Molecular Characterization of *E. coli* Isolated from Internal Organs of Dead Chicken

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SEMESTER: JULY-DECEMBER, 2018

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IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Dedicated

To My

Beloved Teachers

and

Family Members

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ABSTRACT

Escherichia coli is amongst the common pathogenic bacteria that affects chicken production and commonly found in the gastrointestinal tract of healthy worm blooded mammals including chicken. In this study we determined the occurrence of *Escherichia coli* from internal organs of dead chickens by cultural, biochemical, molecular characterization and their antimicrobial resistance by Kirby- Bauer disc diffusion method. A total of 131 internal organ samples (intestine - 34 liver- 24, heart -23, Lungs-20 and oviduct- 28) were collected. Out of 131 samples 41.22% (n=54) were found to be associated with *E. coli*. Isolated *E. coli* were positive to 16s rRNA gene band PCR (584bp). Sero grouping of *E. coil* were performed by slide agglutination test using commercial *E. coil* specific polyvalent 0 (A-I) antisera, *E. coli* O group B (Factor 0: 8, 19, 84) antisera and *E. coli* O group D (Factor 0: 2, 55, 78) antisera. Among the 36 isolates, 38.88% (n=21) belonged to serogroup B and rest of the isolates 61.11% (n=33) to serogroup D. The most prevalent serogroup identified in this study was serogroup D. The isolated *E. coil* was subjected to antimicrobial susceptibility test. All isolates of *E. coil* were susceptibile to ciprofloxacin, norfioxacin, streptomycim and gentamicin. Out of 54 isolates 100% *E. coil* were resistant to erythromycin, ampicillin, penicillin, amikacin, cephalexin, vancomycin and tetracycline. The finding of the present study revealed the prevalence of multidrug resistant *E. coli* in the samples of the study area. Antimicrobial drug resistance is becoming a major threat to global public health. It is not only a threat in the treatment of poultry diseases associated with *E. coli* but also a potential public health hazard to individual who consume poultry products.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	:	Negative
#	:	Identifying number
%	:	Percentage
@	:	At the rate of
+	:	Positive
µg	:	Microgram
µl	:	Microlitre
^{0}C	:	Degree of celcius
Ag	:	Antigen
Assist	:	Assistant
BA	:	Blood agar
BD	:	Bangladesh
BGA	:	Brilliant green agar
EMB	:	Eosin methylene blue
ER	:	Erythromycin
et al.	:	Associated
etc	:	Etcetera
FAO	:	Food and agricultural organization
Gm	:	Gram

H_2O_2	:	Hydrogen peroxide
H_2S	:	Hydrogen sulphide
HSTU	:	Hajee Mohammad Danesh Science and Technology University
i.e.	:	That is
Ltd	:	Limited
M.S	:	Master of Science
MC	:	Mac-Conkey agar
MSA	:	Mannitol Salt agar
MI	:	Milliliter
MIU	:	Motility indole urease
MR	:	Methyl red
NA	:	Nutrient agar
NB	:	Nutrient broth
No.	:	Number
PBS	:	Phosphate buffer saline
Prof.	:	Professor
PSS	:	Physiological saline solution
RPM	:	Rotation per minute
SC	:	Subcutaneous

SE : Standard error

SL : Serial number

LIST OF ABBREVIATIONS AND SYMBOLS (Contd.)

spp : Species

SSA : Salmonella-Shigella agar

v/v : Volume by volume

vp : Voges-Proskauer

w/v : Weight by volume

CHAPTER 1

INTRODUCTION

Bangladesh is an agriculture based country. Poultry rearing is considered superior to the others in agricultural sector because of an almost assured in a relatively short period of time. Poultry industry which has started during 1980s is an excellent agribusiness (Haque *et al.,* 2001). Over the last decades surprising development has been occurred in this sector (Rahman, 2003). It has become a vital sector for it's generating employment, creating additional income and improving the nutritional level of the country. This sector provides fulltime employment to about 20% and partial employment to about 50% of the rural people (Alam *et al.,* 2003).

The poultry population in Bangladesh is estimated to be 221.39 millions of chickens and 41.23 millions ducks (DLS, 2009). About 50,000 chicken farms and 26,000 duck farms have already been set up in private sector in addition to the Government farms. At present , there are more than 130 hatcheries producing 0.476 million day-old-chicks per week and about one million commercial layer and broiler farms supplying 0.6 million kg of poultry meat and 9.9 million table eggs per weeks (Kabir *et al.,* 2005). Development of poultry sector in Bangladesh is being hampered by a number of factors, of which the diseases are considered as the major factor causing 30% mortality of chicken per year (Das *et al.,* 2005). Intestinal bacteria play an important role on health through their effects on gut morphology, nutrition, pathogenesis of intestinal diseases and immune responses (Samad, 2005). Economic impacts in broilers result from reduced growth, increased feed conversion rates, respiratory disease, mortality, treatment cost and condemnations, while in layers, losses are associated with decreased growth rates, mortality and egg production. The development of *E. coli* infection in poultry there are numerous external and internal factors, such as bad bio-security and poor hygiene; improper incubation; management

disturbances; psychological factors, environmental issues, water quality, subclinical intestinal/respiratory infections, parasite infestations, post vaccinal reactions; immunosuppressive viruses, respiratory viruses, hormonal responses to stress. Keeping *E. coli* out of the flock is not practical because intestinal colonization's universal. However, reducing the numbers of *E. coli* through water, feed, environmental sanitation, and good air quality, and protecting the flock from factors, especially viral infections, reduce the likelihood of colibacillosis. Pelleted feed has fewer *E. coli* than mash, rodent droppings are a source of pathogenic *E. coli*, and contaminated water can contain high numbers of the organism. Recontamination of finished feed should not be overlooked. Chlorination of drinking water and use of closed (nipple) watering systems have decreased the occurrence of colibacillosis and condemnation for airsacculitis.

Among the bacterial diseases, it has been reported that *E. coli* is amongst the common pathogenic bacteria that affects chicken production (Kithsiri *et al.,* 2013; Biswas *et al.,* 2006). *Escherichia coli* are a widely studied bacterium and partially harmless microorganism that thrive in the gut of humans and poultry. Chicks can be infected with *E. coli* by vertical transmission through infected parents or by horizontal transmission through hatcheries, sexing in contaminated hatcheries, cloacal infection and transportation of equipment and feed (Dozois *et al.,* 2000). There are >3100 *E. coli* serovars distributed throughout the world (Vogel, 2000).

The genus *Escherichia* is phylogenic ally clustered in the family of Enterobacteriaceae (JanBen *et al.,* 2001; Maurer *et al.,* 1998). *E. coli* is characterized as ubiquitous, Gram-negative, intracellular, straight rod shaped, non encapsulated, facultative, non-spore forming, and motile with flagella (Colle, 1996; Adams *et al.,* 2000). *E. coli* is potentially responsible for various pathogenic processes in man and animal including poultry (Freeman, 1985). In birds, pathogenic *E. coli* strains have been associated with a range of diseases including septicemia,

enteritis, coligranuloma, omphalitis, arthritis, salpingitis, and complicated air sacculitis (Cheville and Arp, 1978; Vandekerchove *et al.,* 2004). Their role in chronic respiratory diseases in meat chicken breeds has been documented (APHA *et al.,* 1937). *E. coli* is one of the most important aetiological agents causing diseases in poultry which leads to significant economic losses related to high mortality, poor weight gain of infected chicken and poor carcass quality (Ewers *et al.,* 2004). The *E. coli* infection in poultry is usually considered as a secondary infection, which is triggered by various predisposing factors particularly environmental factors including poor ventilation, overcrowding and other biological predisposing factors such as viral or parasitic infections (Vandekerchove *et al.,* 2004).

Appropriate farm management approaches have been suggested to overcome *E. coli* problems in poultry farm but several studies reveal that management approaches are not applicable to prevent incidence of *E. coli* infection in the farms. Serotyping remains the most frequently used diagnostic method in different laboratories, with only limited identification (Tortora *et al.,* 1992). Furthermore, antibiotics that are incorporated into selective media may inhibit the less commonly encountered serotypes. Antibiotics are extensively used in poultry industry either as a growth promoter or to control infectious diseases. The rise in antibiotic resistance has been reported in the past two decade in many countries including Bangladesh (Kapil, 2004). Thus, antibiotics have been introduced into poultry populations mainly for disease treatment for many years. Antibiotics also have been used to control disease outbreaks including *E. coli* infection, thus reducing morbidity and mortality rates due to the infections. Even though antibiotics are highly recommended for therapeutic purpose, certain classes of antibiotics were also used for sub therapeutic reasons to prevent occurrence of disease outbreaks in farms. Emergence of multidrug resistant *E. coli* in chicken arise due to the improper use of antibiotics, thereby reducing the clinical efficacy to antibiotics commonly used in human and veterinary medicine (Hasina *et al.,* 2006).

To minimize the bacterial load in poultry farm strict hygienic measure, legislation with government and social awareness are required. Many producers now expect Veterinarians to be an integral part of their quality assurance programs of foods of animal origin (Hubbert *et al.,* 1996). There are many research works on *E. coli* in the world and Bangladesh regarding the presence of *E. coli* in poultry (Mamun *et al.,* 2017; Kamaruddin *et al.,* 2003; Islam *et al.,* 2003; Rahman *et al.,* 2004; kutubuddin *et al.,* 1973; Khan *et al.,* 2005; Mostafa *et al.,* 1998). Afroja *et al.* (2018) conducted a study to find out the presence of *E. coli* in 131 dead chicken internal organ samples, out of 131, 54 samples were shown positive for *E. coli* (41.22%). The presence of *E. coli* in poultry also revealed by a number of researchers (Hofstad *et al.,* 1992; Hyeon *et al.,* 2012; De *et al.,* 2012) in comparison very limited research works had been carried out in Bangladesh concerning *E. coli* in poultry so far. (Hossain *et al.,* 2015) recently conducted a study to colibacillosis in commercial chickens in Bangladesh. From 55 of layer dead chicken samples 87% *E. coli* were isolated. 87% *E. coli* were also isolated from 15 liver samples by Khalid *et al.,* (1990). 87% (n=13) bacterial isolates out of 11 samples were identified as *E. coli* from lungs by Ewers *et al*. (2003). This indicated a high rate of *E. coli* contamination in poultry and dead bird internal organs of Bangladesh. Therefore, *E. coli* status of a farm needs to be determined for its proper control and management (Akond *et al.,* 2009).

But few works have been done yet in Bangladesh to identify the *E. coli* from different internal organ of dead chicken at a time. Therefore, the present study was designed to isolate and identify *E. coli,* as well as serogroping the isolated *E. coli*.

Considering the above situation the main objectives of the present research work were:

I. To isolate and identify *E. coli* from internal organ of dead chickens using cultural, biochemical and molecular techniques.

II. Serogrouping of *E. coli* present in various internal organ of dead chickens.

III. To determine antibiotic susceptibility and resistance patterns of *E. coli.*

CHAPTER-2

REVIEW OF LITERATURE

The objective of this chapter is to collect selective reviews of the research works accomplished in relation to the present study. Literatures on *Escherichia coli* collected from different internal organs of dead bird related to this study are reviewed:

Abdul *et al.,* (2017) studied to determine the prevalence of colibacillosis in chicken in poultry farms in Mymensingh and Tangail districts. Isolation, identification, and antibiogram profile of *Escherichia coli* were also performed. A total of 25 chickens manifested clinical signs of colibacillosis were collected from five different poultry farms during natural outbreaks. In broiler, the prevalence of csolibacillosis was 0.84%, and in layer, prevalence was 0.80%. The prevalence of colibacillosis was 1.0% and 0.5% in 25-30 days old and 31-35 days old broiler, respectively. In case of layer birds, the prevalence was 0.6% in 40-45 days old bird and 1% in 46-50 days old bird. Identity of the *E. coli* isolate of chicken was confirmed by sugar fermentation, biochemical tests, and polymerase chain reaction assay. Antibiogram profile of *E. coli* isolate of chicken revealed that it was multidrug resistant (resistant against two antibiotics, such as ampicillin and cefalexin). Data of this study suggest that colibacillosis is prevalent in the study areas which underscore the need of implementation of prevention and control measure against this disease.

Gerson *et al.,* (2009) reported that avian pathogenic *Escherichia coli* (APEC) strains cause a great diversity of diseases in birds and are responsible for great economic losses in the avian industry. To date, several studies have been carried out to better understand the APEC pathogenesis for a possible development of tools which could prevent the economics losses caused by these strains. This review discusses the virulence factors described do date to be expressed by these

strains and the advances made to understand and identify virulence determinants present in APEC.

Oguttu *et al.,* (2008) investigated that food animals increases the prevalence of antimicrobial drug resistance among their enteric bacteria. It has been suggested that this resistance can in turn be transferred to people working with such animals, *e.g.* abattoir workers. Antimicrobial drug resistance was investigated for *Escherichia coli* from broilers raised on feed supplemented with antimicrobials, and the people who carry out evisceration, washing and packing of intestines in a high-throughput poultry abattoir in Gauteng, South Africa. Broiler carcasses were sampled from 6 farms, on each of which broilers are produced in a separate 'grow-out cycle'. Per farm, 100 caeca were randomly collected 5 minutes after slaughter and the contents of each were selectively cultured for *E. coli.* The minimum inhibitory concentration (MIC) of each isolate was determined for the following antimicrobials: doxycycline, trimethoprim, sulphamethoxazole, ampicillin, enrofloxacin, fosfomycin, ceftriaxone and nalidixic acid. The same was determined for the faeces of 29 abattoir workers and 28 persons used as controls. The majority of isolates from broilers were resistant, especially to antimicrobials that were used on the farms in the study. Overall median MICs and the number of resistant isolates from abattoir workers (packers plus eviscerators) tended to be higher than for the control group. However, no statistically significant differences were observed when the median MICs of antimicrobials used regularly in poultry and percentage resistance were compared, nor could an association between resistance among the enteric *E. coli* from packers and those from broilers be demonstrated.

Vounba *et al.,* (2018) investigated that avian pathogenic *Escherichia coli* (APEC), a subset of extra intestinal pathogenic *E. coli* (ExPEC), are the etiologic agent of avian colibacillosis, one of the main causes of economic losses in the poultry industry. The aim of this study was to characterize *E. coli* isolated from diseased chickens in Senegal to elucidate their virulence potential and antimicrobial resistance (AMR).

A total of 58 isolates, each from a separate farm, were characterized for AMR, virulence, and AMR genes, phylogroup, serogroup, biofilm formation, and pulsed-field gel electrophoresis, and for two isolates, whole genome sequencing (WGS). Fifty isolates (86.2%) were multidrug resistant. Many AMR genes were detected, including variants of blaCTX-M encoding resistance to third-generation cephalosporins (five isolates 8.6%). Most fluoro quinolonenon susceptible isolates were carriers of mutations in gyrA (Ser83Leu, Asp87Asn, and/or Asp87Tyr) and/or parC (Ser80Ile) genes. Forty-nine (84.5%) isolates exhibited at least one of the virulence markers of APEC, among which 23 (39.7%) were defined as virulent APEC. In addition, 10 isolates, of which 9 were defined as APEC, carried virulence profiles corresponding to ExPEC. Seven isolates, of which six were classified as ExPEC, belonged to phylo-sero group F-O25, and following WGS of two of these isolates, were found to belong to the serotype O25:H1 and to the sequence type ST624. Some isolates classified as ExPEC, including F-O25, were found to strongly produce biofilm, suggesting their capability to persist for long time in the environment. F-O25-isolates, although found in different widely separated farms, formed a single cluster that included clones, suggesting that these isolates may have originated from a common source. Taken together, these results suggest that some *E. coli* involved in chicken colibacillosis in Senegal may pose a human health risk.

Wang *et al.,* (2013) investigated that the resistance of avian *Escherichia coli* to commonly used clinical antibiotics in Hebei Province. It is of significance to reveal the extent and mechanism of drug resistance, as well as to prevent and control colibacillosis. We investigated the resistance of 132 *E. coli* isolates to 5 kinds of antibiotics (including β-lactams, aminoglycosides, tetracyclines, sulfonamides, and chloramphenicol) using the Kirby- Bauer drug susceptibility test, and resistance genes were detected by PCR. The results showed that the *E.coli* had a higher resistance rate to

ampicillin, cephalotin, gentamicin, streptomycin, tetracycline, doxycycline and florfenicol, but it had higher sensitive rate to of the resistance genes, the *TEM*, *aac* (3)-IIa, *tet*A, *Sul1*, *Sul2*, and *forI* had higher positive rates. Therefore drug resistance of avian *E. coli* in Hebei Province is very serious. The resistance mechanisms may include structure changes of the target enzyme mediated by multiresistance genes, and resulting in reduced affinity and increased efflux of antibiotics.

Zahraei *et al.,* (2006) studied to determine that antimicrobial agents were used extremely in order to reducing the enormous losses caused by *Escherichia coli* infections (colibacillosis) in Iran poultry industry. In this investigation fifty avian pathogenic *Escherichia coli* (APEC) strains isolated from broiler chickens with colisepticemia and examined for susceptibility to antimicrobials of veterinary and human significance. *In vitro* antibiotic activities of 32 antibiotic substances against the isolates were determined by disc diffusion test (Kirby Bauer method).Multiple resistances to antibiotics were observed in all the isolates. The highest rate of resistance was against Nalidixic acid (100%), Lincomycin (100%), Erythromycin (97%), Oxytetracyclin (95%), Chlortetracycline (95%), Tetracycline (94%) Doxycycline (88%), Difloxacin (83%), Neomycin (81%), Streptomycin (81%), Trimethoprim Sulphamethoxazole (80%), Kanamycin (77%), Enrofloxacin (76%), Norfloxacin (68%), Ciprofloxacin (67%), Chloramphenicol (67%), Furazolidone (66%), Nitrofurantoin (6%), Amoxicillin (53%) and Ampicillin (47%). Resistance to Gentamicin wasn't observed and to Amikacin, Cefazolin, Colistin, Tobramycin, Ceftizoxime, Cefixime, Lincospectin, Ceftazidime and also Florfenicol were low. This study showed resistance rate against the antibiotics that are commonly used in poultry is very high but against them that are only used in human or less frequently used in poultry is significantly low. These findings confirm significant increase in the incidence of antimicrobial resistance in the *E. coli* strains is most probably due to increased use of antibiotics as feed additives for growth promotion and prevention of

diseases, use of inappropriate antibiotics for treatment of diseases, resistance transfer among different bacteria and possible cross resistance between antibiotics used in poultry. This study also showed that the prevalence of Quinolone-Resistant *Escherichia coli* (QREC) is very high in broiler farms in Tabriz province. The high presence of QREC from broiler chickens probably is due to overuse of enrofloxacin in these farms for therapeutic purposes. The present study suggests introduction of surveillance programs to monitor antimicrobial resistance in pathogenic bacteria is strongly needed because other than animal health problems, transmission of resistant clones and resistance plasmids of *E. coli* from food animals (especially poultry) to humans can occur.

Nfongeh *et al.,* (2018) investigated the prevalence of *Escherichia coli* O157:H7 from chicken droppings produced by free ranged and poultry birds at different locations within Cross River State, Nigeria and their susceptibility to commonly used antibiotics. A total of 360 cloacal swab samples each were randomly collected from poultry (confined) and free ranged (unconfined) chickens. Standard cultural, biochemical, and serological (latex agglutination) methods were used to isolate *E. coli* O157:H7. The isolates were subjected to antimicrobial susceptibility testing using disc diffusion method. Out of a total number of 360 anal swab samples collected from poultry and free-ranged chicken, 24 (6.67%) strayed and 7 (1.94%) poultry samples were positive for *E. coli* O157:H7 and the prevalence values differed significantly ($p<0.05$) among the group of birds .Values for age category among the free-ranged birds also differed significantly ($p<0.05$) with strayed chicks (1-3weeks old) having highest value of 10.89%. Out of the 9 isolates screened, 8 (88.89%) were resistant to tetracycline, 7 (77.78%) to ampicillin and nitrofurantoin and 6 (66.67%) to chloramphenicol. All isolates were resistant to at least one antibiotic. This study reveals that the intestinal track of chicken harbors the bacterial pathogen hence interventions are needed to reduce transmission of *E. coli* O157:H7 via poultry products.

Reem *et al.,* (2016) compared the efficiency of compared the efficiency of PCR detection to culture dependent isolation of *E. coli,* as a model, from the liver and intestinal contents. Further, the incidence differences of *E. coli* isolation from the liver and intestine. Samples were collected from birds suffering from respiratory manifestation and/or diarrhea. Ninety *E. coli* isolates were recovered from 60 birds (52 intestinal and 38 liver samples) by bacteriological culture on selective broth and selective agar. PCR was performed using *pho-A* gene as a general marker for *E. coli* on DNA directly purified from the samples and assigned PCR (a). Negative PCR (a) samples were cultured on broth and another PCR was done, assigned PCR (b). Bacteriological isolation was more sensitive than PCR (a) indicating that inhibitors in the samples could have reduced or totally blocked the amplification capacity of PCR (a), which limited its diagnostic usefulness. PCR (b) was more sensitive than PCR (a) and more practical than bacteriological isolation. Detection of three virulence genes; *iut-A, iss* and *tsh,* showed genotypic variations of avian pathogenic *E. coli* (APEC) isolated from the liver and the intestine. In conclusion, an enrichment step results in more sensitive PCR than culture performed one. Further, general genes rather than virulence genes should be used as an indicator for *E. coli* detection in infected materials.

Maria *et al.,* (2001) reported that *Escherichia coli* O157: H7 has emerged as a new pathogen, causing worldwide disease, death and economic loss. Different studies have revealed important survival characteristics of this pathogen, although there are divergent criteria about its ability to survive in various mayonnaise formulations. We studied the effect of different mayonnaise concentrations (0%, 18 %, 37 % and 56 %) (weight/weight) over the survival of the bacterium in common foods from a neotropical environment (Costa Rica). High Colony Forming Units (CFU)/ml] and low *E. coli* populations (104-106 CFU/ml) were inoculated, (three replicates) in meat, chopped cabbage and poultry, and mixed with commercial mayonnaise to obtain the

concentrations specified. They were incubated at 12 ºC for 24, 48 and 72 hr. The *E. coli* O157: H7 enumeration was done according to a standard methodology. Populations of *E. coli* O157: H7 showed an increasing trend during the first incubation period (48 hr), in all the preparations, regardless of the fat concentration used. Our data indicate that *E. coli* O157: H7 is capable of surviving and growing in meat, cabbage and poultry mixed with mayonnaise, independently of its concentration.

Momtaz *et al.,* (2012) studied to detect the distribution of antibiotic-resistant genes in *Escherichia coli* isolates from slaughtered commercial chickens in Iran by PCR. The investigated genes included *aadA1, tet* (A)*, tet* (B)*, dfrA1, qnrA, aac (3)-IV, sul1, bla* SHV*, bla* CMY*, ere* (A)*, catA1* and *cmlA*. According to biochemical experiments, 57 isolates from 360 chicken meat samples were recognized as *E. coli*. The distribution of antibiotic-resistance genes in the *E. coli* isolates included *tet* (A) and *tet* (B) (52.63%), *dfrA1, qnrA, catA1* and *cmlA* (36.84%) and *sul1* and *ere*(A) (47.36%), respectively. Nine strains (15.78%) were resistant to a single antimicrobial agent and 11 strains (19.29%) showed resistance to two antimicrobial agents. Multi-resistance which was defined as resistance to three or more tested agents was found in 64.91% of *E. coli* strains. The results indicate that all isolates harbour one or more of antibiotic resistance genes and that the PCR technique is a fast, practical and appropriate method for determining the presence of antibiotic-resistance genes.

Arshad *et al., (2008) experimented that Peganum harmala* seed extracts have been frequently reported to possess antibacterial potential through in vitro studies, but in vivo studies have acquired less attention. The present study was therefore designed to investigate its efficacy on the course of colibacillosis and effects of long-term feeding on selected parameters of general health in chickens. Two experiments were conducted in this regard. Experiment 1 (a pilot study) was performed to determine the dose of a field strain of *Escherichiacoli* (O1:K1) required to induce clinical symptoms in 4- and

15-d-old specific-pathogen-free chickens. A successful induction of colibacillosis, in terms of clinical signs, mortality, and pathological lesions in addition to reisolation of the pathogen was observed by inoculating 4- and 15-d-old chicks with 4.3 log10 and 6.4 log10 cfu of *E. coli*, respectively, by intraperitoneal injection. Using these doses experiment 2 (main study) consisting of a single experiment with 3 parts was performed. Parts A and B generated the information regarding efficacy of the extract against infection in 4- and 15-d-old chickens applying different treatment schemes, whereas the effects of continuous feeding of the extract were assessed in part C. Whereas no protective effect of the extract could be recorded in young chickens, significant differences ($P < 0.05$) with regard to BW, clinical score, gross lesion score, and total granulocyte counts were observed in 15-d-old birds. Bacterial recovery per gram of tissue and reisolation frequency were lower in treated birds. The continuous feeding of the extract for 6wk resulted in an augmentation in relative liver weight and depletion in alkaline phosphatase, protein, albumin, and globulin. It can be concluded that the crude extract of *Peganum harmala* possesses limited antimicrobial activity against *E. coli* in vivo and longterm continuous feeding may induce undesired effects. Furthermore, the study underlines the value of in vivo experiments and the diverse picture that herbal products, in this case *Peganum harmala*, may deliver by testing them against specific pathogens.

Cason *et al.,* (2000) investigated water samples from a commercial broiler processing plant were tested for coliforms, *Escherichia coli,* and salmonellae to evaluate the numbers of suspended bacteria in a multiple-tank, counter flow scalder. Water samples were taken from each of three tanks on 8 different days after 6-week-old broilers had been processed for 8 h. Coliforms and *E. coli* were counted using Petrifilm, and the most probable number (MPN) of salmonellae was determined both in water samples and in rinses of defeathered carcasses that were removed from the processing line immediately after taking the water samples. Mean coliform concentrations in tanks

1, 2, and 3 (the last tank that carcasses pass through before being defeathered) were 3.4, 2.0, and 1.2 log10 (CFU/ml), respectively. *E. coli* concentrations followed the same pattern with means of 3.2, 1.5, and 0.8 in tanks 1, 2, and 3, respectively, with significant differences (*P*, 0.02) in the concentrations of both coliforms and *E. coli* between the tanks. Sixteen of 24 scald-water samples were positive for salmonellae with a geometric mean of 10.9 MPN/100 ml in the positive samples. Salmonellae were isolated from seven of eight water samples from both tanks 1 and 2, but in only two of eight water samples from tank 3, the last tank that carcasses pass through. It appears that most bacteria removed from carcasses during scalding are washed off during the early part of scald.

Kubra *et al.,* (2015) studied to evaluate lesions seen in the tissues in terms of histopathology and to investigate the apoptotic cells seen in the tissues when *E. coli* outbreak occurred in a poultry farm. A total of 48 Lohmann White strains (53 weeks old) were submitted to the laboratory for necropsy. Microbiologic and histopathology examinations were done on the samples taken from tissues. Apoptotic cells were determined in all of the tissues. The number of apoptotic cells increased as the tissue damage increased.

Akinlabi *et al.,* (2008) experimented *Escherichia coli (E. coli)* isolated from septicaemic clinical cases between October 2005 and March 2006 from eleven poultry farms in Abeokuta, South West Nigeria were tested for their in vitro antimicrobial drug sensitivity pattern. The *E. coli* were isolated from two hundred and fifty samples comprising of intestine, kidneys, lungs, hearts, ovary, spleen and colo-rectum from diseased chickens (mostly commercial layers) submitted for post-mortem examination. Eight of the poultry farms studied disclosed that they had used between 3 to 7 different antimicrobial agents either for treatment, prophylaxis or as growth promoters within the studied period. The result indicated that the 39 *E. coli* isolates showed nineteen different multidrug-resistant patterns to Nitrofurantoin (100 µg), Cefuroxime (20 µg), Norfloxacin (10 µg),

Cotrimoxazole (50 µg), Ciprofloxacin (5 µg), Nalidicic acid (30 µg), Chloramphenicol (10 µg), Ampicillin (10 µg, 25 µg), Ofloxacin (5 µg), Penicillin G (5 i.u), Amoxylin (20 µg), and Cloxacilin (5 µg, 10 µg) discs that were tested.

Jakee *et al.,* (2012) isolated the pathogenic strains that cause distinct syndromes of diarrheal disease. In this study we used a collection of 28 *E. coli* isolated from cattle, buffaloes and chicken obtained in the same geographical area to perform a detailed analysis of the molecular epidemiology of O157 and non O157 strains by using PCR for identifying general similarities and differences in the genetic composition of *E. coli* populations. The isolates showed a degree of diversity in PCR of *stx1*, *stx2* and *eae.* 12 strians had *stx1* gene, 16 strains had *stx2* gene and 10 strains had *eae* gene.76.92% of cattle isolates had *stx2* and 38.46% possessed both *stx1* and *stx2*, also *stx1* was detected from 38.46%of the examined cattle strains. Using restriction enzyme (RE) it is clear that between 3 to 6 fragments were obtained with hind III digested DNA of chicken and buffalo isolates and between 3 to 5 fragments of cow isolates. Among EcoR-I between 5 to 9 fragments were obtained with digested genomic DNA of chicken and cattle and 7 to 9 fragments of buffalo isolates. Hind-III and EcoR-I ribotyping could reveal only minor differences in non O157:H7 strains belonging to the same serotypes. This indicates that ribo typing which is regarded to be a useful tool for epidemiological investigation, was not able to discriminate between STEC isolates belonging to the same serotype. It is believed that in future a better understanding of moleculer diversity of *E. coli* strains of different sources would provide and assist the design of approaches to epidemiological studies.

Christian *et al.,* (2018) investigated to evaluate the prevalence, AR patterns and the characterization of relevant resistance genes in Extended Spectrum β-lactamases (ESBL) and Amp C *E. coli* from large poultry farms in Ecuador. Sampling was performed from June 2013 to July 2014 in 6 slaughterhouses that slaughter broilers from 115 farms totaling 384 flocks. Each sample of collected caeca was streaked onto

TBX agar supplemented with cefotaxime (3 mg/l). In total, 176 isolates were analyzed for antimicrobial resistance patterns by the disk diffusion method and for *bla*CTX-M, *bla*TEM, *bla*CMY, *bla*SHV, bla*KPC*, and *mcr-1* by PCR and sequencing. ESBL and AmpC *E. coli* were found in 362 flocks (94.3%) from 112 farms (97.4%). We found that 98.3% of the isolates were multi-resistant to antibiotics. Low resistance was observed for ertapenem and nitrofurantoin. The most prevalent ESBL genes were the *blaCTX-M* (90.9%) *blaCTX-M-65*, *blaCTX-M-55* and *blaCTX-M-3* alleles. Most of the AmpC strains presented the *blaCMY-2* gene. Three isolates showed the *mcr-1* gene. Poultry production systems represent a hotspot for antimicrobial resistance in Ecuador, possibly mediated by the extensive use of antibiotics. Monitoring this sector in national and regional plans of antimicrobial resistance surveillance should therefore be considere.

Ronco *et al.,* (2017) studied to investigate the genetic diversity among *E. coli* isolates collected on poultry farms with colibacillosis issues, using whole genome sequencing. Hundred and fourteen bacterial isolates from both broilers and broiler breeders were whole genome sequenced. The majority of isolates were collected from poultry with colibacillosis on Nordic farms. Subsequently, comparative genomic analyses were carried out. This included in silico typing (sero- and multi-locus sequence typing), identification of virulence and resistance genes and phylogenetic analyses based on single nucleotide polymorphisms. In general, the characterized poultry isolates constituted a genetically diverse population. However, the phylogenetic analyses revealed a major clade of 47 closely related ST117 O78:H4 isolates. The isolates in this clade were collected from broiler chickens and breeders with colibacillosis in multiple Nordic countries. They clustered together with a human ST117 isolate and all carried virulence genes that previously have been associated with human uropathogenic *E. coli.* The investigation revealed a lineage of ST117 O78:H4 isolates collected in different Nordic countries from diseased broilers and breeders. The data indicate that the closely

related ST117 O78:H4 strains have been transferred vertically through the broiler breeding pyramid into distantly located farms across the Nordic countries.

Xie *et al.,* (2016) investigated cefotaxime-resistant *E.coli* isolates were obtained from retail meat products purchased in Shenzhen, China, during the period November 2012 to May 2013. Thirty seven of these 55 isolates were found to harbor a *bla*CTX-M gene, with *bla*CTX-M-1 group being the most common type. The *bla*CMY-2 was detected in 16 isolates, alone or in combination with other ESBL determinants. Importantly, the *fosA3* gene, which encodes fosfomycin resistance, was detected in 12 isolates, with several being found to reside in the conjugative plasmid which harbored *bla*CTX-M gene. The insertion sequence IS*26* was observed upstream of some of the *bla*CTX-M-55 and *fosA3* genes. Conjugation experiments showed that *bla*CTX-M from 15 isolates were transferrable, with IncI1 and IncFII being the most prevalent replicons. High clonal diversity was observed among the *bla*CTX-M-producers, suggesting that horizontal transfer of the *bla*CTX-M genes among *E. coli* in retail meats is a common event, and that such strains may constitute an important reservoir of *bla*CTX-M genes which may be readily disseminated to other potential human pathogens.

Vandyousefi *et al.,* (1989) studied *E coli* strains, isolated from clinical materials of diseased chickens are reported. About 1119 strains belong to 12 serological groups, 086, 0111, 026, 02a, 055, 0128.084, 021, 078, 0119, 04a, and 0126. The results of pathogenicity tests using the isolated avian serotypes. A syndrome resembling naturally occurring coli septicaemia was reproduced by the intravenous inoculation of the *E. coli* serotypes, most commonly isolated from field cases. The results of invitro sensitivity tests showed that all serotypes were sensitive to Furazolidone and Nalidixique acid .

Robab *et al.,* (2003) designed to determine their resistance to antimicro-bial agents used in Tehran poultry industry. By using Mast

Diagnostic kit only 06 serotype was identified. Multiple resistances to antibiotics were observed in all isolates. The highest rate of resistance was against Tetracycline (94%), followed by Rifampicin (90%), and Oxytetracycline (80%). Fifty *E. coli* isolates showed 10 different patterns of resistance to the antimicrobial agents used in this study. The most com-mon antimicrobial resistance pattern of these isolates was Enrofloxacin / Trimethoprim-Sulphamethoxazole / Tiamulin / Flumequine / Sulphadiazine / Oxytetracycline / Rifampicin. The present study confirms significant increase in the incidence of resistance in *E. coli* isolated from poultry which is probably due to increased use of antibiotics as feed additives for growth promotion and prevention of dis-ease, resistance transfer among different bacteria and possible cross resistance between antibiotics used in domestic animals and those used in human medicine.

Nwankwo *et al.,* (2014) designed to determine the cloacal feacal carriage and occurrence of antibiotic resistant *E. coli* isolates from chicken fed with and without antibiotic supplemented feeds. Cloacal feacal swabs (n = 200) were aseptically obtained from two poultry farms in Abakaliki metropolis, Ebonyi state of Nigeria, and these were inoculated on MacConkey and cystine-lactose-electrolyte-deficient (CLED) media and incubated at 37°C for 18 to 24 h. Suspected colonies of *E. coli* growing on the agar media were subcultured, purified and further characterized using standard microbiology techniques. Antibiogram was investigated using the Kirby-Bauer disk diffusion method as per the clinical laboratory standards institute (CLSI) criteria. A total of 45 *E. coli* was isolated from the 200 cloacal feacal swab samples used for this study. Overall, 28% of *E. coli* were isolated from chicken fed with feed supplemented with antibiotics while only 17% of *E. coli* was isolated from chicken that received feed without antibiotics supplements. All the *E. coli* isolates showed varying rates of resistance and susceptibility to the tested antibiotics. Our results strongly reveal the occurrence of antibiotic resistant *E. coli* from chicken fed with and without antibiotic supplemented feeds. It is

very critical that the continuous use of antibiotics in poultry production be strictly monitored, controlled and discouraged in order to contain the emergence and spread of antibiotic resistant bacteria through poultry production.

Mahmoud *et al.,* (2015) designed to assess the contamination of enterovirulent *Escherichia coli* with table eggs at Mansoura, Egypt. A total of 100 commercially available table eggs were randomly collected from various groceries and supermarkets at Mansoura, Egypt. The samples were screened for the presence of *E. coli* through conventional bacteriological and biochemical analyses followed by confirmation by polymerase chain reaction. Overall, 18% (n=18/100) samples were found to be contaminated with one or more *E. coli* isolates. All possible *E. coli* colonies (n=52) appeared on MacConkey agar plates during the screening process were picked for further analysis. Among the 52 suspected isolates, 24 were confirmed as *E. coli*, which were further serotyped using polyvalent *E. coli* antisera. In this study, 9 different *E. coli* serotypes namely O78, O114, O2, O44, O1, O125, O128, O124 and O26 were identified. Out of these 9 serological strains, 5 (O78, O2, O44, O125, O124 and O26) were positive for *eae* gene, and 3 (O44, O1 and O128) were positive for *stx2* gene. Two serological strains (O44 and O1) were positive for both *stx1* and *eae* genes, while O125 and O114 were positive for *stx2* and *eae* genes. Two strains (O78 and O128) were found to be positive for all three genes (*stx1*, *stx2* and *eae*).Ensuring hygienic measures can effectively reduce the microbial load from table eggs.

Chabba *et al.,* (2014) studied to evaluate the effect of partially protected sodium butyrate (PSB) on performance, digestive organs, intestinal villi and *E. coli* development in broilers chickens. Nine hundred twenty four one-day-old mixed Cobb® chicks were divided in 3 treatments with 7 replicates each in a randomized block design. Treatment T1 was a control diet without any growth promoter, treatment T2 was the control diet plus colistin at 100,000 IU/kgBW and treatment T3 was the control diet with PSB at 700 ppm. Chicks were

fed in mash form for 3 phases: starter (1-14 days), grower (15-28 days) and finisher (29-42 days). There were no significant differences on performance among all treatments in starter phase. Chicks fed PSB in grower and finisher phases had the highest weight gain and the best feed conversion ratio. Relative digestive organs weights were not affected by treatment in any phase. Jejunum and small intestine relative lengths of birds fed PSB and colistin at 14 days were longer than those of birds fed control diet. Jejunal villi of birds fed PSB and colistin at 42 days were higher than those in birds fed the control diet. Colistin produced the deepest crypts and the lowest villi height/crypt depth ratios in all intestinal segments at 14 days. Intestinal *E. coli* growth was not affected by any treatment. These data indicate that partially protected sodium butyrate and colistin improves performance, colistin as an antibiotic growth promoter and PSB by improving intestinal villi development in broilers chickens.

Roberto *et al.,* (2000) studied that newly hatched specific pathogen-free chicks were dosed with a suspension of Bacillus subtilis spores prior to challenge with Escherichia coli O78:K80, a known virulent strain associated with avian colibacillosis, 24 h later. A single oral inoculum of 2:5 _ 108 spores was sufficient to suppress all aspects of *E. coli* O78:K80 infection. Colonisation of deep organs was reduced by a factor of over 2 log10 whilst colonisation of the intestine, as measured by direct caecal count, was reduced over 3 log10. Shedding of *E. coli* O78:K80 was measured by semi-quantitative cloacal swabbing and was reduced significantly for the duration of the experiment, 35 days. B. subtilis persisted in the intestine although with decreasing numbers over the same period. Challenge with the same dose 5 days after pre-dosing with spores overcame any suppressive effect of the spores.

Ashgan *et al.,* (2015) experimented to evaluate the prevalence and the molecular detection characterization of *E. coli* serotype O157:H7 recovered from raw meat and meat products collected from Saudi Arabia. During the period of 25th January 2013 to 25th March 2014,

370 meat samples were collected from abattoirs and markets located in Riyadh, Saudi Arabia ‘‘200 raw meat samples and 170 meat products’’. Bacteriological analysis of the meat samples and sero-typing of the isolated *E. coli* revealed the isolation of 11 (2.97%) strains of *E. coli* O157:H7. Isolation of *E. coli* O157:H7 in raw beef, chicken and mutton were 2%, 2.5%, and 2.5%, respectively, however, there was no occurrence in raw turkey. The incidences of *E. coli* O157:H7 in ground beef, beef burgers, beef sausage, ground chicken and chicken burgers were 5%, 10%, 0.0%, 5% and 0.0%, respectively. The multiplex PCR assay revealed that 3 (27.27%) out of 11 *E. coli* O157:H7 isolates from raw beef.

Esther *et al.,* (2008) stated that *E. coli* infections in avian species have become an economic threat to the poultry industry worldwide. Several factors have been associated with the virulence of *E. coli* in avian hosts, but no specific virulence gene has been identified as being entirely responsible for the pathogenicity of avian pathogenic *E. coli* (APEC). Needless to say, the chicken would serve as the best model organism for unraveling the pathogenic mechanisms of APEC, an extra intestinal pathogen. Five-week-old white leghorn SPF chickens were infected intra-tracheally with a well characterized APEC field strain IMT5155 (O2:K1:H5) using different doses corresponding to the respective models of infection established, that is, the lung colonization model allowing re-isolation of bacteria only from the lung but not from other internal organs, and the systemic infection model. These two models represent the crucial steps in the pathogenesis of APEC infections, including the colonization of the lung epithelium and the spread of bacteria throughout the bloodstream. The read-out system includes a clinical score, path morphological changes and bacterial load determination. The lung colonization model has been established and described for the first time in this study, in addition to a comprehensive account of a systemic infection model which enables the study of severe extra intestinal pathogenic *E. coli* (ExPEC) infections. These in vivo models enable the application of various

molecular approaches to study host–pathogen interactions more closely. The most important application of such genetic manipulation techniques is the identification of genes required for extra intestinal virulence, as well as host genes involved in immunity in vivo. The knowledge obtained from these studies serves the dual purpose of shedding light on the nature of virulence itself, as well as providing a route for rational attenuation of the pathogen for vaccine construction, a measure by which extra intestinal infections, including those caused by APEC, could eventually be controlled and prevented in the field .

Roseliza *et al.,* (2016) investigated that Colibacillosis is an important disease affecting the poultry industry in many countries, caused by the Avian Pathogenic *E. coli* (APEC): it manifests as various clinical signs. It contributes significantly to economic loss for poultry farmers as a result of high mortality and morbidity in poultry. To overcome this, antibiotics have been widely used to eliminate *E. coli* infection in poultry farms in recent years. Treatment with antibiotics has been considered as a vital regimen to control *E. coli* infection at the farm level for many years. However, high frequency of antibiotic resistance of *E. coli* isolates from chicken has become the centre of attention due to public health importance. The aim of the present study is to determine the multidrug resistant profiles of *E. coli* strains isolated from chicken. *E. coli* isolates obtained from clinical cases were re-identified and classified by conventional methods. Multidrug resistant profiles against 13 different antibiotics of 125 *E. coli* isolates were determined by using disk diffusion method according to Clinical Laboratory Standard Institute (CLSI). Antibiogram revealed that 81.6%of the *E. coli* isolates showed multidrug resistant profiles to different antibiotics. Most of the *E. coli* isolates were highly resistant to erythromycin (52.8%), followed with tetracycline (52.0%), spectinomycin (39.2%), trimethoprim (38.4%) and flumequin (37.6%). Out of 125 isolates tested, 19.2% were resistant to more than eight antibiotics, with one isolates found to be multidrug resistant to most of antibiotics except polymyxin B. These findings also demonstrated that

most of the isolates were susceptible to antibiotics commonly used for *E.coli* infections treatment in poultry with lowest resistant score against polymyxin B (92.8%) and colistin (92.0%). Moderate resistant profiles were observed towards amoxycilin (25.6%), apramycin (16%), kanamycin (8.8%) and streptomycin (8.0%). High percentage of multidrug resistance was found among the *E. coli* isolated from chicken as an indicator to more serious problems in animal health.

Shaohui *et al.,* (2014) studied to elucidate the extent of food contamination by enter hemorrhagic *Escherichia coli* (EHEC) O157 in Eastern China. A total of 1100 food and animal fecal samples were screened for EHEC O157. Molecular characterization of each isolate was determined. EHEC O157 was isolated as follows: pig feces, 4% (20/500); cattle feces, 3.3% (2/60); chicken feces, 1.43% (2/140); pork, 2.14% (3/140), milk1.67% (1/60); and chicken meat, 1.67% (1/60). A genes were present in 26.7% (8/30), 40% (12/30), 63.3% (19/30), and 50% (15/30) of the O157 isolates, respectively. Molecular typing showed that strains from fecal and food samples were clustered into the same molecular typing group. Furthermore, the isolates from pork and pig feces possessed the same characterization as the clinical strains ATCC35150 and ATCC43889. Biofilm formation assays showed that 53.3% of the EHEC O157 isolates could produce biofilm. However, composite analyses showed that biofilm formation of EHEC O157 was independent of genetic background. Animal feces, especially from pigs, serve as reservoirs for food contamination by EHEC O157, it is important to control contamination by EHEC O157 on farms and in abattoirs to reduce the incidence of food borne infections in humans.

Emmanuel *et al.,* (2013) studied to demonstrate extended spectrum betalactamases (ESBLs) production from Escherichia coli isolates of poultry origin in Abakaliki, Ebonyi State, Nigeria. A total of 200 feacal and cloacal swab samples from broiler chickens were analyzed in this study. Swab samples were cultured and incubated at 37oC for 18-24 hrs, and colonies growing on agar plates were identified by standard microbiology techniques. Antibiogram was conducted on all isolates by

Kirby-Bauer disk diffusion method and ESBL production was evaluated as per the double disk synergy test (DDST) method. ESBL production was detected in 16 *E. coli* isolates from feacal swab samples and 20 *E. coli* isolates from cloacal swab samples. All isolates showed high resistance to ceftazidime, cefotaxime, sulphamethoxazole-trimethoprim, ampicillin, gentamicin, ciprofloxacin, tetracycline and ofloxacin. The frequency of ESBL production in *E. coli* isolates from broiler chickens in this study has clinical implications for the treatment of bacterial related diseases in human population, thus the need to control the use of antibiotics for non-human purposes in this environment.

Manges *et al*., (2015) experimented that the shared pathogenic potential of human ExPEC and avian pathogenic *E. coli* suggest that these extraintestinal *E. coli* may be derived from the same bacterial lineages or share common evolutionary roots. The consistent observation of specific human ExPEC lineages in poultry or poultry products, and rarely in other meat commodities, supports the hypothesis that there may be a poultry reservoir for human ExPEC. The time lag between human ExPEC acquisition (in the intestine) and infection is the fundamental challenge facing studies attempting to attribute ExPEC transmission to poultry or other environmental sources. Even whole genome sequencing efforts to address attribution will struggle with defining meaningful genetic relationships outside of a discrete food-borne outbreak setting. However, if even a fraction of all human ExPEC infections, especially antimicrobial-resistant ExPEC infections, is attributable to the introduction of multidrug-resistant ExPEC lineages through contaminated food product (s), the relevance to public health, food animal production and food safety will be significant.

Joseph *et al*., (2014) reported that the response of some hematological parameters in neonatal albino rats to different filtrate

dosages of cell free extracts of *Escherichia coli* 0157:H7 obtained from human, cattle and chicken sources were investigated. At higher levels of extracts administration [1.0ml and 2.0ml], Packed cell volume (PCV), Hemoglobin count [HBC] and Red Blood cell Count [RBC] had highest decrease with human faeces isolates [IHF] from 49.9 to 48.0%, 12.0-10.4g dl-1 and 7.7 to 7.6 × 109 ml^{-1} respectively. White Blood count [WBC], Erythrocytes sedimentation Rate [ESR), Lymphocytes and Neutrophils counts on the other hand had highest increase with cattle isolates [ICT] ranging from 4.0 to 8.2 × 106 ml^{-1}, 3.0 to 6.0×106 ml^{-1}, 2.20 to 3.80 × 106 ml^{-1} and 1.60 to 3.90 × 106 ml^{-1} respectively. Statistical analysis showed significant difference [$P<0.05$] in the effects of the filtrate dosages on neutrophils and PCV in all the isolates. Significant difference [$p<0.05$] on the effects of the various dosages of human isolates [IHF] poultry [IPB] and cattle [ICT] isolates on both WBC and ESR were also observed . However, no significant difference was observed [$P>0.05$] with regards to the general effects of isolates on the hematological parameters studied. Cell free extracts of *E. coli* 0157:H7 used were found to have an overall negative effect on the hematological indices. Albino rats can therefore be recommended for use as a reliable small animal model to study host early responses as well as the role of bacterial virulence factors in the induction of hematological diseases.

Victor *et al*., (2015) determined the occurrence of *Escherichia coli* in feces of healthy free-range chickens in Grenada, by culture and their antimicrobial resistance by Kirby-Bauer disc diffusion method. A total of 202 *E. coli* isolates from cloacal swabs were tested for susceptibility to antimicrobial drugs of veterinary and human importance. Of the 202 *E. coli* isolates, 35.1% (71 of 202) showed resistance to at least one out of the 12 antibiotics tested whereas 31% (22 of 71) showed resistance to two or more antibiotics. The most common resistance observed was against tetracycline (29.7%), followed by ampicillin (6.9%), gentamicin (5.9%), and trimethoprim-sulfamethoxazole (3%) while the least common resistance observed was against amoxicillin-clavulanic acid

(0.5%), cephalothin (1%), and ciprofloxacin (1%). Since the sampled chickens do not receive any medication, it is evident that the environment is the plausible source of resistant bacteria. There is a risk of resistant bacteria being transmitted to humans via contaminated poultry products and the environment.

Hemsley *et al*., (1967) investigated the biochemical and serological findings of *E. coli* strains of avian origin isolated mainly in Ontario (95%). A considerable increase in the incidence of disease associated with *E. coli* has become evident in Ontario. Perhaps this is due, in part, to increased awareness of the disease entities described in the literature. There is little doubt that coli septicemia and other *E. coli* disease syndromes are difficult to define because of their complex pathogenesis. In this respect, an effort was made to isolate strains of *E. coli* from cases in which it was considered significant as a cause of disease, as done by workers in Great Britain. The association of certain carbohydrate fermentation patterns, particularly of dulcitol and salicin, was studied in relation to the virulence of the organisms.

CHAPTER-3

MATERIALS AND METDODS

The present research work was conducted between July, 2017 to December, 2018 in the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh for the identification of bacteria from dead chicken internal organs by different microbiological methods and molecular characteristics was conducted in the NIB (National Institute of Biotechnology) for confirmation. The detailed outline of materials and methods are given below.

3.1 Materials

3.1.1 Selection of study area

Samples of different internal organ of dead bird were collected from different poultry farms under sadar, Dinajpur district of Bangladesh (Basherhat, Mohabalipur, Gobindapur) and brought to the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University for laboratory analysis.

3.1.2 Collection of sample

Samples were collected from different internal organs of dead poultry of different age and brought to the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur. Sterile container was use during collection of sample. A total of 131chickens samples were collected for the present research work.

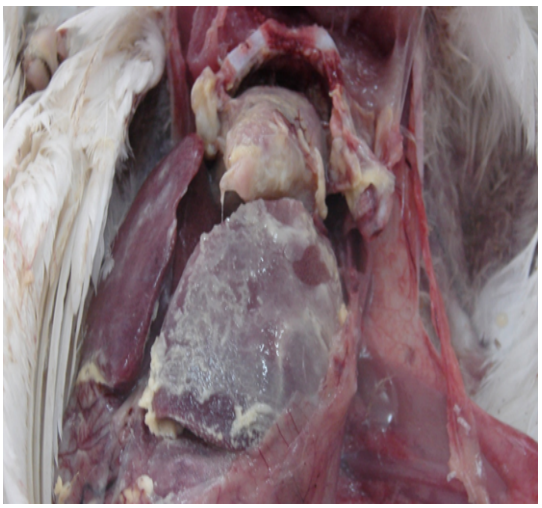


Figure no 1: Extreme perihepatitis caused by *E. coli*

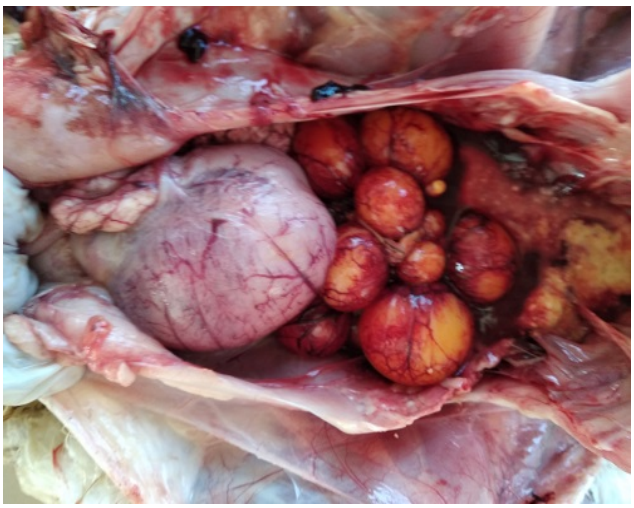


Figure no 2: Collection of oviduct sample of chicken

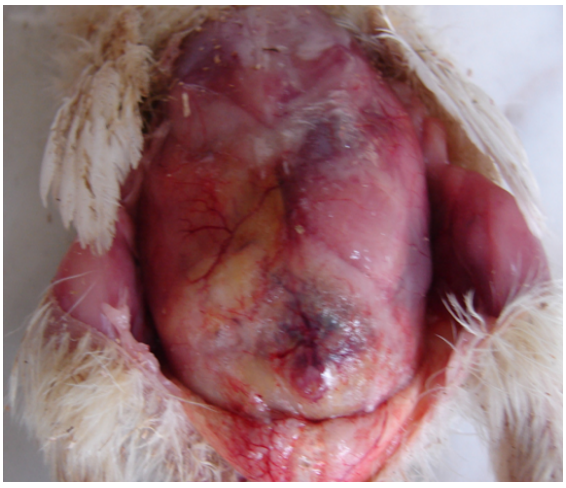


Figure no 3. Omphalitis caused by

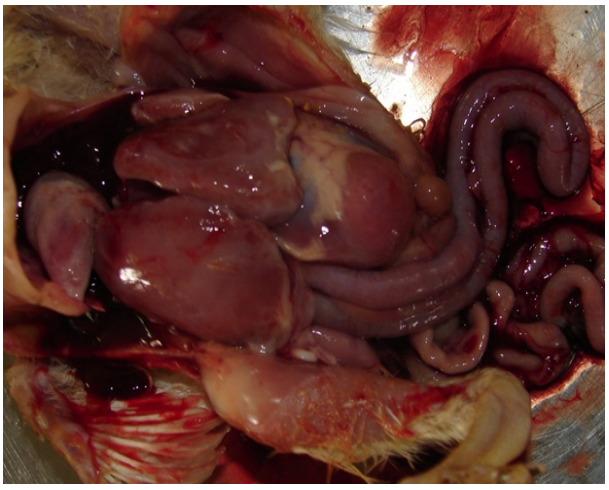


Figure no 4: Septicemia caused

Figure no 5: Collection of intestinal sample of chicken from

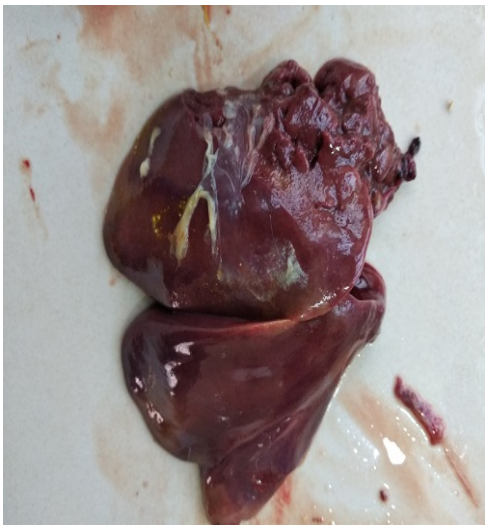


Figure no 6: Collection of liver sample of chicken from farm

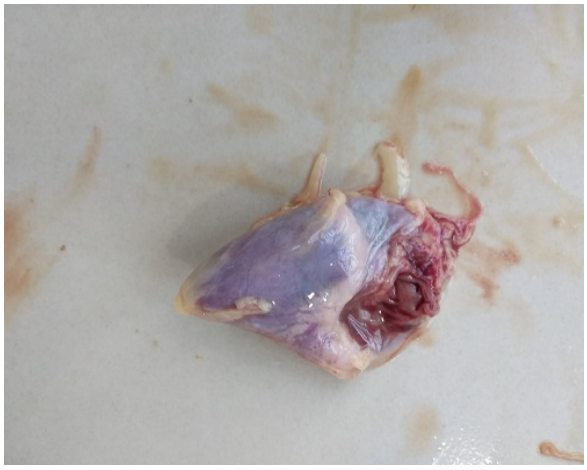


Figure no 7: Collection of heart sample of chicken from farm

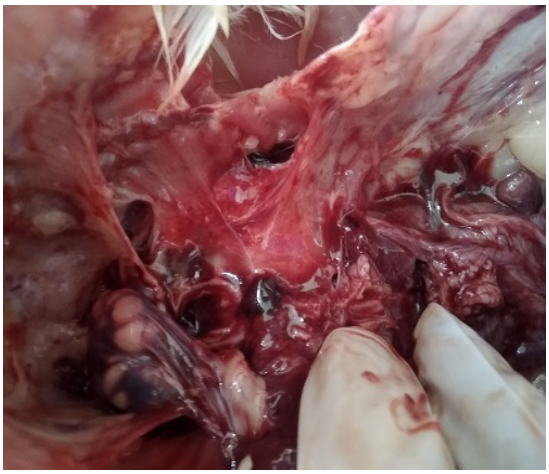


Figure no 8: Collection of lung sample of chicken from farm

3.1.3 Transportation and preservation of samples

The samples were carried out to the laboratory in an ice box contained ice and processed for the isolation, identification and characterization of bacteria subsequently. And the remaining samples were stored at 4ºC for future use.

3.1.4 Media for culture

Different bacteriological culture media and reagents were used for isolation and identification and also propagation of bacteria from different internal organs of dead chicken samples. Those culture media and reagents that were used in this experiment are mentioned below:

3.1.4.1 Liquid media

1. Nutrient broth (Difco)
2. 1% Pepton Water (Difco)
3. Pepton broth (Difco)

1. Nutrient broth

Nutrient broth was used to grow the organisms from the samples collected from the study areas before performing biochemical test and antibiotic sensitivity test (Cheesebrough. 1984).

3.1.4.2 Solid media for culture

1. Nutrient agar medium, (HI-MEDIA, India)
2. MacConkey agar medium, (Difco)
3. Eosin Methylene Blue, (EMB) (HI-MEDIA, India)
4. Salmonella-Shigela agar (Difco)
5. Blood agar medium, (HI-MEDIA, India)
6. Mueller-Hinton agar (Difco)

Solid media

1. Nutrient agar medium, (HI-MEDIA, India):
   Nutrient agar is used for cultivating of non-fastidious microorganisms (Cheesbrough, 1985).
2. Eosin Methylene Blue, (EMB) (HI-MEDIA, India):
   EMB contains dyes that are toxic for Gram positive bacteria and bile salt which is toxic for Gram negative bacteria other than coliforms. EMB is the selective and differential medium for coliforms. Escherichia coli: Blue-black bulls eye; may have green metallic sheen. Escherichia coli colonies grow with a metallic sheen with a dark center.
3. MacConkey Agar medium, (Difco):
   A differential medium for the isolation of coliforms and intestinal pathogens in water and biological specimens. Mac-Conkey agar is a culture medium designed to selectively grow Gram-negative bacteria and differentiate them for lactose fermentation. Lactose-fermenting organisms grow as pink to brick red colonies with or without a zone of precipitated bile. Non-lactose fermenting organisms grow as colorless or clear colonies (Cheesbrough, 1985).
4. Salmonella-Shigela Agar (Difco)
   Salmonella Shigella Agar (SS Agar) is a selective and differential medium widely used in sanitary bacteriology to isolate Enterobacterieace from feces, urine, and fresh and canned foods that produce Clear, colorless and transparent colony (Cheesbrough, 1985).
5. Mueller-Hinton agar (Difco)
   Mueller Hinton Agar is used for the determination of susceptibility of microorganisms to antimicrobial agents. It has become the standard medium for the Bauer Kirby method and its performance is specified by the NCCLS.

3.1.4.3 Media for biochemical test

1) Sugar fermentation broth

2) Oxidase test
3) Catalase test
4) Indole broth
5) Methyl red broth
6) Voges-proskauer broth
7) Simmon's citrate agar (HI-MEDIA, India)
8) Triple sugar iron agar (HI-MEDIA, India)
9) Motility indole urease (HI-MEDIA, India)

3.1.5 Reagent

1. Crystal violet dye
2. Gram's iodine
3. Alcohol
4. Safranin
5. Saline
6. Iodine solution
7. Kovac's reagent
8. Methyl- red solution
9. 3% H_2O_2
10. P – Amino dimethylanilin oxalate
11. Phenol red
12. Phosphate buffered saline (PBS) solution
13. Potassium tellurite (1%)

3.1.6 Glassware and appliances

The different types of important equipment used for this work are listed as follow down-

1. Distilled water
2. Sterile bent glass or plastic spreader ods.
3. Micropipette (1-5µl; 5-50µl; 10-100µl; 50-500µl; 100-1000µl)
4. Freeze (-20°C)
5. Refrigerator (4^0C)
6. Spirit lamp
7. Water bath

8. Vortex Mixture
9. Labeling tape
10. Experimental test tube
11. Stopper
12. Petri dish
13. Conical flask.
14. Durham's tube
15. Slide
16. Microscope
17. Cotton
18. Immersion oil
19. Toothpick
20. Autoclave
21. Thermometer
22. Incubator
23. Jar
24. Beaker
25. Cylinder
26. Electric balance
27. Filter paper
28. Spirit lamp and
29. Bacteriological loop etc.

3.1.7 Materials used for bacterial genomic DNA isolation

- TE buffer
- 10% (w\v) Sodium dodecyl sulfate (SDS)
- 20 mg\ml protinase k (stored in small single-use aliquots at -20^{0}C)
- 3 M Sodium Acetate, pH 5.2
- 25:24:1 Phenol/Chloroform/Isoamyl alcohol
- Isopropanol
- 70% Ethanol
- 95% Ethanol
- 1.5 ml microcentrifuge tubes.

3.1.8 Materials used for polymerase chain reaction

Table no 1: PCR reaction mixture for 16s rRNA.

Master Mix	12.5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Nano Pure Water	9.5 µl
DNA	2.0 µl
Final Volume	25 µl

- Primers used for PCR:

E coli Primer sequences 5'- 3'	PCR Product size	Source
16SrRNA *E coli*:(16E1)5- GGG AGT AAA GTT AAT ACC TTT GCT C-3 (F)	584 bp	Primary: Tsen *et al*-1998 Secondary: Gautam *et al*-2012 Indian j ani. Sci.,82(2):204-208
16SrRNA *E coli*:(16E2):5-TTC CCG AAG GCA CAT TCT-3 (R)		
16SrRNA *E coli*:(16E3):5-TTC CCG AAG GCA CCA ATC-3 (R)		

- Product size: 584bp
- Thermal Cycler (Thermo cycler, ASTEC, Japan)
- 2% agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask
- Electrophoresis apparatus (Biometra standard power pack P 2T)

- 100 bp DNA size marker
- Bromphenicol blue of loading bufter.
- Ethidium bromide (0.5 µg/ml)
- Distilled water
- UV trans-illuminator

3.1.9 Antimicrobial sensitivity discs

- To determine the drug sensitivity pattern of different bacterial isolate with different types of commercially available antimicrobial discs (Oxoid Ltd., UK) were used. The followings are the antibiotics that were tested against, the selected organism with their disc concentration.

Table no 2: Antimicrobial agents with their discs concentration.

S/N.	Name of antimicrobial agents	Disc concentration (µg /disc)
1.	Cloxacillin (COX)	1µg
2.	Vancomycin (VA)	30 µg
3.	Cephalexin (CN)	30 µg
4.	Ciprofloxacin (CIP)	5µg
5.	Amoxicillin (AMX)	30µg
6.	Chloramphenicol (C)	30µg
8.	Ampicillin (AMP)	25µg
9.	Penicillin (P)	10 units
10.	Amikacin (AK)	30µg
11.	Tobramycin (TOB)	10µg
13.	Kanamycin (K)	30µg
14.	Gentamycin (GEN)	10µg
15.	Neomycin (N)	30µg
16.	Colistin (CL)	10µg
18.	Norofloxacin (NX)	10µg
21.	Erythromycin (E)	15µg

Note: µg = Microgram

3.2 Methods

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

3.2.1 Experimental layout

The experimental work was divided into three steps: The first step was performed for the isolation and identification of the organisms of the collected sample using cultural, staining and biochemical characteristics. The second step was performed to molecular characterization for selective isolates by 16s rRNA gene sequencing. The third step was conducted for the determination of antibiotic sensitivity and resistant pattern of isolated organisms of various samples by using different antibiotic discs available in the market. The layout of the diagrammatic illustration of the present study is shown in figure 9..

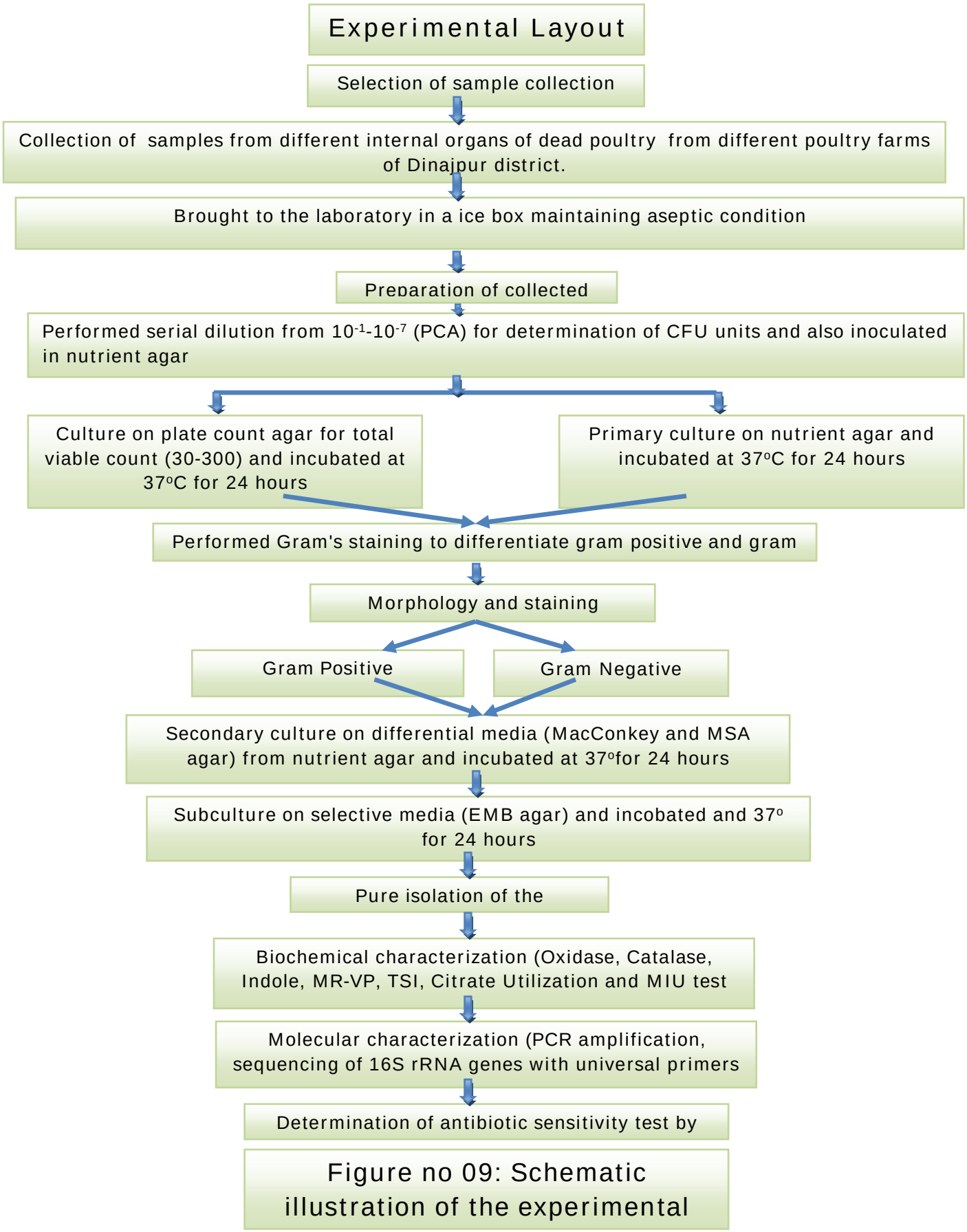


Figure no 09: Schematic illustration of the experimental

3.2.2 Laboratory preparations

All items of required glassware including test tubes, pipettes plate, slides, cylinder, flasks, conical flasks, glass and vials soaked in a household dishwashing detergent solution ('Trix' Recket and Colman Bangladesh Ltd.) overnight. Contaminated glassware was disinfected with 2% sodium hypochlorite solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized either by dry heat at 160^0C for 2 hours or by autoclaving for 15 minutes at 121^0C under 15 lbs pressure per sq inch. Autoclaved items were dried in a hot air oven over at 50^0C. Disposable plastic (items e.g. micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50^0C for future use.

3.2.3 Sampling and processing of samples

Proper care was taken during the sampling procedure to prevent contamination of sample. The samples tubes were completely tied at the time of sampling that prevent contamination. After came to the laboratory 1ml urine sample was taken from each sample and mixed with 9ml PBS (Phosphate Buffer Solution) in each test tube for serial dilution (10-1 to 10-7) and 50 µl samples were seeded on nutrient agar, plate count agar, using spread plate method. The plating was done in the laminar flow to maintain aseptic conditions and the medium were then incubated at 37^0C for 24 h.

3.2.4 Plan of the experiment work at a glance

All of those samples were collected from different Poultry Farm of Dinajpur District with a ice containing ice box. Then all of the samples were transferred to the microbiological laboratory of department of Microbiology, HSTU, Dinajpur, Bangladesh. Samples were primarily inoculated into Nutrient agar. Subsequently Nutrient agar, Blood agar, EMB agar, SS agar, MacConkey agar were employed and specific

biochemical tests were done for isolation and identification of bacteria. Molecular characterization also performed for genome sequencing. At last performed antibiotic sensitivity test with the pure isolated bacteria.

3.2.5 Preparation of culture media

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

3.2.5.1 Nutrient broth media

Thirteen grams of dehydrated nutrient broth (HI-MEDIA, India) was suspended into 1000 ml of distilled water and boil to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121°C and 1.2 kg/cm^2 pressure for 15 minutes. The sterility of the medium was checked by incubating at 37°C for overnight and stored at 4°C in aerator for further use. (Cater 1979).

3.2.5.2 Nutrient agar media

Twenty eight grams of nutrient agar powder (HI-MEDIA) was dissolved in 1000 ml of cold distilled water in a flask. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile Petridish and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use. (Cater 1979).

3.2.5.3 Blood agar media

Forty grams of Blood agar base (HI-MEDIA, India) powder was dissolved in 1000 ml of distilled water. The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes and 45°C. Then 5-10 % sterile defibrinated blood was added to the medium and distributed to sterile petridishes and allowed to solidify. After

solidification of the medium in the petridishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use. (Cater 1979).

3.2.5.4 Eosin methylene blue agar

Thirty six grams of EMB agar base (HI-MEDIA, India) was added to 1000 ml of water in a flask and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121°C for 15 minutes and I to 50°C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petridish sized and allowed to solidify. After solidification of the medium in the petridishes, these were incubated at 37° C for overnight to check their sterility and petridishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use. (Cater 1979).

3.2.5.5 MacConkey agar

51.5 grams MacConkey agar base (HI- MEDIA, India) powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121°C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^{0}C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass Petridishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petridishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petridishes, these were incubated at 37°C for overnight to check their

sterility and used for cultural characterization or stored at 4°C in refrigerator for future use. (Cater 1979)

3.2.5.6 Salmonella Shigela (SS) agar

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by boiling for 5 minutes. After boiling the medium was put into water bath at 45^0- 50^0C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (12°C) for 15 minutes. Cool to 50°C and aseptically add 50 ml concentrated Egg Yolk Emulsion (FD045) and 3 ml sterile 3.5% Potassium Tellurite solution (FD047) or 50 ml Egg Yolk Tellurite Emulsion (FD046). If desired add rehydrated contents of 1 vial of BP Sulpha Supplement (FD069). Alternatively 1 vial of Fibrinogen Plasma Trypsin Inhibitor Supplement (FD195) may be used per 90 ml medium in place of Egg yolk Tellurite Emulsion (FD046) for identification of coagulase, positive Stapylococci. Mix well and pour into sterile Petri plates. (Baird-Parker AC., 1962).

3.2.5.7 Mueller-Hinton agar (Difco)

Suspended 38 gm of the medium in one liter of distilled water. Then heated with frequent agitation and boil for one minute to completely dissolve the medium. Then autoclaved at 121°C for 15 minutes. Pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth.

3.2.5.8 Sugar media

The medium consists of peptone water of which fermentable sugar was added to the proportion of 1%. One gram of Bacto peptone (HI-MEDIA) and 0.5 gram of sodium chloride were added in 100 ml of distilled water. The medium was boiled for 5 adjusted to P^H 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2% solution was added to peptone water and then in 5 ml into cotton plugged test tubes containing Durham's fermentation and placed invertedly. These were then sterilized by autoclaving at 1.2 kg /cm^2 121°C for 15 minutes. The sugars used for fermentation were prepared separately 10% solution in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A mild heat was necessary to dissolve the sugar. The sugar solutions were sterilized in Arnold steam sterilizer at 100°C for 30 minutes for 3 consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture containing 4.5 ml sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it at for 24 hours (Cater 1979).

3.2.6 Preparation of reagents

3.2.6.1 Methyl-Red solution

The indicator MR solution was prepared by dissolving 0.1 gram of Bacto methyl-red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water. (Merchant and Parker 1967).

3.2.6.2 Methyl-Red

A quantity of 17 gms of MR-VP medium (HI-MEDIA) was dissolved in 1000 ml of distilled water, dispensed in 2 ml amount in each tube and the tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight o check their sterility and then in refrigerator for future use. (Merchant and Parker, 1967).

3.2.6. 3 Alpha-Naphthol solution

Alpha-naphthol solution was prepared by dissolving 5 gram of alpha-naphthol in 100 ml of 95% ethyl alcohol (Merchant and Parker, 1967).

3.2.6.4 Potassium hydroxide solution (H_2O_2)

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of potassium hydroxide crystals in100 ml of cooled water. (Merchant and Parker, 1967).

3.2.6.5 Kovac's reagent

The solution was prepared by mixing 25 ml of concentrated hydrochloric acid in 5 ml of Amyl alcohol and 5 gram of paradmethyl-aminobenzyldehide crystals were added to this mixture. This was then kept in a flask equipped with rubber cork for future use (Merchant and Parker, 1967).

3.2.6.6 Phosphate buffered saline solution

For preparation of Phosphate buffered saline (PBS) solution, 8 gram of sodium chloride, 2.89 gram of disodium phosphate, 0.2 gram of potassium chloride and 0.2 gram of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121c maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 pH. (Cheesbrough, 1984)

3.2.6.7 Crystal violet solution (0.5%)

Dissolve 500 mg crystal violet mixed 75 ml distilled water then added 25 ml methanol and store at room temperature which acts as a primary coloring agent. (Isenberg,1995).

3.2.6.8 Gram's iodine

Dissolve 6.7 gram of potassium iodide in 100mL of demonized water, add 3.3 g of iodine; stir to dissolve, then dilute to 1 L. Store in a bottle which acts as a mordant. (Isenberg; 1995).

3.2.6.9 Safranin

Dissolve 0.1 g safranin in 75 mL of distilled water, then dilute to 100 ml. Filter before use which acts as a counter stain. (Isenberg., 1995)

3.2.7 Isolation of bacteria

3.2.7.1 Procedure for isolation and identification of bacterial pathogens

Primary culture on nutrient agar

With the help of sterile inoculating loop the collected samples were directly inoculated into nutrient agar and incubated at 37^{o}C for 24 hours. The incubated media were then examined for growth of bacteria.

Inspection: Growth of microorganisms and their colony characteristics were recorded according to procedures described by (Carter, 1979).

Secondary culture on differential media

The organisms were inoculated into MacConkey agar, EMB agar and incubated at 37^{o}C for 24 hours. The incubated media were then examined for growth of bacteria.

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

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

Step -3. The inoculums was spread over the remainder of the plate by drawing the cooled, sterilized loop across the part of the inoculated area, then streaking in a single direction in each parallel line. This

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

Culture on selective media

EMB agar for *E. coli.*

3.2.7.2 Isolation of bacteria by colony morphological characteristics

Each of the colonies was seen for size, shape, pigment, diameter, form, margin and elevation.

3.2.7.3 Gram's staining

Gram's staining method was followed to study the morphological and staining characteristics bacteria and to provide information about the presumptive bacterial identification as per recommendation of Merchant and Packer (1967). A pure colony of each isolates was picked and gram staining was performed. Then the shape, arrangement and gram reaction of the isolates were observed under microscopic field.

Gram's staining method

- Clean glass slides were obtained.
- Using sterile technique, smear of each of the organisms were prepared by placing a drop of normal saline on the slide, and then transferring each organism separately on the slide with a sterile, cooled loop and mixing organism by means of circular of the inoculating loop.
- Smears were allowed to air-dry and then heat fixed in the usual manner.

- Smears were flooded with crystal violet and let stood for one minute then washed with tap water.
- Smear were gently flooded with gram’s iodine mordant and let stood one minute. Gently washed with tap water.
- 95% alcohol-acetone was added for 10 seconds, gently washed with tap water.
- Safranin was added as counter stain for 1 minute.
- Gently washed with tap water and dry by air.
- Then examined under microscope with high power objects (100 X) using immersion oil.

Gram’s staining observation –

- Gram positive: Dark purple
- Gram Negative: pale to dark red
- Cocci: Round shape
- Bacilli: Rod shape

3.2.8 Biochemical tests

Isolated organism with supporting growth characteristics of suspected identified by biochemical test. Several types of biochemical tests were performed in this study to confirm the specific bacteria that are as follow down:

1) Sugar fermentation test
2) Oxidase test
3) Catalase test
4) Indole test
5) Methyl red (MR) test
6) Voges-Proskauer (VP) test
7) Simmon's citrate
8) Triple sugar iron (TSI) agar
9) Mortility indole urease (MIU) test

3.2.8.1 Sugar fermentation test

- The sugar fermentation test was performed by inoculating a loop full of NB culture of the organisms into each tube containing five basic sugars (e.g. dextrose, sucrose, lactose, maltose and mannitol) separately and incubated for 24 hours at 37°C acid production was indicated by the color change from reddish to yellow in the medium and the gas production was noted by the appearance of gas Bubbles in the inverted Durham's tube (Cheesbrough, 1985).

3.2.8.2 Oxidase test

- The oxidase test uses Kovac's reagent (a 1% [wt/vol] solution of N, N, N', N' – tetramethyl-ρ-phenylenediamine dihydrochloride) to detect the presence of cytochrome c in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. The oxidase test can be performed on filter paper or on a swab (Cheesbrough, 1985).

3.2.8.3 Catalase test

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

3.2.8.4 Indole test

- Two milliliter of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°c for 48 hours. Kovac's reagents (0.5ml) were added, shake well and examined after 1 minute. A red color in the reagent layer indicated indole test

positive. In negative case there is no development of red color (Cheesbrough, 1985).

3.2.8.5 Methyl red test (MR)

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

3.2.8.6 Voges-Proskauer test (VP)

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

3.2.8.7 Simmon's citrate agar (SCA)

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

3.2.8.8 Triple sugar iron agar (TSI)

- This tube medium is used to identify Gram negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985)
- Glucose fermentation – indicated by yellow butt
- Lactose fermentation – indicated by yellow slant

- Hydrogen sulfide production – indicated by blackening of the medium
- Gas production – indicated by presence of a crack, bubble or gas space
- pH indicator – phenol red
- Hydrogen sulfide indicator – ferric ammonium citrate with sodium thiosulfate. (H_2S)

3.2.8.9 Mortility indole urease (MIU) test

MIU medium is a semisolid medium used in the qualitative determination of motility, production of indole and urease decarboxylase. MIU medium is used for the differentiation of the family Enterobacteriaceae. The organisms tested must ferment glucose for proper performance of the medium. The medium contains a small amount of agar allowing for detection of motility (Cheesbrough, 1985).

3.2.9 PCR amplification, sequencing of 16S rRNA genes with universal primers and phylogenetic analysis of *Escherichia coli*

3.2.9.1 Basic protocol of bacterial genomic DNA isolation

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

3.2.9.2 Genomic DNA isolation

3.2.9.3 DNA extraction procedure

3.2.9.4 Materials

- TE buffer
- 10% (w/v) sodium dodecyl sulfate (SDD)
- 20mg/ml proteinase K (Stored small single- use aliquots at -20°C

- 3 M Sodium acetate, PH 5.2
- 25:24:1 Phenol/ Chloroform/Isoamyl alcohol
- Isopropanol
- 70% Ethanol
- 95% Ethanol
- 1.5 ml microcentrifuge tubes

3.2.9.5 Procedure

1. Inoculate a 25 ml of liquid culture with the bacterial strain of interest .Grow in conditions appropriate for that stain until the culture is saturated.
2. Spin 1.0 ml of the overnight culture in a microcentrifuge tube for 5 minutes at 10000 rpm.
3. Discard the supernatant.
4. Repeat this step. Drain well into a kimwipe.

Resuspend the pellet in 467µl TE buffer by repeated pipetting. Add 30µlof 10% SDS and 3µl of 20 mg /ml proteinase K to give a final concentration of 100mg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 30 min to 1 hour at 37 °C

5. Add and approximately equal volume (500µl) of phenol/Chloroform/ Isoamyl alcohol. Mix thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phases are completely mixed.
6. Then centrifuge the tube at 12000 rpm for 10 minutes.
7. Remove aqueous, viscous supernatant (~400µl) to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of Phenol/ Chloroform/Isoamyl alcohol, extract thoroughly and spin in a microcentrifuge at 10000 rpm for 5 min.
8. Transfer the supernatant to a fresh tube (~400µl).
9. Add 1/10th volume of 3 M Sodium acetate and mixed.
10. Add 0.6 volumes of Isopropanol to precipitate the nucleic acids, keep on ice for 10 min.
11. Centrifuge at 13500 rpm for 15 min.
12. Decant the supernatant.

13. Wash the obtain pellet with 1 ml of 95% ethanol for 5 min. Then centrifuge at 12000 rpm for 10 min.
14. Decant the supernatant.
15. Dry the pellets well as there is no alcohol.
16. Resuspend the pellet in 50µlof TE and then 7.5µlof RNase. Store DNA at 4°C for short term and at -20 °C for long term.

3.2.9.6 Concentration of extracted DNA & purity

Sometimes isolated genomic DNA may contain a large amount of RNA & pigments, which usually cause over estimation of DNA concentration on a UV spectrometer. Quantification of DNA is a very important step to know the amount of DNA that is present when performing the techniques such as PCR. When assessing DNA purity it is important to understand that while A260/A280 ratio is easy to determine & is the most widely used method, it is not particularly robust. DNA absorbs so strongly at 260 nm that it takes significant protein contamination to have a noticeable effect on A260/A280 ratio. On the other hand, the A260/A280 ratio is a particularly robust method for assessing DNA contamination of protein preparations. Because DNA & RNA are so similar, spectrophotometer cannot be used to detect contamination of DNA by RNA and vice versa. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA & a ratio below 1.70 is generally accepted as ethanol, contamination or other salts are present.

3.2.9.7 DNA concentration and purification determining procedure

- At first switched on computer that attached with thermo scientific Nano Drop 2000 spectrophotometer machine and opened the nucleic acid measuring programmed (Nano drop 2000 software) that was installed before.
- Then opened the lid of this spectrophotometer machine and wiped upper and lower pedestal properly with ultra-pure water

and lint free laboratory tissues so that no contamination are present.

- 2 µL of TE (without DNA samples) was dropped on the lower pedestal of the spectrophotometer machine for banking cycle. TE was used because it acts protecting buffer of DNA from degradation. It should be kept on mind that same chemicals should be placed on pedestal that was used as dissolving chemical of that DNA. It was recommended that an aliquot of the blanking buffer be measured as if it were a sample. This confirms that the instrument was working well and that dried down sample on the pedestal is not a concern. To run a blanking cycle, performing the following :

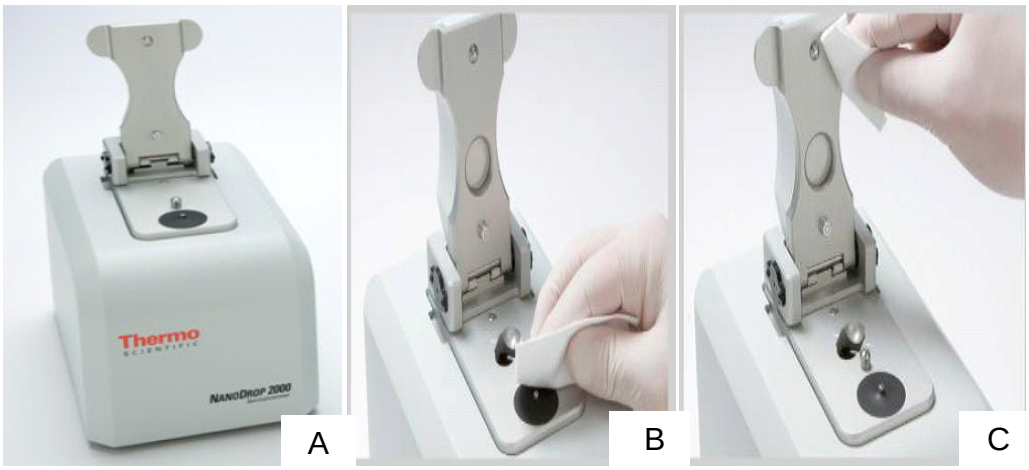


Figure 10: Thermo scientific nano drop 2000 spectrophotometer A, B and C wipe the upper and lower pedestal of

- Loaded an aliquot of the blank onto the lower measurement pedestal and lower the sampling arm.
- Clicked blank to measure and store the reference spectrum.
- Analyzed a fresh replicate of the blank as though it were a sample by choosing measure. The result should be a spectrum that varies no more than 0.04A (10 nm absorbance equivalent).

- Wiped the blank from both measurement pedestal surfaces with a dry laboratory wipe and repeat the process until the spectrum is within 0.04A (10 nm path).

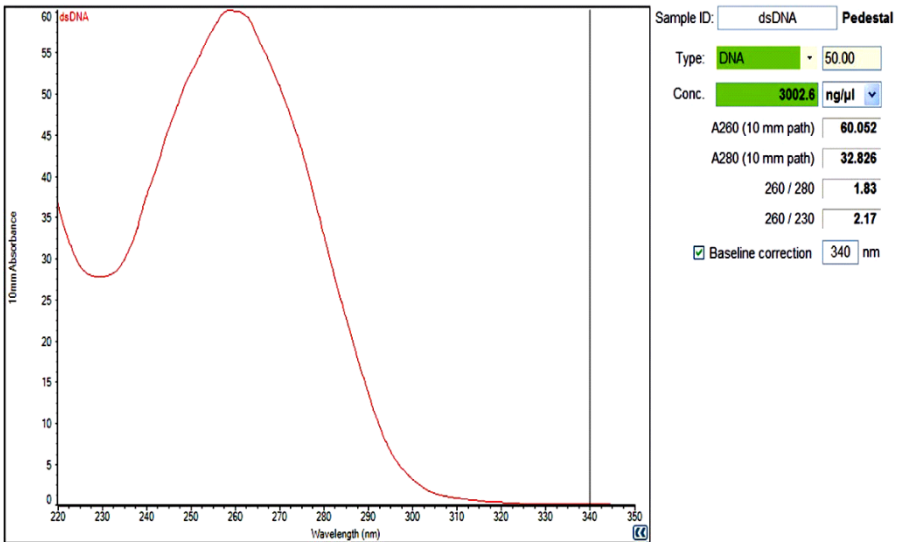


Figure no 11 (a): Software output, showing both spectra and numerical data.

- After blanking the machine with TE buffer then 2µL of DNA sample containing TE buffer was placed on the lower pedestal of the spectrophotometer machine and clicked the nucleic acid measurement button to measure the DNA concentration (ng/µL) and absorbance ratio (260nm/280nm) of DNA that express the actual purity and concentration of DNA.
- A graph was also visualized that showing the purity and concentration of DNA.
- After measuring all the samples the upper and lower pedestal was wiped properly with ultra-pure water and closed the software and last shutdown the computer.

Sample	Ratio	Con. (ng/µl)
1	1.65	317.9
2	1.69	351.4
3	1.56	202.6

3.2.9.8 Primer and PCR amplifications

Primer for *E coli* identification.

Table no 3 (a): Primer sequences and their sources

E coli Primer sequences 5'- 3'	PCR Product size	Source
16SrRNA *E coli*:(16E1)5- GGG AGT AAA GTT AAT ACC TTT GCT C-3 (F)	584 bp	Primary: Tsen *et al*-1998 Secondary: Gautam *et al*-2012 Indian j ani. Sci.,82(2):204-208
16SrRNA *E coli*:(16E2):5-TTC CCG AAG GCA CAT TCT-3 (R)		
16SrRNA *E coli*:(16E3):5-TTC CCG AAG GCA CCA ATC-3 (R)		

3.2.9.9 Polymerase chain reaction (PCR)

3.2.9.10 Principles of PCR

PCR is based on the mechanism of DNA replication in vivo: dsDNA is unwound to ssDNA, duplicated and rewound. This technique consists of repetitive cycle of:

- Denaturation of the DNA through melting at elevated temperature to convert double –stranded DNA to single - stranded DNA
- Annealing (hybridization) of two oligonucleotides used as primers to the target DNA
- Extension of the DNA chain by nucleotide addition from the primers using
- DNA polymerase as catalyst in the presence of Mg^{2+} ions

3.2.9.11 The PCR reaction mixture

The PCR reaction was performed in 25 µl reaction scale. The reaction consisted of 12.5 µl of 2x master mix (GENE Amp Fast PCR Master mix (2x)). About 2µl sample (samples were diluted at 50 (ng/µl), 0.2 µl Taq DNA polymerase, 0.5µl forward primer, 0.5µl reverse primer were used. 9.3µl molecular grade water was added to make final volume of 25µl.

Thermal conditions

3.2.9.12 Precautions of PCR

- PCR tubes were numbering carefully.
- All work was carried out in bio-safety cabinet class II to avoid contamination.
- Melt the vial containing all PCR reaction components.
- All components were taken in correct amounts into tube on PCR cooler box.
- All tubes were Spin down or gently pipetting.

3.2.9.13 Preparation of 2% agarose gel electrophoresis

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones. In addition to size separation, nucleic acid fractionation using agars' gel electrophoresis can be an initial step for further purification of a band of interest.

Electrophoresis through agarose is a standard used to identity and purity of DNA fragments. The technique is simple, rapid to perform & capable of resolving fragments of DNA that cannot be separated by other procedure.

3.2.9.14 Electrophoresis

3.2.9.15 Process of electrophoresis:

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

- When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
- The gel stained in ethidium bromide (0.5µg/ml) for 10 minutes, in a dark place.
- The gel was distained in distilled water for 10 minutes. The distained gel was placed on the imaging system in the dark chamber of the image documentation system.

The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in an USB flash drive

For agarose gel electrophoresis, 2% agarose gel was prepared (for tray size 11cm x 10 cm, required 70 ml of agarose gel for thickness of 0.5 cm). For casting the gel the following steps are followed:

- Initially 1.4 gm agarose powder was weight out and placed into a 250 ml conical flask.
- Then 70 ml of electrophoresis buffer (1x TBE buffer) was added into the flask.
- The flask was then placed into a microwave oven. Using a low to medium setting, the time was set for 2 minute. The oven was stopped & swirled the container gently to suspend the agarose which was not dissolved. The solution was heated again for 1 minute to dissolve small translucent agarose particles
- When the agarose solution was cooled to about 50°C (the flask was cooled enough to hold comfortably with bare band), 7µL to 10 mg/ml solution of ethidium bromide was added so that in the gel the concentration of ethidium bromide was maintained as 1µg/ml (the final concentration of ethidium bromide in the melted agarose solution may be in the range of 0.5-1.0 µg/ml) & mixed well by gentle shaking to make DNA visible under ultraviolet light & poured into gel tray

3.2.9.16 Comb set-up

- The ends of the gel casting tray was selected with casting dams. It is recommended to seal the dams tightly with gel casting tray in order to prevent leakage.
- The gel tray was leveled on a leveling table or working bench using the leveling bubble.
- The comb was then placed into the appropriate groove and slot of the tray.
- The molten agarose (about 50°C) was poured onto tray. Hot agarose (above 60°C) might cause the comb to warp or craze & would decrease the life time of the comb. Warping might also result in sample wells of uneven depth. Air bubbles were removed by pushing away to the side using a disposable tip.
- The gel was allowed to solidify at room temperature for 20-30 minutes.
- The comb was removed carefully from the solidified gel.
- The casting dams were removed from the edges of the gel tray, so that the gel does not slide off the tray.

3.2.9.17 Loading sample in the wells

- A sheet of aluminum fuel paper was taken & 2µL of gel loading buffer (6x GLB) placed on it. 10µL of PCR products was mixed with 2µL GBL and pipetting gently. The total volume of loading sample was 10µL that was loaded into the well. Loading volume is dependent upon the type of comb used (i.e. well thickness and length) & thickness of the gel. The prepared samples were then loaded slowly to allow them to sink to the bottom of the wells. A molecular weight marker DNA was also loaded on side of the gel.
- The tray was placed by keeping the gel horizontal onto the base of the electrophoresis chamber, so that the sample wells were near the cathode (negative end generally marked as black). DNA sample would migrate towards the anode (positive end generally marked as red) during electrophoresis.

- Sufficient amount of 1x TBE buffer (about 600 ml) was added to cover the gel to a depth of about 5 mm /0.5 cm (1-5 mm may be used). The volume of electrophoresis buffer should not be above maximum buffer mark on electrophoresis system.
- Electrophoresis was carried out at 150 volts for 1 hour. The separation process was monitored by the migration of the dye in the loading buffer. When the bromophenol blue dye had reached about three-fourths (3/4) of the gel length, the electrophoresis was completed and stopped.

3.2.9.18 Documentation of the DNA sample

After electrophoresis the gel was taken out carefully from the electrophoresis chamber and placed on UV trans illuminator (WUV-L50, Korea) for primary checking the DNA bands & then placed into the high performance gel documentation chamber (UVD1-254) for further checking and picture storage.

Procedure

- Inoculate a 25 ml of liquid culture with Pseudomonas spp. Grow in conditions appropriate for *E. coli* until the culture is saturated.
- Spin 1.0 ml of the overnight culture in a micro centrifuge tube for 5 minutes at 10000 rpm.
- Discard the supernatant.
- Repeat this step. Drain well onto a kimwipe.
- Re-suspend the pellet in 467 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ ml Proteinase k to give a final concentration of 100 µg/mg Proteinase k in 0.5% SDS. Mix thoroughly and incubate 30 min for 1 hr at 37^0C.
- Add an approximately equal volume (500 µl) of Phenol/Chloroform/Isoamyl alcohol. Mix thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phase are completely mixed.
- Then centrifuge the tubes at 12000 rpm for 10 minutes.

- Remove aqueous, viscous supernatant (400 µl) to a fresh microcentrifuge tube , leaving the interface behind. Add an equal volume of Phenol/Chloroform/Isoamyl alcohol extract thoroughly and spin in a microcentrifuge at 10000 rpm for 5 min.
- Transfer the supernatant to a fresh tube (400 µl).
- Add 1/10th volume of 3 M sodium acetate and mix.
- Add 0.6 volumes of isopropanol to precipitate the nucleic acids, keep on ice for 10 minutes.
- Centrifuge at 13500 rpm for 15 minutes.
- Discard the supernatant.
- Wash the obtained pellet with 1 ml of 95% ethanol for 5 minutes. Then centrifuge at 12000 rpm for 10 minutes.
- Discard the supernatant.
- Dry the pellets as there is no alcohol.
- Resuspend the pellet in 50 µl of TE and then 7.5 µl of RNase. Store DNA at 4^{0}C for short term and at -20^{0}C for long term.

3.2.9.19 PCR Condition:

Table no 3 (b): Condition of PCR.

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

3.2.9.20 Nucleotide sequence accession number and BLAST analysis

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

3.2.9.21 Chain-termination methods (Sanger sequencing)

Steps of Sanger sequencing using ABI 3130 Genetic analyzer

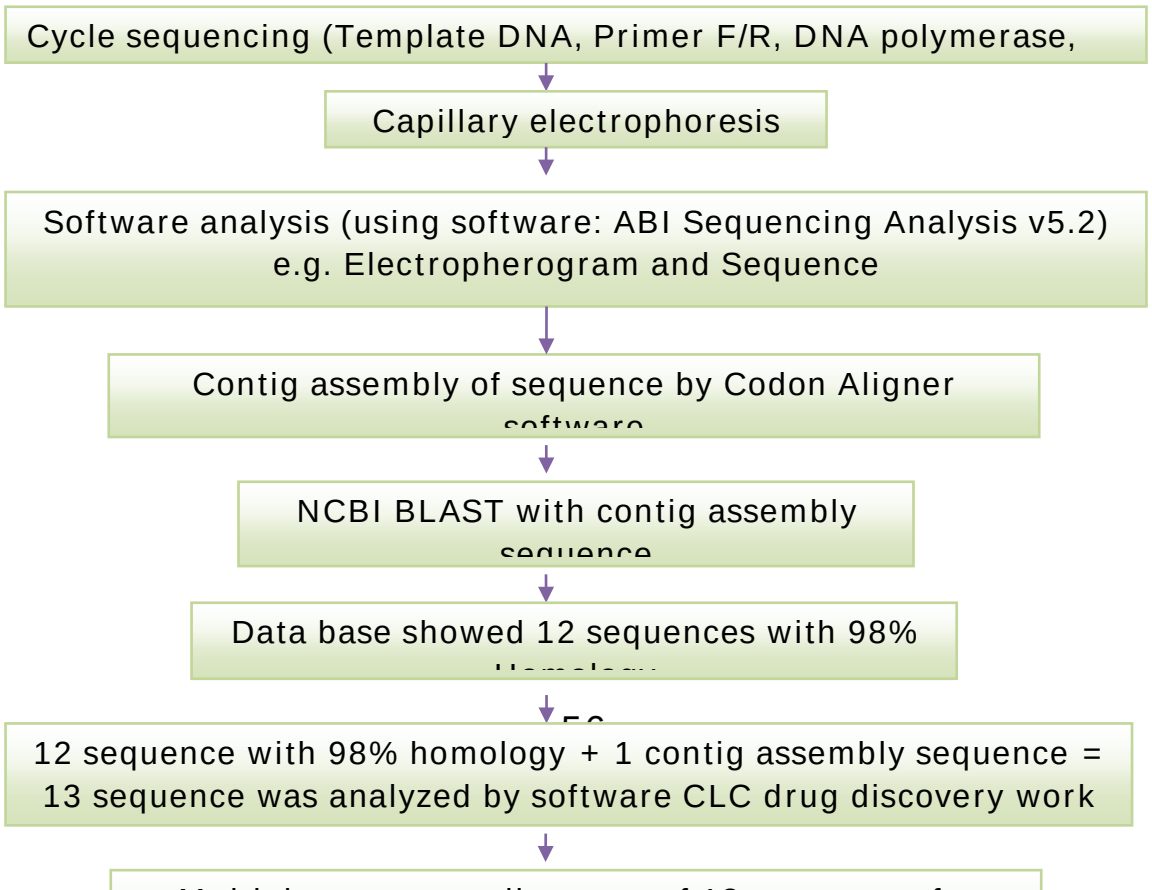


Figure no 11 (b): Steps of Sanger sequencing using ABI 3130 Genetic analyzer

Figure no 12: Preparation of sample for PCR test at National Institute of Biotechnology in Dhaka

3.2.10 Antibiotic sensitivity tests

Kirby-Bauer (K-B) antibiotic sensitivity testing:

Materials:

- Test tube rack
- Bunsen burner
- Inoculating loop or needle
- Forceps
- Sterile swabs
- Mueller-Hinton or Nutrient agar plates
- Antibiotic disks
- Stock broth cultures of experimental bacteria
- 35°C to 37°C non-CO_2 incubator

Antimicrobial sensitivity test was performed according to the procedure Kirby-bauer disk diffusion susceptibility test protocol suggested by Leigh (1990). First developed in the 1950s, it was refined and by W. Kirby and A. Bauer, then standardized by the World Health Organization in 1961. The Kirby-Bauer (K-B) disk diffusion test is the most common method for antibiotic resistance/susceptibility testing. Sensitivity to antibiotics was studied on Mueller-Hinton agar plate used the different types of commercial antibiotic discs. After the discs were placed on the plates, the plates were inverted and incubated at 37°C for 16 to 18 hours. After incubation, the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the undersurface of the plate without opening the lid. The value was compared with the zone-size table. The zones of growth inhibition were provided by Clinical and Laboratory Standards Institute (CLSI, 2007). Isolates were classified as susceptible, intermediate and resistant categories based on the standard

interpretation tables updated according to the Clinical and Laboratory Standards Institution (CLSI, 2007).

Table no 4: Antimicrobial agents with their disc-concentration

Sl. No	Antimicrobial Agents	Symbol	Disc concentration (µg/disc)	Diameter of zone of inhibition (ZOI)		
				Resistant	Intermediate	Susceptible
1	Ampicillin	AMP	25	≤13	14-16	≥17
2	Amoxicillin	AM	30	≤13	14-17	≥18
3	Amikacin	AK	30	≤14	15-16	≥17
4	Cephalexin	CN	30	≤14	-	≥14
5	Ciprofloxacin	CIP	5	≤20	21-30	≥31
6	Gentamycin	GEN	10	≤12	13-14	≥15
7	Kanamycin	K	30	≤13	14-17	≥18
8	Penicillin	P	10	≤11	12-21	≥22
9	Erythromycin	E	15	≤13	14-17	≥18
10	Vancomycin	VA	30	≤9	10-11	≥12
11	Chloramphenicol	C	30	≤12	13-17	≥18
12	Cloxacillin	COX	1	≤15	16-18	≥19
13	Tobramycin	TOB	3O	≤10	-	≥12
14	Cliostin	CL	10	≤10	-	≥11
15	Neomycin	N	30	≤12	13-16	≥17
16	Cloxacillin	COX	1	≤10	11-12	≥13
17	Norofloxacin	NX	10	≤10	-	≥11
18	Cefotaxime	CTM	30	≤10	-	≥11
19	Ofloxacin	OFX	2	≤12	13-5	16

Source: CLSI- 2013 [Note: S=Sensitive, R=Resistant, I=Intermediate]

3.2.11 Maintenance of stock culture

After completion of characterization of bacterial pathogens it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of the isolated E. coli was stored in 10% glycerin and used as stock culture. The equal volume of 10% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at 37ºC. The isolated organisms were given code name for convenience. (Buxton and Fraser, 1977).

3.2.12 Statistical analysis

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

3.2.13 Questionnaire for exposure assessment of *E. coli* in poultry farm

Date:

Information for sample collection from poultry farm and hatchery Name and addresses of the farm/farm owner:

Farm code :

Date of DOC received :

Source of DOC (hatchery name) :

Source of Feed (feed mill) :

Type of bird (Broiler/ Layer) :

Age of bird :

Expected date of bird sale :

Any medicine/chemicals were given to the flock while sampling? : Y/N If Yes, please mentions the name of medicine and dose:

Sample ID:		
Sample type	Sample ID	Remarks
Chicken intestine		
Chicken liver		
Chicken heart		
Chicken lung		
Chicken oviduct		

Farmer Address	
Name:	Village/Area/Ward:
Road #:	Post Office:

Union/city corporation/Pouroshava:	Upazila/PS:
Zila/District:	Division:

CHATER-4

RESULTS

4.1 Results of isolation and identification of *E. coli* bacteria by different bacteriological methods

The present research was designed to determine the isolation, characterization and antibiogram study of bacterial pathogens isolated from internal organs of dead chicken at Dinajpur district of Bangladesh. The collected samples were subjected to various bacteriological examinations such as cultural, biochemical techniques and antibiotic sensitivity pattern in the laboratory of the department of Microbiology, HSTU, Dinajpur, for isolation and identification of bacteria. A total of 131 samples were collected from different internal organs for this study. Out of 131 samples, 54 were found to be positive for *E. coli* and a total of 54 isolates were isolated. Result of morphological, staining, cultural, biochemical, molecular, antibiotic sensitivity pattern and percentage of incidence of isolated *E. coli* bacteria are shown in Table no. 5

4.1.1 Results of cultural examination

After inoculation of dead chicken internal organs on Nutrient agar and Nutrient broth, it was observed that mat growth was found in Nutrient agar and turbidity in Nutrient broth.

Table no 5: Results of cultural, morphological, staining and motility characteristics of *E. coli*

Name of bacteria	Staining Characteristics	Name of Media	Colony Characteristics	Motility Characteristics
E. coli	Gram negative large rod shaped pink color.	Nutrient broth	Turbidity	*E. coli* is highly motile and will show turbidity throughout the tube
		Nutrient Agar	Large, mucoid, white colony.	
		Mac-Conkey's Agar	Produce large mucoid rose pink colony.	
		EMB agar	Transmitted light blue black center with a narrow, clear edge. Blue-green metallic sheen With reflected light.	

		Tryptic soy agar	Small white colony.	
		Salmonella-Shigella Agar	*E. coli* on plate with rose pink colony	

4.1.1.1 Nutrient broth

Nutrient broth inoculated with the samples revealed the growth of *E. coli* after 24 hours of incubation at 37^0C. The growth of *E. coli* was indicated by the presence of turbidity.

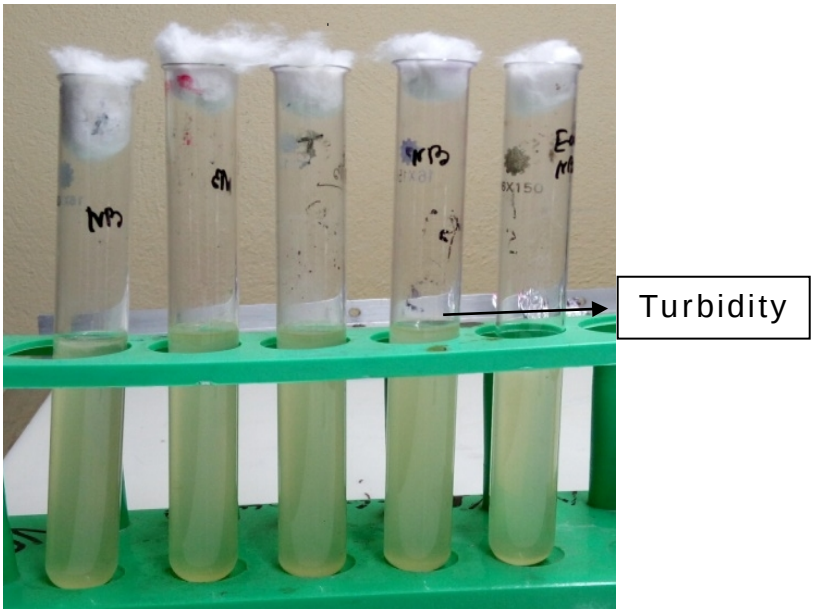


Figure no 13: On Nutrient broth *E. coli* shows turbidity

4.1.1.2 Nutrient agar

Nutrient agar plates spread with the samples revealed the growth of bacteria after 24 hours of incubation at 37^oC aerobically and were indicated by the

growth of circular, small smooth, convex, and gray white or yellowish colonies.

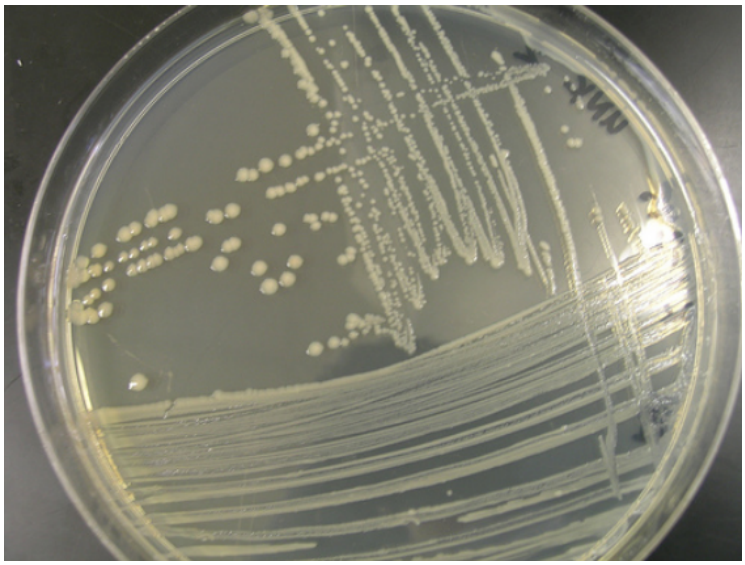


Figure no 14: Culture of organism on Nutrient Agar plate with circular, small smooth, convex and gray white colonies

4.1.1.3 Mac-Conkey Agar

Mac-conkey agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated the colorless colonies after prolonged incubation rose pink color colonies.

Figure no 15: *E. coli* on Mac-Conkey agar plate with pink color colony

4.1.1.4 Eosin methylene blue agar

Eosin methylene blue agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by the growth of smooth, circular , black color center with blue-green metallic sheen color colonies.

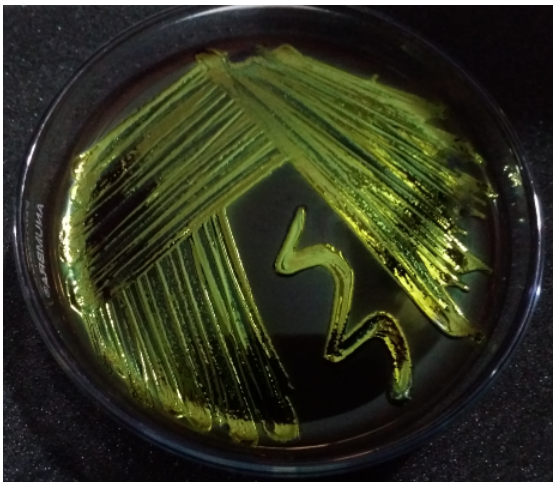


Figure no 16: *E. coli* on EMB agar plate with blue green metallic sheen color colony

4.1.1.5 Salmonella-Shigella agar

Salmonella-shigella agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by transparent colonies, pink color colony.

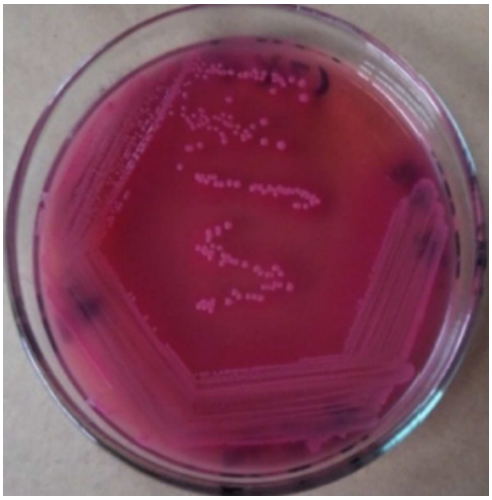


Figure no 17: *E. coli* on Salmonella-Shigella agar plate with rose pink colony

4.1.1.6 Tryptic soy agar

Eosin methylene blue agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by the growth of Small white colony.

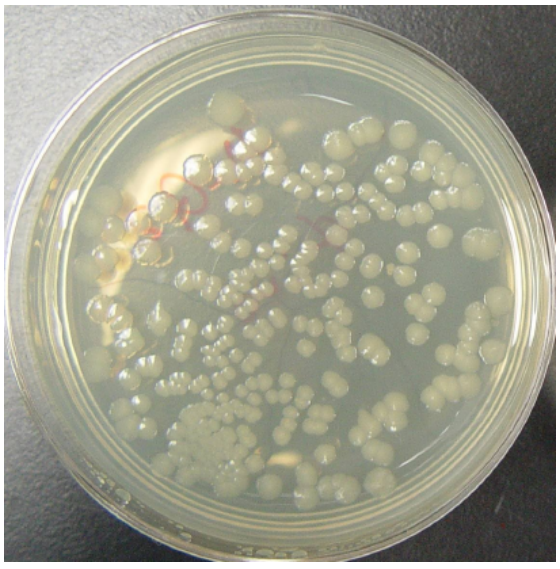


Figure no 18: *E. coli* on Tryptic soy agar plate with small white colony

4.1.2 Results of Gram's staining by microscopic examination:

Microscopic observation was performed to observe the morphology of the isolates. Under microscopy gram negative *E. coli* was found. Gram negative *E. coli* was curved, slender, rod shape and also showed pink color colonies under 100X microscopy.

Figure no 19: *E .coli* showing Gram negative (pink colour) rod shape under 100X microscopy

4.1.3 Results of motility test

The motility test was performed according to the method described by Cowan (1985) to differentiate motile bacteria from the non-motile one. Before performing the test, a pure culture of the *E. coli* isolates was allowed to grow in Nutrient broth. One drop of cultured broth was placed on the cover slip and placed inverted over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the concave depression of the hanging drop slide for better attachment of the cover-slip to prevent air current and evaporation of the fluid. The hanging drop slide was then examined carefully under 100x objective of a compound microscope using immersion oil. The motile and non-motile organisms were identified by observing motility in contrasting with the movement of bacteria. The motile *E. coli* were isolated by observing motility in contrasting with Brownian movement of *E. coli*.

Figure no 20: *E. coli* is highly motile and will show turbidity throughout the tube

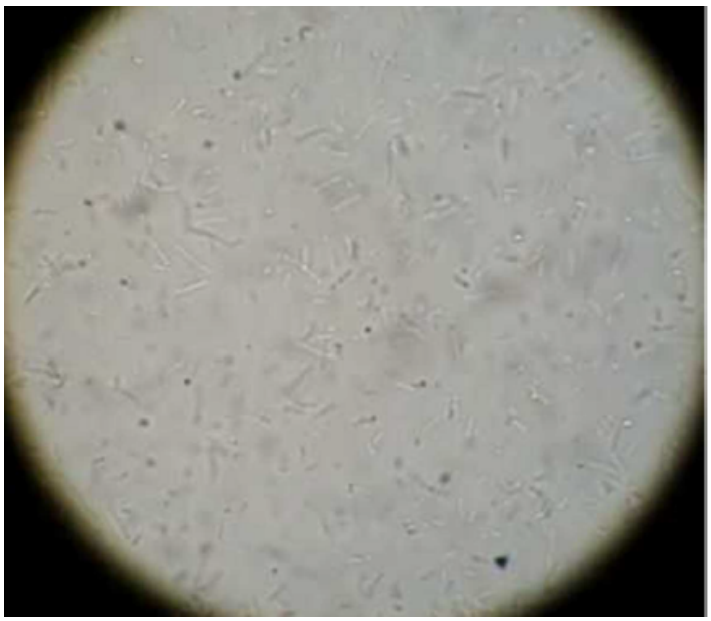


Figure no 21: *Escherichia coli* showing motility in hanging drop slide.

4.1.4 Results of different biochemical tests

Biochemical test was performed to confirm the E. coli bacteria that isolated from internal organs of dead chickens. After seen the colony characters on different media and also Gram's staining identify the

bacteria for biochemical test. Table no. 6 showed the biochemical test results.

Table no 6: Results of different biochemical tests

Name of isolate	OX	CT	IN	MR	VP	SC	TSI		MIU	
							Slant	Butt	M	I
E. coli	-	+	+	+	-	-	A(yellow)	A(yellow)	+	+

Legends:+ = positive, - = negative, A=acid, OX= oxidase, CT=catalase, IN= indole, MR= methyl-red, VP= voges-proskauer, SC= simmon's citrate, TSI= triple sugar iron, MIU= motility indole urease.

4.1.4.1 Oxidase test

E. coli; was negative result for oxidase test.

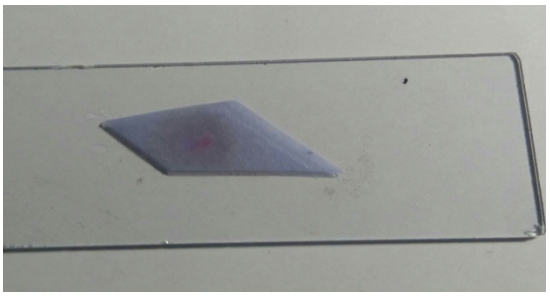


Figure no 22: Oxidase test negative *E. coli*

4.1.4.2 Catalase test

All the isolates of *E. coli* were found positive as the isolates produced bubbles in catalane test.

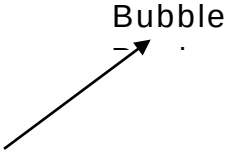


Figure no 23: Catalase positive for *E. coli* (left) and control (right)

4.1.4.3 Methyl- Re t

E. coli was positive to MR test. The test was conducted by inoculating colony of the isolated *E. coli* in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of methyl red solution was added. A bright red coloration was produced.

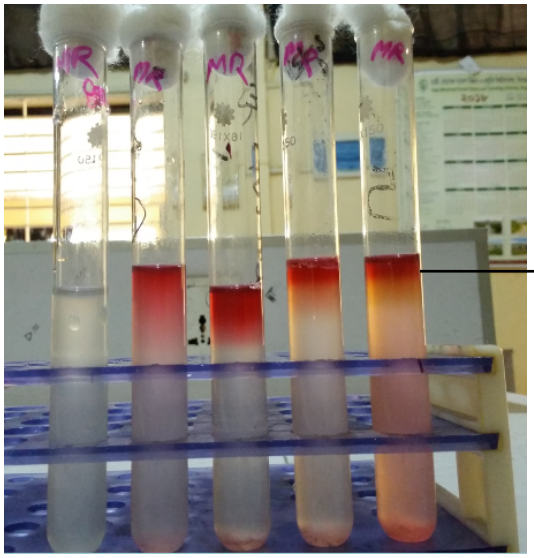


Figure no 24: Methyl-Red test was positive result indicated by the changed of medium to bright red color inoculated (right), and left is control.

4.1.4.4 Voges-Proskaur test

E. coli; was negative result by indicated no rose coloration for Voges-Proskaur test.

Figure no 25: Voges - Proskauer test indicated negative result by no change to rose red colour.

4.1.4.5 Indole test

E. coli was positive results by indicated cherry red coloration for indole test.

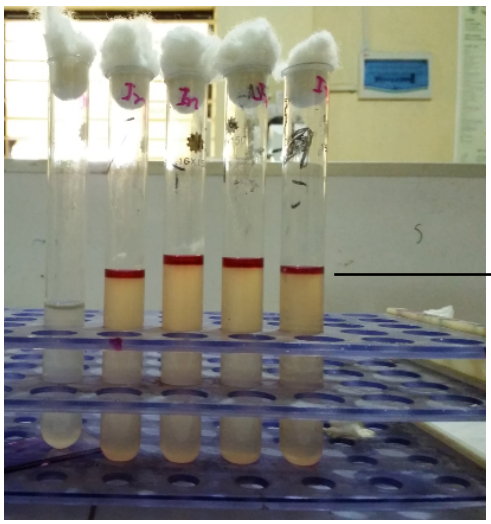
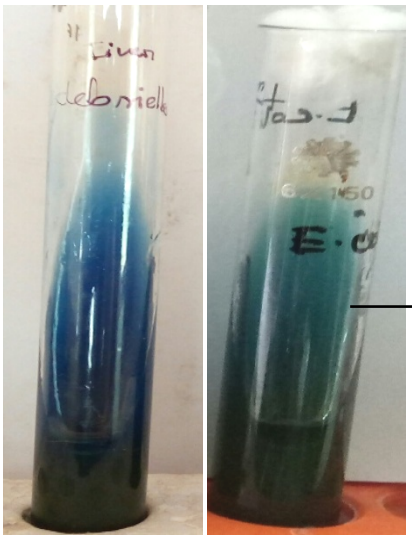


Figure no 26: Indole test showing positive result by red coloration of the medium colour inoculated (right); and negative result indicated by the no red coloration of the medium for control (left).

4.1.4.6 Simmons citrate test

E. coli; was negative results by no changed medium green to blue coloration for simmons citrate test.



Control Negative

Figure no 27: Simmons citrate test showing negative result (right) and control (left).

4.1.4.7 TSI (Triple Sugar Iron) test:

E. coli was showed yellow coloration both butt and slant.

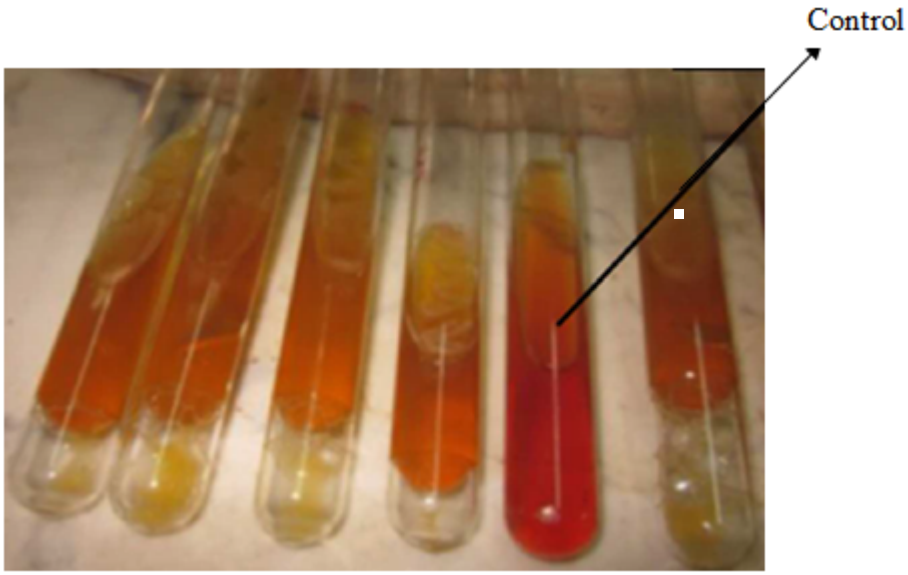
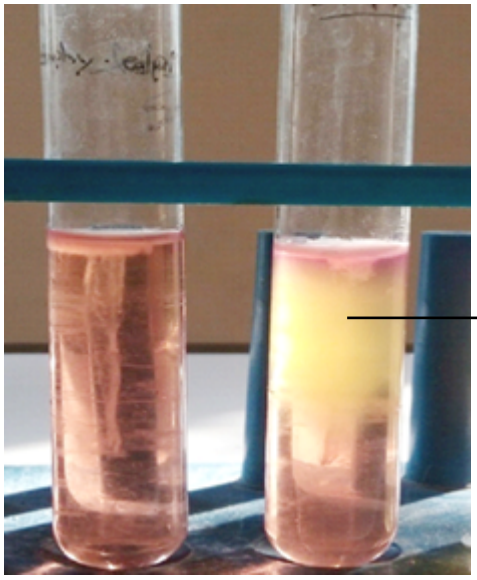


Figure no 28: Triple sugar iron test

4.1.4.8 MIU (Motility Indole Urease) test

E. coli; was positive results by medium changed to colorless & yellow coloration for MIU test.



Control Positive

Figure no 29: Motility Indole Urease test showing control (left), and positive (right)

4.1.4.8 Sugar fermentation test

All the isolates of *E. coli* were fermented dextrose, glucose, sucrose, lactose and manitol.

Table no 7: Biochemical reaction patterns of *E. coli* sugar fermentation test

Bacteria	Sugar fermentation properties				
	Dextrose	Glucose	Sucrose	Lactose	Manitol
E. coli	+	+	+	+	+

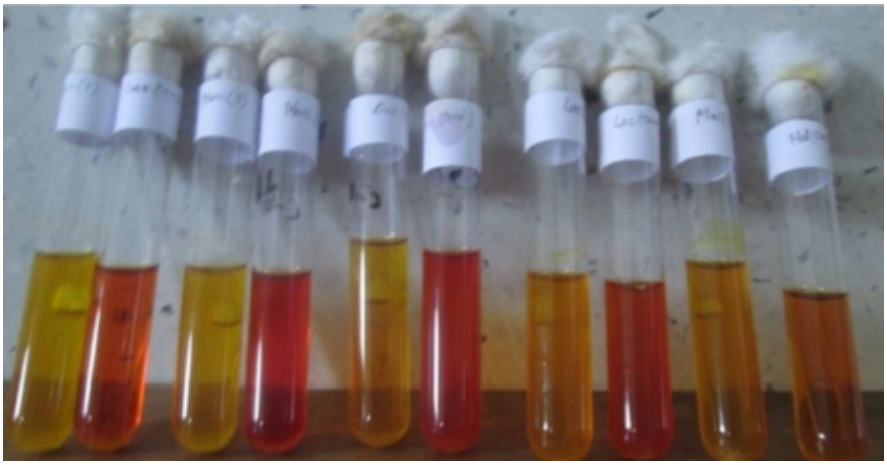


Figure no 30: *E. coli* fermented dextrose, glucose, sucrose, lactose, mannitol that were designated by color change and production of acid and gas in Durhum's tube (right) and control (left)

4.1.5 Result of PCR amplification, sequencing of 16S rRNA genes with universal primers and phylogenetic analysis of *Escherichia coli*

Out of 131 samples, positive cases of *E. coli* was present in 54 cases and the percentage was very high (40%) from all identified isolates. 16S rRNA gene region was amplified with the universal primers, Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3') Reverse primer- 1492R (5' TACCTTGTTACGACTT 3'). PCR Amplification band was found at 584 bp.

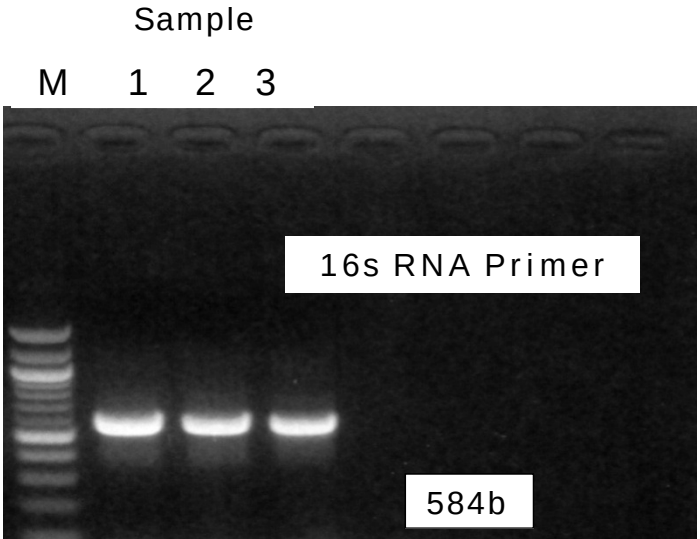


Figure no 31: Result of amplification of 16S rRNA gene region of

M: Marker, 2kb DNA ladder

Note: PCR= Polymerase Chain Reaction, kb= kilo base, bp=base pair.

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4.1.5.1 Electropherogram for *E. coli*

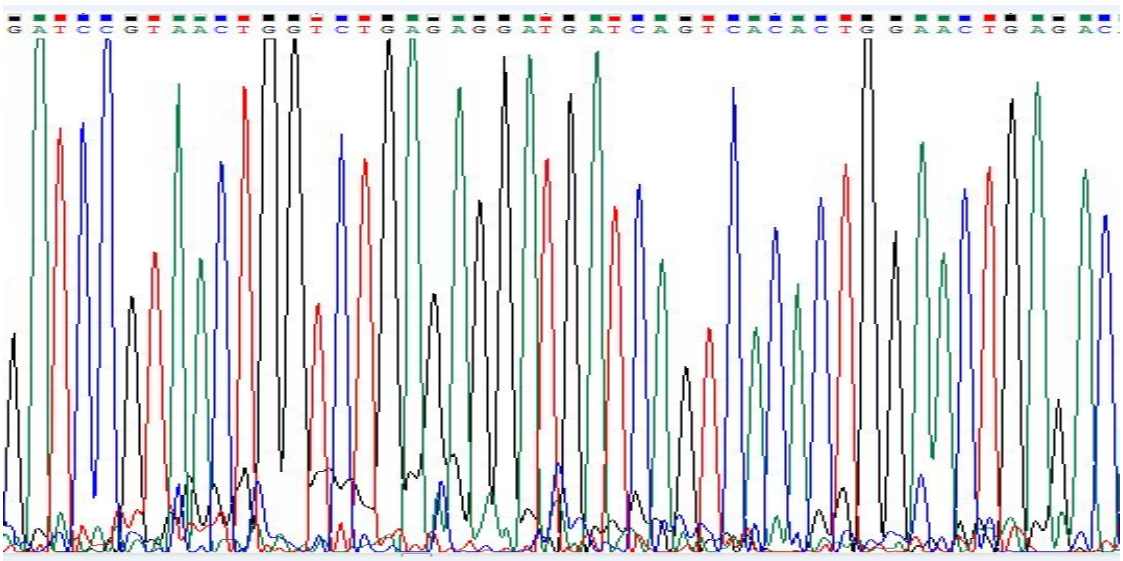

Figure no 32: Electropherogram of 16s rRNA gene sequence of *E.coli*

4.1.5.2 Contig sequence of *E.coli*

AATATCCATGACGGAGAAAACACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATTCCCGGGCTCAACCT
GGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTA
GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG
GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAA

GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTA
ACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTC
AAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTT
TCAGAGATGAGAATGTGCCTTCGGGAAAGTT

4.1.5.3 Phylogenic tree analysis of *E.coli*

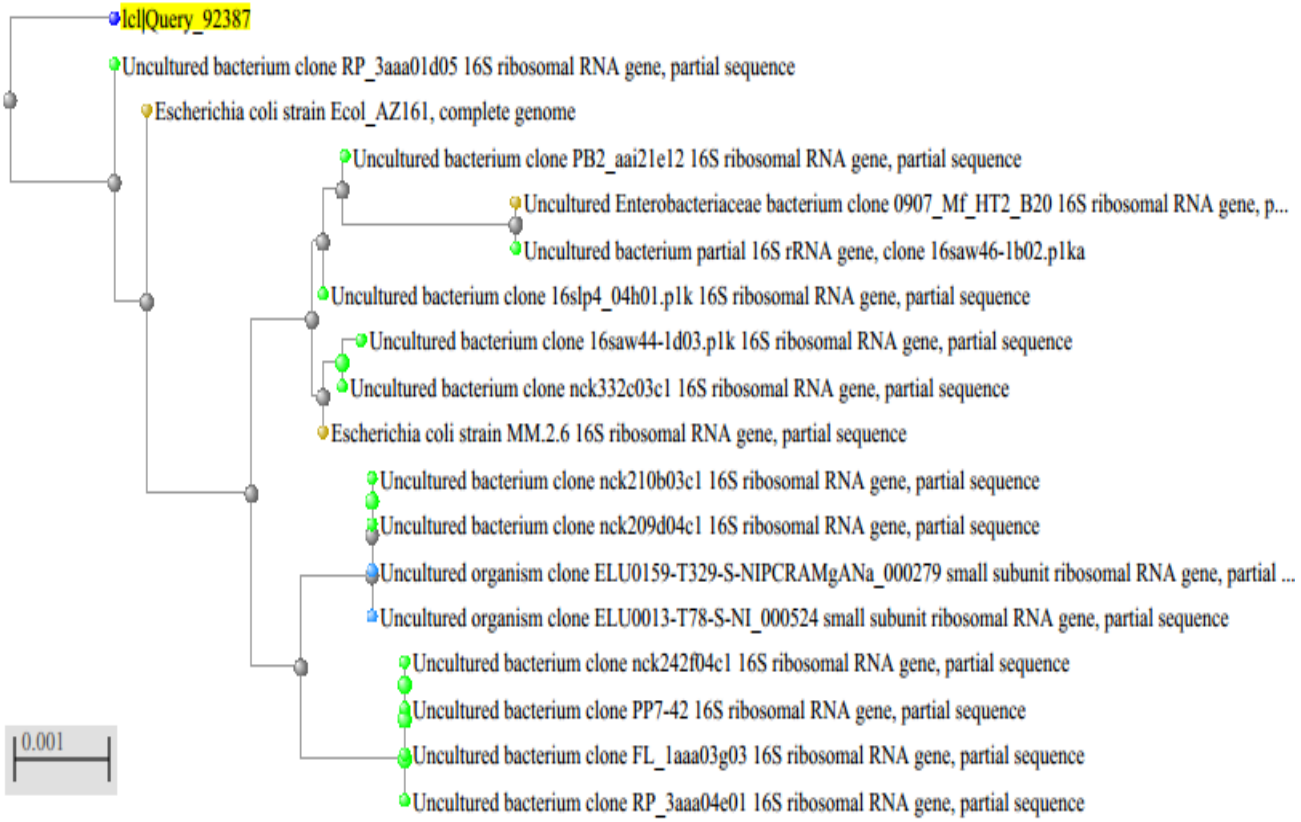

Figure no 33: Phylogenic tree analysis of *E.coli*

4.1.6 Results of *E. coli* obtained from internal organs of dead chickens

Out of 131 dead internal organs samples, positive cases of *E. coli* were found to be positive 54 (41.22%) and total 77 (58.78%) samples were negative.

Table no 8: Frequency of *E. coli* in dead chickens

Total number of cases	No. of positive Cases	Positive Percentage	No. of Negative Cases	Negative Percentage
131	54	41.22%	77	58.78%

4.1.6.1. Results of isolated *E. coli* from chicken intestine

Results of isolation and identification of *E. coli* in dead chicken intestine are presented in Table no 8. A total of 34 samples were collected and 30 samples were positive for *E. coli.*

Table no 09: Results of *E. coli* in dead chicken intestine

Type of sample	Placement	Sample No.	Sample ID	Result
				E. coli
	Basherhat	1	5 July 031030173CI1	Positive
		2	5 July 031030125CI2	Positive
		3	5 July 031030115CI3	Positive
		4	5 July 031030201CI4	Negative
		5	5 July 031030820CI5	Positive
		6	12 July 031030135CI6	Positive
		7	12 July 031030190CI7	Positive

Chicken intestine		8	12 July 031030289CI8	Positive
		9	12 July 031030383CI9	Positive
		10	12 July 031030112CI10	Positive
	Mohabali-pur	11	12 July 031030175CI11	Positive
		12	12 July 031030756CI12	Positive
		13	19 July 0310301768CI13	Positive
		14	19 July 031030765CI14	Positive
		15	19 July 031030315CI15	Negative
		16	19 July 031030188CI16	Positive
		17	19 July 031030567CI17	Positive
		18	19 July 031030175CI18	Positive
		19	26 July 031030563CI19	Positive
		20	26 July 031030906CI20	Negative
		21	26 July 031030586CI21	Positive

		22	26 July 031030798CI22	Positive
	Gobinda-pur	23	26July 031030102CI23	Positive
		24	26July 031030324CI24	Positive
		25	31 July 031030424CI25	Positive
		26	31 July 031030476CI26	Positive
		27	31 July 031030479CI27	Positive
		28	31 July 031030409CI28	Negative
		29	31 July031030097CI29	Positive
		30	06 Aug 031030176CI30	Positive
		31	06 Aug 031030187CI31	Positive
		32	06 Aug 031030189CI32	Positive
		33	06 Aug 031030009CI33	Positive
		34	06 Aug 031030090CI34	Positive

4.1.6.2 Results of isolated *E. coli* from chicken liver

Results of isolation and identification of *E. coli* in dead chicken liver are presented in Table 10. A total of 23 samples were collected and 06 samples were positive for *E. coli*

Table no 10: Results of isolated *E. coli* in chicken liver

Type of sample	Placement	Sample No.	Sample ID	Result
				E. coli
	Basherhat	1	8 July 031030115CL1	Positive
		2	8 July 031030115CL2	Negative
		3	8 July 031030115CL3	Positive
		4	8 July 031030115CL4	Negative
		5	8 July 031030115CL5	Negative
		6	8 July 031030115CL6	Negative
		7	8 July 031030115CL7	Negative
		8	8 July 031030115CL8	Negative
		9	12 July 031030115CL9	Positive
		10	12 July 031030115CL10	Negative
	Mohabali-	11	12 July	Negativ

Chicken liver	pur		031030115CL11	e
		12	12 July 031030115CL12	Negative
		13	16 July 031030115CL13	Negative
		14	16 July 031030115CL14	Positive
		15	16 July 031030115CL15	Negative
		16	16 July 031030115CL16	Negative
		17	16 July 031030115CL20	Negative
		18	23 July 031030115CL21	Positive
		19	23 July 031030115CL22	Negative
	Gobindapur	20	23July03103011CL23	Negative
		21	23July03103015CL24	Negative
		22	23 July03103011CL25	Negative
		23	23 July03103011CL26	Positive

4.1.6.3 Results of isolated *E. coli* in chicken heart

Results of isolation and identification of *E. coli* in chicken heart are presented in Table 11. A total of 25 samples were collected and 07 samples were positive for *E. coli.*

Table no 11: Results of isolated *E. coli* in chicken heart

Type of Sample	Placement	Sample No.	Sample ID	Result *E. coli*
Chicken heart	Basherhat	1	08Jul021020104CH1	Positive
		2	08Jul021020104CH2	Negative
		3	08Jul021020104CH3	Negative
		4	08Jul021020104CH4	Negative
		5	08Jul021020104CH5	Positive
		6	08Jul021020104CH6	Negative
		7	08Jul021020104CH7	Negative
		8	07Aug021020302CH8	Negative
	Mohabali-pur	9	17Aug02120302CH9	Negative
		10	7Aug021020302CH10	Negative
		11	7Aug021020302CH11	Negative
		12	7Aug021020302CH12	Positive
		13	14Aug021020302CH3	Negative
		14	19Aug021020302CH	Positive

	Gobindapur		14	
		15	19Aug021021100CH15	Negative
		16	19Aug020203110CH16	Negative
		17	19Aug021020110CH17	Negative
		18	19Aug021020110CH18	Negative
		19	26Aug021020110CH19	Positive
		20	26Aug021020110CH20	Positive
		21	26Aug021020110CH21	Negative
		22	26Aug021020102CH22	Negative
		23	26Aug021020102CH23	Negative
		24	26Aug021020102CH24	Negative
		25	26Aug021020102CH25	Positive

4.1.6.4 Results of isolated *E. coli* in chicken lung (airsac)

Results of isolation and identification of *E. coli* in chicken heart are presented in Table 12. A total of samples were collected and 07 samples were positive for *E. coli.*

Table no 12: Results of isolated *E. coli* in chicken lung (air sac)

Type of Sampl	Placement	Sample No.	Sample ID	Result *E. coli*
	Basher-hat	1	08Jul021020104CLN1	Negative
		2	08Jul021020104CLN2	Negative

Chicken lung		3	08Jul021020104CLN3	Positive
		4	08Jul021020104CLN4	Negative
		5	08Jul021020104CLN5	Negative
		6	08Jul021020104CLN6	Negative
		7	08Jul021020104CLN7	Negative
		8	07Aug021020302CLN8	Negative
	Mohabali-pur	9	17Aug02120302CLN9	Positive
		10	7Aug021020302CLN10	Negative
		11	7Aug021020302CLN11	Negative
		12	7Aug021020302CLN12	Positive
		13	14Aug021020302CLN13	Negative
	Gobinda-pur	14	19Aug021020302CLN14	Positive
		15	19Aug02102110CLN15	Negative
		16	19Aug020203110CLN16	Negative
		17	19Aug021020110CLN17	Negative
		18	19Aug021020110CLN18	Negative
		19	26Aug021020110CLN19	Positive
		20	26Aug021020110CLN20	Negative

4.1.6.5 Results of *E. coli* in chicken oviduct

Results of isolation and identification of *E. coli* in chicken heart are presented in Table 13. A total of 22 samples were collected and 04 samples were positive for *E. coli.*

Table no 13: Results of *E. coli* in chicken oviduct

Type of	Placement	Sampl	Sample ID	Result

sample		e No.		*E. coli*
Chicken oviduct	Basher-hat	1	5 July 031030173CO1	Negative
		2	5 July 031030125C02	Negative
		3	5 July 031030115CO3	Negative
		4	5 July 031030201C04	Negative
		5	5 July 031030820C05	Positive
		6	12 July 031030135CO6	Positive
		7	12 July 031030190CO7	Negative
		8	12 July 031030289CO8	Negative
		9	12 July 031030383CO9	Negative
		10	12 July031030112CO 10	Negative
		11	12 July031030175CO 11	Negative
	Mohabali-pur	12	12 July031030756CO 12	Negative
		13	19 July031031768CO 13	Negative
		14	19 July031030765CO 14	Negative
		15	19 July031030315CO 15	Negative
		16	19 July031030188CO 16	Negative
		17	19 July 031030567C017	Negative
		18	19 July031030175CO 18	Positive
		19	26 July031030563CO 19	Negative

		20	26 July031030906CO 20	Negative
	Gobinda-pur	21	26July 031030102CO21	Negative
		22	26July 031030324CO22	Negative
		23	31 July031030424CO 23	Positive
		24	31 July031030476CO 24	Negative
		25	31 July031030479CO 25	Positive
		26	31 July031030409CO 26	Negative
		27	31 July031030097CO 27	Negative
		28	06 Aug031030176CO 28	Positive

Legends:

CI=Chicken intestine, C=Chicken liver, CH=Chicken heart, CLN=Chicken lungs, CO=Chicken oviduct.

4.1.7 Summary of the results of *E. coli* obtained from different internal organs

A total of 47 samples were collected from 5 farms of Basherhat, Sadar, Dinajpur district and 17 samples were positive for *E. coli*. (Table 14). The positive (%) samples of isolated *E. coli*. in collected chicken intestine, chicken liver, chicken heart, chicken lungs and chicken oviduct were 9 (90%), 3(30%), 2 (25%), 1 (12.5%) and 2 (18.18%) respectively (Table 14).

A total of 41 samples were collected from 8 farms of Mohabalipur, Sadar, Dinajpur district and 16 samples were positive for *E. coli*.

(Table14). The positive (%) samples of isolated *E. coli*. in collected chicken intestine, chicken liver, chicken heart, chicken lungs and chicken oviduct were 8 (83.33%), 2(20%), 1 (20%), 2 (40%) and 2 (11.11%) respectively (Table 14).

A total of 43 samples were collected from 7 chicken farms of Gobindapur, Sadar, Dinajpur district and 21 samples were positive for *E. coli*. (Table14). The positive (%) samples of isolated *E. coli*. in collected chicken intestine, chicken liver, chicken heart, chicken lungs and chicken oviduct were 11 (91.66%), 1(25%), 4 (33.33%), 2 (28.57%) and 3 (37.5%) respectively (Table 14).

A total of 131 samples were collected from 5 chicken farms of Basherhat, Mohabalipur and Gobindapur of Sadar, Dinajpur district and 54 (41.22%) samples were shown positive for *E. coli* (Table 15). Out of 131 samples, 34 chicken intestines, 24 chicken liver, 25 chicken heart, 20 chicken lungs, 28 chicken oviduct were subjected for *E. coli*(Table 14). Out of 34 chicken intestines samples 30 (88.23%) shown positive, out of 24 chicken liver samples 6 (25%) shown positive, out of 25 chicken heart samples 7 (28%) shown positive, out of 20 chicken lungs samples 5 (25%) shown positive and out of 6 chicken oviduct samples 6 (21.42%) shown positive for *E. coli*(Table 14). The highest percentages of *E. coli* were observed in collected samples of chicken intestines 11 (91.66%) from Gobindapur, Sadar of Dinajpur district (Table 14).

In this study, a total of 131 samples were collected from 5 chicken farms of Basherhat, Sadar, Dinajpur district, 8 chicken farms from Mohabalipur, sadar, Dinajpur district and 7 chicken farms of Gobindapur, sadar, Dinajpur district in Bangladesh. Out of 131 samples, 54 (41.22%) samples have shown positive for *E. coli* (Table 14).

Table no 14: Summary of isolated *E. coli* from internal organs of dead chicken

Placement (no. of farms)	No of samples collected						*E. coli*					
							No. of isolates (%)					
	CI	CL	CH	CLN	CO	Total	CI	CL	CH	CLN	CO	Isolates
Baserhat (5)	10	10	8	8	11	47	9 (90)	3 (30)	2 (25)	1 (12.5)	2 (18.18)	17 (36.17)
Mohabalipur (8)	12	10	5	5	9	41	10 (83.33)	2 (20)	1 (20)	2 (40)	1 (11.11)	16 (34.04)
Gobindapur (7)	12	4	12	7	8	43	11 (91.66)	1(25)	4 (33.33)	2 (28.57)	3 (37.5)	21 (48.83)
Gross total	34	24	25	20	28	131	30 (88.23)	6 (25)	7 (28)	5 (25)	6 (21.42)	54 (41.22)

Legends:

CI=Chicken intestine, C=Chicken liver, CH=Chicken heart, CLN=Chicken lungs, CO=Chicken oviduct.

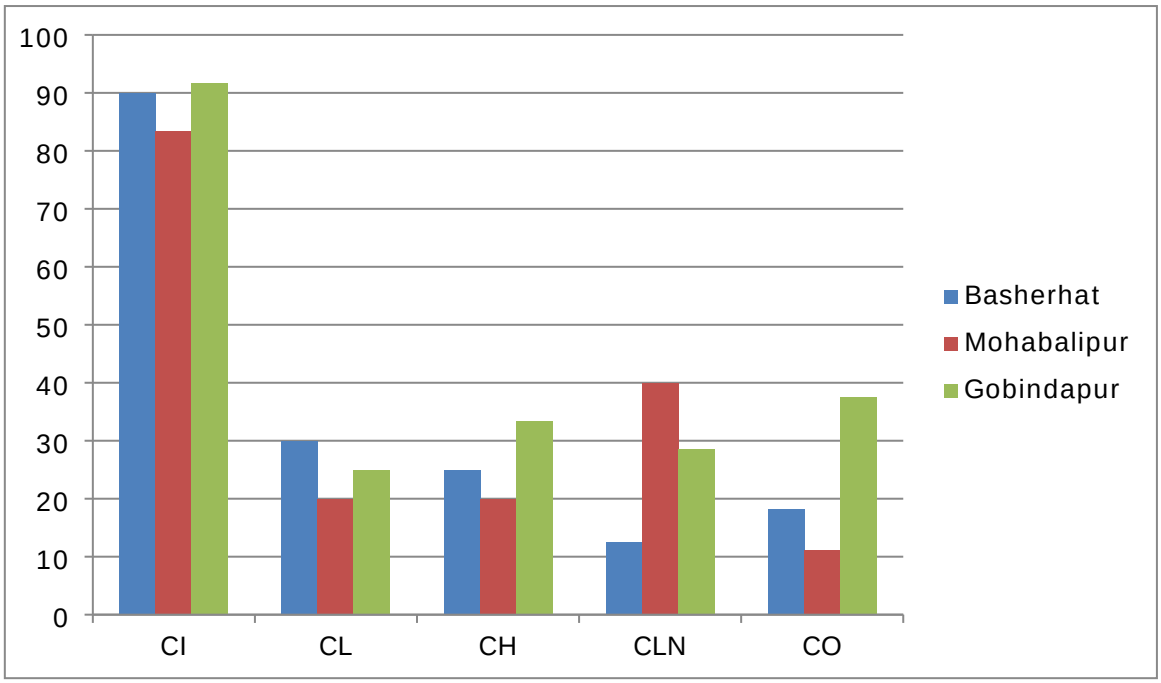

Figure no. 34: Summary of isolated *E. coli* from different internal organs of dead chickens

Table no 15: Total *E. coli* isolated from Basherhat, Mohabalipur, Gobindapur of sadar, Dinajpur district.

Sample collection area	No. of samples collected	No. of *E. coli* isolated	% of *E. coli* detected
Basherhat	47	17	36.17
Mohabalipur	41	16	39.02
Gobindapur	43	21	48.83
Total	131	54	41.22

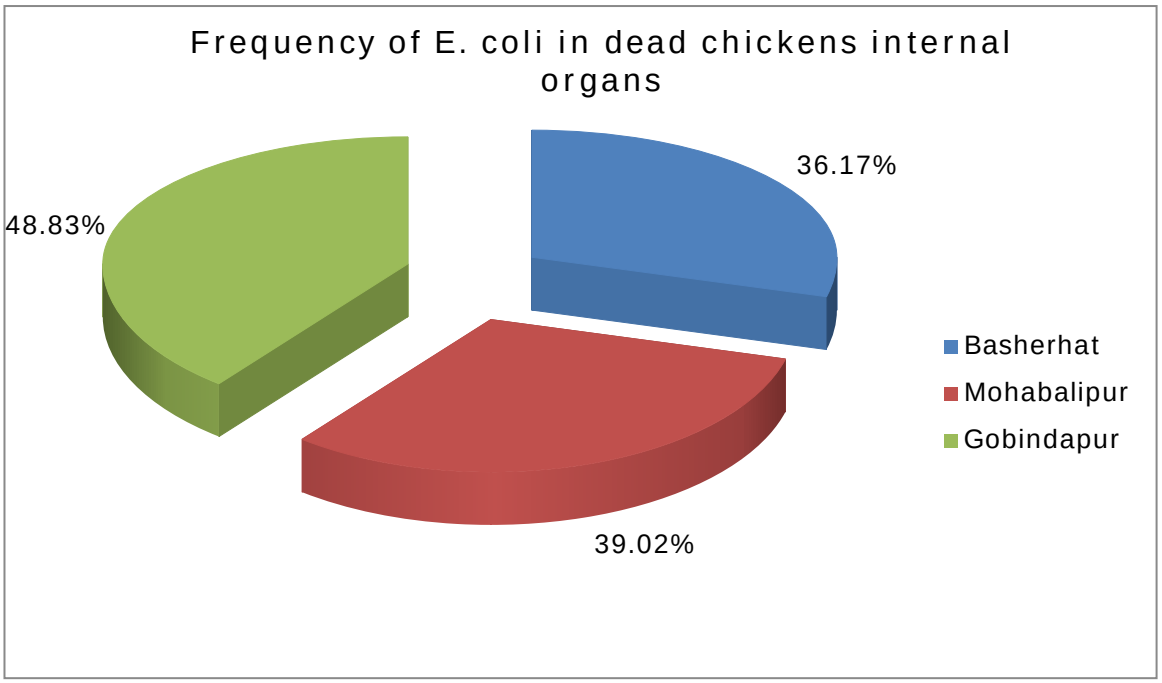

Figure no. 35: Frequency of *E. coli* in dead chickens internal organs

4.1.7.1 Result of *E. coli* serogrouping

Serogrouping of *E. coli* isolates was performed by slide agglutination test using commercial *E. coli* specific polyvalent O (A-I) antisera, *E. coli* O group B (Factor O: 8, 19, 84) antisera and *E. coli* O group D (Factor O: 2, 55, 78) antisera (S & A Reagent Lab).The test was performed according to the protocol supplied by the manufacturer. All isolates were positive to *E. coli* Poly A-I antisera and then some were positive to *E. coli* O group B antisera and some were positive to *E. coli* O group D antisera as presented in Table 16, and 17.

Table no 16. Serogrouping of *E. coli* isolates by slide agglutination test

Isolate no.	Result		
	Poly A-I	Group B (O:8,19,84)	Group D (O:2,55,78)
1	Positive	Negative	Positive
2	Positive	Negative	Positive
3	Positive	Negative	Positive
4	Positive	Positive	Negative
5	Positive	Positive	Negative
6	Positive	Negative	Positive
7	Positive	Negative	Negative
8	Positive	Negative	Positive
9	Positive	Positive	Negative
10	Positive	Negative	Positive
11	Positive	Negative	Positive
12	Positive	Negative	Positive
13	Positive	Positive	Negative
14	Positive	Negative	Positive
15	Positive	Positive	Negative
16	Positive	Positive	Negative
17	Positive	Negative	Positive
18	Positive	Negative	Positive
19	Positive	Positive	Negative
20	Positive	Negative	Positive
21	Positive	Negative	Positive
22	Positive	Negative	Positive
23	Positive	Negative	Positive
24	Positive	Negative	Positive
25	Positive	Positive	Negative
26	Positive	Negative	Positive
27	Positive	Negative	Positive
28	Positive	Positive	Negative
29	Positive	Positive	Negative
30	Positive	Positive	Negative
31	Positive	Negative	Positive

32	Positive	Positive	Positive
33	Positive	Negative	Positive
34	Positive	Negative	Positive
35	Positive	Positive	Positive
36	Positive	Negative	Positive
37	Positive	Positive	Positive
38	Positive	Positive	Positive
39	Positive	Negative	Negative
40	Positive	Negative	Positive
41	Positive	Negative	Negative
42	Positive	Negative	Negative
43	Positive	Negative	Positive
44	Positive	Negative	Negative
45	Positive	Negative	Positive
46	Positive	Positive	Negative
47	Positive	Negative	Negative
48	Positive	Positive	Positive
49	Positive	Negative	Negative
50	Positive	Positive	Negative
51	Positive	Negative	Positive
52	Positive	Positive	Positive
53	Positive	Positive	Negative
54	Positive	Positive	Positive

Table no. 17: Summary of *E. coli* serogrouping

Isolates	No. (%) of *E. coli* isolates		
E. coli (n=54)	Poly A-I	Group B (O:8,19,84)	Group D (O:2,46)
	54 (100%)	21 (38.88%)	33 (61.11%)

4.1.8 Results of antimicrobial study

4.1.8.1. Results of antimicrobial susceptibility of *E. coli*

All 54 *E. coli* isolates were subjected to antimicrobial susceptibility testing against 17 selected antibiotics. The results of

susceptibility analysis showed that all the 54 (100%) *E. coli* isolates were susceptible to gentamicin, norfloxacin, ciprofloxacin and streptomycin. All isolates of *E. coli* 100% (n=54) were resistant to tetracycline and erythromycin, Ampicillin, Amikacin, Cephalexin, Penicillin, Vancomycin whereas averages 25 (46.29%) isolates were susceptible, 15 (27.77%) isolates were intermediate and 12 (22.22%) isolates were resistant to amoxicillin. Another 33(61.11%) isolates were susceptible, 9 (16.66%) isolates were intermediate and 21 (38.88%) isolates were resistant to azithromycin. Results are presented in Table 19 and Figure 16a, 16b.

Table no 18: Antimicrobial susceptibility pattern of *E. coli* by disk diffusion method

Antimicrobial agents	No. (%) of *E. coli* isolates		
	S	I	R
Amoxicillin	33 (61.11%)	9 (16.66%)	12 (22.22%)
Azithromycin	15 (27.77%)	18 (33.33%)	21 (38.88%)
Ciprofloxacin	54 (100%)	0 (0%)	0 (0%)
Erythromycin	0 (0.0%)	0 (0%)	54 (100%)
Gentamicin	54 (100%)	0 (0%)	0 (0.0%)
Norfloxacin	54 (100%)	0 (0%)	0 (0.0%)
Streptomycin	54 (100%)	0 (0%)	0 (0%)
Tetracycline	0 (0.0%)	0 (0%)	54 (100%)
Chloramphenicol	29 (53.70%)	11 (20.37%)	14 (25.92%)
Ampicillin	0 (0.0%)	0 (0%)	54 (100%)
Amikacin	0 (0.0%)	0 (0%)	54 (100%)
Cephalexin	0 (0.0%)	0 (0%)	54 (100%)
Colistin	13 (24.07%)	31 (57.40%)	10 (18.51%)
Penicillin	0 (0.0%)	0 (0%)	54 (100%)
Neomycin	43 (79.62%)	6 (11.11%)	5 (9.25%)
Kanamycin	17 (31.48%)	0 (0%)	40 (74.07%)
Vancomycin	0 (0.0%)	0 (0%)	54 (100%)

Legends: S= Susceptible; I= Intermediate; R= Resistance

4.1.8.2. Results of antimicrobial resistance pattern of *E. coli*

The results of antimicrobial resistance patterns of *E coli* are summarized in Table 20. Out of 54 *E coli* 17 (31.48%) were resistant to 2 agents E-TE. 10 (18.51%) were resistant to 3 agents E- AMX-TE. 12 (22.22%) were also resistant to 4agents E-AZM-TE. Another 15 (27.77%) were resistant to 5 agents AMX-AZM-E-TE-PE. The isolated *E coli* were detected as multidrug resistant isolates as presented in Table 19 and Figure 40(a) and 40 (b).

Table no 19: Results of antimicrobial resistance pattern of *E. coli* by disc diffusion method

Isolates	Resistance profiles	No. of isolates (%)
E. coli (n=54)	No resistance demonstrated	–
	Resistant to 2 agent (E-TE)	17 (31.48%)
	Resistant to 3 agents (E- AMX-TE)	(18.51%)
	Resistant to 4 agents (E-AZM-TE)	(22.22%)
	Resistant to 4 agents (AMX-AZM-E-TE-PE)	(27.77%)
	Total resistant isolates	54 (100%)

Legends: AMX=Amoxicillin, AZM=Azithromycin, E=Erythromycin, GEN=Gentamicin, CIP=Ciprofloxacin, NOR=Norfloxacin, TE=Tetracycline, S=Streptomycin, PE=Penecillin.

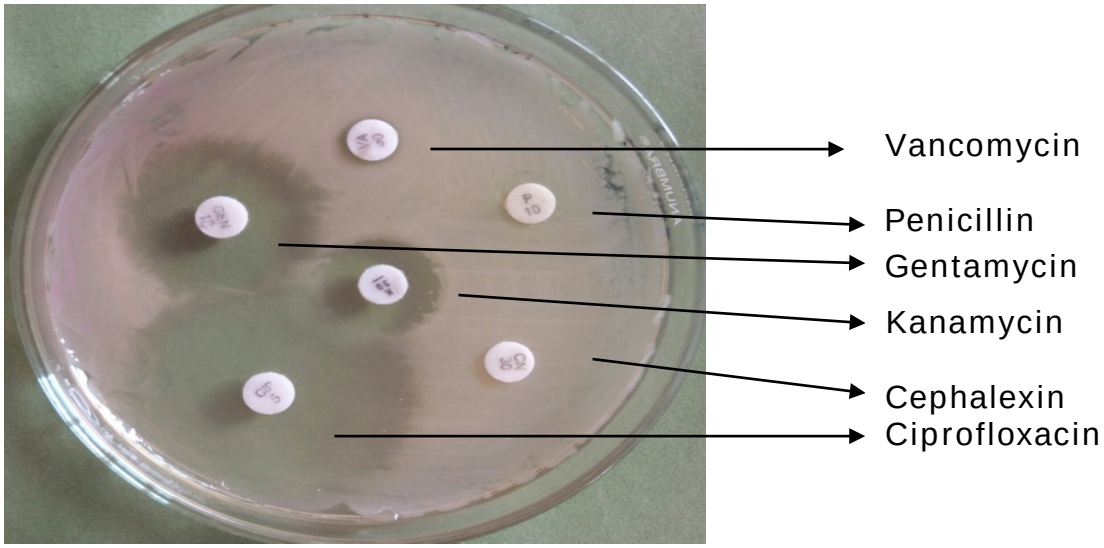

Figure no 36 (a): Antibiotic sensitivity test for *E. coli*

Legends: S = Sensitive

I=Intermediate

R= Resistant

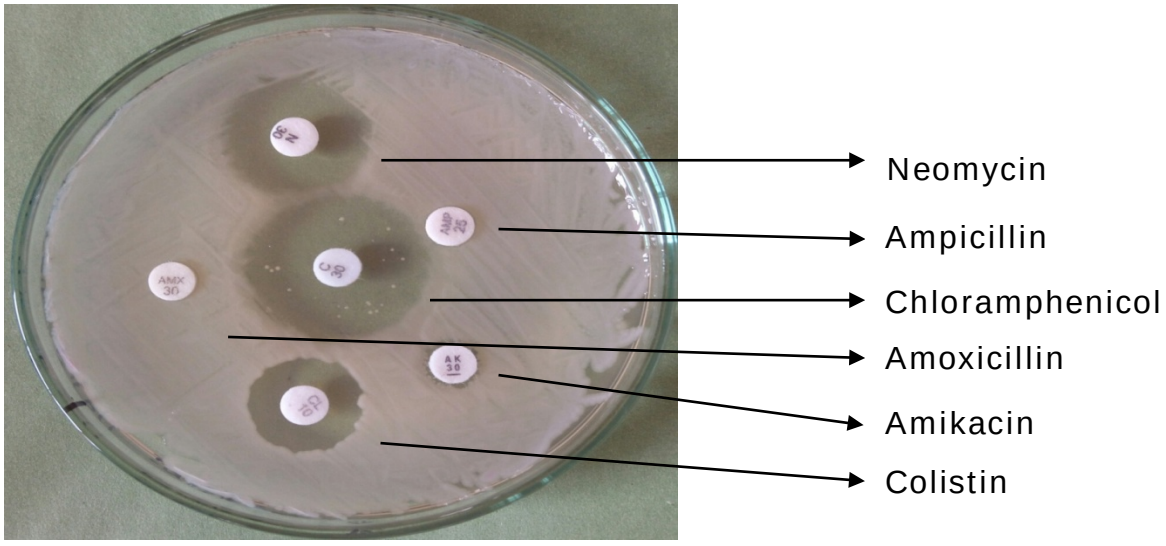

Figure no 36 (b): Antibiotic sensitivity test for *E. coli*

Legends: S = Sensitive

I=Intermediate

R= Resistant

CHAPTER 5

DISCUSSION

The present study was carried out for the isolation and molecular characterization of *E. coli* isolated from internal organs of dead chickens of Basherhat, Mohabalipur and Gobindapur sadar of Dinajpur district of Bangladesh. The samples (Chicken in testine, chicken liver, chicken heart, chicken lungs and chicken oviduct) were collected and brought to the Microbiology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur for the cultural and morphological studies as well as characterization of *E. coli.* PCR was performed at National Institute of Bangladesh (NIB) with genus specific 16s rRNA gene (584 bp). Serogrouping of *E. coli* isolates were performed by slide agglutination test using commercial *E. coli*-specific polyvalent O (A-I) antisera, *E. coli* O group B (Factor O: 8, 19, 84) antisera, and *E. coli* O group D (Factor O: 2, 55, 78) antisera (S & A Reagent Lab). Finally, antibiogram study was done to evaluate the sensitivity and resistance patterns of the *E. coli* with available and mostly used antibiotics and some certain used antibiotics. The cultural characterization of *E. coli* isolates from dead chickens internal organs were produce rose pink colour smooth opaque, colorless colony in MacConkey agar, and with circular, small smooth, low convex and gray white colonies in nutrient agar which were similar to (Edberg*et al.*1982; Edwards *et al.*1972*;* Buxton and Freser *et al.* 1977), yellowish green colonies surrounded by an intense yellow green zone on BG agar (Ali *et al.* 2015; Akond *et al.* 2009) and characteristic metallic sheen colonies on the EMB agar (Horvath *et al.*1974*)*. Differences in colony morphology manifested by the isolates may be due to loosing or acquiring some properties by the transfer of host or choice of host tissue as observed by Cowan and Freeman *et al.* (1985).In Gram's staining, the morphology of the isolated *E. coli* exhibited pink coloured, small rod shaped, arranged in single or paired Gram negative bacilli and in the hanging drop

technique all the isolated *E. coli* revealed motility as observed by several authors (Buxton and Fraser, 1977; Freeman, 1985; Jones, 1987). Reactions in TSI agar slant revealed yellow slant and butt with gas but no production of hydrogen sulphide gas was observed which supports the finding of Buxton and Fraser (1977). In this study, all the isolates of *E. coli* fermented dextrose, maltose, lactose, sucrose and mannitol with the production of both acid and gas (Ali *et al*. (1998). The results of Catalase, MR and indole test of the *E. coli* isolates were positive but V-P test was negative which are in agreement with the reports of Buxton and Fraser (1977) and Honda *et al*. (1982).

In this study, biochemical tests were performed for the identification of *E. coli* which were also used by several researchers (Brown *et al.* (2004). Thomas *et al.,* 1988; Sarada *et al*., 1999). In carbohydrate fermentation test, the isolates that fermented glucose, maltose and produced acid and gas also ferment lactose those indicated positive for *E. coli*as was stated by Buxton and Fraser (1977). The isolates were positive for Methyl Red test but negative for VP test indicating characteristics of *E. coli.* test which was similar with the statement of (Brown *et al.* (2004). In indole test, all the test isolates (n=54) developed red color that indicated the *E. coli*. The isolates were also positive to indole test and this was similar with the findings of Sarada *et al*. (1999).Motility test was elementary basis for the detection of motile and non-motile. *E. coli*. In motility test, 54 isolates were motile.

In this study molecular characterization was done by PCR, in which 16s rRNA gene was amplified for the detection of isolated *E. coli*. All conditions and results found in the PCR was related by the findings of the several authors (Chen J. & Griffiths (2001), and Bej and Steffan, 1990; Lin and Tsen, 1996). The present study was attempted to investigate the presence of *E. coli* in chicken sadar, Dinajpur district in Bangladesh.

E. coli were isolated from 34 apparently healthy dead bird intestine samples and positive isolates were 88.23% (n=30). In this study the incidence of *E. coli* in dead bird intestine organs was closely similar with the results reported by several researchers (Hossain *et al.,* 2015; Sudershan *et al.,* 2012; Ahmed *et al.,* 2009; Zhao *et al.,* 2001).

There was presence of 25.00% (n=6) in collected samples of chicken liver (24) from dead bird liver samples and this findings was very close to the findings of several researchers (Khalid *et al.,* 1990). This finding also indicated that transmission of *E. coli* occured to the chick that was similar to the findings reported by several authors (Kaul *et al*., 1992; Shah *et al*., 1992).

In this study 28.00% (n=7) of *E. coli* were positive in chicken heart samples (n=25). The result was supported by several authors (Ewers *et al*., 2003; Gross *et al.,* 1991). In chicken lungs samples 25.00% (n=5) of *E. coli* were positive samples (n=20). The prevalence of *E. coli* in chicken lungs was closely similar to the findings of Ewers *et al*. (2003). There was also 21.42% (n=6) *E. coli* were present in chicken oviduct samples (n=28). The results of this study were closely related with the results of several authors (Mukhopadhyaya *et al.,* 1992*;* Bandyopadhay *et al*., 1984). About 34 chicken intestine samples were collected for the research work. Among34 chicken intestine samples 88.23% (n=30) samples were positive for *E. coli*. Among three places higher percentage observed 91.66% (n=11) in Gobindapur, sadar, Dinajpur district of Bangladesh. The results of this study were in close relation with the results of several researchers (Kabir *et al*., 2010; Amara *et al*., 1995; Sarker *et al*., 2012). Chicken intestine samples have been used to provide evidence of persistent intestinal colonization by *E. coli* in individual birds reported by Nolan *et al*. (2013); Kim *et al*. (1996).

Among the 131 collected samples from three different places of sadar, Dinajpur district, the total *E. coli* were isolated as 41.22% (n=54). The isolated *E. coli* in Basherhat was 36.17% (n=17) and in Mohabalipur

was 34.04% (n=16) and the isolated *E. coli* in Gobindapur was 48.83% (n=21). The results of this study were closely related with the results of several authors (Talha *et al.*, 2001; Kabir, 2010).

From the collected 131 samples, 54 *E. coli* were isolated. Among the 54 isolates, 38.88% (n=21) belonged to serogroup B and 61.11% (n=33) isolates belonged to serogroup D. The most prevalent serogroup identified in this study was serogroup D. The results of serogrouping corelated with the results of motility test where total 54 isolates were motile. These findings were in agreement with the result reported by several researchers (Cloud *et al.*, 1985; La *et al.*, 2002; and Shuchismita *et al.*, 2007).

In this study it was revealed that *E. coli* were sensitive to ciprofloxacin, gentamicin, norfloxacin and streptomycin. This result was supported by a number of researchers (Jakaria *et al.,* 2012; CLSI *et al.,* 2007; Chen *et al.,* 2014; Blanco *et al.,* 1997). Out of the 54 *E. coli* isolates 100% (n=54) were resistant to tetracycline which was similar to the report of (Ahmed *et al.,* 2013; Jiang *et al.,* 2011). 17 (31.48%) were resistant to 2 agents E-TE. 10 (18.51%) were resistant to 3 agents E-AMX-TE. 10 (18.51%) were also resistant to 3 agents E-AZM-TE. Another 15 (27.77%) were resistant to 4 agents AMX-AZM-E-PE. Similar studies were also observed by several researchers (Jakaria *et al.*, 2012; Islam *et al.*, 2004; Hossain *et al.,* 2005; Olarinmoye *et al.,* 2013; Tapan *et al.,* 2012). Resistant profile of *E. coli* were recorded some multi drug resistant *E. coli* which was similar to the result of some researchers (Jakaria *et al.*, 2012; Obeng *et al.*, 2012).

In the limited attempt, samples were collected from only three places of sadar, Dinajpur district for isolation and identification of *E. coli* .So, investigations on other places of Dinajpur district as well as other districts will be required to identify the *E. coli* associated with commercial poultry production.

In relation to the present study, further study might be performed on the following aspects:

i. Genomic studies about genes responsible for pathogenicity and drug resistance of *E. coli.*

ii. Molecular characterization of the isolated *E. coli* by pulsed field gel electrophoresis (PFGE)

CHAPTER 6

CONCLUSIONS

In the context of this study it may be concluded that internal organs collected from dead chickens are reservoirs for the *E. coli*. They might make the birds vulnerable for easy access of infection. The bacterial pathogens may passes through the faeces to the environment and can easily spread to other animals, humans via soil, food and water. The finding of the present study revealed the prevalence of multidrug resistant *E. coli* in the samples of the study area. Antimicrobial drug resistancy is becoming a major threat to global public health. It is not only a threat in the treatment of poultry diseases associated with *E. coli* but also a potential public health hazard to individually that consume poultry products. Considering the findings of this research work, it may be concluded that:

a) The presence of *E. coil* from internal organs of dead chicken were observed.
b) The highest occurrence of *E. coil* observed at intestine of dead chicken
c) The most prevalent *E. coli* serogroup identified in this study was *E. coli* serogroup D.
d) The findings of the present study revealed the presence of multidrug resistant of *E. coil* from internal organs of dead chicken.

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APPENDICES

APENDIX 1

A. Composition of different media

Nutrient broth

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

Nutrient agar

Ingredients	g/L
Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	20.0
Final pH (at 25°C)	7.1±0.1

3. Mac Conkey agar

Peptone	17.0 gm
Protease peptone	3.0 gm
Lactose	10 gm
Bile salts	1.5 gm
Sodium chloride	5.0 gm
Agar	13.5 gm
Neutral Red	0.03 gm
Crystal violet	0.001 gm
Final pH (at 25^0C)	7.1 ± 0.2

4. Blood Agar

Blood Agar	60 gm
Distilled Water	1000 ml
Bovine blood	5.00 ml
Or Nutrient agar	500 ml
Sterile Defibrinated blood	25 ml

5. Salmonella-Shigella agar

Peptic digest of animal tissue	5.00 gm
Beef extract	5.00gm
Lectose	10.00 gm
Bile salts mixture	8.50 gm
Sodium citrate	10.00 gm
Sodium thisulphate	8.50 gm
Ferric citrate	1.00 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	15 gm
Disstilled water	1000 ml
Final pH (at 25^0C)	7.0 ± 0.2

6. Eosine methylene blue agar

Ingredients	g/L
Peptone	100
Lactose	10.0
K2HP04	2.0
Eosin	0.4
Methylene blue	0.065
Agar	20.0
Final pH	6.8±0.2

7. Muller- HingtonAgar g/L

Beef infusion	300.00 gm
Casein acid hydrolysate	17.50 gm
Starch	1.50 gm
Agar	17.00 gm
Final pH (at 25^{o}C)	7.3 ± 0.1 gm

8. *Stapylococcus* medium No. 110 g/L

9. Mannitol Salt Agar

10. Cetrimide Agar Base

Composition**

Ingredients	Gms / Litre
Pancreatic digest of gelatin	20.000
Magnesium chloride	1.400
Potassium sulphate	10.000
Cetrimide	0.300
Agar	15.000

Final pH (at 25°C) 7.2±0.2

11. Tryptic Soy Agar

12. Baird Parker Agar

Ingredients	Gms / Litre
Casein enzymichydrolysate	10.000
Meat extract B#	5.000
Yeast extract	1.000
Glycine	12.000
Sodium pyruvate	10.000
Lithium chloride	5.000
Agar	20.000

Final pH (at 25°C) 7.0±0.2

13. Mitis Sali varius Agar Base

Formula / Liter Supplement (# 7989)

Enzymatic Digest of Casein	15 g	Tellurite Supplement (1%) Chapman	
Enzymatic Digest of Animal Tissue	5 g	(Potassium Tellurite, 100 mg)	
Sucrose	50 g		
Dextrose	1 g		
Dipotassium Phosphate	4 g		
Trypan Blue	0.075 g		
Crystal Violet	0.0008 g		
Agar	15 g		

Final pH: 7.0 ± 0.2 at 25°C

Ingredients	Grams/Litre
Casein peptone (pancreatic)	15.0
Soya peptone (papainic)	5.0
Sodium chloride	5.0
Agar	15.0

Final pH 7.3 +/- 0.2 at 25°C

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.

14. Plate Count Agar

15. Sugar media

a. Peptone water

Bacto-peptone	10.0 gm
Sodium chloride	5.0 gm
0.5% Phenol red	0.1 ml
Distilled water	1000 ml

b. Sugar solutions

Individual sugar	5 gm
Distilled water	100 ml

c. Sugar media preparation

Peptone water	4.5 gm
Sugar solution	0.5 ml

16. Simmons citrate agar

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

17. TSI Agar slant

Ingredients	3.00 gm
Lab Lamco Powder	
Yeast extract	3.00 gm
Peptone	20.00 gm
Sodium chloride	5.00 gm
Lactose	10.00 gm
Sucrose	10.00 gm
Glucose	1.00 gm
Ferric citrate	0.3 gm

Sodium thiosulphate	0.3 gm
Phenol red	0.3 gm
Agar	12.00
Distilled water	1000
Final pH	7.4 +/- 0.2 (at 25°C)

Store dehydrated powder, in a dry place, in tightly-sealed containers at 2-25°C.

18. MR- VP medium (Himedium, India)

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

19. Indole medium

20. MIU medium

APPENDIX 2

B. Preparation of reagents

1. Peptone water

Peptone	1gm
Distilled water	1000 ml

2. Kovac's reagent for indole preparation

P- dimethyl aminobenzaldehyde	5 gm
Amyl alcohol	75 gm
Conc. HCL	25 ml

3. V-P reagent-1

   5% alpha- naphthanol in absolute ethyl alcohol

4. V-P reagent-2

   40% potassium hydroxide containing 0.3% creatine. The ingredient was dissolved by heating gently over a steam bath.

   When in solution, added 0.052 gm of cotton blue dye

5. Methyl red Solution

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

6. Phenol red solution

   0.2% aqueous solution of phenol red

7. Phosphate buffered saline solution

Sodium chloride	8.0 gm
Disodium hydrogen Phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium dihydrogen phosphate	0.2 gm
Distilled water to make	1000 ml

8. Gram stain solution

A. Stock Crystal violate

Crystal violate	10.0 gm
Ethyl alcohol (95%)	1000 ml

B. Stock oxalate solution

Ammonium oxalate	1.0 gm
Distilled water	1000 ml

C. Crystal violet working solution

20 ml of solution no. (a) Mixed with 80 ml of solution no. (b). Additional dilution was made when desired.

D. Ethyl alcohol 250 ml

E. Acetone 250 ml

F. Counterstain

Safranin	2.5 ml
Ethyl alcohol (95%)	100 ml

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

9. Oxidase reagent

Tetramethyl-p- phenylenediamine 0.1 ml

Distilled water 10 ml

10. 3% Hydrogen per oxide (H_2O_2) for catalase test

H_2O_2 3 ml

Distilled water 97 ml

11. Potassium Tellurite 3.5%

Components (1 mL per vial)

Potassium Tellurite 0.35g

Distilled water 1mL

Shelf life of prepared cultured medium

BA..... The plates may be stored at 2-8^{0}C

MA..... 4 weeks at 2-8^{0}C

NA/NB..... Up to 2 years in cool dark place

SS agar..... 6 weeks at 2-8^{0}C