67% and group C was 13.33%.

The TVC/g fecal sample found on group A, group B and group C were 9.8×10^6, 4.9×10^4 and 3.4×10^3 respectively. *E. coli* load on three different samples of 3 groups of sheep were not uniform but varried quite considerably. The *E. coli* counts/g sample on group A, group B and group C were 4.24×10^6, 2.4×10^4 and 5.5×10^2 respectively and *Salmonella spp.* load were 3.2×10^3, 2.1×10^2 and 1×10^2 CFU/g sample on group A, group B and group C respectively. Group A had highest bacterial load (6.99 Log 10/g) and group C had lowest bacterial load (3.53Log10/g).

Enteric bacteria from human and animal feces can be found in surface waters; the fecal bacteria are brought into aquatic environments mainly through treated or untreated wastewater release, surface runoffs and soil leaching (James *et al,* 2003). *Salmonella* is a gram-negative, rod-shaped bacterium that lives in the intestinal tracts of infected animals and humans (Jay et al. 2005). They are microscopic pathogens that pass from the feces of people or animals to other people or other animals (USDA, 2012). When contaminated food and water are ingested by humans or animals, the bacteria are once again passed through the fecal-oral route perpetuating the cycle. Fecal indicator organisms are typically used to demonstrate the potential presence or absence of groups of pathogens associated with wastewater or sewage sludge (Kator and Rhodes, 2003).

EM Moriarty *et al.* (2011) reported that there was an increase in enterrococcal concentrations by upto 3 orders of magnitude, with peak concentrations recorded between 11 and 28 days after deposition. *E. coli* concentrations increased in 3 out of four seasons by upto 1.5 orders of magnitude, with peak concentrations recorded between 8 and 14 days after deposition if they found water during the period of dehydration. For this reason the animals pastured on low land had higher microbial load and those pastured on high land had lower concentration because on low land there was high chance of recontamination.

E. coli and *Salmonella spp.* cause diarrhea in sheep and sheep become unhealthy. Total viable bacterial load of 10 different fecal samples of sheep whose age almost same but having different body weight. The total viable counts were 4.25 x 10^{11}CFU/gm feces, 4.24 x 10^{11} CFU/gm feces, 4.5 x 10^{11} CFU/gm feces, 5.05 x 10^{11} CFU/gm feces, 5.07 x 10^{11} CFU/gm feces, 6.67 x 10^{11}CFU/gm feces, 7.1 x 10^{11}CFU/gm feces, 7.56 x 10^{11} CFU/gm feces, 7.58 x 10^{11}CFU/gm feces, 8.08 x 10^{11} CFU/gm feces respectively (Table 1). Highest total vible colony found 8.08 x 10^{11} CFU/gm feces of a sheep which body weight was 7kg, and lowest count 4.24 x 10^{11} CFU/gm feces of a sheep which body weight was 9kg. Study also showed that the average *E. coli* counts/gm of 10 fecal sample were 2.02 x 10 ^{11}CFU/gm, 2.24 x 10^{11} CFU/gm, 2.2444 x 10^{11} CFU/gm, 1.785 x 10 ^{11}CFU/gm, 3.03 x 10^{11} CFU/gm, 3.29 x 10^{11} CFU/gm,4.04 x 10 ^{11}CFU/gm, 4.55 x 10 ^{11}CFU/gm, 5.05 x 10 ^{11}CFU/gm, 2.03x 10 ^{11}CFU/gm of fecal sample respectively, where highest count was 5.05 x 10 ^{11}CFU/gm of feces of a sheep, body weight 7kg and lowest count was 1.785 x 10 ^{11}CFU/gm of feces of a sheep with body weight 8.5 kg. Here i count The average colony of *Salmonella spp /*gm of 10 fecal sample were 1.7 x 10 ^{11}CFU/gm, 1.97x 10 ^{11}CFU/gm, 1.77 x 10 ^{11}CFU/gm, 2.02 x 10 ^{11}CFU/gm, 3.54 x 10 ^{11}CFU/gm, 2.4 x 10^{11} CFU/gm,2.53x 10 ^{11}CFU/gm, 1.87 x 10 ^{11}CFU/gm, 2.02 x 10 ^{11}CFU/gm, 2.07x 10^{11} CFU/gm of fecal sample respectively and highest colony found 3.54 x 10 ^{11} CFU/gm of feces of a sheep, body

weight 8 kg and lowest was 1.7 x 10^{11} CFU/gm of feces of a sheep whose body weight 9.5 kg.

It can be said that higher bacterial load lower body weight and lower bacterial load higher body weight.

Characterization results of the study indicated that the contaminated by bacteria. An clear finding of the colony characteristics of the isolates were observed. The feces isolates were able to produce pale red colour colony on BGA agar, characteristic metallic sheen colony on EMB agar and bright pink colored colony on MacConkey agar.

In Gram's staining, the organisms revealed gram negative, pink colored with rod shaped appearance and arranged in single or in pair were suspected as *E. coli* which supported by Zinnah *et al*. (2007) and *salmonella spp* exhibit Gram negative, small rod, single or paired in arrangement under microscope which was supported by the other researchers (Cheesbrough, 1985).

In the present study, biochemical tests which were used for characterization of bacterial pathogens also used by Kara *et al*. (2004). *E. coli* gave positive reaction to indole, Motility Indole Urease, Methyl red and salmonella isolates from sheep feces was indole negative, not produced red color, absence of turbidity on MIU test indicated non-motile organism, on TSI agar slant produce yellow butt and slunt, MR test positive but VP test negative which supported by American Type Culture Collection.

CHAPTER VI

CONCLUSIONS

The present study was conducted for determination of bacterial loads on sheep feces and growth performance of sheep, sample were collected from three unions of Gobindagonj Upazilla, Gaibandha, a total of 45 fecal samples of sheep from 165 animals randomly. sheep are of different body weight, a series of tests were performed for the isolation, identification and total viable count of different types of bacteria and digital weight balanced (Mega digital scale) were used to know the body weight, birth weight of sheep. Different types of ordinary, enriched and selective media such as nutrient agar, Plate count agar, MacConkey agar, Eosin methylene blue agar, Triple sugar iron agar, Brilliant green agar, Nutrient agar and Triple Sugar Iron agar slant were used for the determination of the cultural characteristics of the different types of isolates. Biochemical properties of the isolated bacteria were studied by MR, VP and indole tests.

From the present study, it was concluded that sheep having higher microbial load have lower body weight.

In the context of this study, it may be concluded that,

- Total viable count of organisms was successfully performed from different fecal samples.

- *E. coli* and Salmonella spp. were present and could be isolated from fecal samples.

- *E. coli* and salmonella spp was successfully isolated and confirmed by different bacteriological agar media and biochemical reaction.
- Birth weight and body weight could be measured accurately.
- Hygienic measure should be taken to ensure that the collection, handling, processing, distribution and storage of sample.

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