ISOLATION AND IDENTIFICATION OF BACTERIA FROM ENVIRONMENTAL SAMPLE OF LIVE BIRD MARKET AND THEIR ANTIBIOGRAM STUDY

A THESIS

BY

ABDIRAHMAN AHMED MOHAMED

REGISTRATION NO. 1605583

SEMESTER: JULY-DECEMBER, 2017

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MASTER OF SCIENCE (M.S.)

IN

MICROBIOLOGY

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DECEMBER, 2017

Dedicated

To

My Beloved Parents and Teachers

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ABSTRACT

The research work was conducted in order to isolate, identify, characterize and to detect antibiotic resistance pattern of *Salmonella spp., E. coli, Klebsiella spp. and Shigella spp.* found in the environmental samples. A total of 30 environmental samples were collected from live bird market of Dinajpur district and brought to the Microbiology laboratory of Hajee Mohammad Danesh Science and Technology University for bacteriological examination. The isolates were detected by standard bacteriological tests including cultural tests, staining and biochemical tests. The study revealed that the total prevalence of *E. coli, Salmonella spp., Klebsiella spp. and Shigella spp.* in environmental samples were 26.7%, 33.3%, 16.7% and 23.3% respectively. The results also suggest that total samples pose significant alarming for the public health issue if not maintain proper hygienic steps in place. The study of antibiotic resistance pattern showed a number of pathogenic isolates to be drug resistant and drug sensitive. Antibiotic sensitivity test revealed that *Salmonella spp., E. coli* and *Shigella spp*. were sensitive to Chloramphenicol (100%) while *Klebsiella spp.* was resistant to Chloramphenicol (100%). *E. coli, Klebsiella spp.* and *Shigella spp.* were resistant to Erythromycin (100%) while *Salmonella spp.* was found intermediate to Erythromycin (60%). So it can be suggested that the antibiotic resistance properties of isolated bacteria can cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

Key words: *E. coli, Salmonella spp., Klebsiella spp., Shigella spp.,* Environmental sample, Antibiotic resistance.

LIST OF ABBREVIATIONS AND SYMBOLS

%	:	Percentage
/	:	Per
-	:	Negative
+	:	Positive
<	:	Less than
>	:	Greater than
µg	:	Microgram
µl	:	Micro liter
^{0}C	:	Degree of Celsius
BD	:	Bangladesh
C	:	Chloramphenicol
C	:	Cefixime
CIP	:	Ciprofloxacin
CL	:	Colistin
E	:	Erythromycin
E. coli	:	Escherichia coli
e.g.	:	Example
EMB	:	Eosin Methylene Blue
et al.	:	Associated
Fig	:	Figure
Gm	:	Grams
H_2S	:	Hydrogen sulfide
Hrs	:	Hours
HSTU	:	Hajee Mohammad Danesh science and Technology University

LIST OF ABBREVIATIONS AND SYMBOLS (Cont...)

Ib	:	Pound
I	:	Intermediate
Kg	:	Kilogram
KOH	:	Potassium hydroxide
L	:	Lactose
MC	:	MacConkey
Mg	:	Milligram
Min	:	Minutes
MIU	:	Motility Indole Urease
Ml	:	Milliliter
Mm	:	Millimeter
MR	:	Methyl Red
NA	:	Nutrient agar
NB	:	Nutrient broth
No.	:	Number
PBS	:	Phosphate buffered saline
R	:	Resistant
S	:	Sensitive
SL	:	Serial
Spp	:	Species
SSA	:	Salmonella Shigella Agar
TSI	:	Triple sugar iron
USEPA	:	US Environmental Protection Agency
VP	:	Voges Proskauer

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CHAPTER I

INTRODUCTION

Bangladesh is an agriculture based country. About 80% people of the country are dependent on agriculture either directly or indirectly. The poultry industry comprising of commercial poultry mainly broiler and layers plays an important role in the economy of Bangladesh. There are approximately 163.50 million of poultry including ducks reared throughout Bangladesh (Giasuddin *et al.,* 2002). Little is known about the bacterial presence in the poultry environment such as in poultry litter and in the poultry house air (Saleh *et al.,* 2003).

Reports by Grill and Best (1998) and Ruff (1992) have listed animal feed as one of the sources of microorganisms to animals. Poultry feeds are referred to as complete feeds as they are designed to contain all the nutritional materials needed for proper growth, meat and egg production in birds. Various brands of poultry feeds are in existence depending on the functions they perform in the birds. Thus, there are growers, finishers, layers, starters among others. Poultry feeds can potentially become contaminated with food borne pathogenic microorganisms during harvesting and eventual marketing of the bagged feeds. Poultry feeds contaminated with bacteria pathogenic to humans can contribute to human food borne illness through the feed-poultry-food-human chain. The production of poultry feeds requires microbiological safety regulations to escape microbial contamination of the product. Prominent among these microorganisms, the bacteria *Salmonella* and *E. coli* infections of poultry have been shown to be of critical importance in Bangladesh. However, the pathogens discharged from the chicken contaminate the litter, feed, water and thus the nearby birds. The rapid growth of the poultry industry has resulted in the production of massive quantities of poultry wastes. The advancement of poultry industry in Bangladesh is interrupted by a number of constraints, of which major one is outbreak of disease causing about 30% mortality of chickens in every year (Ali, 2004).

The major causes of bacteria are *Escherichia coli*, *Salmonella*, etc. *E. coli* is a major pathogen of commercially produced poultry causing *Colibacillosis* all over the world.

E. coli is Gram-negative, rod-shaped, flagellated, motile, oxidase negative, facultative anaerobes. Salmonellosis is one of the most important diseases that cause serious economic loss due to mortality and reduced egg production (Khan *et al.,* 2005).

Salmonella spp. are Gram negative, small rod-shaped, non-spore forming, non-capsulated, aerobic and facultative anaerobic organisms and classified under the family Enterobacteriaceae (OIE, 2000).

The *Salmonella spp.* is potentially responsible for various pathogenic processes in man and animal including poultry (Freeman, 1985).

Evidence has been presented that poultry feeds may be a common and very important source of paratyphoid organisms. The level of *Salmonella* contamination in poultry feeds is normally low; however, it has been shown that even one organism per 15 grams of feed can produce infection (Harry and Brown, 1974).

Salmonellosis in poultry resulted in continuous increase of public health problems as stated by Corrier *et al*., (1990).

Klebsiella pneumonia is a common opportunistic pathogen that causes human infections. It can be widely distributed not only in the respiratory and intestinal tracts of humans and animals but also in a variety of environments and vectors. This pathogen can cause pneumonia, respiratory tract infections, urinary system infections, septicemia and other diseases (Struve *et al.,* 2004).

Shigella spp. is a genus of gram-negative, facultative anaerobic Enterobacteriaceae that includes *S. dysenteriae, S. flexneri, S. boydii,* and *S. sonnei. Shigella* species have highly evolved invasive systems that enable the bacteria to invade and multiply within the human intestinal epithelia, ultimately leading to severe inflammatory colitis called bacillary dysentery or *shigellosis (*Faruque *et al*., 2010).

Considering the above points the present study was undertaken with the following objectives:

i. To isolate and identify the bacterial pathogens from environmental samples of live bird market.
ii. To detect the sensitivity pattern of the commercially available antimicrobial agent to those identified isolates.

CHAPTER II

REVIEW OF LITERATURES

Andoh *et al.* (2016) conducted a cross-sectional study of poultry flocks in Ghana and suggested that the reduced detection rates in dust compared to feces could be due to relatively high temperatures in dusts, a consequence of the climatic conditions. Moreover, sampling strategies that are appropriate to detect positive flocks in the European setting, with a negligible number of positive flocks, may not be effective in high prevalence situations, as in Nigerian chicken layer farms, where a relatively similar prevalence of *Salmonella* infection was revealed for all the matrices sampled.

D. Hailu *et al.* (2016) reported that *E. coli* is a commensal bacterium in humans and animals and has a wide range of hosts. It is commonly present in the environment and considered an indicator of fecal contamination in food and water. It can acquire, maintain, and transmit resistance genes from other organisms in the environment. *E. coli* serotype O157:H7 is an enterohaemorrhagic strain, which was initially recognized as a reservoir of potential *E. coli* with zoonotic potential that could be transferred directly from birds to humans.

Abbas *et al.* (2016) investigated that isolation of bacteria from birds he observed that, the result revealed that isolation rate was (63%) for *Staphylococcus aureus*, (66%) for *Staphylococcus* group D, (49%) for *Escherichia coli,* (6%) for *E. coli* O157, (11%) for *Salmonella spp*., (18%) for *Shigella spp.,* (14%) for *Vibrio spp*., (10%) for *Auromonas spp.,* (8%) for *Plesimonas Shigelloides,* (30%) for *Klebsiella spp.,* (2%) for *Nocardia spp.*

Barbiour *et al.* (2015) stated that *Salmonella* serovars can be present in feces excreted by healthy animals and may be transferred to raw foods of animal origin through contamination during slaughtering and processing

Adenyanju and Ishola (2014) conducted antibiotic sensitivity test of *E. coli* isolates of poultry by using different Gram negative antibiotics. Nitrofurantion and Augmentin showed a decrease in their sensitivity to isolates than they normally should. *E. coli* showed 100% resistance to Augmentin and Amoxicillim.

Furtula *et al.* (2013) collected 12 surface water and 28 ground water samples in the Abbotsford area of British Columbia, Canada, near poultry farms and berry farm's that used poultry litter as fertilizer, as well as a reference site in a residential area in Port Moody, British Columbia. They also collected litter samples from two different poultry farms, one broiler farm and one layer farm. *Enterococci* were isolated from these samples and tested for resistance to 16 clinical antibiotics. Overall, 86% of litter isolates, 58% of surface water isolates and 100% of ground water isolates were resistant to more than one antibiotic. Resistance to Lincomycin, Tetracycline, Penicillin and Ciprofloxacin in poultry litter isolates was high as 80.3%, 65.3%, 61.1% and 49.6%, respectively. Resistance in the surface water to the same antibiotics was 87.1%, 24.1%, 7.6% and 12.9%, respectively.

Ramya *et al.* (2013) described that sensitivity of *Salmonella Enteritidis* was 100% for Ciprofloxacin followed by Chloramphenicol and Amikacin (96%), Genatmycin (90%), Amoxicillin (82%), Streptomycin (80%), Tetracycline (76%), Nalidixic acid (68%), Ampicillin (58%) and Sulfonamide (10%). The resistance was higest for Sulfonamide (76%) followed by Ampicillin (32%), Nalidixic acid (30%) and 6-20% for Gentamycin, Amoxicillin and Tetracycline.

Onah Gloria *et al.* (2013) used pour plate plating technique 0.1ml of water sample was pipetted into petri dishes and already prepared agar (Nutrient agar and MacConkey agar) were poured into the peridishes, swirled for a homogeneous mixture, allowed to solidify and incubated at 37oC for 24 hrs. Presumption coliform counts were carried out by using double strength lactose and single strength lactose broth in test tubes and these were incubated at 37oC for 24 hrs. The result showed high counts of microorganism in well water while pipe-borne water had low counts. The bacterial isolated are *Citrobacter spp*., *Escherichia coli* and *Klebsiella Spp.*

Okwori *et al.* (2013) stated that enteric organisms potentially transmissible to humans were sub-cultured and identified based on their morphological and biochemical characteristics. Prevalence results at start and finish points obtained revealed six (6) bacterial organisms as follows: *Escherichia coli* (100%), *Salmonella paratyphi A* (72.8-77.8%), *Proteus mirabilis* (61.1-83.3%), *Campylobacter* species (5.6%), *Citrobacter ferundii* 13(16.7-33.3%), and *Yersinia enterocolitica* (22.4%). The presence of these pathogenic bacteria has public health significance because of the nature and use of their location (being a park) and its proximity to human dwellings.

Hilari WH *et al.* (2013) obtained a total of 508 isolates from 300 fecal samples: 172 *E. coli* (33.9% of isolates; 57.3% of individuals), 153 *Enterobacter* spp. (30.1% of isolates; 51.0% of individuals), 89 *Klebsiella* spp. (17.7% of isolates; 29.7% of individuals), 59 *Citrobacter* spp. (11.6% of isolates; 19.7% of individuals), 21 *Proteus vulgaris* (4.2% of isolates; 7.0% of individuals), 5 *Providencia alcalifaciens* (0.98% of isolates; 1.67% of individuals), 5 *Serratia* sp. (0.98% of isolates; 1.67% of individuals), 3 *Hafnia aivei* (0.59% of isolates; 1.00% of individuals) and 1 *Salmonella* spp. (0.20% of isolates; 0.33% of individuals). *Escherichia coli* isolates were subsequently tested for susceptibility to the following antibiotics: Amoxillin (70.93% of the isolates were resistant), Ampicillin (75.58%), Ciprofloxacin (23.25%), Chloramphenicol (33.14%), Doxycycline (64.53%), Enrofloxacin (41.28%), Tetracycline (69.19%) and Sulfonamide (71.51%). Multi resistance to three and four groups of antibiotics occurred in 40 samples (23.25%) and 4 samples (2.32%), respectively. These results demonstrate that illegally traded birds are carriers of potentially pathogenic bacteria, including *E. coli* strains with antimicrobial resistance.

Amin *et al.* (2012) evaluated that the antibiotic resistance pattern of *Escherichia coli* strains isolated from animals to ascertain the levels of antibiotic resistance pervasiveness. A total of 28 *E. coli* strains were isolated from facial samples and the antibiotic resistance pattern of *E. coli* strains was determined by means of disk diffusion assay. The resistance pattern determined for all strains was amoxicillin, streptomycin, cefepime, azteronam, amoxicillin/clavunac acid, ciprofloxacin and ceftriaxone. About 50-75% of the strains were reported as resistant to more than five antibiotics (multidrug-resistant). This might result in broadening of the antibiotic resistance canvas among animals and from animals to human taking the animal food products or living in close contact with them.

Hemen *et al.* (2012) isolated *Shigella, Salmonella* and *Escherichia coli* from poultry litter and tested their antibiotic sensitivity patterns against Septrin, Chloramphenicol, Sparfloxacin, Ciprofloxacin, Amoxycillin, Augmentin, Gentamicin, Pefloxacin, Tarivid and Streptomycin. *Escherichia coli* were found to be resistant against 8 out of 10 drugs against which their antibiotic sensitivity pattern was tested followed by *Shigella* (6 out of 10) and *Salmonella* (3 out of 10). *Shigella* and *Salmonella* were completely resistant to Chloramphenicol, Augmentin, Pefloxacin, Amoxycillin. *Shigella* was also resistant to all the antibiotics except Septrin and

Ciprofloxacin. Percentage antibiotics susceptibility pattern of Gram negative bacteria showed that all bacterial isolates (100%) were resistant to Chloramphenicol while most of the isolates were susceptible to Amoxycillin.

Silva HG *et al.* (2012) A total of 267 samples were analyzed, including fecal samples from zoo animals and rodents, insects (Muses domestics and Periplaneta Americans) and samples of the zoo animal's food. Salmonella was detected in 11.6% of the samples analyzed. Characterization of the isolates was performed with serotyping and pulsed-field gel electrophoresis. The following serovars were isolated: *S. san diego, S. oranienburg*, *S. weltevreden, S. braenderup, S. derby*, S. 6,7, Men: x:- and S. 3,10, H:r:- The isolates showed seven pulsed-field gel electrophoresis patterns with a Jaccard coefficient 0.75 indicating a possible common origin. The prevalence of asymptomatic infections caused by *Salmonella* spp. in zoo animals was high. These findings demonstrate the diversity of *Salmonella* serovars in several captive wild animal species.

Karin H *et al.* (2011) found that the non-typhoidal *Salmonella* represents an important human and animal pathogen worldwide. Most human Salmonellosis cases are food borne, but each year infections are also acquired through direct or indirect animal contact in homes, veterinary clinics, zoological gardens, farm environments or other public, professional or private settings.

Ghanbarpour *et al.* (2011) determined that Phylogenetic background and the virulence gene profiles of *Escherichia coli* isolates from colisepticemic and feces of healthy (AFEC) broiler chickens. In this study, 253 *E. coli* isolates including 161 avian pathogenic *E. coli* (APEC) and 112 AFEC isolates were examined by PCR. In general, 253 *E. coli* isolates distributed among group A (51.8%), B1 (15.8%), B2 (8.7%), and D (23.7%). Ten (8.9%) AFEC isolates segregated into B1 phylo-group and 102 (91.1%) isolates fell into six different Phylogenetic subgroups and total prevalence of *E. coli* was 63.64% in that study from colisepticemic fecal samples.

Pipes SM, Vigre H, Makela P, Hall T. (2010) estimated that the most important food sources for *Salmonellosis* cases were eggs (32%) and poultry meat (15%) in USA. For the pathogens, a large proportion of cases could not be linked any source. Among illnesses that could be attributed to a source, 58% of *Salmonellosis* cases were attributed to eggs, and 29% of *Salmonellosis* cases were attributed to chicken. Results also revealed regional differences in the relative importance of specific sources. Nevertheless, the presented source attribution approach

can be applied to other food-borne pathogens and easily adaptable to countries having and appropriate number of reported outbreaks.

Alexandre G *et al.* (2010) estimated that occurrence of vancomycin resistant enterococci (VRE) in fecal samples of ostriches from a farm of Southern Portugal, the mechanisms implicated and the associated virulence factors, 13 years after the banning of the glycopeptide avoparcin as animal growth promoter in the European Union. Fifty-four fecal samples of ostriches were inoculated in Slanetz-Barley supplemented with vancomycin (4μg/mL) for VRE recovery. Susceptibility to 11 antibiotics was performed by disk-diffusion agar method in recovered VRE isolates.

Faruque *et al*. (2010) stated *Shigella* is a genus of gram-negative, facultative anaerobic Enterobacteriaceae that includes *S. dysenteriae, S. flexneri, S. boydii,* and *S. sonnei. Shigella* species have highly evolved invasive systems that enable the bacteria to invade and multiply within the human intestinal epithelia, ultimately leading to severe inflammatory colitis called bacillary dysentery or *shigellosis.*

Ahmed *et al.* (2009) isolated sixty-nine *Escherichia coli* and 10 *Salmonella,* from retail chicken meat in Hiroshima prefecture, Japan, the samples were assayed for antimicrobial susceptibility, the presence of integrons and antimicrobial resistance genes.

Maiko S *et al.* (2009) described that imported animals, especially those from developing countries, may constitute a potential hazard to native animals and to public health. In this study, a new flock of lesser flamingos imported from Tanzania to Hiroshima Zoological Park were screened for multidrug-resistant Gram-negative bacteria, integrons and antimicrobial resistance genes. Thirty-seven Gram-negative bacterial isolates were obtained from the flamingos. Seven isolates (18.9%) showed multidrug resistance phenotypes, the most common being against; ampicillin, streptomycin, tetracycline, trimethoprim/sulfamethoxazole and nalidixic acid. Molecular analyses identified class 1 and class 2 integrons, beta-lactamase-encoding genes, blaTEM-1 and blaCTX-M-2 and the plasmid-mediated quinolone resistance genes, qnrS and qnrB. This study highlights the role of animal importation in the dissemination of multidrug resistant bacteria, integrons and antimicrobial resistance genes from one country to another.

Costa *et al.* (2009) reported under field conditions, three commercial antimicrobials were subsequently prescribed to 16,000 broiler chickens during their rearing period, via drinking water using sub therapeutic levels from for 3 days. A control group of 16,000 broilers was placed in the same controlled environment poultry house. This study provides clear evidence that a sequential medication of a broiler flock, with different antimicrobial classes during short periods of time for prophylactic objectives, was accompanied by a dramatic increase in both antimicrobial resistance rates and phenotype diversity of *E. coli* strains.

Hanning *et al.* (2009) observed that food borne *Salmonella spp.* is a leading cause of food borne illness in USA each year. Traditionally, most cases of *Salmonellosis* were thought to originate from meat and poultry products. However, an increasing number of *Salmonellosis* outbreaks are occurring as a result of contaminated produce. Several produce items specifically have been identified in outbreaks, and the ability of *Salmonella* to attach or internalize into vegetables and fruits may be factors that make these produce items more likely to be sources of *Salmonella.* This review examines outbreaks of *Salmonella* due to contaminated produce, the potential sources of *Salmonella,* and possible control measures to prevent contamination to produce.

Turblin (2009) conducted a study belonging to the family of *Enterobactericeae*, *Escherichia coli* is a coccobacillus gram-negative (2-3 x 0.6 μm), non spore-forming and able to grow in aerobic and non-aerobic condition. It can have a capsule. Most strains are motile and have peritrichous flagella. The basic metabolic characteristics of this enterobacteria are to be Catalase (+) and oxidase (-), as well as to ferment glucose and reduce nitrate into nitrites. The genus *E. coli* is closely related to the genus *Shigella.*

Ahmed *et al.* (2008) conducted a study to determine the seroprevalence of *Salmonella* infections in poultry. This study covered cultural prevalence with isolation and identification of the causal agent and pathological lesions in different organs produced by *Salmonella* in layer farms of Mymensingh district during July to December 2007. The materials were blood samples; liver and cloacal swabs of live and dead birds. The used methods are whole blood agglutination test by commercially available *Salmonella* antigen kit, culture in different media, Gram's staining, motility test basic 5 sugar fermentation test and histopathology. The overall seroprevalence was 45.9% in live bird and the rate of seroprevalence decreased with advancement of age of birds. The cultural prevalence in seropositive group was 71% and in seronegative group was 59%. In

dead bird, the cultural prevalence in liver was 64% and from cloaca 57%. A total 160 isolates were characterized, among them 64.2% were *Salmonella pullorum,* 22.3% were *Salmonella gallinarum* and 13.5% were Paratyphoid group of *Salmonellae*.

Chaiba *et al.* (2008) conducted a study to estimate the occurrence and distribution of *Salmonella* in raw chicken meat and giblets (liver and gizzard) on the Moroccan market. From November 2005 to November 2006, a total of 576 samples were collected from retailers. Of these, 144 samples were from popular market, 144 from a supermarket at Meknes (center-south Morocco). Of the total 576 samples examined. *Salmonella* was detected in 57 (9.90%) of the samples analyzed. Among chicken samples examined high proportion of gizzard (13.88%), liver (11.11%), leg (8.33%) and breast (6.25%) were contaminated with *Salmonella.*

Eglezos *et al.* (2008) determined the bacteriological profile of raw, frozen chicken nuggets manufactured at a chicken processing facility in Queensland, Australia using a total of 300 frozen batches, *Escherichia coli* and *Salmonella* over a period of 4 years. The mean of the aerobic plate count was 5.4 log CFU/g, and counts at the 90^{th}, 95^{th}, and 99^{th} percentiles were 5.7, 5.9, and 6.5 log CFU/g, respectively. The maximum number of bacteria detected was 6.6 log CFU/g. E. coli prevalence was 47%, and of the positive samples, the mean was 1.9 log CFU/g; counts at the 90^{th}, 95^{th}, and 99^{th} percentiles were 2.3, 2.4, and 2.8 log CFU/g, respectively. There was a significant relationship ($P < 0.05$) between season and both aerobic plate counts and *E. coli* counts, and no correlation between *E. coli* counts and *Salmonella* prevalence. Their study provides valuable data on the bacteriological quality of raw, frozen chicken nuggets.

Wu *et al.* (2008) investigated antibiotics susceptibility of *Escherichia coli* isolated from duck in China. The susceptibility rate of isolates producing ESBLs against third generation cephalosporin and synthetic penicillin were lower than 42.9 but the resistance rates were rather than 28.6%, the resistance rates against amoxicillin, ampicillin and ceftiofur were 100%.

Wei *et al.* (2008) reported that most *E. coli* was poorly susceptible to ampicillin, amoxicillin, ciprofloxacin, and third generation cephalosporins. Most *E. coli* was susceptible to gentamycin and aztreonam. Clinical laboratories needed to determine of beta-lactamase-producing *E. coli* isolates. Gentammicin and aztreonam were the first candidate drugs for infections caused by beta-lactamase-producing *E. coli.*

Smet *et al.* (2008) conducted research work using a total of 295 ceftiofur-resistant *Escherichia coli* isolates obtained from 489 cloacal samples collected at five different Belgian broiler farms and evaluated the diversity of this resistance at the farm level. Strains were examined for resistant against beta-lactam antibiotics and other antimicrobial agents by using disk diffusion tests. Three different beta-lactam resistance phenotypes suggested the presence of an extended-spectrum beta-lactamase (ESBL), a class C beta-lactamase, or other combination of ESBL with a class C beta-lactamase. Seventy-six percent of these isolates also showed acquired resistance to other antimicrobial agents. They show that ceftiofur-resistant *E. coli* strains are often present in cloacal samples of broilers at the farm level in Belgium. The diversity of broad-spectrum beta-lactamases among these isolates is high, and they may act as a reservoir of ESBL and ampC genes.

Someya *et al*. (2007) reported *colibacillosis* from layer chickens in a commercial egg-producing farm in Western Japan. Three flocks of chicken at 18-21 weeks of age were affected during the initiation of egg lay. Postmortem examination revealed pericarditis, perihepatitis, airsacculitis, subcutaneous inguinal lesion, and injured cloaca. *Escherichia coli* were isolated from the lesions of the affected birds. The results suggested that certain *E. coli* virulence genes and host factors, such as the initiation of egg lay may be associated with occurrence of *colibacillosis.*

Ruiz and Bushansingh (2007) reported the effects of dietary addition of a mannan oligosaccharide (Bio-Mos) or purified lignin (Alcell lignin) as alternatives to virginiamycin on the microbial populations in the caecum and litter, and caecal *Escherichia coli* after challenge were determined in broiler chickens. A total of 800 day-old male broiler chicks were divided into 5 treatments and 4 replicates with 40 birds each. The groups were fed a negative control diet, a positive control diet (11 mg/kg virginiamycin), Bio-Mos diets (0.2% in starter and 0.1% in grower diets) and Alcell lignin diets (1.25 and 2.5%) for 42 days. Birds were challenged with *E. coli* at 21 days of age, after which caecal and litter microbial populations were evaluated at 28 and 42 days of age. They found Bio-Mos reduces the caecal population of *E. coli* and increases the population of beneficial lactobacilli and bifidobacteria, and is more advantageous compared to virginiamycin for broiler diets.

Trample *et al.* (2007) examined five clinically normal fowls from three farms (farm A, farm B, and farm C), and five dead fowls with lesions of peritonitis from each of the same three commercial egg-laying operations from bacterial culturing. *Escherichia coli* were isolated from the cloaca in 14 of 15 healthy fowls and from all 15 fowls with peritonitis. Oviducts of normal fowls did not contain *E. coli* (0/15) whereas oviducts from 13 of 15 hens with peritonitis were positive for this pathogen. No lesions and no *E. coli* (0/15) were found in the peritoneal cavity of healthy hens, but peritonitis lesions from 13 of 15 dead fowls yielded *E. coli.*

Savita and Malik (2007) conducted a survey to determine the association of various serotypes of *Escherichia coli* and *salmonella* with diarrhea and its source of spread among poultry in central India. The occurrence of salmonella in diarrheic chickens and only one litter sample. The prevalence rates were 8.69 and 3.22%, respectively. The reported the prevalence of *Salmonella* infection was seen up to 8 weeks of age, whereas *E. coli* infection was seen up to more than 12 weeks of age.

Ahmed *et al.* (2007) collected and tested 366 *Escherichia coli* strains from 10 host groups and surface waters for the presence of 15 virulence genes. The virulence genes includes eaeA, VT1, 2 and 2e, LT1, ST1 and 2, Einv gene, EAgg gene, CNF1 and 2, papC, O111 and O157 side chain LPS. Of the 262 strains obtained from nine different hosts including six strains from humans, two from horses, eight from dogs, two from ducks, five from cattle, seven from chickens, four from pigs, two from sheep and three from deer and remaining 104 strains obtained from water samples.

Nasrin *et al*. (2007) isolated bacteria from litter were *E. coli, Bacillus* spp. and *S. aureus.*

USEPA (2006) described *E. coli* are a species of bacteria that is a subgroup of total coliforms and faecal coliform. *E.coli* is generally found in human and warm blooded-animal intestinal tracks, which are used to indicate the recent presence of fecal pollution of water samples.

Yang *et al.* (2005) reported that *Shigella* have evolved from different strains of *E. coli* and have become highly specific human pathogens through extensive convergent evolution involving gain and loss of functions.

Connor & Schwartz (2005) described *Salmonella typhi* is the aetiological agent of typhoid fever, while paratyphoid fever is caused by S. Paratyphi A, B and C. Since the clinical symptoms of paratyphoid fever are indistinguishable from typhoid fever, the term 'enteric fever' is used collectively for both fevers, and both *S. Typhi* and *S. Paratyphi* are referred as typhoid *Salmonella.*

Biswas *et al.* (2004) analyzed 335 organ samples collected from 6 upazila of 4 districts namely, Gaibandha, Sirajong, Sherpur and Netrokona to identify the endemic bacterial, viral and fungal diseases affecting semi-scavenging chickens. Analyses of the organ samples revealed that three viral, three bacterial, and one fungal disease were prevalent in the study areas, namely, New castle disease, Fowl pox, Infectious bursal disease; *Salmonellosis*, *Colibacillosis*, *Fowl cholera* and *Aspergillosis* with the prevalenve of 15.5%, 9.3%, 3.9%, 3.0, 8.7%, 5.1, and 1.2%, respectively.

Habibur Rahman *et al.* (2004) conducted to study the incidence and gross pathological alteration produced by *Salmonella gallinarum* in and surrounding areas of Hyderabad city. For this all the affected organ was collected and brought to the laboratory for detail study. In the affected organs, the rate of incidence of *Salmonella gallinarum* was recorded 36.5%. The organs which showed positive reaction towards the *Salmonella gallinarum* infection were 27 ovaries (13.5%), livers 21 (10.5%), spleen 21 (10.5%) and kidneys only 4 (2%).

Struve *et al.* (2004) described *Klebsiella pneumonia* is a common opportunistic pathogen that causes human infections. It can be widely distributed not only in the respiratory and intestinal tracts of humans and animals but also in a variety of environments and vectors. This pathogen can cause pneumonia, respiratory tract infections, urinary system infections, septicemia and other diseases.

Nayak *et al.* (2003) conducted a comprehensive ecological survey from April 1997 to June 1999 on turkey flocks (F1 to F4), maintained in farm condition in USA. The authors collected turkey cloacal and crop content, litter, air, feed, feeder and environmental swab samples from affected turkeys. *Salmonella* was isolated of feed and 1% of feeder samples. They identified *S. heiderberg* (65%) & *senftenberg* (19%), *S. muenster* (10%) & *S. anatum* (3%) and *S. Worthington* (3%) among the total isolates.

Wei *et al.* (2003) stated earlier *Shigella* and *Escherichia coli* were classified in the same genus because of their genetic similarity. Recent studies reveal that 175 of the total 3235 open reading frames were exclusive for S. flexneri as the result of the comparative genomic study between the two organisms.

Takaya *et al.* (2003) stated *Salmonella* bacteria enter the digestive tract via contaminated water or food; they tend to penetrate the epithelial cells lining the intestinal wall. *Salmonella* pathogenicity islands (SPIs) encode for type III secretion systems, multi-channel proteins that allow *Salmonella* to inject its effectors across the intestinal epithelial cell membrane into the cytoplasm. The bacterial effectors then activate the signal transduction pathway and trigger reconstruction of the actin cytoskeleton of the host cell, resulting in the outward extension or ruffle of the epithelial cell membrane to engulf the bacteria. The morphology of the membrane ruffle resembles the process of phagocytosis.

EscobarParamo *et al.* (2003) recorded that *Shigella* strains were put in a different genus from E. coli because of their medical significance, human host interactions, pathogenicity, physiology (failure to ferment lactose or decarboxylate lysine) and serological characteristics.

Xu *et al.* (2003) investigated alimentary and respiratory micro flora in cases of distemper and parvovirus infection, they stated that *E. coli*, *Salmonella* and *Staphylococcus* existed in 45 fecal samples from two cases with diarrhea. *Staphylococcus* and *Klebsiella pneumonia* existed in 36 nasal secretion samples from distemper cases mainly with respiratory symptoms. In an antibiotic sensitivity test, bacteria from faces and nasal secretion cultures showed the highest sensitivity rates of 83.33% and 86.33% to amikacin (from 8 antibiotic tested). In a united antibiotic sensitivity test, chloramphenicol and amikacin showe the highest synergism rates of 58.82% and 63.4% to bacteria from faces and nasal seretion cultures respectively.

Mishra *et al.* (2002) isolated *E. coli* from domestic poultry. They isolated fifty strains of *E. coli* from 250 specimens like heart blood, intestinal contents, liver, lung, ovaries, peritoneal fluids, spleen, and unabsorbed yolk of poultry. Majority was isolated from heart blood and serotype O78 was the most predominant isolates and prevalence was 61.22%.

Hossain *et al.* (2002) showed that the antibiotic sensitivity pattern of isolates were highly sensitive to chloramphenicol, ciprofloxacin, kenamycin and cephalexin and an increasing trend of resistance was recorded in both broiler and layer isolates. It may be concluded from the results of this study that the high resistance of *E. coli* to antibiotics constitutes a threat to poultry industry in Bangladesh.

Pakpinyo *et al.* (2002) reported the prevalence of enteropathogenic *E. coli* (EPEC) in poultry enteritis-mortality syndrome (PEMS) in turkey in North California, USA.

Ichida *et al*. (2001) observed that poultry farms may or may not remove the old litter before placement of every new flock. Several poultry farms visited in the present study remove poultry litter only twice a year, placing fresh litter shavings on top of the old litter, which may allow the microbial activity to sufficiently compost the litter.

Bogaard *et al.* (2001) determined the percentage of fecal samples containing resistant *Escherichia coli* and the proportion of resistant fecal *E. coli* in three poultry populations with genotype of ciprofloxacin-resistant isolates from these eight populations from turkey meat by pulsed-field gel electrophoresis (PFGE) after small digestion. They reported that the proportion of samples containing resistant *E. coli* and the percentages of resistant *E. coli* were significantly higher in turkeys and broilers than in the laying hen population and multi-resistant isolates were common in turkey and broiler but absent in laying hen. They also concluded that resistant clones and resistance plasmids of *E. coli* were commonly transmitted from poultry to humans.

Wasfy MO *et al.* (2000) described that the *Campylobacter, Salmonella,* and *Shigella* remain major contributions to acute enteric infections; few studies on these pathogens have been conducted in Egypt. From 1986 to December 1993, 869 *Salmonella, Shigella* and *Campylobacter* strains were isolated from stool specimens from 6,278 patients, presenting to Abbassia Fever Hospital, Cairo, Egypt, with acute enteric infections. *Salmonella* predominated, totaling 465 isolates, followed by *Shigella* with 258 isolates, and *Campylobacter* with 146 isolates. Of the *Shigella* isolates, 124 were *Shigella flexneri,* 49 were *S. sonnie,* 47 were *S. dysenteriae* (mainly serotype 1, 2, and 3), and 38 were *S. boydii. Campylobacter* spp. comprised 92 *Campylobacter jejuni* and 54 *C. coli* isolate. Isolation of *Salmonella* was highest during the months of February-March, June-July, and October-November, while that of *Shigella* was maximal from July to

October. Isolation of *Campylobacter* increased during May-June and again during August-October. Although *Salmonella* was sensitive to amikacin, aztreonam, ceftriaxone, and nalidixic acid, it was however, resistant to erythromycin, strepromycin, ampicillin, chloramphenicol, and tetracycline.

Cardamone *et al.* (2000) isolated *Salmonella spp.* From feed samples, water samples, egg samples and also fecal samples of chicken on a chicken farm in Palermo, Italy.

Wray and Wray (2000) described the genus *Salmonella* is named after the American bacteriologist Daniel E. Salmon who together with Smith isolated in 1886 bacteria from pigs (now known to be *Salmonella Cholerasuis*) which the horse considered to be the cause of Swine fever (hog cholera).

Dhruba *et al.* (1999) isolated the *salmonella* organism using 5 fermentation reactions. Nearly all isolates were positive for mannitol; maltose, and dextrose with the production of acid with different degrees while lactose and sucrose are not fermented.

Bass *et al.* (1999) shown a high prevalence of integrons in *E. coli* isolated from broiler chickens and culture of the litter in the study showed that coliforms were present in 250,000 CFU/g of litter.

Braun *et al.* (1998) compared several conventional cultural procedures for the isolation of *Salmonella* from the fecal samples of layer flocks. They concluded that the use of selective enrichment in tetrathionate broth followed by plating on modified semi-solid Rappaport Vassiliadis medium resulted in a significantly, higher solution rate of Salmonella compared to Brilliant Green and Xylose lysine deoxycholate medium.

Martin *et al.* (1998) suspected that susceptible to infection by pathogenic microorganisms such as *Listeria monocytogenes, Salmonella* and *Campylobacter* spp. that may be present in poultry litter.

Ali *et al.* (1998) described the colony characteristics of *E. coli.* The authors found metallic sheen on the EMB agar, rose pink colony on the MacConkey agar and pinkish colony on the SS agar medium. The authors also characterize *E. coli* isolated from feces and urine of animals and human.

Ingraham *et al.* (1998) studied the effect of temperature on *E. coli.* The normal temperature range extends from 21 to 37°C. The maximum temperature at which serial growth could be sustained is 49°C.

Martin *et al.* (1998) observed that there are concerns regarding the safety of feeding poultry litter to cattle due to potential infection by pathogenic microorganisms that may be present in poultry litter. Many pathogenic strains such as *Listeria monocytogenes*, *Salmonella, Campylobacter spp.,* and *Bordetella spp.* have been found in poultry litter samples.

Khan *et al.* (1998) stated that *Salmonellosis* in poultry causes heavy economic loss through mortality and reduced production.

Blanco *et al.* (1997) analyzed that the absence of enterotoxigenic *Staphylococci*, enterotoxigenic *E. coli,* enteropathogenic *E. coli,* and *E. coli* O157 in poultry litter was not unexpected due to their reported low frequency or absence in poultry flocks.

Haque *et al.* (1997) isolated, identified and produced colored *pullorum* antigen in Bangladesh for the rapid whole blood test. For this study postmortem examination (PM) of 350 chickens were performed in Dhaka, Bangladesh during 1995. The isolates were characterized by cultural, morphological, biochemical, and serological tests. They identified 40 isolated *S. pullorum* and *S. gallinarum.*

Sasipreeyajan *et al.* (1996) detected *Salmonella* in 13 broilers flocks, 15 layer flocks and 7 parent breeder flocks in Thailand from October 1991 to August 1992. *Salmonella* were isolated from cloacal swabs, feces and litter from all broiler and breeder flocks and 87% of the layer-flocks.

Holt *et al. (*1994) stated that *Salmonella* is an important genus of the family Enterobacteriaceae. Members of the genus are Gram-negative, facultative anaerobes and inhabit the intestinal tract of man and animals. They may be recovered from a wide range of hosts such as poultry, swine, human, foods and environment. Members of the genus *Salmonella* may be pathogenic to wild or domestic animals and human.

McDade *et al.* (1994) demonstrated the worldwide distribution of *E. coli* in nature. They observed the ability of *E. coli* to survive on the surface of glass, ceramic tile, asphalt tile and rubble tile.

Award and Thiemann (1993) carried out a survey for monitoring *Salmonella* infection throughout the production period in laying farms under floor and cage systems and hatcheries. They found *Salmonella* in the poled intestinal samples at slaughter from 30% farms with floor husbandry and from 20% of the farms with caging; *Salmonella* was isolated in unhatched chicks and in one of these *Salmonella* was isolated from the surface of a ventilator.

Haque *et al.* (1991) found that Poultry is essential to the national economy of Bangladesh and the welfare of human beings. Several constraints such as the diseases, poor husbandry, low productivity and shortage of feed affect the optimal performance of this industry in Bangladesh.

Corrier *et al*. (1990) stated that *Salmonellosis* in poultry resulted in continuous increase of public health problems.

Liiadis and Lordanidis (1990) performed bacteriological examination of 8500 birds submitted for PM examination in northern Greece during a period of 6 years yielded 721 isolates of *Salmonella,* 610 of which were *S. gallinarum* originated from 133 flocks of fowls and 57 flocks of turkeys.

Kotova *et al*. (1988) conducted study on human developing the *Salmonella* carrier state (*S. enteritidis* and *S. dublin*) after acute *salmonellosis* and as a result of occupational exposure to poultry. The *Salmonella* species most frequently isolated from poultry employees was *S. typhimurium*, while *S. newport*, *S. enteritidis* and *S. Dublin* were also isolated.

Barrow *et al.* (1987) observed mortality among 1 day-old chicks following oral administration of *S. typhimurium* varied considerably with strain and with breed. Virulent strains first multiplied in the liver and spleen. Death probably resulted from anorexia and dehydration. The major virulence factor was invasiveness.

Girao *et al* (1985) isolated *Salmonella* from feedstuffs, rations and samples collected from chicks with various health problems. They isolated 6 species of *S. gallinarum* and 22 species of *S. pullorum* from 37 *Salmonella* isolated from 77 diseased flocks.

Edward and Ewing (1985) stated that fermentation of *E. coli* produced gas from glucose, indole, lysine, arabinose, mannitol, ortho-nitrophenyl galactoside, trehalose and xylose was found in *E. coli.*

Freeman *et al.* (1985) mentioned that *E. coli* is widely distributed in the intestinal tract of man and animals. For this reason, they often employed as indicator of fecal pollution of water supplies.

Levine *et al.* (1984) stated that most of the strains of *E. coli* lived as benign commensals, many perhaps all were opportunistic pathogens of humans and animals. The authors also revealed that *E. coli* is the infecting organism in more than 80% of cases of urinary tract infections, which included a symptomatic bacteriuria, cystitis and pyelonephritis.

Black *et al.* (1981) said, in the third world countries, ETEC was associated with traveler's diarrhea. It was the major and best characterized group of *E. coli,* which came to prominence in the late 1960s and 1970s, largely on the basis of work carried out in Calcutta by Gorbach Sack, and co-workers. It was a major cause of infants' diarrhea less developed countries. Some reports of infants prospectively followed-up by frequent household surveillance suggest that as many as two to three clinical ETEC infections per child per year occur during the first two to three years of life. One of the bacterial cause of dehydrating infant diarrhea in developing areas, and an infection correlated with adverse nutritional consequences. It was also the agent most frequently responsible for traveler's diarrhea.

Hofstad *et al.* (1978) found that paratyphoid infections are economically among the most important bacterial disease of the hatching industry and result in high death losses among all types of young poultry.

Buxton and Fraser (1977) stated that the first member of the group *Salmonella* was the typhoid *bacillus*, originally observed in human tissues by Eberth in 1880 cultured by Graftkey in 1884.

Buxton and Fraser (1977) mentioned that *E. coli* was a normal inhabitant of the intestinal tracts of vertebrates, including man. Under certain conditions, the number of these organisms in the intestines undergoes a marked and rapid increase, and this may be associated with definite signs of ill health and even death.

Harry and Brown (1974) presented that poultry feeds may be a common and very important source of paratyphoid organisms. The level of *Salmonella* contamination in poultry feeds is normally low; however, it has been shown that even one organism per 15 grams of feed can produce infection.

Merchant and Packer (1967) reported that in 1892 Loeffler isolated organism from a natural outbreak of a typhoid like disease in mice which he called *Bacillus typhimurium*. Sal. Pestis caviae which was isolated from an intestinal infection of Guinea pig by Wherrx in 1908 is now a synonym of Sal. typhimurium. The authors also stated that in 1921 Schermer and Ehlirch isolated an organism from ewe which had aborted. The authors found the organism belonged to the paratyphoid group: consequently they named it *Bacillus paratyphi abortus ovis*.

Merchant and Packer (1967) performed some biochemical tests for *E. coli* and concluded that most *E. coli* fermented lactose, reduced nitrates and were methyl-red positive. Approximately 10% of them were late lactose fermenters and some of them were non-lactose fermenters.

CHAPTER III

MATERIALS AND METHODS

The study was conducted for isolation and identification of bacteria from environmental sample of live bird market. The research work was carried out in the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur-Bangladesh, during the period of July to December, 2017. The detailed description of the materials and methods are given below:

3.1 Materials

3.1.1 Study Area

Environmental samples were collected from live bird market in Dinajpur district, Bangladesh.

3.1.2 Study Population

A total of 30 environmental samples were collected from live bird market for bacteriological examination with antibiogram study and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh.

3.1.3 Laboratory preparation

All items of glass wares including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides, vials and agglutination test tubes in a household dishwashing detergent solution (Trix, Recket and Colman Bangladesh Ltd.) for overnight, contaminated glassware's were disinfected in 2% sodium hypo chloride solution prior to cleaning. The glassware's were then cleaned by brushing, washed thoroughly and finally sterilized either by dry heat at 160°C for 2 hours or by autoclaving for 15 minutes at 121°C under 15 lbs pressure by square inch. Autoclaved items were dried in a hot air oven over 50°C. Disposable plastic were (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50°C for future use.

3.1.4 Necessary instruments

In this research work following instruments were used:

Test tubes, Petri-culture dishes, Conical flasks, Pipette, Slides, Microscope, Sterilized cotton, Immersion oil, Bacteriological incubator, Jar, ice boxes, Measuring balance, Hand gloves, Sprit lamps, Match lighter, Inoculating loop & Inoculating needle, Stop watch, Glass spreader and test tube rack.

3.1.5 Bacteriological media used for culture

3.1.5.1 Nutrient Broth (NB)

Nutrient broth was used to grow the bacteria from the samples.

3.1.5.2 Nutrient agar (NA)

Nutrient agar (NA) medium was used to grow the bacterial positive sample from the nutrient broth.

3.1.5.3 MacConkey agar (MC)

MacConkey agar (MC) medium was used for the identification of organisms under the family of Enterobacteriaceae through studying fermentation characteristics.

3.1.5.4 Eosin Methylene Blue (EMB) agar

Eosin methylene blue (EMB) agar medium was used as selective media for growth of *E. coli* and *Klebsiella spp*.

3.1.5.5 Salmonella-Shigella (SS) agar

Salmonella-Shigella medium was used as selective medium for *salmonella spp.* and *Shigella spp.*

3.1.5.6 Mueller Hinton agar

Mueller Hinton agar was used for antimicrobial sensitivity test of microorganisms like *E. coli, Klebsiella spp., Shigella spp.* and *Salmonella spp.*

3.1.6 Media used for Biochemical tests

- Basic Sugar media (dextrose, lactose, sucrose, manitol and maltose)
- Triple sugar iron (TSI) agar slant
- Buffered glucose broth
- MIU agar base
- Peptone water
- Methyl red (MR)
- Voges Proskauer (VP)
- Indole test.

3.1.7 Chemicals, Reagents and solutions

The following reagents were used for conducting the biochemical tests and microscopic test during identification:

- Gram's stain
- 1% Eosin capsular stains
- Methylene Blue stain
- Methyl red (MR)
- Voges- Proskaur (VP) solution
- A-naphthol
- Kovac's reagent
- Phosphate buffered saline
- Glycerine
- Hexisol hand rub

3.1.8 Antibacterial Sensitivity Discs

The antibiotic susceptibility pattern of all the isolated bacteria from each sample as well as from control samples was determined using the disk diffusion method according to the Bauer - Kirby technique (Bauer *et al*., 1966).

The followings are the antibiotics that were tested the selected organisms.

Table 1: Antibacterial agents and their disc concentration

Antibacterial agents	Symbol	Disk concentration (mcg/ disc)
Chloramphenicol	C	30 mcg/disc
Ciprofloxacin	CIP	5 mcg/disc
Colistin	CL	10 mcg/disc
Erythromycin	E	15 mcg/disc
Cefixime	CFM	5 mcg/disc
Neomycin	N	30 mcg/disc
Kanamycin	K	30 mcg/disc
Vancomycin	VA	30 mcg/disc
Gentamycin	GEN	10 mcg/disc
Penicillin	P	10 mcg/disc

3.2 Methods

3.2.1 Experimental layout

The environmental samples were collected from live bird market of Sadar, Dinajpur district, Bangladesh for bacteriological analysis with antibiogram study of the isolated bacteria. The experimental layout illustrated in figure 1.

EXPERIMENTAL LAYOUT

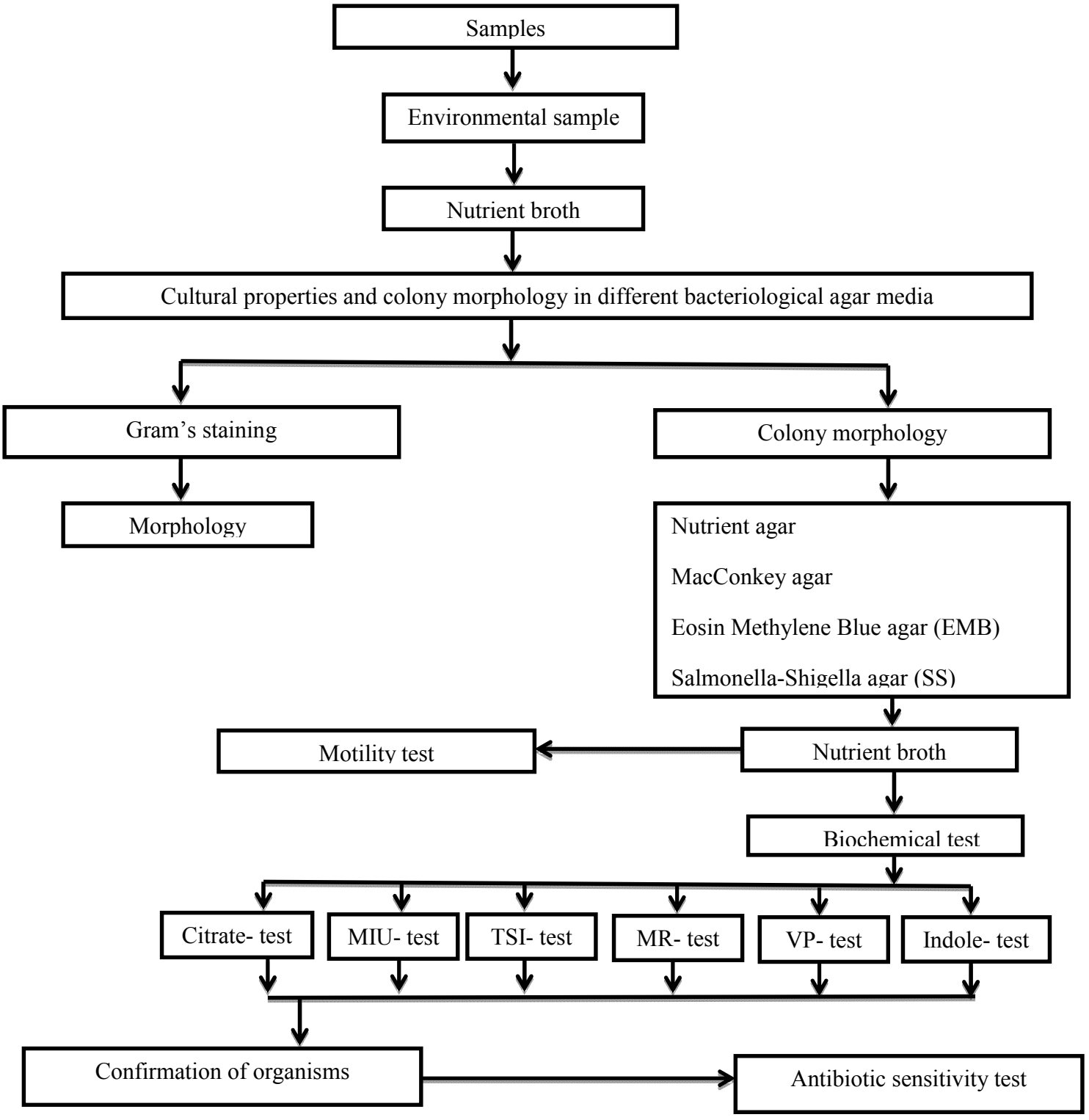


Figure 1: Sketch to show the experimental design

3.2.2 Preparation of various bacteriological culture media

3.2.2.1 Nutrient broth

This was prepared by dissolving 13gms of dehydrated nutrient broth (Hi- media Laboratories Pvt. Ltd. India) into 1000 ml distilled water and sterilized by autoclaving at 121°C under pounds pressure per square inch (1.2kg/CM2) for 15 minutes (Buxton and Fraser, 1977). Then the broth was dispended into tubes (10 ml/tubes). The tubes were then stored at 4°C in the refrigerator until used.

3.2.2.2 Nutrient agar

28 grams of Bacto- Nutrient agar (Hi- Media laboratories Pvt. Ltd.) was added to 1000 ml distilled water in a flask and heated to boil for dissolving the medium completely. The medium was then sterilized by autoclaving at 121°C maintaining a pressure of 15 pounds/sq. inch (1.2 kg/cm2) for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to cool down its temperature.

3.2.2.3 MacConkey agar medium

51.50 grams powder of MC agar base (Hi- Laboratories Pvt. Ltd.) 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 121°C maintaining a pressure of 15 pounds /sq. inch (1.2 kg/cm2) for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to decrease its temperature.

3.2.2.4 Salmonella- Shigella (SS) agar medium

60 grams powder of SS agar base (Hi- Media Laboratories Pvt. Ltd.) was added to 1000 ml distilled water in a flask and heated to boil for dissolving the medium completely. The medium was the sterilized by autoclaving at 121°C maintaining a pressure of 15 pounds/sq. inch (1.2kg/cm2) for 15 minutes. After autoclaving the medium was put into water bath of 45°C to cool down its temperature.

3.2.2.5 Eosin Methylene Blue agar

36 grams of EMB agar base (Hi- Media Laboratories Pvt. Ltd.) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in 10ml quantities in sterile glass petridishes (medium size) and in 15ml quantities in sterile glass petridishes to form a thick layer therein. To accomplish the surface of quite dry, the medium was allowed to solidify for about 2 hours with the covers of petridishes partially removed. The sterility of the medium was judged and used to store at 4°C in refrigerator for further use.

3.2.2.6 Simmon Citrate agar

24.28 grams of Simmons Citrate agar base (Hi-Media Laboratories Pvt. Ltd) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in 10ml quantities in sterile glass petridishes (medium size) and in 15ml quantities in sterile glass petridishes (larger size) to form a thick layer therein. To accomplish the surface of quite dry, the medium was allowed to solidify for about 2 hours with the covers of petridishes partially removed. The sterility of the medium was judged and used to store at 4°C in refrigerator for further use.

3.2.2.7 Triple Sugar Iron (TSI) agar slant

A quantity of 6.5 grams of Bacto TSI agar (Hi-Media Laboratories Pvt. Ltd) was suspended in 1000 ml of distilled water and heated up to boiling to dissolve the medium completely. The solution was then distributed into tubes plugged with cotton plugs and sterilized in an autoclave at 121°C at a pressure of 15-pounds/sq. inch (1.2kg/cm2) for 15 minutes. After sterilization, the tubes were kept in slanting position in such a manner so as to allow a generous butt after solidification. The tubes were then incubated at 37°C for overnight to check their sterility and then stored in a refrigerator for future use (Cheesebrough 1984).

3.2.2.8 Methyl-Red Voges-Proskauer (MR-VP) broth

A quantity of 1.7 grams of Bacto MR-VP medium was dissolved in 1000 ml of distilled water dispensed in 5 ml in each test tube and then the tubes were autoclaved at 121°C at a pressure of 15-pounds/sq inch (1.2kg/cm2) for 15 minutes. After autoclaving, the tubes containing medium

were incubated at 37°C for overnight to check their sterility and then stored in a refrigerator for future use (Buxton and Fraser. 1977).

3.2.2.9 Motility Indole Urease (MIU)

A quantity of 24 grams of Bacto MIU medium was dissolved in 1000 ml of distilled water dispensed in 5 ml in each test tube and then the tubes were autoclaved at 121°C at a pressure of 15-pounds/sq inch (1.2kg/cm2) for 15 minutes. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and then stored in a refrigerator for future use.

3.2.2.10 Mueller Hinton agar

Suspended 38 grams of agar powder into 1000 ml of distilled water. Heat for boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

3.2.3 Preparation of reagents

3.2.3.1 Methyl-Red solution

The indicator methyl red solution was prepared by dissolving 0.1 grams of Bacto methyl-red in 300ml of 95 percent alcohol and diluting to 500ml with the addition of 200ml of distilled water (Cheesbrough, 1984).

3.2.3.2 Phosphate buffered saline solution

For preparation of PBS, 8 grams of NaCl, 2.89 grams of disodium hydrogen phosphate (Na2HPO4.12H2O), 0.2 grams of KCl and 0.2 grams of potassium hydrogen phosphate (KH2PO4) were suspended in 1000 ml of distilled water. The solution was heated to dissolve the ingredients completely. The solution was then sterilized by autoclaving at 121°C maintaining a pressure of 15-pounds/sq. inch for 15 minutes and stored at 4-8°C until use. The pH of the solution was measured by a pH meter maintained at 7.0-1.2 (Cheesbrough, 1984).

3.2.3.3 1% Peptone water

Dispended 10 gm of peptone water agar powder in 1000 ml of distilled water and boiled at 95°C for few minutes and then poured over 20 ml solution dispersed into sterilized plastic tubes and stored at 4-8°C.

3.2.4 Sample collection and sample processing

30 environmental samples were collected from live market in Dinajpur district, Bangladesh. Samples were collected by using cotton swab with test tube and kept jar, ice boxes. Then it was inoculated into Nutrient broth and incubated at 37°C for 24 hours. After 24 hours it was streaked into Nutrient agar and MacConkey agar medium by inoculating loop.

3.2.5 Techniques followed for the isolation and identification of bacteria

3.2.5.1 Culture of samples into different bacteriological media

3.2.5.1.1 Nutrient broth (NB)

Samples were inoculated into nutrient broth (NB) and incubated at 37°C for 24 hours.

3.2.5.1.2 Nutrient agar (NA)

Samples were inoculated into nutrient agar (NA) and incubated at 37°C for 24 hours.

3.2.5.1.3 MacConkey agar (MC)

The cultivated organisms from nutrient agar (NA) were inoculated directly into MacConkey agar and incubated at 37°C for 24 hours.

3.2.5.1.4 Eosin Methylene Blue (EMB) agar

Lactose fermenting pink colony from MacConkey agar was sub-cultured into EMB agar and incubated at 37°C for 24 hours.

3.2.5.1.5 Salmonella-Shigella (SS) agar

The non lactose fermenting colorless colony from the MacConkey agar was sub-cultured on SS agar and incubated at 37°C for 24 hours.

3.2.6 Examination of plates

3.2.6.1 Gross colony study

Morphological characteristics (shape, size, surface texture, edge elevation, color, opacity) developed after 24 hours of incubation were carefully studied as described by Merchant and Packer (1967) and recorded.

3.2.6.2 Gram's staining

The representative bacterial colonies were characterized morphologically using Gram's stain according to the method described by Merchant and packer (1967).

The procedure was as follows:

1. A small colony was picked up from Nutrient agar plates with a bacteriological loop, smeared on separate glass slide and fixed by gentle heating.

2. Crystal violet was then applied on each smear to stain for 2 minutes and then washed with running water.

3. Few drops of Gram's iodine were then added to act as mordent for 1 minute and then washed with running water.

4. Then acetone alcohol was added to decolorize for 15 seconds and then washed with running water.

5. After washing with water, safranin was added as counter stain and allowed to stain for 1 minute.

6. The slides were then washed with water, bottled and dried in air and then examined under microscope with high power objective (100X) using immersion oil.

3.2.7 Identification of bacterial isolates by using specific biochemical tests

Isolated organisms and growth characteristics of *E. coli* and *Klebsiella spp.* on EMB agar, *Salmonella spp.* and *Shigella spp.* on SS agar were subjected to various biochemical tests named TSI agar slant reaction, MR-VP, MIU, Indole reaction and Citrate utilization test were carried out for identification of suspected isolates.

3.2.7.1 TSI agar slant test

The TSI agar slant was used to detect the lactose, sucrose and dextrose fermenters. The medium also helped to determine the ability of the organisms to produce 1-12S. The organisms under this study were heavily seeded with a platinum needle over the surface of the slants and stabbed into the butt of the tubes containing TSI agar. After an anaerobic incubation of 24 hours at 37°C the tubes were examined for changes in the slant or in the butt or in both places.

3.2.7.2 Simmon citrate utilization test

Selected colony was inoculated on Simmon citrate agar. The medium was incubated for overnight at 37°C for 24 hours.

3.2.7.3 Voges-Proskauer (VP) test

The organisms were inoculated into 5 ml sterile buffered glucose broth. It was incubated at 37°C for 72 hours. Then 3ml of a-napthol was added and followed by 1ml of potassium hydroxide. Then they were mixed well and waited for 30 minutes for the slow development of a pink color for positive cases. There is no development of a pink color for negative cases (Cheesbrough, 1984).

3.2.7.4 Methyl Red (MR) test

The organisms were inoculated into 5 ml sterile buffered glucose broth. It was incubated at 37°C for 48 hours. Then 2-3 drops of methyl red solution was added. The result shown the red color in the upper part of the test tubes in positive cases and in negative cases there was no development of yellow color (Cheesbrough, 1984).

3.2.7.5 Indole test

One colony form a pure culture of the bacterium was inoculated into 5ml of peptone water and incubated for 24 hours. After incubation 1ml of Kovac's reagent was added, shaked well and examined after 1 minute. A red color in the reagent layer indicated indole positive. In negative case there is no development of red color (Cheesbrough, 1984).

3.2.7.6 Motility Indole Urease (MIU) test

Suspected colony inoculated in to the separate tube containing MIU medium. The medium was incubated at 37°C for 24 hours.

3.2.8 Characterization of Bacteria

Individually isolated colonies of the same morphology were selected from appropriate agar plates, cloned and checked for purity of growth prior to characterization of the respective genera and species. Characterization of the respective genera and species were done on the basis of morphological, cultural, biochemical and serological reaction. The classification and specification of organisms was based on the scheme presented in Bergey's Manual of Systematic Bacteriology (Holt, 1985).

3.2.9 Maintenance of stock culture

3.2.9.1 Agar slant method

The organisms isolated were inoculated into the nutrient agar slants and incubated at 37°C for 24 hours in a bacteriological incubator and then examined for growth. Then the sterile mineral oil was poured into the tubes until the colonies were covered completely. The tubes were sealed off with paraffin wax and kept at room temperature for future use. By this method, bacteria can be preserved with no deviation of their original characters for few months (Buxton and Fraser, 1977).

3.2.10 Antibiotic sensitivity test

3.2.10.1 The Kirby-Bauer disc diffusion method

All aspects of the Kirby-Bauer procedure are standardized to ensure consistent and accurate results. Because of this, a laboratory must adhere to these standards. The media used in Kirby-Bauer testing must be Mueller-Hinton agar at only 4 mm deep, poured into either 100mm or 15mm Petri dishes. The pH level of the agar must be between 7.2 and 7.4.

3.2.10.2 Procedure

1. Label the covers of each of the agar plates with the name of the test organism to be inoculated.
2. Using a septic technique, inoculated all agar plates with their respective test organisms as follows:
    - ❖ Dip a sterile cotton swab into a well mixed saline test culture and excess inoculums by pressing the saturated swab against the inner wall of the culture tube.
    - ❖ Using the cotton swab streaked the entire agar surface horizontally, vertically and around the outer edge of the plate to ensure a heavy growth over the entire surface.
3. Allowed all culture plates to dry for about 5 minutes
4. Using the sensi-disc-dispenser, applied the antibiotics discs by placing the dispenser over the agar surface and pressing the plunger, depositing the discs simultaneously onto the agar surface. If dispensers are not available, distribute the individual discs at equal distances with forceps dipped in alcohol and flamed.
5. Gently pressed each disc down with the wooden end of a cotton swab or sterile forceps to ensure that the discs adhere to the surface of the agar.
6. Incubated all plate cultures in an inverted position for 24 to 48 hours at 37°C.

3.2.10.3 Reading Antibacterial Disc Plates and Interpreting Results

After 24 hours of incubation, each plate was examined if plate was satisfactory streaked, and the inoculum was corrected, the resulting zone of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies were apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using sliding calipers or a inches above a black, nonreflecting background and zones are measured in millimeter (mm) from the upper surface of the agar illuminated with reflected light, with the cover removed (EUCAST, 2015).

CHAPTER IV

RESULTS

The result described below includes the isolation and identification of *E. coli, Shigella spp., Klebsiella spp.* and *Salmonella spp.* of collected samples. This study were attempted to look for the differences, among the isolates. A total of 30 environmental samples were collected from live bird market, Dinajpur district, Bangladesh.

4.1 Isolation and identification of *E. coli, Shigella spp., Klebsiella spp.* and *Salmonella spp.*

4.1.1 Results of Cultural examinations

The individual cultural characteristics of isolated bacteria are presented in **(table-2)**

4.1.1.1 Ordinary media

4.1.1.1.1 Culture on Nutrient agar

Small, round and smooth colony was found **(Plate: 3).**

4.1.1.2 Differential media

4.1.1.1.2 Culture on MacConkey agar

MacConkey agar plates streaked separately with the organisms from nutrient agar revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically.

Lactose fermenting strains grow as red or pink and may be surrounded by a zone of acid precipitated bile **(Plate: 4).**

Non-lactose fermenting strains *Shigella spp.* and *Salmonella spp.* are colorless and transparent; typically do not alter appearance of the medium **(Plate: 5).**

4.1.1.3 Selective media

4.1.1.3.1 Culture on Eosin Methylene Blue (EMB) agar

EMB agar plates streaked separately with the lactose fermenting organisms from MacConkey agar revealed the growth of *Klebsiella spp.* and *E. coli* bacteria after 24 hours of incubation at 37°C aerobically.

The greenish-black colonies with metallic sheen on EMB agar indicated *E. coli* **(Plate: 6).**

The pinkish mucoid colonies indicated *Klebsiella spp.* **(Plate: 8).**

4.1.1.3.2 Culture on Salmonella-Shigella *(*SS) agar

SS agar plates streaked separately with the non-lactose fermenting organisms from MacConkey agar revealed the growth of *Salmonella spp.* and *Shigella spp.* bacteria after 24 hours of incubation at 37°C aerobically.

The black colonies identified as *Salmonella spp.* **(Plate: 10).**

Translucent and colorless colonies identified as *Shigella spp.* **(Plate: 12).**

Table 2: Characterization of isolated bacteria by cultural properties

Name of media used for culture	***E. coli***	***Salmonella spp.***	***Klebsiella spp.***	***Shigella spp.***
Nutrient agar	Smooth, circular, white to grayish colony with peculiar fetid odor	Small, round and smooth colony	Smooth, circular, white colony	Small, circular, colorless, smooth and translucent
MacConkey agar	Rose pink lactose fermenter colony	Colorless, pale, and translucent colony	Mucoid pink lactose-fermenter colony	Colorless, pale non-lactose fermenter colony
Eosin Methylene Blue (EMB) agar	Moist circular colonies with dark centers yellow green metallic sheen	No growth	Pinkish mucoid colony	No growth
Salmonella-Shigella (SS) agar	No growth	Translucent and smooth colony with dark black centers	No growth	Translucent, colorless colonies

4.1.2 Results of Gram's staining

Isolated bacterial pathogens were stained by Gram's staining techniques for microscopic study **(Table-3).**

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored, short plumped rods *E. coli* arranged in single, pairs or short chains. **(Plate: 7).**

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored, small rod shaped *Klebsiella spp.* arranged in single, pairs or short chains. **(Plate: 9).**

The microscopic examination of Gram's stained smears from SS agar revealed Gram-negative, pink colored, very short plumped rods *Salmonella spp*. arranged in single, paired or short chains. **(Plate: 11).**

The microscopic examination of Gram's stained smears from SS agar revealed Gram-negative, pink colored, small rod shaped *Shigella spp.* arranged in single, pairs or short chains. **(Plate: 13).**

Table 3: Characterization of isolated bacteria by Gram's staining technique

Gram's Staining				
Color	**Shape**	**Arrangement**	**Gram's staining reaction (+/-)**	**Identification**
Pinkish color	Short plump rods	Single, paired or short chain	Gram negative	*E. coli*
Pinkish color	Very short plump rods	Single, pairs or short chain	Gram negative	*Salmonella spp.*
Pinkish color	Small rod-shaped	Single, paired or short chain	Gram negative	*Klebsiella spp.*
Pinkish color	Small rod-shaped	Single, paired or short chain	Gram negative	*Shigella spp.*

4.1.3 Results of Biochemical tests

The isolated organisms were confirmed by different biochemical tests.

Table: 4. Identification of *E. coli* by different biochemical tests

Biochemical test	Change of the media	Results	Plate no.
Indole test	Pink color ring at the top of the media	Positive	14
MR test	Red color	Positive	15
VP test	No color change	Negative	16
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	17
TSI	Butt-Yellow Slant-Yellow	Acid and Gas production (+) H_2S (-)	18
Citrate Utilization test	No color change	Negative	19

Legends: B=Butt, S=Slant, MR = Methyl-Red test, VP = Voges-Proskauer test, TSI = Triple Sugar Iron, MIU = Motility Indole Urease, + = Positive reaction, - = Negative reaction

Table: 5. Identification of *Salmonella spp.* by different biochemical tests

Biochemical test	Change of the media	Results	Plate no.
Indole test	No color change	Negative	14
MR test	Red color	Positive	15
VP test	No color change	Negative	16
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	17
TSI	Butt-Yellow Slant-Yellow	No Acid and Gas production (-) H_2S (+)	18
Citrate Utilization test	Prussian blue color	Positive	19

Legends: B=Butt, S=Slant, MR = Methyl-Red test, VP = Voges-Proskauer test, TSI = Triple Sugar Iron, MIU = Motility Indole Urease, + = Positive reaction, - = Negative reaction

Table: 6. Identification of *Klebsiella spp.* by different biochemical tests

Biochemical test	Change of the media	Results	Plate no.
Indole test	No color change	Negative	14
MR test	No color change	Negative	15
VP test	No color change	Negative	16
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	17
TSI	Butt-Yellow Slant-Yellow	Acid and Gas production (+) H_2S (-)	18
Citrate Utilization test	Prussian blue color	Positive	19

Legends: B=Butt, S=Slant, MR = Methyl-Red test, VP = Voges-Proskauer test, TSI = Triple Sugar Iron, MIU = Motility Indole Urease, + = Positive reaction, - = Negative reaction

Table: 7. Identification of *Shigella spp.* by different biochemical tests

Biochemical test	Change of the media	Results	Plate no.
Indole test	Pink color ring at the top of the media	Positive	14
MR test	Red color	Positive	15
VP test	No color change	Negative	16
MIU test	Diffuse, hazy growth, slightly opaque media	Positive	17
TSI	Butt-Yellow Slant-Yellow	No Acid and Gas production (-) H_2S (-)	18
Citrate Utilization test	No color change	Negative	19

Legends: B=Butt, S=Slant, MR = Methyl-Red test, VP = Voges-Proskauer test, TSI = Triple Sugar Iron, MIU = Motility Indole Urease, + = Positive reaction, - = Negative reaction

4.2 Maintenance of stock culture

The stock culture was maintained following the procedures of Choudhury *et al.* (1987). During the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of the isolated organisms were stored in sterilized 80% glycerin and used as stock culture. The equal volume of 80% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored -80°C in a freezer for future use

4.3 Prevalence of isolated bacteria in environmental samples

The total prevalence of *E. coli, Salmonella spp., Klebsiella spp. and Shigella spp.* in environmental samples was 26.7%, 33.3%, 16.7% and 23.3% respectively.

Table 8: Prevalence of *E. coli, Salmonella spp., Klebsiella spp.* and *Shigella spp.* in environmental samples (n=30)

Environmental samples	No. of isolates	Percentage (%)
E. coli	8	26.7%
Salmonella spp.	10	33.3%
Klebsiella spp.	5	16.7%
Shigella spp.	7	23.3%
Total	30	100%

4.4 Observation of Antibiotic Sensitivity Test

Antimicrobial susceptibility testing was performed using Muller-Hinton agar (Hi-Media Laboratories Pvt. Ltd) plates as recommended by the Clinical and Laboratory Standards Institute.

4.4.1 Antibiotic sensitivity test of *Salmonella spp.*

The antibiotic sensitivity test revealed that the isolated *Salmonella spp.* was sensitive to Chloramphenicol (100%) and Colistin (80%). The isolates were resistant to Ciprofloxacin (100%) and Cefixme (100%) while Erythromycin (60%) was found intermediate.

Table 9: Results of Antibiotic sensitivity test of *Salmonella spp.* (n=10)

Antibacterial agents	Disk concentration (mcg/ disc)	No. and Percentage (%) of isolates		
		Sensitive	Intermediate	Resistance
Chloramphenicol	30 mcg/disc	(10) 100%	(0) 0%	(0) 0%
Ciprofloxacin	5 mcg/disc	(0) 0%	(0) 0%	(10) 100%
Colistin	10 mcg/disc	(8) 80%	(0) 0%	(2) 20%
Erythromycin	15 mcg/disc	(0) 0%	(6) 60%	(4) 40%
Cefixime	5 mcg/disc	(0) 0%	(0) 0%	(10) 100%

(Legends: C= Chloramphenicol, CIP= Ciprofloxacin, CL= Colistin, E= Erythromycin, CFM= Cefixime, R= Resistant, S= Sensitive, I= Intermediate).

4.4.2 Antibiotic sensitivity test of *E. coli*

The antibiotic sensitivity test revealed that the isolated *E. coli* was resistant to Ciprofloxacin (100%), Erythromycin (100%) and Cefixime (100%) while the isolates were sensitive to Chloramphenicol (100%) and Colistin (75%).

Table 10: Results of Antibiotic sensitivity test of *E. coli* (n=8)

Antibacterial agents	**Disk concentration (mcg/ disc)**	**No. and Percentage (%) of isolates**		
		Sensitive	**Intermediate**	**Resistance**
Chloramphenicol	30 mcg/disc	(8) 100%	(0) 0%	(0) 0%
Ciprofloxacin	5 mcg/disc	(0) 0%	(0) 0%	(8) 100%
Colistin	10 mcg/disc	(6) 75%	(0) 0%	(2) 25%
Erythromycin	15 mcg/disc	(0) 0%	(0) 0%	(8) 100%
Cefixime	5 mcg/disc	(0) 0%	(0) 0%	(8) 100%

(Legends: C= Chloramphenicol, CIP= Ciprofloxacin, CL= Colistin, E= Erythromycin, CFM= Cefixime, R= Resistant, S= Sensitive, I= Intermediate).

4.4.3 Antibiotic sensitivity test of *Klebsiella spp.*

The antibiotic sensitivity test revealed that the isolated *Klebsiella spp.* was sensitive to Ciprofloxacin (80%), Colistin (100%) and Cefixime (100%) while the isolates were resistant to Chloramphenicol (100%) and Erythromycin (100%).

Table 11: Results of Antibiotic sensitivity test of *Klebsiella spp.* (n=5)

Antibacterial agents	**Disk concentration (mcg/ disc)**	**No. and Percentage (%) of isolates**		
		Sensitive	**Intermediate**	**Resistance**
Chloramphenicol	30 mcg/disc	(0) 0%	(0) 0%	(5) 100%
Ciprofloxacin	5 mcg/disc	(4) 80%	(0) 0%	(1) 20%
Colistin	10 mcg/disc	(5) 100%	(0) 0%	(0) 0%
Erythromycin	15 mcg/disc	(0) 0%	(0) 0%	(5) 100%
Cefixime	5 mcg/disc	(5) 100%	(0) 0%	(0) 0%

(Legends: C= Chloramphenicol, CIP= Ciprofloxacin, CL= Colistin, E= Erythromycin, CFM= Cefixime, R= Resistant, S= Sensitive, I= Intermediate).

4.4.4 Antibiotic sensitivity test of *Shigella spp.*

The antibiotic sensitivity test revealed that the isolated *Shigella spp.* was sensitive to Chloramphenicol (100%) and Ciprofloxacin (100%). The isolates were resistant to Erythromycin (100%) and Cefixime (100%) while Colistin (43%) was found intermediate.

Table 12: Results of Antibiotic sensitivity test of *Shigella spp.* (n=7)

Antibacterial agents	Disk concentration (mcg/ disc)	No. and Percentage (%) of isolates		
		Sensitive	Intermediate	Resistance
Chloramphenicol	30 mcg/disc	(7) 100%	(0) 0%	(0) 0%
Ciprofloxacin	5 mcg/disc	(7) 100%	(0) 0%	(0) 0%
Colistin	10 mcg/disc	(3) 43%	(4) 57%	(0) 0%
Erythromycin	15 mcg/disc	(0) 0%	(0) 0%	(7) 100%
Cefixime	5 mcg/disc	(0) 0%	(0) 0%	(7) 100%

(Legends: C= Chloramphenicol, CIP= Ciprofloxacin, CL= Colistin, E= Erythromycin, CFM= Cefixime, R= Resistant, S= Sensitive, I= Intermediate).

Laboratory Picture gallery

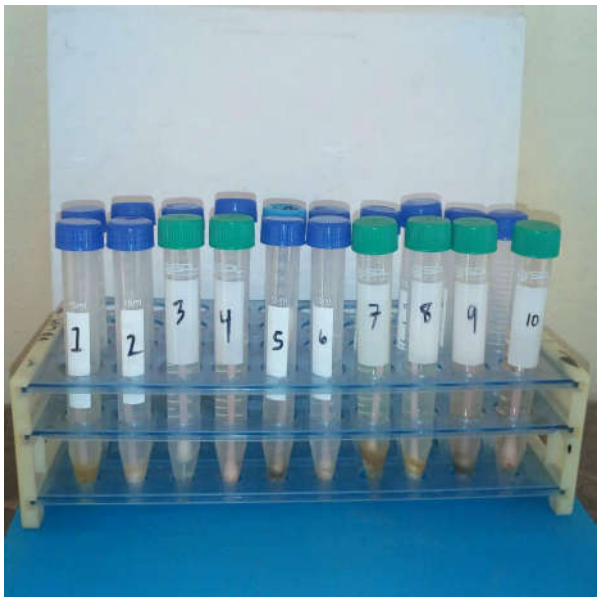


Plate 1: Collected samples from live bird

Plate 2: Growth of microorganisms on Nutrient broth (left) compared with control test tube

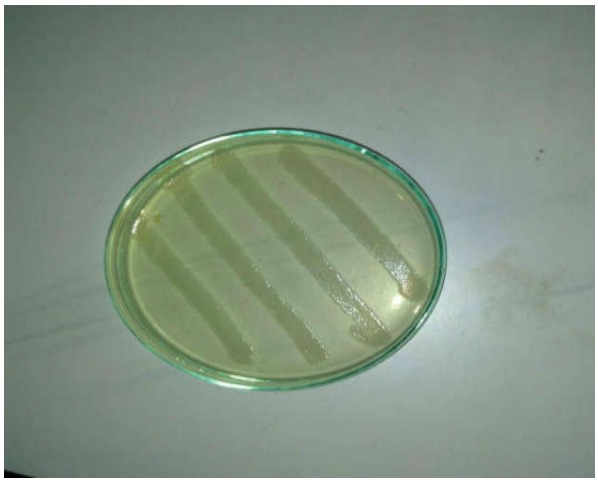


Plate 3: Growth of microorganisms on Nutrient agar (left) compared with control petridish (right)

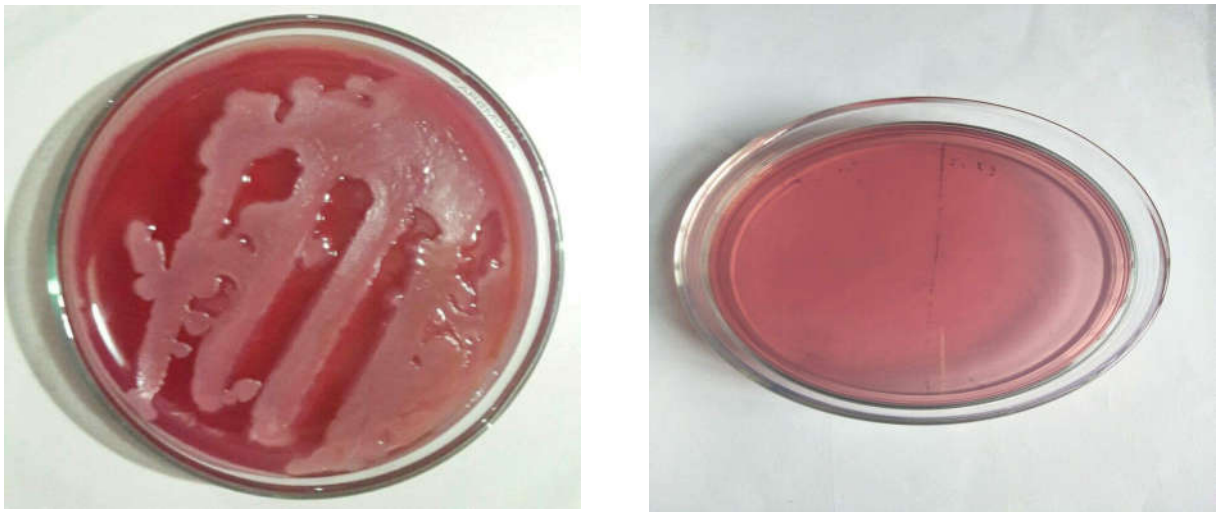


Plate 4: Lactose fermented organisms grows on MacConkey agar (left) compared with control petridis (right)

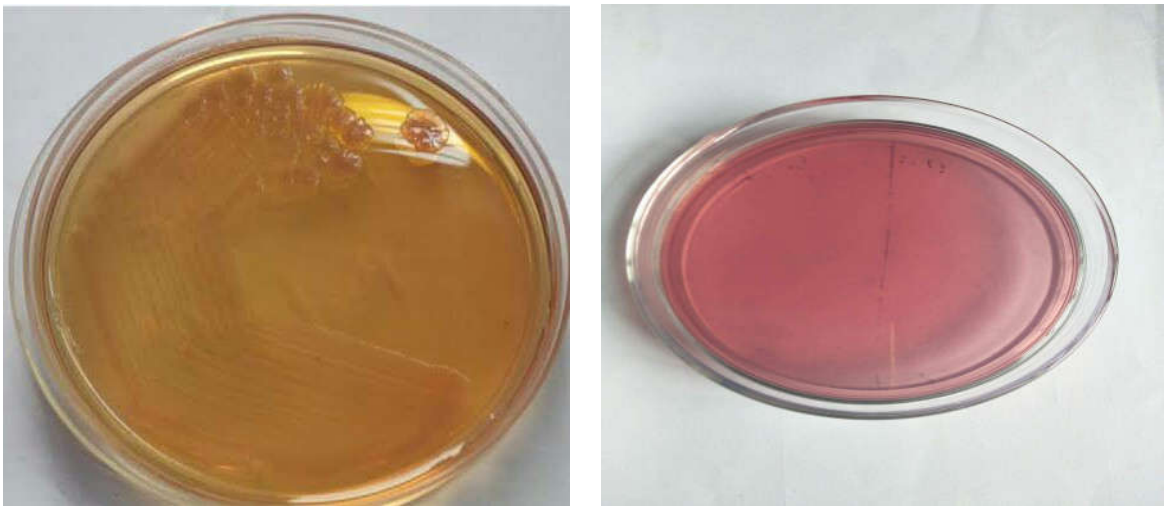


Plate 5: Non-lactose fermented organisms grows on MacConkey agar (left) compared with control petridis (right)

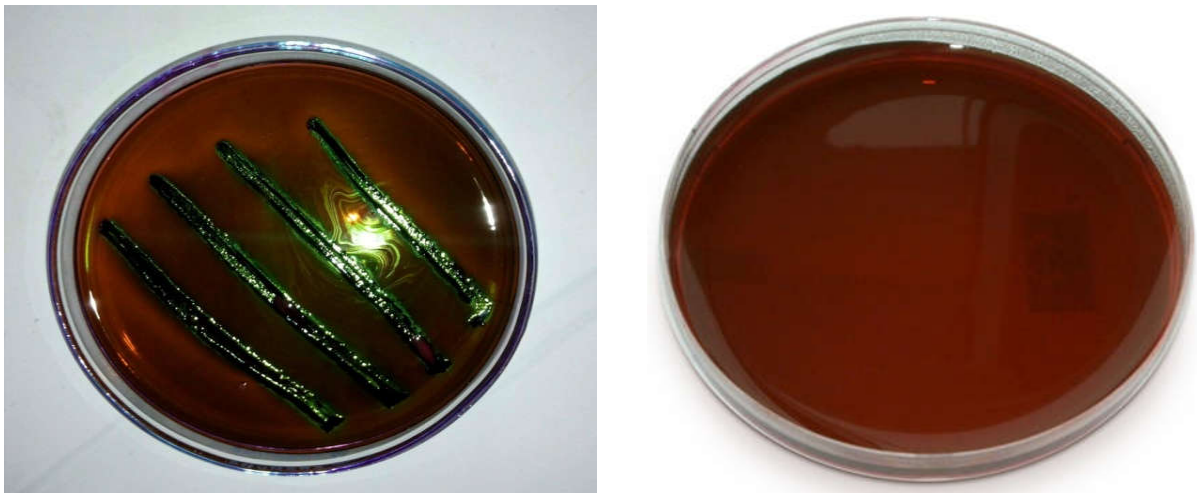


Plate 6: *E. coli* grows on Eosin Methylene Blue (EMB) agar produced metallic sheen colonies (left) compared with control petridis (right)

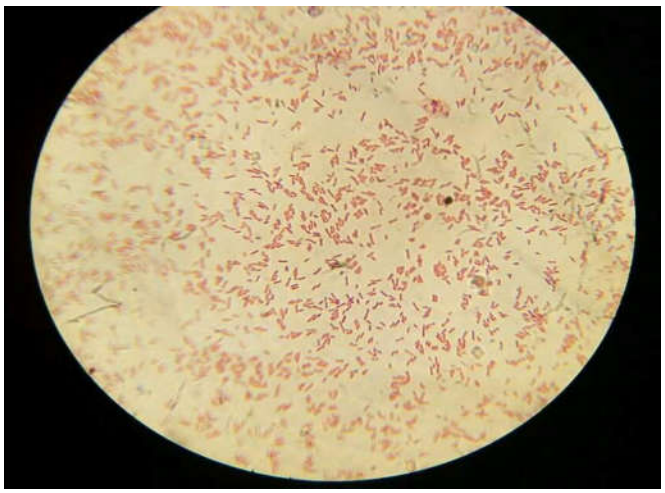


Plate 7: Gram-negative *E. coli*, pink colored, short plump rods, single, paired or in short chain seen under microscope

Plate 8: *Klebsiella spp.* grows on Eosin Methylene Blue (EMB) agar produced pink colonies (left) compared with control petridis (right)

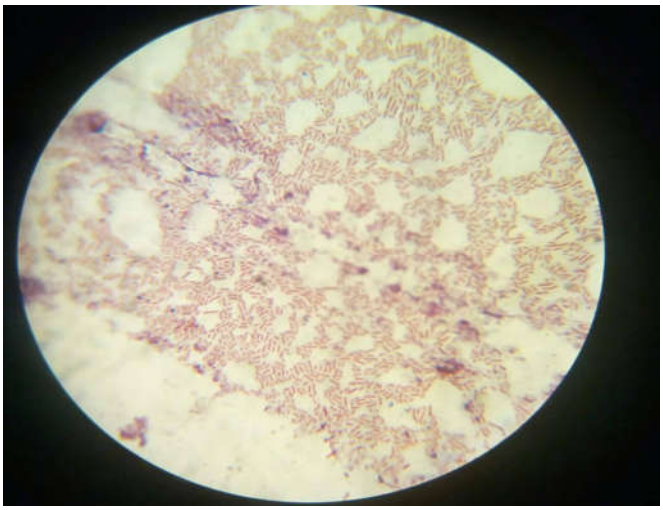


Plate 9: Gram-negative *Klebsiella spp.,* pink colored, short rods, single or paired seen under microscope

Plate 10: *Salmonella spp.* grows on Salmonella-Shigella (SS) agar produced black colonies (left) compared with control petridis (right)

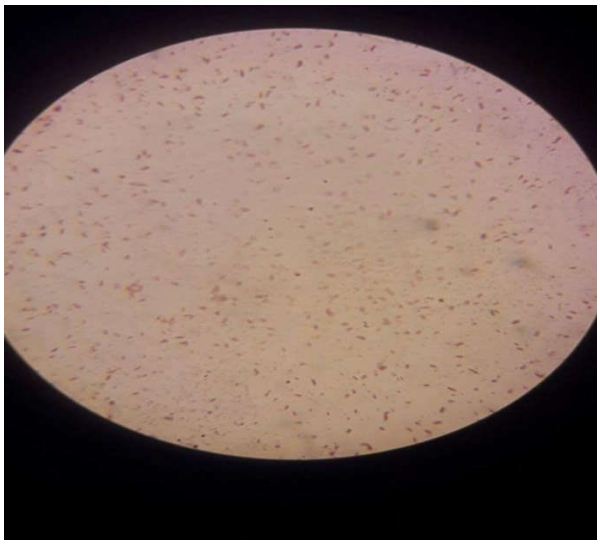


Plate 11: Gram-negative *Salmonella spp*., pink colored, very short plump rods, single or paired seen under microscope

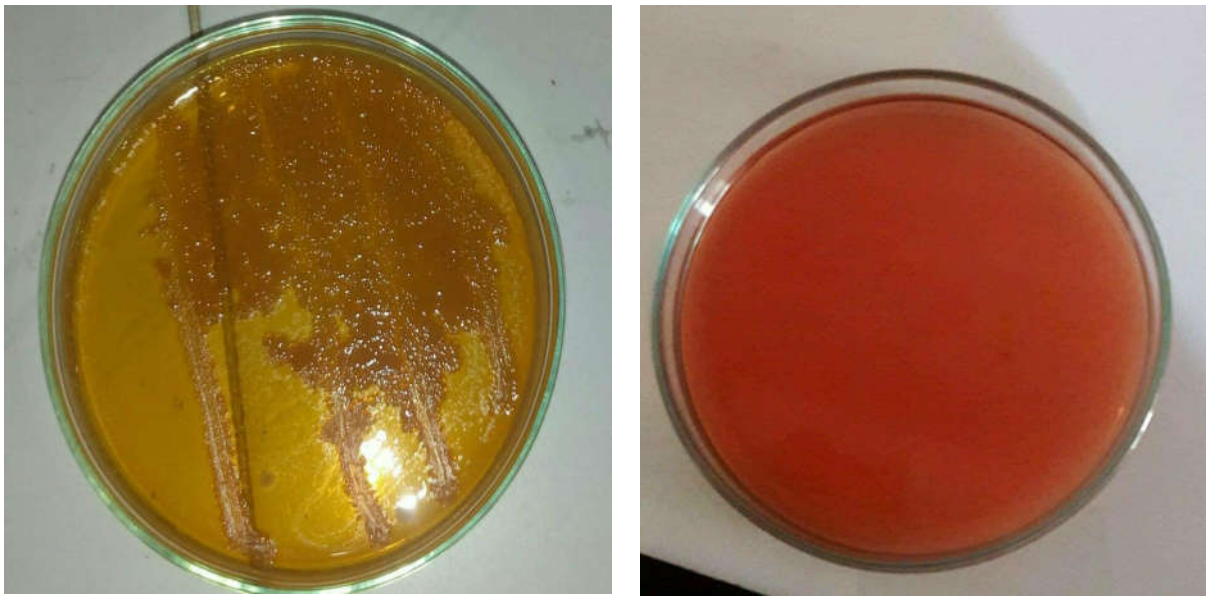


Plate 12: *Shigella spp.* grows on Salmonella-Shigella (SS) agar produced colorless colonies (left) compared with control petridis (right)

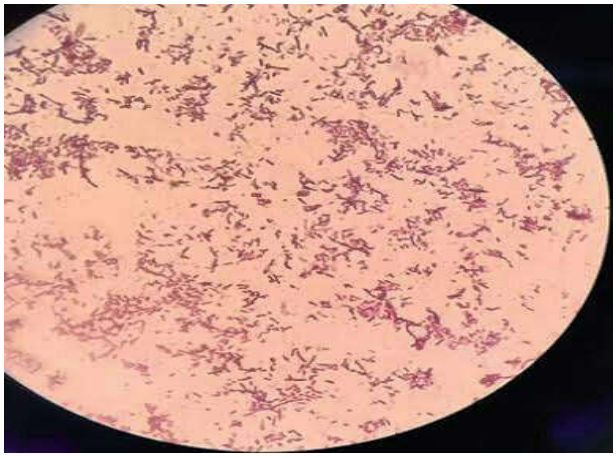
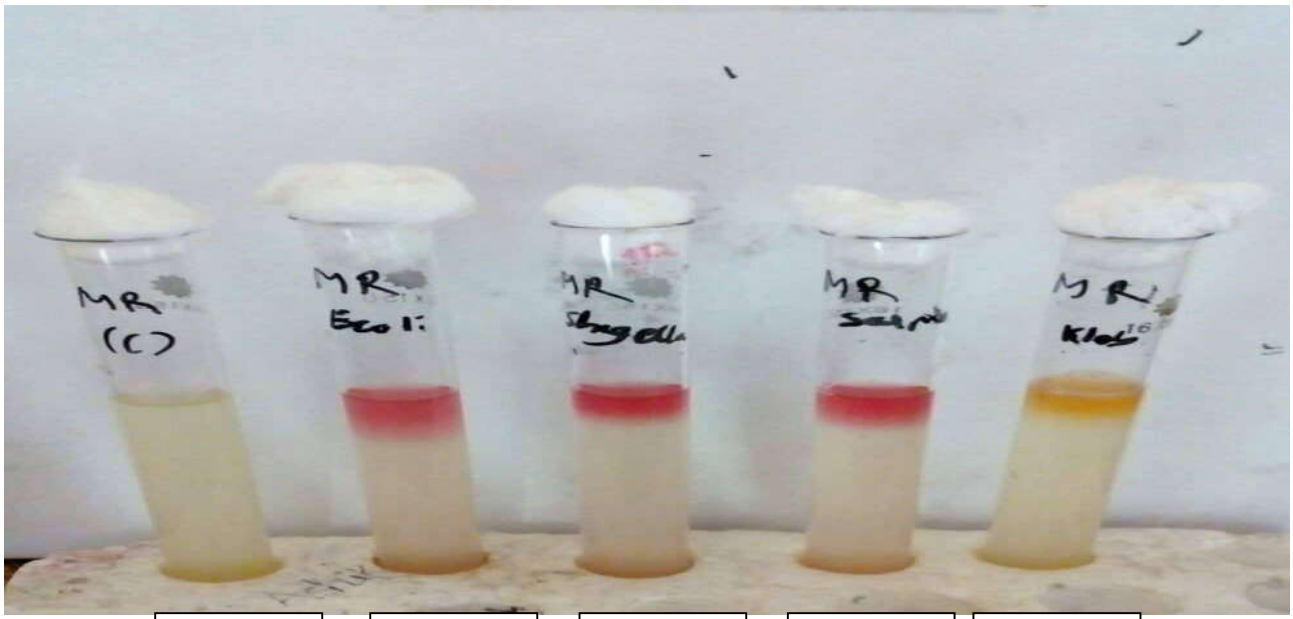


Plate 13: Gram negative *Shigella spp.,* pink colored, small rod-shaped, single, paired or short chain seen under microscope

Control	A	B	C	D

Plate 14: Indole test results (right) A= *Shigella spp.* (positive), B= *E. coli* (positive), C= *Salmonella spp.* (negative), D= *Klebsiella spp.* (negative), and uninoculated control (left)



Control	A	B	C	D

Plate 15: MR test results (right) A= *E. coli* (positive), B= *Shigella spp.* (positive), C= *Salmonella spp.* (positive), D= *Klebsiella spp.* (negative), and uninoculated control (left)

Plate 16: VP test results (right) A= *Shigella spp.* (negative), B= *E. coli* (negative), C= *Salmonella spp.* (negative), D= *Klebsiella spp.* (negative), and uninoculated control (left)

Plate 17: MIU test results (right) A= *Salmonella spp.* (positive), B= *Shigella spp.* (positive), C= *E. coli* (positive), D= *Klebsiella spp.* (positive), and uninoculated control (left)

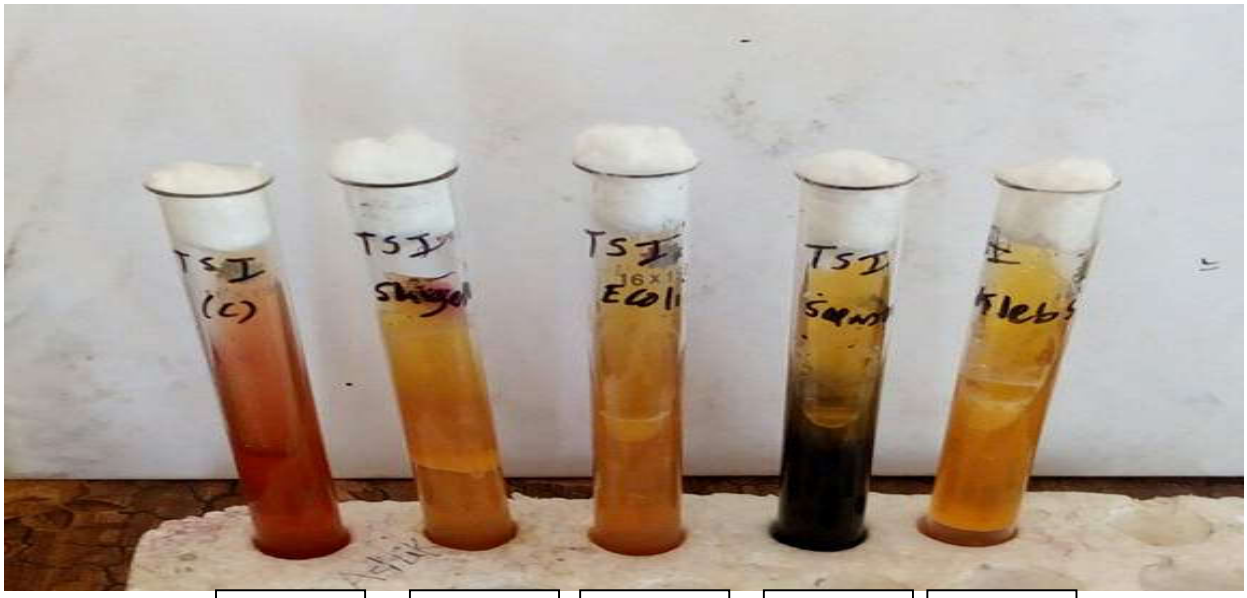


Plate 18: TSI test results (right) A= *Shigella spp.* [No acid and gas production (-), H_2S (-)], B= *E. coli* [Acid and gas production (+), H_2S (-)], C= *Salmonella spp.* [No acid and gas production (-), H_2S (+)], D= *Klebsiella spp.* [Acid and gas production (+), H_2S (-)], and uninoculated control (left)

Plate 19: Citrate utilization test results (left) A= *Shigella spp.* (negative), B= *E. coli* (negative), C= *Klebsiella spp.* (positive), D= *Salmonella spp.* (positive), and uninoculated control (right)

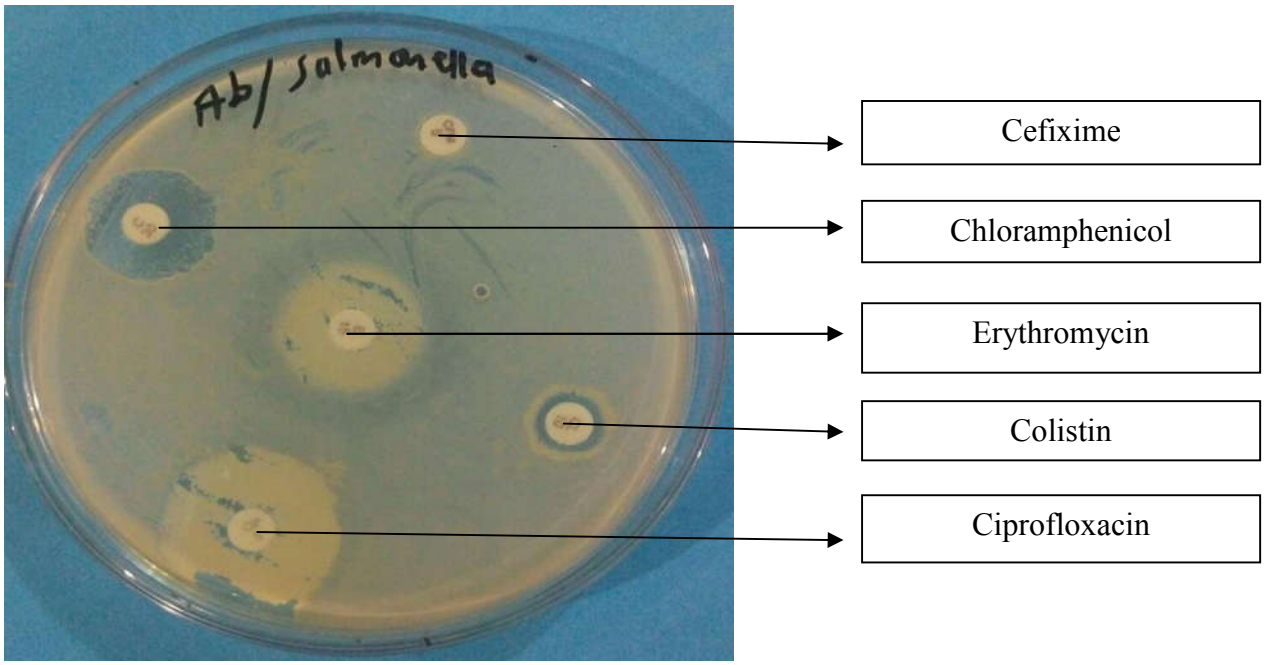


Plate 20: Antibiotic sensitivity test results of *Salmonella spp.* on Mueller-Hinton agar

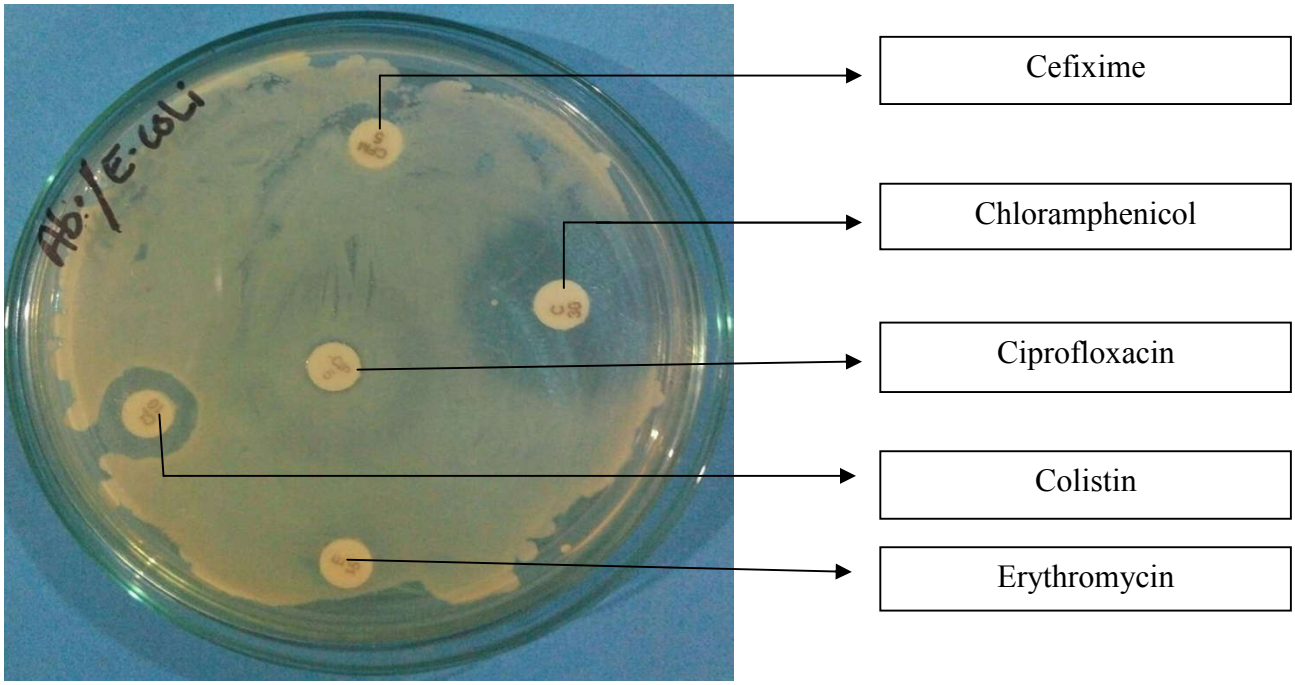


Plate 21: Antibiotic sensitivity test results of *E. coli* on Mueller-Hinton agar

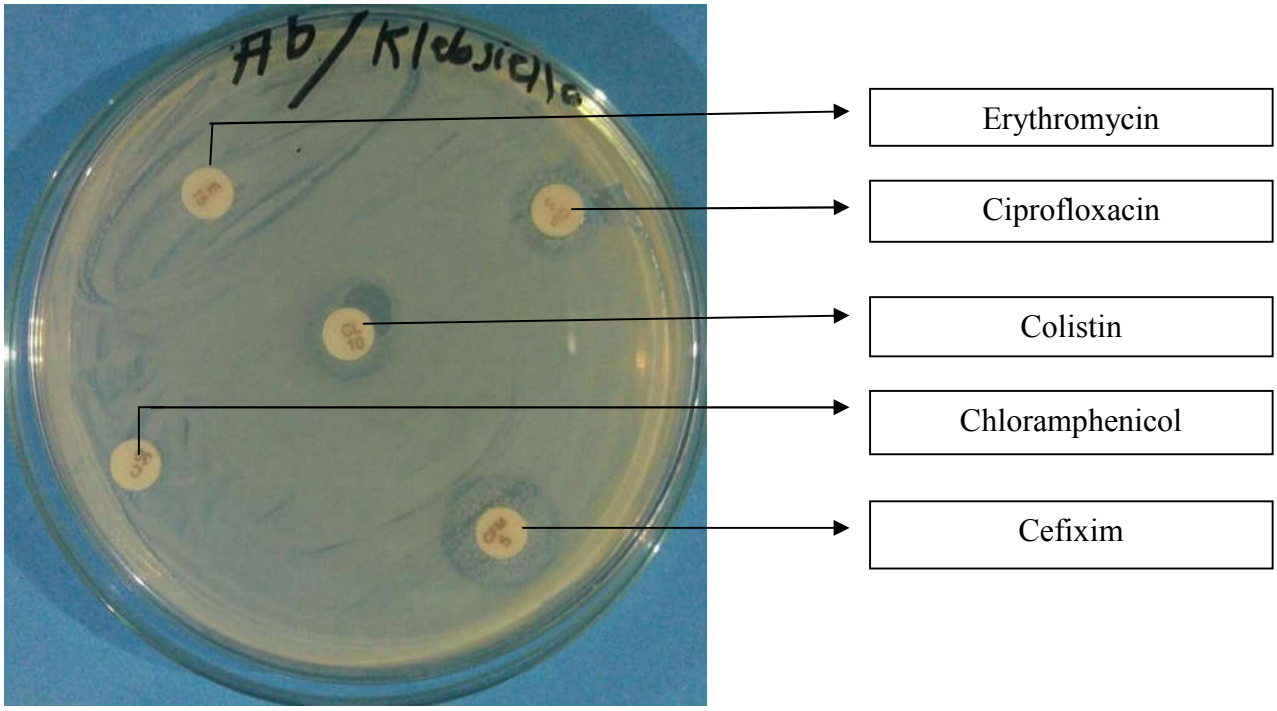


Plate 22: Antibiotic sensitivity test results of *Klebsiella spp.* on Mueller-Hinton agar

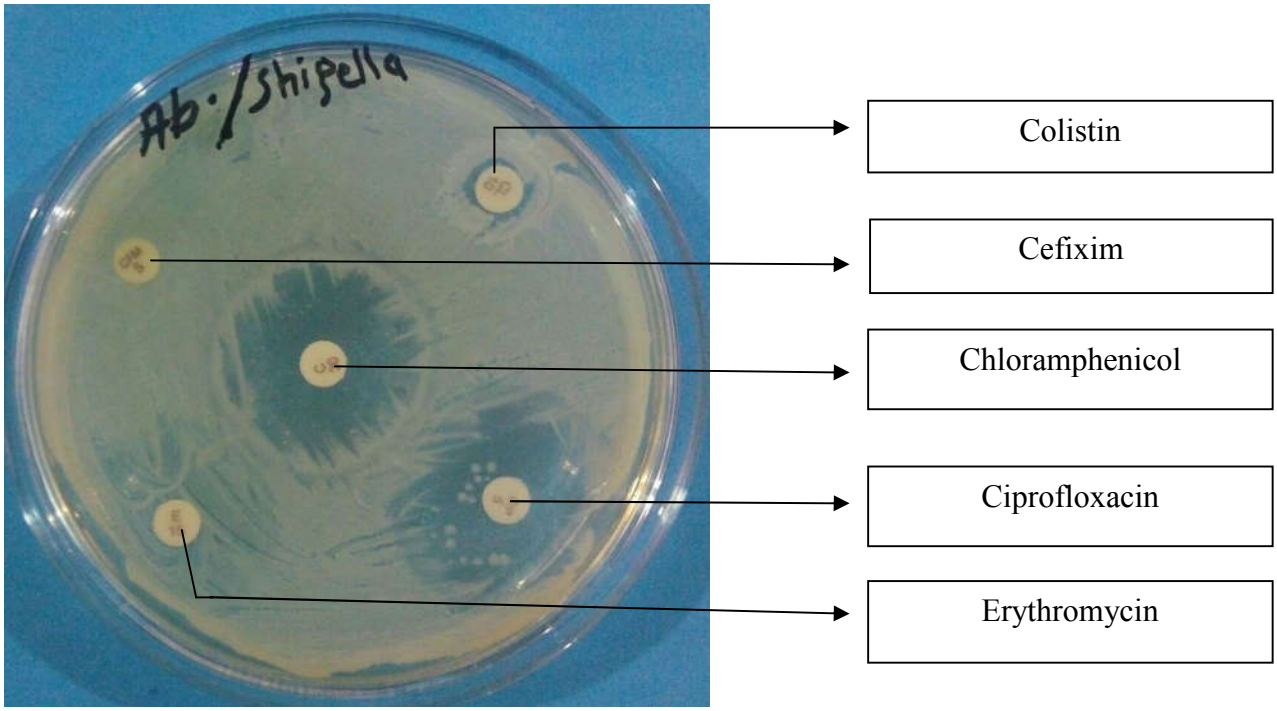


Plate 23: Antibiotic sensitivity test results of *Shigella spp.* on Mueller-Hinton agar

CHAPTER V

DISCUSSION

The present study was conducted for isolation and identification of bacteria from environmental sample with antibiogram study during the period from July to December, 2017. In this study there were 30 environmental samples collected from live bird market in Sadar, Dinajpur district and brought to the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University for bacteriological examination. Isolation and identification of bacteria were confirmed by their colony on different cultural media, staining characteristics and biochemical tests. The bacteriological media employed in this study were selected according to the findings of the authors (Buxton and Fraser, 1977 and Nazir *et al*., 2005). The study revealed that the total prevalence of *E. coli, Salmonella spp., Klebsiella spp. and Shigella spp.* in environmental samples was 26.7%, 33.3%, 16.7% and 23.3% respectively.

The frequency distributions of bacterial isolates in different samples were found variable. Results of the present study indicated that all four different types of bacteria were not present in the same collected from environment sample which was more or less similar to the findings of Sharifuzzaman *et al.*, 2014.

In Gram's staining, the morphology of the isolated Gram-negative, pink colored, short plumped rods *E. coli* arranged in single, pairs or short chains, *Klebsiella spp.* is a Gram-negative, pink colored, small rod shaped, arranged in single, pairs or short chains, *Salmonella spp*. is a Gram-negative, pink colored, very short plumped rod, arranged in single, paired or short chains and *Shigella spp.* is a Gram-negative, pink colored, small rod shaped, arranged in single, pairs or short chains which was supported by Buxton and Fraser, 1977.

In this study, biochemical tests were MIU positive for all isolates. *E. coli, Salmonella spp*. and *Shigella spp.* were MR positive while isolates of *Klebsiella spp.* were MR negative. All isolates were VP negative. Isolates of *E. coli* and *Shigella spp.* were Indole positive while *Salmonella spp.* and *Klebsiella spp.* were Indole negative and these were supported by Cheesebrough, 1984.

The antibiotic sensitivity was indicated by diameter of the zone of growth inhibition by specific antibiotic supported by EUCAST, 2015.

Antibiotic sensitivity pattern of isolated *E. coli* and *Salmonella spp.* was performed against seven commonly used antibiotics belonging to different groups. From antibiotic sensitivity study it was observed that *E. coli* was sensitive to Colistin, moderately sensitive to Ciprofloxacin and these findings were similar to the reports of Nazer *et al*. (2005).

Hemen *et al*. (2012) isolated *Shigella spp., Salmonella spp.* and *Escherichia coli* from poultry litter and tested their antibiotic sensitivity patterns against Septrin, Chloramphenicol, Sparfloxacin, Ciprofloxacin, Amoxycillin, Augmentin, Gentamicin, Pefloxacin, Tarivid and Streptomycin. *Escherichia coli* were found to be resistant against 8 out of 10 drugs against which their antibiotic sensitivity pattern was tested followed by *Shigella spp.* (6 out of 10) and *Salmonella spp.* (3 out of 10). *Shigella spp.* and *Salmonella spp.* were completely resistant to Chloramphenicol, Augmentin, Pefloxacin, Amoxycillin. *Shigella spp.* was also resistant to all the antibiotics except Septrin and Ciprofloxacin. Percentage antibiotics susceptibility pattern of Gram negative bacteria showed that all bacterial isolates (100%) were resistant to Chloramphenicol while most of the isolates were susceptible to Amoxycillin.

In this study, *Salmonella spp., E. coli* and *Shigella spp*. were sensitive to Chloramphenicol (100%) while *Klebsiella spp.* was resistant to Chloramphenicol (100%).

E. coli, Klebsiella spp. and *Shigella spp.* were resistant to Erythromycin (100%) while *Salmonella spp.* was found intermediate to Erythromycin (60%).

CHAPTER VI

SUMMARY AND CONCLUSION

The present study was conducted in order to isolate, identify and characterize with the antimicrobial resistance pattern of isolated bacteria from environmental samples from live bird market, Dinajpur district. All the samples were bacteriologically examined for the isolation and identification.

The study revealed that the total prevalence of *E. coli, Salmonella spp., Klebsiella spp. and Shigella spp.* in environmental samples was 26.7%, 33.3%, 16.7% and 23.3% respectively.

In this study, *Salmonella spp., E. coli* and *Shigella spp*. were sensitive to Chloramphenicol (100%) while *Klebsiella spp.* was resistant to Chloramphenicol (100%).

E. coli, Klebsiella spp. and *Shigella spp.* were resistant to Erythromycin (100%) while *Salmonella spp.* was found intermediate to Erythromycin (60%).

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

i. The presence of *E. coli, Salmonella spp., Klebsiella spp*. and *Shigella spp.* in most of the samples are public health concern.
ii. The antibiotic resistance properties of isolated bacteria can cause serious health hazards because of ineffective treatment of the sufferers by the commonly prescribed antibiotics.

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APPENDICES

APPENDIX 1

Composition of Different Media

1. Nutrient broth (Hi-Media)

Ingredients:	**(g/L)**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (25°C)	7.4±0.2

2. Nutrient agar (Hi-Media)

Ingredients:	**(g/L)**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (25°C)	7.4±0.2

3. MacConkey agar (Hi-Media)

Ingredients:	**(g/L)**
Peptic digest of animal tissue	17.00
Protease peptone	3.0
Lactose monohydrate	10.00
Bile salt	1.5
Sodium chloride	5.0
Agar-agar	15.00
Neutral red	0.03
Final pH (25°C)	7.1± 0.2

4. Salmonella-Shigella agar

Ingredients:	**(g/L)**
Peptic digest of animal tissue	5.0
Beef extract	5.0
Lactose	10.00
Bile salts mixture	8.50
Sodium citrate	10.00
Sodium thiosulphate	8.50
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	15.00
Distilled water	1000 ml
Final pH (25°C)	7.0±0.2

5. Eosine Methylene Blue agar (Hi-Media)

Ingredients:	**(g/L)**
Peptic digest of animal tissue	10.00
Lactose	5.0
Sucrose	5.0
Di-potassium phosphate	2.0
Eosin-Y	0.40
Methylene blue	0.065
Agar	20.00
Final pH (25°C)	7.2±0.2

6. Simmon's Citrate agar

Composition:	**(g/L)**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.00
Bacto bromothymol blue	0.08
7.3±0.1	

7. TSI agar (Hi-Media)

Ingredients:	**(g/L)**
Peptic digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast extract	3.0
Beef extract	3.0
Lactose	10.00
Sucrose	10.00
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol red	0.024
Agar	12.00
Final pH (25°C)	7.4±0.2

8. Muller Hinton agar

Composition:	**(g/L)**
Beef infusion	300.00
Casein acid hydrolysate	17.500
Starch	1.50
Agar	17.00
Final pH (25°C)	

9. MIU medium base (Hi-Media)

Ingredients:	**(g/L)**
Casein enzymatic hydrolysate	10.00
Dextrose	1.0
Sodium chloride	5.0
Phenol red	0.01
Agar	2.0
Final pH (25°C)	6.8±0.2

10. MR-VP medium (Hi-Media)

Ingredients:	**(g/L)**
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH (25°C)	6.9±0.2

11. Peptone water

Ingredients:	**(g/L)**
Peptone	1.0 gm
Distilled water	1000 ml

APPENDIX 2

Preparation of reagents

1. Kovac's reagent

P-dimethyl aminobenzyl dehyde	5 gm
Amyl alcohol	175 gm
Conc. HCL	25 ml

2. V-P reagent 1

5% alpha-naphtolin absolute ethyl alcohol

3. V-P reagent 2

40% potassium hydroxide containing 0.3 creatine. The ingredients were dissolved by heating gently over steam bath. When in solution add 0.05 gm of cotton blue dye.

4. Phosphate buffered solution

Sodium chloride	8 gm
Disodium hydrogen phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water	1000 ml

5. Methyl red solution

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

6. Phenol red solution

0.2% aqueous solution of phenol red

7. Potassium hydroxide solution

40% aqueous solution of KOH

8. Gram stain solution

a. Stock crystal violet

Crystal violet	10 gm
Ethyl alcohol	1000 ml

b. Stock oxalate solution

Ammonium oxalate	1 gm
Distilled water	1000 ml

c. Lugols iodine solution

Iodine crystal	1 gm
Potassium iodide	2 gm

d. Ethyl alcohol 250 ml
e. Acetone 250 ml
f. Counter stain

Safranine	2.5 ml
Ethyl alcohol (95%)	100 ml

Safranine working solution

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