CHARACTERIZATION OF SALMONELLA ISOLATED FROM DUCK AND PIGEON WITH THEIR ANTIBIOGRAM

A THESIS
BY

MD. MOSHIUR RAHMAN
REGISTRATION NO. 1105113
SESSION: 2011-2012
SEMESTER: JANUARY-JUNE, 2013

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

FACULTY OF VETERINARY AND ANIMAL SCIENCE
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
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Approved as to style and content by

Prof. Dr. Md. Mostafizer Rahman
Research Supervisor

Dr. Md. Shahidur Rahman khan
Research Co-Supervisor

Prof. Dr. Md. Mostafizer Rahman,
Chairman
Examination committee
And
Head of
DEPARTMENT OF MICROBIOLOGY
FACULTY OF VETERINARY AND ANIMAL SCIENCE
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY,
DINAJPUR-5200

JUNE, 2013

DEDICATED

TO

MY

BELOVED PARETS

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June, 2013

ABSTRACT

Salmonellosis is a common problem in poultry farms of our country. Indiscriminate use of antibiotic to control the disease results drug resistance and limits the therapeutic possibilities in the treatment of the disease. A total of 90 samples were collected from liver, spleen and intestine of Duck, Pigeon; either dead or sick, brought for antibiogram study following isolation of *Salmonella*e from those birds during the period from January 2013 to June, 2013 from three upazilla of Dinajpur District of Bangladesh. The collected samples were inoculated to different cultural media (NA, MC agar, EMB agar, SS agar, BGA) for the determination of cultural characteristics of the organism.

The isolated organisms were subjected to Carbohydrate (CHO) fermentation test (Dextrose, Lactose, Sucrose, Maltose, Mannitol), MR-VP and indole test. The positive case was recorded as 39.58% in duck, 28.57% in pigeon, The antibiogram study revealed that the isolates were highly sensitive to Ciprofloxacin, Levofloxacillin, Azithromycin; moderately sensitive to Gentamicin, Colistin sulphate. They are less sensitive to Cephradine, Doxycycline while resistant to Amoxycillin and Tetracycline.

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ACRONYMS USED

-	=	Resistance
-	=	Negative
%	=	Percentage
+	=	Less sensitive;
+	=	Positive
++	=	Moderately sensitive
+++	=	Highly sensitive.
µg	=	Microgram
µl	=	Micro litter
AML	=	Amoxycillin
AZM	=	Azithromycin
B	=	Bacitracin
BA	=	Boold Agar
BD	=	Bangladesh
BGA	=	Brilliant Green Agar
C	=	Celsius
C	=	Chloramphenicol
CAR	=	Carbinicillin
CIP	=	Ciprofloxacin
CN	=	Gentamicin
CP	=	Cephalexine
CS	=	Cloacal swab
CSP	=	Cloacal swab procedure
CT	=	Colistin Sulphate
CT	=	Cotrimoxazole
CTX	=	Cefotaxime
DNA	=	Deoxy ribonucleic acid
DO	=	Doxycycline
E	=	Erythromycin

ACRONYMS USED (CONTD.)

e.g.	=	Example
ELISA	=	Enzyme Linked Immunosorbent Assay
G	=	Gram
G/L	=	Gram/Litter
K	=	Kanamycin
LEV	=	Levofloxacillin
MC	=	Mackonkey
Mg	=	Milligram
MR	=	Methyl red
NA	=	Nutrient Agar
NA	=	Nalidixic acid
°C	=	Degree centigrade
OIE	=	Office International des Epizooties
PBS	=	Phosphate buffered saline
PEF	=	Pefloxacin
pp	=	Page number
S	=	Salmonella
SB	=	Selenite broth
SE	=	Salmonella enteritidis
SL	=	Serial
SP	=	Species
SS	=	Salmonella-shigella
TE	=	Tetracycline
TOB	=	Tobramycin
TS	=	Tracheal swab
TSI	=	Triple Sugar Iron
V-P	=	Voges-Proskaure

CHAPTER I

INTRODUCTION

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INTRODUCTION

Salmonellosis is a disease condition caused by a wide variety of salmonellae servers in various hosts including duck, pigeon which remain serious problem with public health significance throughout the world (Tabaraie *et.* bs,1994). Substantial economic loss results from mortality and poor growth of infected animal, birds and human beings as well as the hazards of transmitting food poisoning with gastroenteritis to human and represents a serious problem for the food industry (cooke, 1990 and Todd, 1990). The Duck and pigeon population of Bangladesh is suffering from various diseases and disease conditions *e.g* salmonellosis, duck viral hepatitis, duck plague, pigeon pox. From reports of the field cases it is assumed that salmonellosis is one of the important diseases among them.

Salmonellae are Gram negative, small rod shaped nonsporeforming, noncapsulated,aerobic and facultatively anaerobic organism and classified under the family *Enterobacteriaceae* (OIE, 2006). The genus salmonellae includes a large group of serologically and biologically related bacilli and as a rule they are motile by means of peritrichous flagella with the exception of salmonellae pullorum and salmonellae gallinarum (Blood et al.,2003).

Among more than 2300 serotypes of Salmonella identified, only 10% of these have been isolated from poultry (Gast, 1997). Chicken are the natural hosts for both. *S. pullorum* and *S. gallinarum* (Snoeyenbos. 1991 and Pomeroy and Nagaraja.1991). Pigeons might be the sources of Salmonellosis in human. Pigeon isolates of Salmonella are Salmonella enterica subsp. serovar typhimurium variant Copenhagen ; Rabsch *et al.*, 2002; Nastasi *et al.*, 1993), S. typhimurium and S. Cerro (Tanaka et al., 2005).

In 1919 Rettger reported a disease which was observed in ducklings from which he isolated an organism typical of the Salmonella group. The following year Rettger and Scoville (1920) described the organism in detail and named it *Bacterium anatis.* However Price and Berry (1962) have shown that *Salmonella anatum* is the most important infectious agent. Few of the ducklings die after three to four weeks of age; the greatest mortality occurs within the first week or 7-10 days. Examination of death duckling revealed no lesion of any pathogenic condition except, perhaps paleness of the tissue as a whole and light body weight (Merchant and Packer, 1967). But the clinical sign may vary from species to species (Radostits *et al.*,

Generally Salmonella organisms are being characterized by using various cultural, biochemical, serological and molecular study (Begum, 2005). Salmonellae giving rise to a large number of serotypes besides leading to variation in pathogenesity of the species and may cause serious confusion in diagnosis of the disease. Antibiotic sensitivity was also performed by Gyurov and Dyako (2000); Sung *et al.*, (2002); Banani *et al.*, (2003); Khan, (2004); Rahman, (2005) to select the antibacterial agents for effective therapeutic purpose of *Salmonellosis.* However, antibiotic treatment of *Salmonellosis* is problematic due to the recent emergence of multi-drug resistant of *Salmonella* strains (Mirza *et al.*, 1996).

Salmonellosis assumes to occur as one of the following forms peracute septicemia, acute enteritis, chronic enteritis or a subclinical carrier state. Common clinical signs are septicemia, fever. enteritis, diarrhea. But the clinical signs may vary from species to species (Blood et al., 2003).

The species and varieties of Salmonellae pathogenic for man, animals chicken, duck, pigeon and are numerous and the disease and hazards they produce have a great economic magnitude. A little bit work on isolation and identification of Salmonellae from pigeon, and duck are observed.

Keeping in consideration the above mentioned ideas the presence research work has been undertaken with the following objectives:

I. To isolate and identify the salmonella species in duck and pigeon from field cases.

II. To study the biochemical characteristics of the isolated salmonella species.

III. To study serotypic characteristics of the isolated salmonella species.

IV. To study antibacterial sensitivity of the isolated salmonella species.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 Historical background

For obtaining the distinct evidences and key information, the whole review of literature related to the research title "Characterization of Salmonella isolates of duck and pigeon and their antibacterial sensitivity study" has been conveniently brought under the following parenthesis :

2.2 Isolation of Solmonella

2.2.1 Isolation of Solmonella in general

Chowdhury, A. *et al.*, (2011) investigated the study was conducted aiming at the isolation and identification of *Salmonella*) from different brands duck feeds sold in Savar, Dhaka, Bangladesh. Seven different duck feeds were subjected to microbiological analysis. All were counted separately on the nutrient Agar media. Hence, bacteria were these samples were analyzed by culturing in different media such as nutrient broth (NB), nutrient Agar (NA), SS Agar (Salmonella-Shigella Agar), BGA (brilliant-green Agar), Mac Conkey, DHL and EMB (eosin methylene blue) media.

Mondal *et al.*, (2008) performed a study with the aim to isolate and identify *Salmonella* organism from diarrhoeic and apparently healthy ducks and to characterize duck *Salmonella* by biochemical test. Antibiotic sensitivity analysis of duck *Salmonella* was performed. A total of 65 cloacal samples were collected from ducks of three different regions such as Char Nilokkhiya, BAU Poultry Farm and Boyra, Mymensingh. Out of 65 samples 9 (13.07%) were found positive. The antibiotic sensitivity pattern showed

that the duck isolates were highly sensitive to ciprofloxacin, kanamycin, nalidixic acid, co-trimoxazole and cephalexin but these isolates were highly resistant to chloramphenicol.

Joanne *et al.*, (2008) were collected 110 fecal samples from 82 farms across the islands of Trinidad and Tobago. Salmonella was isolated from fresh and frozen samples and the serotype of each was determined through bacterial culture. An in-house, nested PCR that detects all pathogenic Salmonella species was utilized in analyzing the samples. Five samples were positive for Salmonella by bacterial culture, whereas 44 were positive by the nested PCR. Of the samples, 87 (79%) gave equivalent PCR results for both enrichment broths—28 were positive for both and 59 were negative for both). species.

Kinde *et al.*, (2005) examined studied the samples including drag swabs, egg belt or egg rollout swabs, fan-blade swabs, rodent organ and intestinal pools, beetle *(Alphitobius diaperinus)* pools, housefly *(Musca domestica)* pools, chicken organ and intestinal pools, and egg pools were obtained for *Salmonella* culture from two flocks from two different commercial layer ranches. The two ranches were purposefully selected for the study based on their previous status of *S. enteritidis* isolation using environmental drag swabs in cooperation with practicing veterinarians. *Salmonella* sp. was isolated from 337 out of 979 (34.42%) non-egg samples. S. *enteritidis* was isolated from samples obtained ranch 1 from manure drag swabs, 4/284 (1.4%); rodent organs, 1/24 (4.2%); and housefly pool cultures 1/21 (4.8%). *S. enteritidis* was isolated from ranch 2 from mouse organ and intestinal pool samples, 1/24 (4.2%).

Tanaka *et al*,. (2005) stated that some public areas in Japan such as parks and gardens can be highly contaminated with pigeon feces. The authors examined levels of four bacterial contaminations in fecal samples from feral pigeons in 7 prefectures isolated *Salmonella typhimuritan* and *S. Cerro* from 17 (3.9%) of 436 samples, The polymerase chain reaction detected *Chlamydia psittaci* and *C pecorum* in 106 (22.9%) of 463 samples, but *E. coli* O-1 57 was not isolated from any of the samples.

Cherry *et al.*, (2004) reported an outbreak of S. *enterica* serovar *typhimurium* in a veterinary clinic in New York, United States of America. Confirmed cases were in one cat, two veterinary technicians, four persons associated with clinic patients and a nurse not related to the clinic. This outbreak emphasizes the importance of strong public health ties to the animal health community.

Majnaric *et al.*, (2003) performed a survey during the year 2002 at the Diagnosis division of the Krizevci Veterinary Institute in Croatia. The analyzed samples (broilers, eggs, and consumer egg layer faeces and hatchery swabs) were delivered by the Veterinary Inspection conforming to the 2002 regulation. They confirmed *Salmonella* in 52 (2.75%) of 1888 samples; 42 (82%) of 9 (19%) other *Salmonellae.*

Nayak *et al.*, (2003) carried out a comprehensive ecological survey from April 1997 to June 1999 on 4 turkey flocks (F, to $_{F4)}$, maintained on fan-n condition in Georgia, USA. The authors collected turkey caecal and crop content, litter, drinker, air, feed, feeder, and environmental swab samples from affected turkeys.

Hossain *et al.*, (2002) isolated *Salmonella* from diarrheic calves. The author stated that the *Salmonella spp.* produced small round and smooth colonies on nutrient agar and opaque, translucent and colorless colonies on SS agar. The organisms produced colourless, pale, transparent colonies on MacConkey agar and small, round, low convex, translucent, pale red colour colony on BGA against pinkish background which was initially green in colour.

Talha *et al.*, (2001) conducted a pathological investigation on the poultry diseases occurring in Mymensingh district, Bangladesh, was conducted during the period from July 1998 to October 1999. A total of 381 samples of either dead or sick birds were collected from different upazillas of Mymensingh district. Diagnosis of different disease conditions was made on the basis of the history, age of birds, clinical signs, gross and microscopic lesions, isolation of organisms and response to treatment

Wray and Wray (2000) described the *Salmonella* is named according to the American bacteriologist Daniel E. Salmon who together with Smith isolated a bacteria in 1886 from pigs (now known to be *Salmonella choleraesuis)* which the authors considered to be the cause of swine fever (hog cholera).

Uzzau *et al.* (2000) stated that Salmonella constitutes a genus of zoonotic bacteria of worldwide economic and health importance. The current view of *Salmonella* taxonomy assign the member of this genus to two species *Salmonella enterica, Salmonella bongori. Salmonella enterica* itself is devided into six sub species, *enterica, salamae, arizonae, diarizonae, indica, and houtenae,* also known as sub species I, II, , III a ,IV, VI respectively.

Dhruba *et al.*, (1999) isolated 25 strains of *S. gallinarum* from diseased broilers layers and cockerels of different age groups farms in West Bengal between 1994 and 1995 and the isolates were characterized by morphological cultural and biochemical examination.

Goneagul *et al.*, (1999) used cloacal swab procedure (ssp) and Drag swab procedure (DSP) to isolate *Salmonella* from 20 breeders, 17 layer and 13 broiler flocks from Bursa in Istanbul and Ixmir districts. *Salmonella* suspected colonies were identified biochemically and serologically. They detected *S. gallinarum (5* cases), *S. typhimurium* (4) and *S. enteritidis* (3) by CSP and *S. gallinarum (5), S. typhimurium (7)* and *S. enteritidis* (6) by DSP. They concluded that DSP in mere reliable and practical than CSP for detecting *Salmonella* infection on chicken.

Gast *et al.*, (1998) reported that direct contact with infected birds and indirect contact with contaminated environmental surfaces are known to be important factors in the dissemination of *Salmonella enteritidis* in poultry flocks, the potential role of airborne transmission is less clearly defined. They considered the mechanism by which *S. enteritidis* might spread between groups of chicks housed in controlled-environment disease transmission cabinets, separated by an unoccupied space that prevented any direct or indirect contact.

Christensen *et al.*, (1994) identified 14 Danish and 5 German isolates of *S. el7tertica serosar Gallinarum hiovar galllinarum* from recent outbreaks in Denmark and Germany. They used phenotypic and genomic methods for the isolation and identification of this species.

Jha *et al.*, (1994) conducted a study on *Salmonellosis* in village chickens and at farms in eastern Nepal. Of 1409 blood samples examined serologically, the prevalence of *Salmonellosis* in fowls at the Tarahara Livestock Farm was 25.7%, 21.3% in the village flocks of Bhojpur and 12% in the village flocks of Dhankuta. Breed prevalence was highest (29.8%) in Giriraja fowls. They found that the highest prevalence was in 51-60 week old chickens. Bacteria isolated form seropositive animals were identified as *S. pullorum.*

Irwin *et al.*,(1990) examined 500 cloacal swabs of broiler chickens, from birds of *50* farms collected at a single abattoir. All were negative for verocytotoxin. 96 different *patterns of resistance to antibacterial agents were found in the 500 E. cloli isolates* selected. *Salmonella spp.* Were recovered from 19 (3.8%) birds and at least Salmonella positive chickens was identified from 7 of the 50 farms.

Prukner *et al.*, (1987) examined 17717 poultry samples and resulted in the isolation of bacteria from 10324 (58%); 82.7% of the isolates were *Escherichia coli* and 7.5% Salmonella. Of the 759 Salmonella isolates 363 (47.8%) were S. *virchow,* 247 (32.5%) *S. enteritidis,* 59(7.8%) *S. typhimurium* and 45 (5.9%) *S. gallinarum.*

Judy and Hart (1968) carried out a survey to detect the carrier of *Solmonella* organisms in cattle slaughtered in northern territory. In that survey samples of mesenteric lymphnodes and faeces from 655 cattle from all districts of the Northern territory were investigated for *Salmonella.* Results showed that 1.5% of faecal samples revealed the presence of *Salmonella* of 5 serotypes, most commonly S. *typhimurium*, S. *chester* and,S. *adelaide.*

Jack *et al.*, (1968) reported that the organism (s. *abortus ovis*) may be pathogenic for mice when injected intraperitoneally at a dose of 2.5x 10^8 but the pathogenicity appears to bevariable for particular isolates. It does not cause death of mice when given by mouth nor does it appear to spread from infected to healthy mice which are housed together.

Wilson and McDonald (1967) recorded nine isolates of S. *typhimurium* from 1573 wild birds of seventy four different species (0.6%) in one survey and eight from 1142 specimens (0.7%) in another.

Stevens *et al.*, (1967) reported that 1,728 incidents of *Salmonella* infection in cattle were encountered by veterinary investigation service in England and Wales, and all but 27 of these infections were associated either with S. *dublin* ot S. *typhimurium*. **Stenberg *et al.*, (1967)** examined 13,061 faecal samples from a herd suspected to have Salmonellosis and stated that only 6.6% of the samples yielded *Salmonello*. S. *typhimuriumwas* isolated from 97% of the positive samples.

O'conner *et al.*, (1967) in the survey at the Dublin abbaitor, examined species of liver, bile, spleen, mesenteric lymphnodes and faeces from 1000 animals and reported that S. dublin was the only serotypes identified and isolated from3.2o/o of the animals examined..

Anon *et al.*, (1965) from a survey in England and Wales reported that S. dublinand S. typhimurium were the connect serotypes encountered in the incidence of calf infection. The serotypes were isolated from raw materials of animal feed stuff and Salmonella infected cattle.

Slavkov *et al.*, (1965) examined 8,739 faecal samples and7,605blood samplesfrom cattle of all ages and found the highest percentage of almonella carrier incalves. The strains of Salmonella isolated from faeces were S.*choleraeszis*, S.*typhimurium*, S. *newport*, S. *dublin*, S. *enteritidis* and also S. *arizona*.

Grau *et al.*, (1965) reported that 87 (45%) of 193 samples of rumen fluid of slaughtered cattle were found to contain Salmonella. Thirty one serotypes were isolated and the most coilrmon serotypes were S. oranienberg (13%), S. veile (12%), S. typhimurium (11%), S. sondiego (9%) and S, saintpaul (7%) representing 52% of the organisms identified.

Zipplies *et al.*, (1964) isolated *Salmonella* from 139 of the 10,592 swabs taken from the surface of carcases, organs chopping boards, scales, towels, sausage meat and meat products in the meat processing plants at the institute of Karl- Mam- Stadt. S. *stanly*, S. *anatum* and ,S. *typhimurium* were mostly isolated from 58, 28 and 18 of the 139 positive samples respectively although S. *bovis morbificans*, S. d*erby*, S. *brandenburg*, S. *barielly*, S. *newington*, S. *newport*, S. *heidenberg*, S. *manchester*, S. *give* and S. *abony* were also isolated from some of the samples. Mixed infections were also present.

Shaffer *et al.*, (1964) had the contention that birds are relatively refractory to S. **typhimurium** infection under natural condition and as adults in the laboratory.

Dack *et al.*, (1963) described 20,179 incidents of S. *typhimurium* in England and Wales in the 1954-1959.

Goodchild and Tucker (1963) recovered *Salmonellae* from eleven (2.85%)cases out of a total of 382 post mortem examinations of British wild birds. In cloacae swabs taken from 551 apparently normal wild birds, *Salmonellae* were found from two (0.39%). The types isolated were,S. *indiana*, S. *menston* and,S. *typhimurium*.

Schaal (1963) observed that the river water is the important source of Salmonellosis of *cattle. The author brought S typhimurium infection under control in a herd of about 160* cattle by exclusion of drinking water obtained from river.

Zwart *et al.*, (1962) investigated the *Salmonella* carrier state of different animals in Ghana and reported that 21.3% of the cattle, 8.3% of the dogs, 8.6% of the rodents, 37.5*o/o* of the lizards and29.6% of the snakes were found to have infected with Salmonella and 11 strains of *Salmonella* with hitherto unknown antigenic patterns were isolated.

Zwart *et al.*, (1962) said that salmonellosis is a world wide problem in people and animals. *Salmonella* organisms are widely distributed in nature such as water, faeces, grains, egg shell, oyster shell etc. According to him, products of plant origin also may contain *Salmonellae* which may be due to their contamination by animals and their faeces.

Elizabeth *et al.*, (1955) isolated Salmonellae from 15% of the mammals, from 7.8% of the birds and from 12.3% of the reptiles studied at the Detroit Zoological Park. Out of the 34 positive cases of birds 9 types of *Salmonellae* were identified as following: S. *typhimttrfuim*, S. *anatum*, S. *enteritidis*, S. *give*, S. *scharzengrund*, S. *pullorum*, S. *sendiego*, S. *champoign* and S. *poona*, in each case, one species was untypable.

Jennings *et al.*, (1954) in a survey of wild bird mortality examined 112 dead specimens of birds and isolated, S. *pullorum* from a magpie and,S. *galinarum* from a curlew and a black bird. He also teviewed that in Australia outbreaks of ovine Salmonellosis have been recorded as being due to drinking water heavily contaminated with droppings of birds like magpies, crows and hawks were shown to be infected with S. *typhimurium.*

Howarth *et al.*, (1954) examined 37 calves obtained from several dairies in the Los Angeles after holding them there for 24 hours and dividing them in two groups consisting of 22 and 15 calves respectively of about 5 days of old. They stated that 12 of 22 calves and 14 of the 15 calves were showing sings of weakness and Diane and 76% and 66% of the ailing calves of the respective groups were shedding *Salmonella* in their faeces.

Ritchie and Clayton (1951) reported the isolation of S. *dublin* mostly with the exception of S. *typhimurium* in one occasion from samples of faeces, bile and liver swabs of healthy cattle of Irish and non Irish origin. S. *dublin* was isolated

Taylor et al., (1950) in a survey of food poisoning cases in Britain found that 91% of the outbreaks were due to *Salmonello* infections of which S. *typhimurium* being responsible for 75%o of the outbreak from l0% and 3.4% of fecal and bile samples respectively from non Irish cattle. Out of the 202 liver swabs from Irish cattle and 171 liver swabs from non Irish cattle examined, 25 and none of the samples revealed S. *dublinrespectively.*

Wolff *et al.*, (1947) also reported incrimination of beef in outbreaks of *Salmonella* infection in man and this was substantiated by the isolation of S. enteritidis from the patients and from the suspected beef.

Arthur *et al.*, (1947) stated that the majority of *Salmonella* occuring primarily as animal and bird pathogens attained importance in human medicine because of their adoption as human pathogens.

2.2.2 Isolation and identification of *Salmonella* from Duck and Pigeon

Frederick *et al.*, *(2012)* investigated that Duck meat is comparable to that of duck despite being meat and it is a close alternative source of protein and other nutrients for humans. Duck meat is high in protein, iron, selenium and niacin; and lower in calories compared to many cuts of beef. This mini-review reports on the production potentials of ducks and the physicochemical composition of selected duck strains. It also reports on world duck population.

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

Dilmaghani *et al.*, (2011) *Salmonella enterica* subspecies *enterica* serovar Typhimurium causes food-borne outbreaks and systemic diseases in humans and animals. *groEL* gene (also known as *mopA* gene in *S.* Typhimurium), possessing conserved sequence, plays an important role in invasion of bacteria. The purpose of present study was to identify the polymorphism of *groEL* gene among different avian in different regions by PCR-RFLP method.

Mondai *et al.*, (2008) examined that This study was aimed at isolating and identifying *Salmonella* from diarrheic and apparently healthy ducks and to characterize duck *Salmonella* by biochemical test. Antibiotic sensitivity analysis of duck *Salmonella* was performed. A total of 65 cloacal samples were collected from ducks of 3 different regions such as Char Nilokkhiya, BAU Poultry Farm and Boyra, Mymensingh. Out of 65 samples, 9 were found positive (13.07%). The antibiotic sensitivity pattern showed that the duck isolates were highly sensitive to ciprofloxacin,

Deng *et al.*, (2008) stated that ducks were subcutaneously infected with a high-virulence strain of *Salmonella enterica* ssp. *enterica* serovar *Enteritidis* (*Salmonella Enteritidis*). The kinetics of the *Salmonella* Enteritidis genomic DNA loads, the immunohistochemical localization of the bacterial antigens, and the histopathological examination in various tissues were investigated.

Mondal *et al.*, (2008) performed a study with the aim to isolate and identify *Salmonella* organism from diarrheic and apparently healthy ducks and to characterize duck *Salmonella* by biochemical test. Antibiotic sensitivity analysis of duck *Salmonella* was performed. A total of 65 cloacal samples were collected from ducks of three different regions such as Char Nilokkhiya, BAU Poultry Farm and Boyra, Mymensing. Out of 65 samples 9 (13.07%) were found positive..

Pennycoot *et al., (2006)* postmortem examinations were carried out on the carcases of 779 wild birds. Salmonellosis was a common cause of death in greenfinches (Carduelis chloris), house sparrows (Passer domesticus) and chaffinches (Fringilla coelebs), and was also responsible for the deaths of other birds such as goldfinches (Carduelis carduelis), feral pigeons and different species of gulls..

Zhang *et al.*, (2006) reported that *Salmonella* is the leading cause of foodborne illnesses in the United States, and *Salmonella enteritidis (SE)* is the second most frequently isolated *Salmonella serovar. A* rapid and simple method for detecting SE in poultry environmental samples is critical for effective control of SE. Food and Drug Administration (FDA) culture method for detecting SE in naturally contaminated environmental samples.

Roy *et al.*,(2006) isolated five hundred sixty-nine *Salmonella* out of 4745 samples from poultry products, poultry, and poultry environment in 1999 and 2000 from the Pacific Northwest. These *Salmonella* were identified to their exact source, and some were serogrouped, serotyped, phage typed, and tested for antibiotic sensitivity..

Hossain *et al.*, (2004) conducted a study to determine the prevalence of poultry diseases in Raishahi. Bangladesh, during the period of January 2001 to February 2002. A total of 327 cases were studied, of which some were sick and the others were dead. Diagnoses of different diseases were made based on the history, clinical findings, pathological findings, age, isolation and identification of causative agents, serology and response to treatment. The incidence rates of infectious bursitis disease (113D), infectious laryngotracheitis (ILT), avian leukosis complex. Marek's disease, duck plague, pullorum disease of birds were 12.53, 0.61, 3.36, 0.61, 2.14 and 5.81%.respectively

Oscar *et al.*, (2004) analyzed the existing data and predictive models were used to define the input settings of a previously developed but modified quantitative risk assessment model (QRAM) for *Salmonella* and whole chickens.

Sujatha *et al.*,(2003) isolated and characterized *Salmonella gallinarurn* from poultry in and around Hyderabad and Secunderabad cities in India. Six isolates of *S. gallinarum* were obtained from 21 clinical samples by employing both preenrichment and selective media. Percentage of positive isolation was 28.67%.

Tibaijuka *et al.*,(2003) found a cross sectional relationship between the presence and prevalence of *Salmonellae in* retail raw chicken meat and giblets (gizzard and liver) in super markets in Addis Ababa (Ethiopia). A total of 301 samples (244 chicken meat, 32 gizzards and 25 livers) were collected from 22 randomly selected super markets and examined for the presence of *Salmonella* were defected from 54 (17.9%) of the 301 samples examined chicken meat and giblet samples in 68.2% (15/22) of the supermarkets were contaminated with *Salmonellae.*

Dhruba *et al.*, (1999) isolated 25 strains of *S. gallinarum* from diseased broilers layers and cockerels of different age groups farms in West Bengal between 1994 and 1995 and the isolates were characterized by morphological cultural and biochemical examination.

Goneagul *et al.*, (1999) used cloacal swab procedure (ssp) and Drag swab procedure (DSP) to isolate *Salmonella* from 20 breeders, 17 layer and 13 broiler flocks from Bursa in Istanbul and Ixmir districts. *Salmonella* suspected colonies were identified biochemically and serologically.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The study was conducted in the Bacteriology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh during the period of January 2013 to June, 2013. The detailed outline of the Materials and Methods are given below:

3.1 Materials

3.1.1Study area

The samples were collected from Pigeon farms and Duck farms of three upazillas of Dinajpur district of Bangladesh. The sample were collected from the selected species and brought to the laboratory of the department of microbiology for conducting the experiment.

3.1.2 Field samples

A total number of 90 field samples comprising liver, spleen faeces and intestinal contents were aseptically collected for the characterization of salmonellae..

3.1.3 Media for culture

3.1.3.1 Nutrient broth (NB)

Nutrient broth (NB) was used to grow the *Salmonella* organisms from the samples collected from the study areas of Dinajpur district.

3.1.3.2 Bacto selenite broth

Bacto selenite broth (SB) was used to grow the *Salmonella* organisms and to minimize the growth of other enterobacteria

3.1.3.3 Nutrient agar (NA)

Nutrient agar (NA) medium was used to grow the *Salmonella* organisms from the collected samples.

3.1.3.4 Salmonella-Shigella (SS) agar

Salmonella-Shigella (SS) agar medium was used as a selective medium for *Salmonella* organism in which *Salmonella* grows easily and shows typical colony characters.

3.1.3.5 MacConkey agar (MC)

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

3.1.3.6 Brilliant Green Agar (BGA)

Brilliant green agar (BGA) medium was used as a selective medium for the identification of *Salmonella* organisms.

3.1.3.7 Eosin Methylene Blue (EMB) agar

Eosin methylene blue (EMB) agar medium was used for the purpose of differential growth of *Salmonella* organisms and other enterobacteria specially *Escherichia coli.*

3.1.4 Media used for biochemical test

In order to identify *Salmonella* the following media were used for biochemical tests: Sugar media (dextrose, lactose, sucrose, mannitol, and maltose), Triple sugar iron (TSI) agar slant, buffered glucose broth, MIU agar base, peptone water.

3.1.5 Chemicals, reagents and solutions

The following reagents were used for conducting the biochemical tests: Gram's stain, Methyl red-Voges-Proskaure (MR-VP) solution, α-napthol, Kovac's reagent, phosphate buffered saline (PBS) solution and glycerin.

3.1.6 Hexisol hand rub

Hexisol hand rub (100 ml bottle) was used for antisepsis prior to swab sample collection from the selected species.

3.1.7 Glass wares and other necessary instruments

In this research work following glass ware and instruments were used: Test tubes (with and without Durham's fermentation tube), Petri-culture dishes, conical flasks, pipette, slides, microscope, sterilized cotton, immersion oil, bacteriological incubator, jar, ice boxes, measuring balance, hand gloves, spirit lamps, match lighter and bacteriological loop, glass spreader and forceps, scissors.

3.1.8 Antibacterial disc

Different antibacterial disc, manufactured by Oxoid Ltd. Basingstoke, Hampshire, England, were selected for the antibacterial sensitivity study against isolated *Salmonella spp.* The antibacterial agents were (Doxycycline, Amoxycillin, Colistin Sulphate, , Cefotaxime, Gentamicin, Ciprofloxacin, Azithromycin, Tetracycline, Levofloxacillin, Bacitracin, which are commonly used in the field condition for the treatment of *Salmonellosis.*

3.2 Methods

3.2.1 Experimental design

The experimental design is schematically presented in figure 1. The entire study is divided into two steps. The first step includes the isolation of Salmonella bacteria and for this different types of samples were collected from duck, pigeon and Duck. The identification were made according to their cultural and morphological characteristic, biochemical properties and by motility test. The second step is antibiogram study of the isolated *Salmonella sp.*

Experimental Design

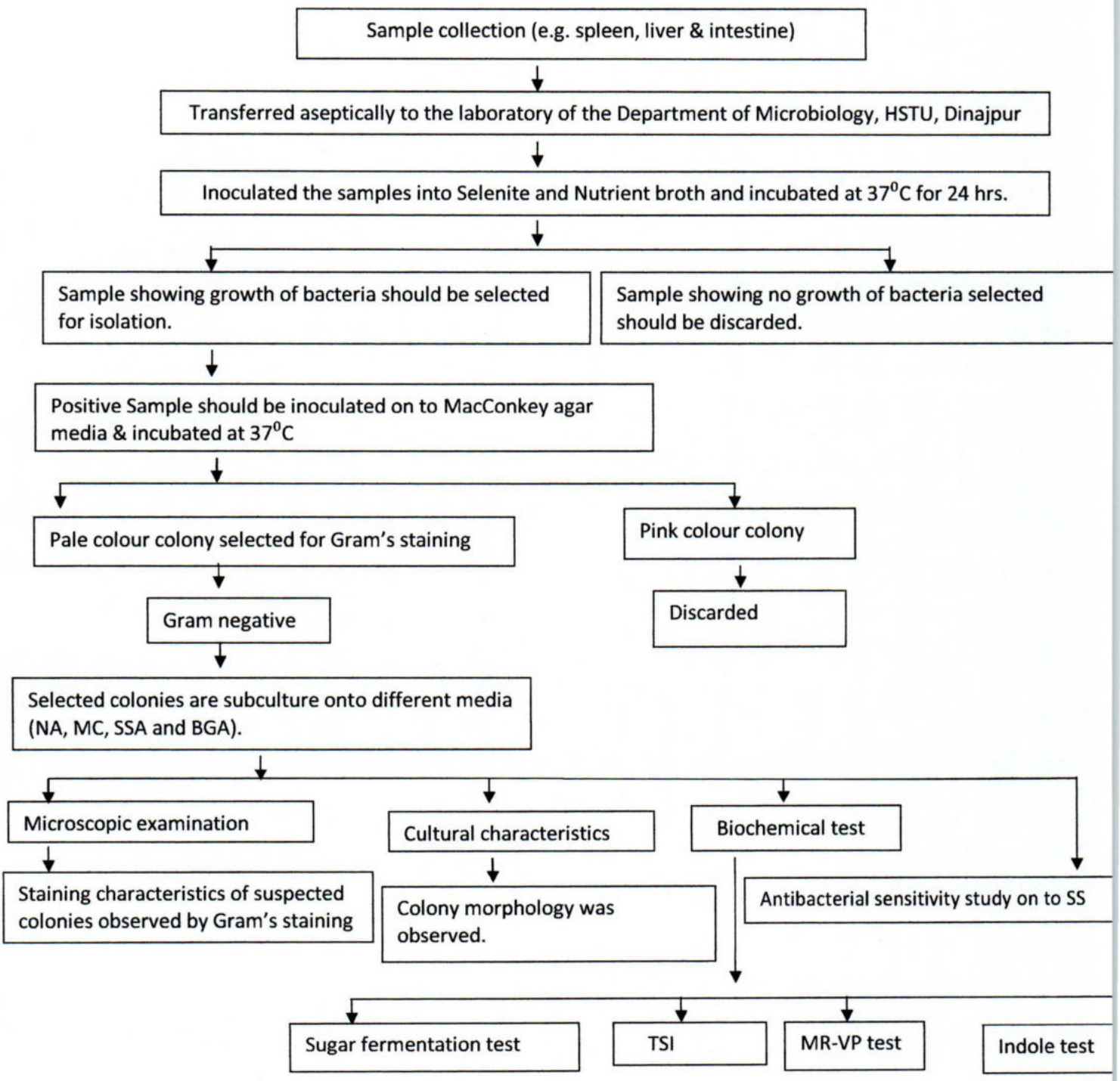

Fig. Schematic Illustration of the experimental design

3.2.2 Collection and transportation of samples

The liver, spleen and intestinal contents were obtained from the study areas for bacteriological study. Sterile cotton swab and sterile NB in sterile glass tube was carried to the sampling area. Liver, spleen and intestinal contents were taken aseptically from the chicken, Duck, and pigeon of different ages. Test tubes containing swab samples were then immediately brought to the laboratory for cultural examination.

3.2.3 Isolation and identification of *Salmonella* organisms

3.2.3.1 Preparation of culture media and reagents

3.2.3.1.1 Nutrient broth

This was prepared by dissolving 13 gms of dehydrated nutrient broth (Hi-media, Laboratories Pvt. Ltd. India) into 1000 ml of distilled water and sterilized by autoclaving at 121°C under 15 pounds pressure per square inch (1.2 Kg/Cm^2) for 15 minutes (Buxton and Fraser, 1977). Then the broth was dispensed into tubes (10 ml/tube). The tubes were then stored at 4°C in the refrigerator until used.

3.2.3.1.2 Bacto-selenite broth

In 100 ml of distilled water, 2.3 gms of dehydrated Bacto-selenite broth (Hi-media, Laboratories Pvt. Ltd. India) was added and heated up to boiling to dissolve the medium. It was then shacked well and distributed in 5 ml. quantities of each of the sterile test tubes closed with cotton plugs sterilized in the autoclave machine and used for cultural characterization or stored at 4°C in refrigerator for further use (Carter, 1979).

3.2.3.1.3 Sugar media

The medium consists of peptone water to which fermentable sugar was added to the proportion of 1 %. Peptone water was prepared by adding one gram of Bacto peptone (Unipath Ltd.) and 0.5 grams of sodium chloride in 100 ml distilled water. The medium as boiled for 5 minutes, adjusted to pH 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2 % solution was added to peptone water and then dispensed in 5 ml amount into cotton plugged fermentation test tubes. These were then sterilized through autoclaving at 121°C at 15-pound pressure per sq inch for 15 minutes. The sugars used for fermentation were prepared separately as 10 % solutions in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A little heat was necessary to dissolve the sugar (Buxton and Fraser, 1977).

The sugar solutions were sterilized in Arnold stream sterilizer at 100°C for 30 minutes for three consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water. Before use, the sterility of the sugar media was examined by incubating it for 24 hours at 37°C.

3.2.3.1.4 Nutrient agar

28 grams of Bacto-Nutrient agar (Hi-Media Laboratories Pvt. Ltd) was added to 1000 ml distilled water in a flask and heated to boil for dissolving the medium completely. The medium was then sterilized by autoclaving at 121°C maintaining a pressure of 15-pounds /sq. inch (1.2 kg/cm^2) for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to cool down its temperature. Then 10 ml of medium was poured into each sterile petridishes and allowed to solidify. After solidification of the medium

in the petridishes, these were allowed for incubation at 37°C for overnight to check their sterility and then stored in a refrigerator for future use (Carter, 1979).

3.2.3.1.5 MacConkey agar medium

It is particularly useful for the isolation of enteric organisms because many other bacterial species are inhibited by sodium Chloride. This medium also has the advantage that it enables to make a distinction between lactose non-fermenting colonies of *Salmonellae* (pale colour) and lactose fermenting colonies of E. coli (pink colour). 51.50 grams powder of MC agar base (Hi-Media Laboratories Pvt. Ltd.) was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121°C maintaining a pressure of 15-pounds /sq. inch (1.2 kg/cm^2) for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to decrease its temperature. After solidification of the medium in the petridishes, the petridishes were allowed for incubation at 37°C for overnight to check their sterility and then stored in a refrigerator for future use (Buxton and Fraser, 1977).

3.2.3.1.6 Salmonella-Shigella (SS) agar medium

Sixty grams powder of SS agar base (Hi-Media Laboratories Pvt. Ltd) was added to 1000 ml distilled water in a flask and heated to boil for dissolving the medium completely. The medium was then sterilized by autoclaving at 121°C maintaining a pressure of 15-pounds /sq. inch (1.2 kg/cm^2) for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to cool down its temperature. Then 10 ml of medium was poured into each sterile petridishes and allowed to solidify. After solidification of the medium in the petridishes, these were allowed for incubation at 37°C for overnight to

check their sterility and then stored in a refrigerator for future use (Cown, 1985).

3.2.3.1.7 Brilliant Green Agar medium

Fifty-eight grams of powder (Hi-Media Laboratories Pvt. Ltd) was dissolved in 1000 ml of distilled water in a flask. It was then gently heated with gentle agitation and brought just to the boiling temperature to dissolve the medium completely. Then it was cooled to 50°C, mixed properly and poured into sterile petridishes (10 ml in each petridishes) and allowed to solidify. Then incubated at 37°C for overnight to check their sterility and stored in a refrigerator for future use (Buxton and Fraser, 1977).

3.2.3.1.8 Eosine Methylene Blue agar

36gms of EMB agar base (Hi-Media Laboratories Pvt. Ltd) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in10 ml quantities in sterile glass petridishes (medium size) and in 15 ml quantities in sterile glass petridishes (larger size) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of petridishes partially removed. The sterility of the medium was judged and used to store at 4°C in refrigerator for further use.

3.2.3.1.9 Simmons Citrate Agar

24.28 grams of Simmons Citrate agar base (Hi-Media Laboratories Pvt. Ltd) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in10 ml quantities in sterile glass petridishes (medium size) and in 15 ml quantities in sterile glass petridishes (larger size)

to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of petridishes partially removed. The sterility of the medium was judged and used to store at 4°C in refrigerator for further use.

3.2.3.1.10 Triple Sugar Iron (TSI) agar slant

A quantity of 6.5 grams of Bacto TSI agar (HiMedia Laboratories Pvt. Ltd) was suspended in 1000 ml of distilled water and heated up to boiling to dissolve the medium completely. The solution was then distributed in tubes plugged with cotton plugs and sterilized in an autoclave at 121^0C at a pressure of 15-pounds/sq. inch (1.2 kg/cm^2) for 15 minutes. After sterilization, the tubes were kept in slanting position in such a manner so as to allow a generous butt after solidification. The tubes were then incubated at 37°C for overnight to check their sterility and then stored in a refrigerator for future use (Cheesbrough. 1985).

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

A quantity of 1.7 grams of Bacto MR-VP medium was dissolved in 100 ml of distilled water dispensed in 5ml amount in each test tube and then the tubes were autoclaved at 121^0C maintaining a pressure of 15-pounds/sq. inch (1.2 kg/cm^2) for 15 minutes. After autoclaving, the tubes containing medium were incubated at 37° C for overnight to check their sterility and then stored in a refrigerator for future use (Buxton and Fraser, 1977).

3.2.3.1.12 Methyl-Red solution

The indicator methyl red solution was prepared by dissolving 0.1 gram of Bacto methyl-red in 300 ml of 95 percent alcohol and diluting to 500 ml with the addition of 200 ml of distilled water (Cheesbrough, 1985).

3.2.3.1.13 Phosphate buffered saline solution

For preparation of PBS, 8 grams of NaCl, 2.89 grams of disodium hydrogen phosphate (Na2HPO4.12H20), 0.2 grams of KCI and 0.2 grams of potassium hydrogen phosphate (KH2PO4) were suspended in 1000 ml of distilled water. The solution was heated to dissolve the ingredients completely. The solution was then sterilized by autoclaving at 121°C maintaining a pressure of 15-pounnds/sq. inch for 15 minutes and stored at 4-8°C until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1985).

3.3 Sampling procedure

Different samples such as liver, spleen and intestinal contents collected from the selected farms were subjected to bacteriological examination for isolation and identification of *Salmonella* spp. as per procedure described by OIE Manual (2000) and Douglas *et al.*, 1998.

3.4 Duck, pigeon, Duck, samples

3.4.1 Liver sample

Liver samples were collected from Duck, pigeon and duck after dissecting them. A spatula was heated until bright red and then touches it to the liver surface to burn. Then a sterile wire loop was inserted into the liver and finally inoculated into test tube containing nutrient or bacto selenite broth and then it was incubated at 37°C for 24 hr for streaking on bacteriological media.

3.4.2 Spleen Sample

Spleen samples were collected from Duck, pigeon and duck after dissecting them. Then a sterile wire loop was inserted to inside the spleen and finally inoculated into test tube containing nutrient or bacto selenite broth and then it was incubated at 37°C for 24 hr for streaking on bacteriological media.

3.4.3 Intestinal contents

Intestinal contents were collected from Duck, pigeon and duck after dissecting them. The intestines were incised with sterile scissors. The contents were collected from the intestinal lumen. These were inoculated into test tube containing nutrient or bacto selenite broth and then these were incubated at 37°C for 24 hr for streaking on bacteriological media.

3.5 Cultivation and isolation of *Salmonella*

After collection, each of the samples was inoculated into each of the freshly prepared NB and SB separately. The tubes were identified properly and then incubated at 37°C for 24 hours aerobically in bacteriological incubator. The incubated tubes were then examined for growth of bacteria and a loopful from all incubated tubes was streaked separately into the NA plates and BA plates and then incubated at 37^0C for 24 hours aerobically in bacteriological incubator for the growth of bacteria. Then, a loopful of bacterial culture from incubated tubes were streaked separately into the SS agar, MC agar, EMB and BGA plates and incubated at 37°C for 24 hours aerobically in bacteriological incubator. Then the plates were examined and studied carefully for the presence of characteristic colonies of *Salmonellae.* The characteristic colonies produced by the isolated *Salmonellae* on SS agar, MC

agar plates are shown in (Table 4, Page 51). The incubated tubes containing bacterial growth were preserved at refrigerator for further use.

The smears were prepared from each of the agar plates. The smears were then fixed and stained with Gram's method of staining and examined under microscope at 100 magnifications for the presence of gram negative rods. BGA plates containing characteristic colonies of Salmonellae were selected for sub-culturing in order to obtain a pure culture of the organisms. The organisms thus obtained as pure culture were selected for subsequent studies.

3.6 Morphological characterization by Gram's staining method

The bacteria obtained from representative *Salmonella* colonies were characterized microscopically using Gram's stain according to the method described by Merchant and Packer (1967). The procedure was as follows:

A small colony was picked up with a bacteriological loop, smeared on a glass bacteriological slide and fixed by gentle heating. Crystal violet solution was then applied on the smear to stain for two minutes and then washed with running water. Gram's iodine was then added to act as mordant for one minute and then again washed with running water. Acetone alcohol was then added for 10-15seconds which act as a decolorizer. After washing with water, safranine was added as counter stain and allowed to stain for 2 minutes. The slide was then washed with water, blotted and dried in air and then examined under microscope with high power objective (100X) using immersion oil (Plate 04, Page 43).

3.7 Identification of isolated *Salmonella by* using specific biochemical tests

For this study carbohydrate fermentation tests, TSI agar slant reaction, MR-VP and Indole reaction test were carried out for identification of suspected *Salmonella.* All the isolates from different sources were tested for the detection of *Salmonella spp.* according to the methods described in the Douglas *et al.*, 1998 and OIE Manual, 2000.

3.7.1 Carbohydrate fermentation test

The carbohydrate fermentation test was performed by inoculating a loopful of NB culture of the organisms into the tubes containing different sugar media (Dextrose, Lactose, Sucrose, Maltose, Mannitol) and incubated for 24 hours at 37°C. Acid production was indicated by the colour change reddish to yellow in the medium

3.7.2 Reaction of the organisms in TSI agar slant

The TSI agar slant was used to detect the lactose fermenters and the saccharose and dextrose fermenters. The medium also helped to determine the ability of the organisms to produce H_2S. The organisms under this study were heavily seeded with a platinum needle over the surface of the slants and stabbed into the butt of the tubes containing TSI agar. After an aerobic incubation of 24 hours at 37°C the tubes were examined for changes in the slant or in the butt or in both places. Pinkish slant and yellow butt or black slant and yellow butt was recorded as the positive reaction for *Salmonella* (Cheesbrough, 1985) (Plate 08, Page 45).

3.7.3 Methyl red test

The test was conducted by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of MR solution was added. A red coloration is positive and indicates an acid pH of 4.5 or less resulting from the fermentation of glucose. A yellow coloration is negative (Cheesbrough, 1985) (Plate 05, Page 44).

3.7.4 Voges-Proskauer test

The organisms were inoculated into 5 ml sterile buffered glucose broth . It was incubated at 37°C for 72 hours. Then 3 ml of α-napthol was added and followed by 1 ml of 40% potassium hydroxide. Then they were mixed well and waited for 30 minutes for the slow development of a pink colour for positive cases. In negative cases there was no development of pink colour (Cheesbrough. 1985).(Plate 10 page 46)

3.7.5 Methyl Red (MR) test

The organisms were inoculated into 5 ml sterile buffered glucose broth. It was incubated at 37°C for 48 hours. Then 2-3 drops of methyl red solution(1 gm of methyl red in 300 ml of alcohol and then 500 ml of distilled water was made) was added. The result was read immediately for red colour in the upper part of test tube in positive cases and in negative cases there was development of yellow colour (Cheesbrough., 1985).

3.7.6 Indole test

One colony from a pure culture of the bacterium was inoculated into 5 ml of peptone water and incubated for 48 hours. Kovac's reagent (1 ml) was added, shaked well and examined after 1 minute. A red colour in the reagent layer indicated Indole. In negative case there is no development of red colour (Cheesbrough. 1985).(Plate 07, Page 45)

3.7.7 Motility Indole Urease test

18 grams of powder of MIU agar base (Hi-Media Laboratories Pvt. Ltd) was added to 950 ml. of distilled water in a flask and heated boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121°C maintaining a pressure of 15 pound per sq. inch for 15 minutes. After autoclaving the medium was put in to water bath of 45°C to decrease the temperature. Then the medium in the test tube was allowed for incubating at 37°C for overnight to check their sterility and then stored in refrigerator for future use. Motility is positive if the entire medium became opaque (semisolid state of the medium permits the bacteria moving). Result is negative if the culture grows only on the stabbing line. In case of indole, a red layer on the surface of the medium appears if the reaction is positive and if the entire medium turns red indicated the urease positive.(Plate 09, Page 46)

3.8 Antibiogram study of the isolated *Salmonella spp*

Susceptibility of isolates of the *Salmonella sp* to different antimicrobial agents was performed to determine the drug sensitivity pattern and to interpret their disease potential. This method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium

throughout the disc. The overnight nutrient broth cultured Salmonella isolates were poured on SS agar and spread uniformly with the help of sterile glass spreader. Antibacterial disc were applied aseptically to the surface of the plate at an appropriate arrangement with the help of sterile forceps and incubated at 37°C for 24 hours, aerobically (Carter, 1979). The following are the antibiotics that were tested against isolated *Salmonella spp.*

Table 1. Antimicrobial agents and their disc concentration

Antimicrobial agent	Disc concentration
Amoxycillin	10µg
Gentamicin	10 µg
Doxycycline	10 µg
Tetracycline	30 µg
Bacitracin	10 µg
Clindamycine	10 µg
Cefotaxime	30 µg
Ciprofloxacin	5 µg
Levofloxacin	5 µg
Erythromycin	5 µg
Colistin sulphate	30µg
Cephradine	10 µg

3.9 Maintenance of stock culture

3.9.1 Agar slant method

The organisms isolated and identified as Salmonella spp. were inoculated onto the SS agar slants and incubated at 37°C for 24 hours and then examine for growth. Then the sterile mineral oil was poured into the tube until the colonies were covered completely. The tube were sealed off with paraffin and kept at room temperature for future use. By this method, bacteria can be preserved with no deviation of their original characters for few months (Buxton and Fraser, 1977).

3.9.2 Sterile buffered glycerin method

Sterile buffered glycerin (20%) was performed by mixing 20 parts of pure glycerin and 80 parts of PBS. Then a loopful of thick bacterial culture was mixed with 20% sterile buffered glycerin in small vials and was preserved at -20°C. This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977).

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

In the present study, liver, spleen intestinal samples from duck, pigeon, were collected from different region of Dinajpur. These samples were subjected to various bacteriological and biochemical examination in the laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU). A total of 90 different samples were tested for the examination. The results are presented below:

4.1.1 Isolation of *Salmonella spp.* from duck sample

Salmonella spp. were isolated and identified from the duck samples (liver, spleen & intestine) after cultivation on, NA, MC agar, BGA agar and SS agar medium. The results of morphological study and cultural characteristics are presented in (Table 2, Page 37). *Salmonella spp.* was detected from nineteen out of fourty eight samples. Among the positive samples, sixteen were from spleen, sixteen were from intestine and sixteen were from liver. The positive samples were collected from different area of Dinajpur district.

4.1.2 Isolation of *Salmonella spp.* from pigeon samples

Salmonella spp. were isolated and identified from the pigeon samples (spleen, liver & intestine) after cultivation on NA, MC agar SS agar, BGA agar, medium. The results of morphological and cultural characteristics are presented in (Table 2, Page 37). *Salmonella spp.* was detected from twelve out of fourty two samples. Among the positive samples fourteen were isolated both from spleen & liver and fourteen were from intestine. The positive samples were collected from different area of Dinajpur district. Each positive sample was treated as an isolates.

Table 2. Result of isolation of Salmonella from liver, spleen and intestine of Duck and Pigeon

Name of species	Name of samples	No. of sample tested	No. of positive cases		No. of isolates of *Salmonella*	Percentages of prevalence (%)
			Sick	Dead		
Duck	Liver	16	2	5	19	39.58
	Spleen	16	2	5		
	Intestine	16	2	3		
Pigeon	Liver	14	3	2	12	28.57
	Spleen	14	2	3		
	Intestine	14	1	1		

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

4.2.1 Results of cultural examination

4.2.1.1 Nutrient broth

Nutrient broth was inoculated separately for observing the growth of bacteria after 24 hours of incubation at 37°C aerobically and positive result was indicated by the presence of turbidity (Plate 6, page 44)

4.2.1.2 Bacto selenite broth

The growth of bacteria in the Bacto selenite broth was confirmed by streaking onto NA and SS Agar. (Plate 12, page 47)

4.2.1.3 Nutrient Agar

Nutrient agar plates streaked separately with the samples revealed the growth of bacteria after 24 hours of incubation at 37^0C aerobically and were indicated by the growth of circular, smooth, opaque, translucent colonies (Plate 02, Page 42).

4.2.1.4 MacConkey (MC) agar

The organisms showed colourless, smooth, pale, transparent colonies when cultured on MacConkey agar (Plate 03, Page 43).

4.2.1.5 Salmonella-Shigella (SS) agar

The organisms exhibited opaque, translucent and colorless colonies when cultured on SS agar (Plate 01 Page 42)

4.2.1.6 Brilliant Green Agar (BGA)

The organisms produced pale pink colour colonies against a pinkish background when cultured on BGA, which was of green color before growth of the organisms (Plate 11, Page 47)

4.2.2 Results of Gram's staining technique

In Gram's staining the organism revealed Gram-negative, pink colour, small rod shaped appearance, arranged in single or paired when observed under microscope (Plate 04, page 43)

Table 3. Morphology, cultural and staining characteristics of isolated *Salmonella spp.*

Isolates	Colony characteristics			Morphology (staining Characters)
	SS agar	MC agar	BG agar	
Duck sample	Opaque translucent colorless smooth round colonies.	Pale, colourless, smooth transparent raised colonies.	Pale, pink color colonies against a pinkish background	Gram negative short rod shaped singly arranged.
Pigeon sample	Opaque translucent colorless smooth round colonies	Pale, colorless smooth transparent raised colonies	Pale, pink color colonies against a pinkish background	Gram negative short rod shaped singly arranged

SS = *Salmonella-Shigella* agar, MC = MacConkey agar, BG=Brilliant Green Agar

4.2.4 Results of biochemical test

4.2.4.1 Fermentation reaction with five basic sugars All the isolates fermented dextrose and mannitol and produced acid and gas or only acid. No fermentation were seen in lactose and sucrose but slight fermentation occurred in maltose. Acid production was indicated by the colour change from reddish to yellow.

4.2.4.2 TSI slant reaction

In the stab culture of TSI agar, the isolated *Salmonella* organisms produced an alkaline reaction (red) in slant and acid reaction (yellow) in the butt and slightly black colour due to H_2S production were also recorded (Plate 08 Page 45).

4.2.4.3 Indole test

All the isolates were Indole positive (Plate07, Page 45).

4.2.4.4 Methyl Red-Voges proskaur (MR-VP) test

All the isolates were MR positive and VP negative (plate 05, page 44)

Table 4. Biochemical characters of the *SaImonellae spp.*

CHO fermentation and other biochemical tests		Result
Dextrose fermentation		+
Lactose fermentation		-
Sucrose fermentation		-
Mannitol fermentation		+
Indole production		+
MR test		+
VP test		-
MIU medium	Motility	±
	Indole	+
	Urea	-

Legends:

+ = Positive; - = Negative; MR= Methyl Red; VP= Voges-Proskauer; MIU= Motility, Indole Urea

4.2.4.5 Motility Indole Urea (MIU)

All the isolates in the MIU medium showed negative result that means the test tubes were clear (non motile), no red colour in the neck (indole negative) and no urease production.

Table 5. Result of CHO fermentation and other biochemical test of the isolated Salmonella

Isolates	Lactose	Dextrose	Sucrose	Maltose	Mannitol	Indole	MR	VP	MIU		
									Motility	Indole	Urea
Duck	-	+	-	+	+	+	+	-	-	+	-
Pigeon	-	+	-	+	+	+	+	-	-	+	-

4.3 Results of antibacterial sensitivity study of the isolated *Salmonella spp*:

Antibiotic sensitivity pattern of isolated Salmonella *spp* were performed against 12 commonly used antibiotics belongings to different groups. After incubation plates were examined and diameters of the zones of inhibition for individual antibacterial agents were designated as highly sensitive (+++), moderately sensitive (++), less sensitive (+) and resistant (-) (Table 03, page 39)

Form the antibiogram study, it was revealed that among the isolated *Salmonella* organism from duck, were highly sensitive to Azithromycin, Ciprofloxacin and Levofloxacillin (Table 03, Page 39). moderately sensitive to Colistin sulphate, less sensitive to Doxycycline, Bacitracin & Erythromycin; Gentamicin, Cefotaxime. and resistance to Amoxycillin & Tetracycline.

Among the isolates of pigeon highly sensitive to Levofloxacillin, Ciprofloxacin, Azithromycin. moderately sensitive to Ccefotaxim, Gentamicin, , less sensitive Bacirtracin & Colistin Sulphate, Doxycycline (Table 03, Page 39)

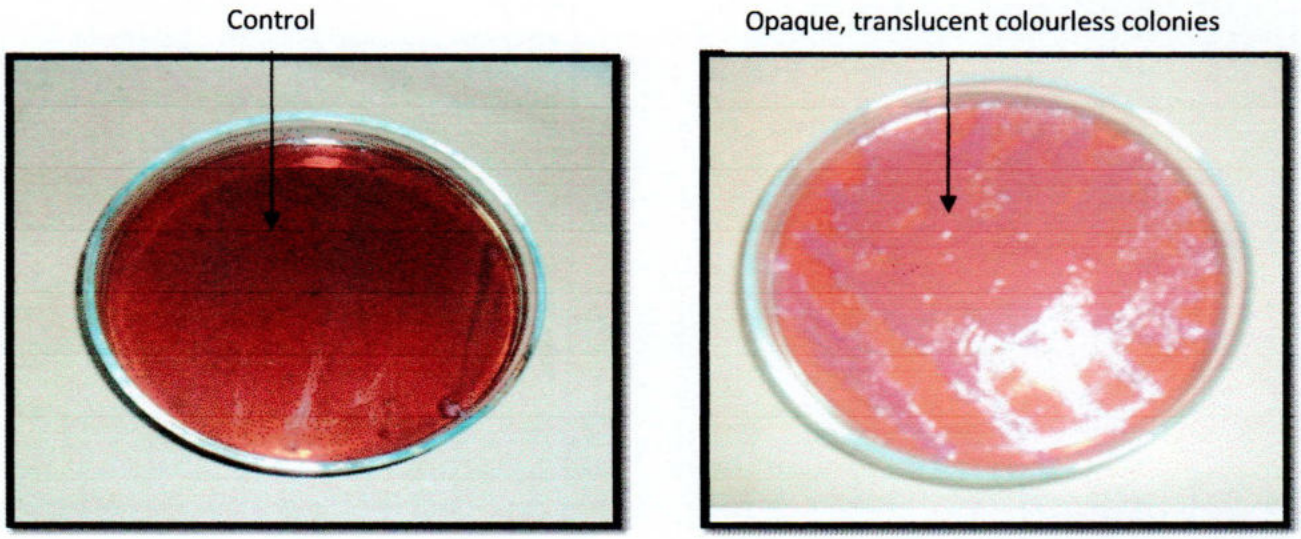

Plate 01: Salmomella-Shigella agar medium

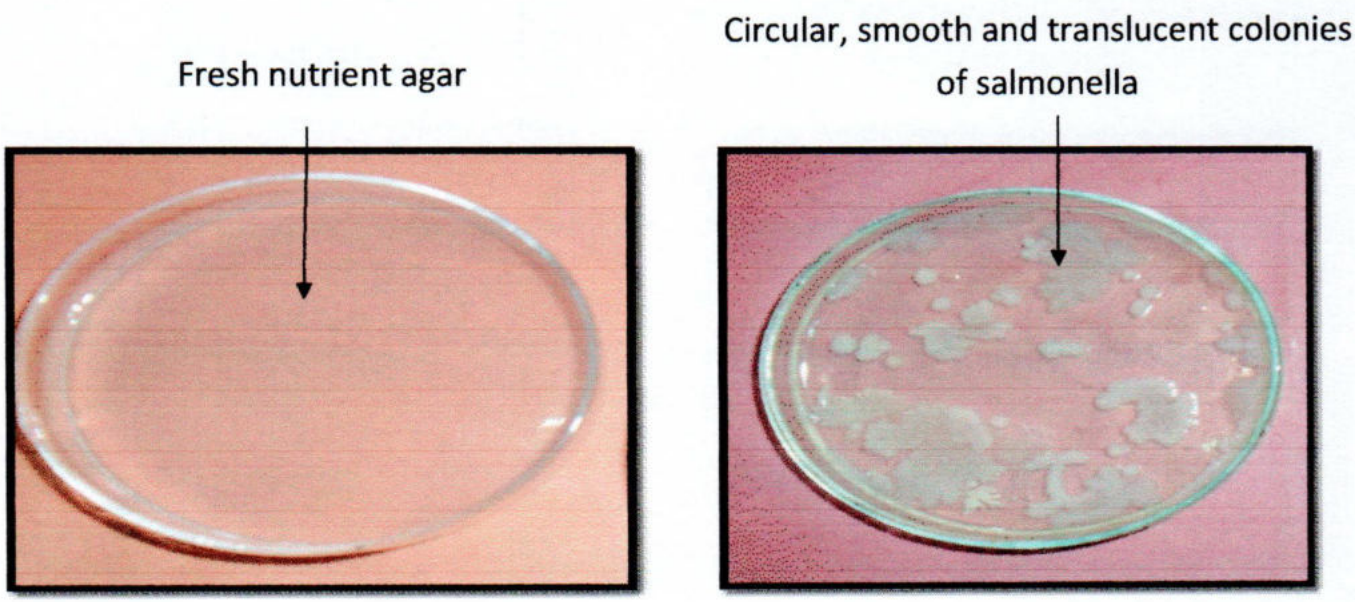

Plate 02: salmonella on nutrient agar medium

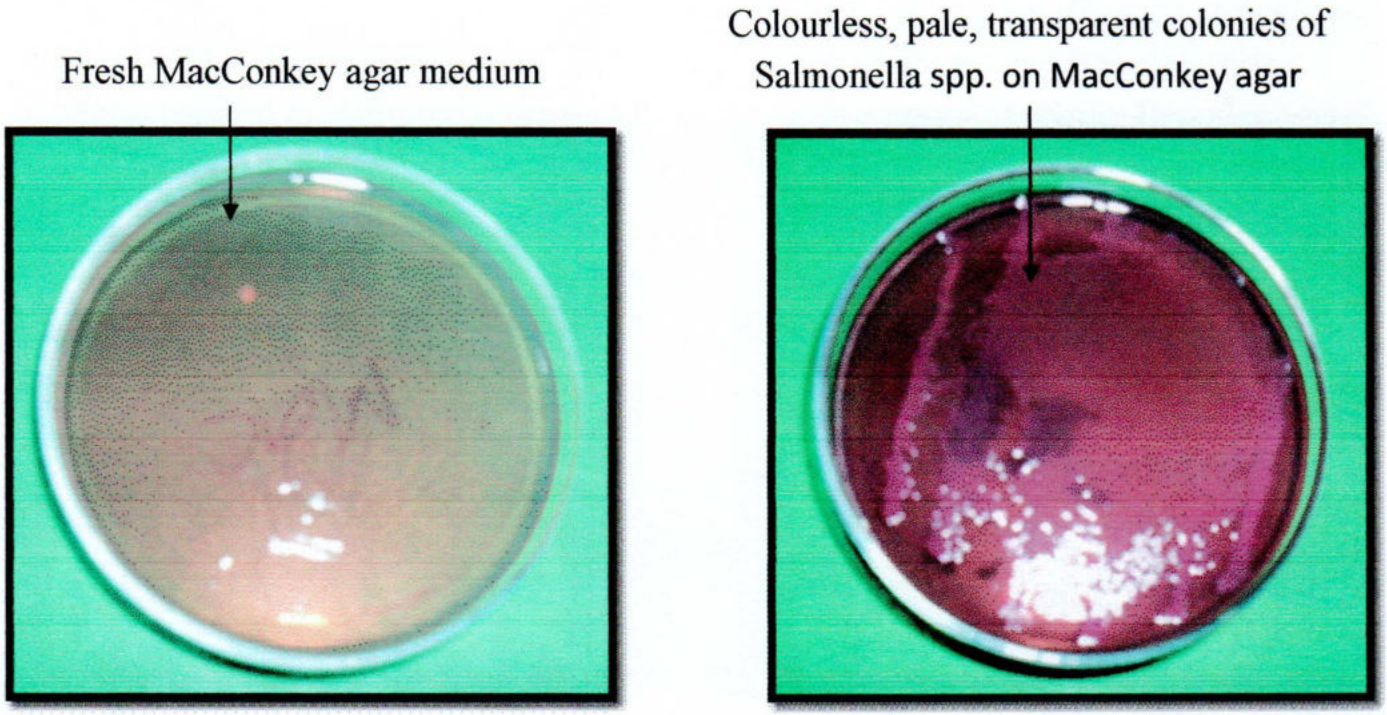

Plate 03: MacConkey agar medium showing growth of bacteria

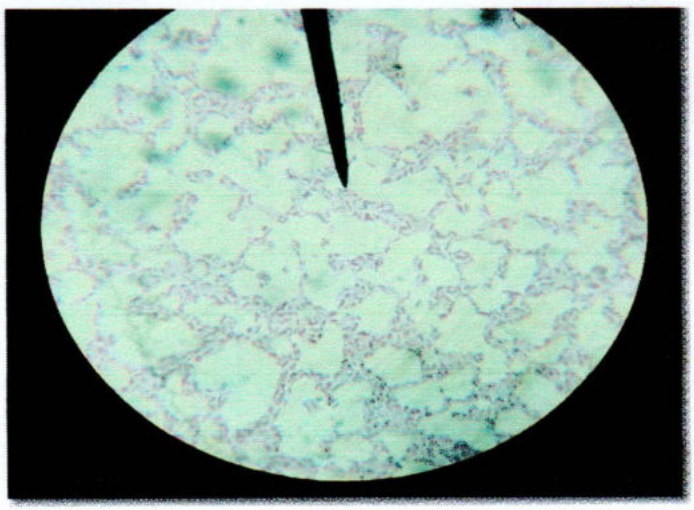

Plate 04: Salmonella organism showing gram negative small rod, arranged single or pairs (grams staining)

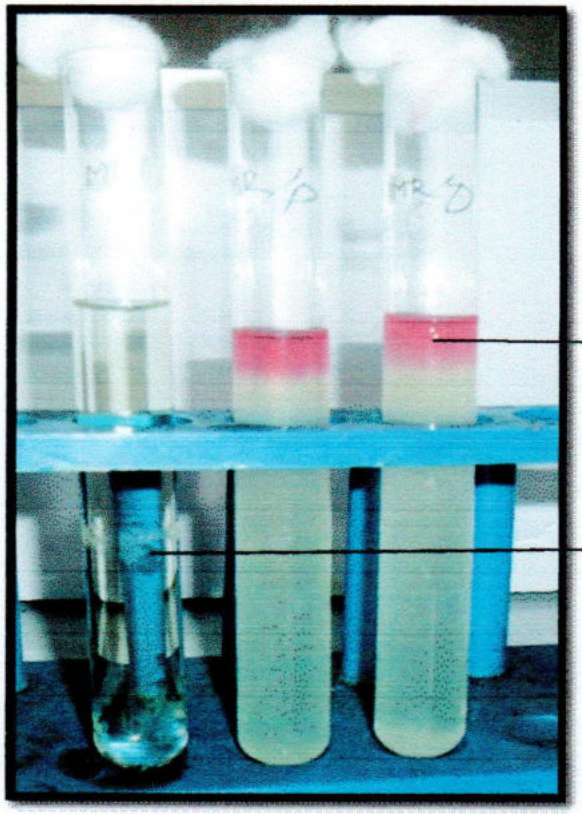

Plate 05: Methyl – Red test

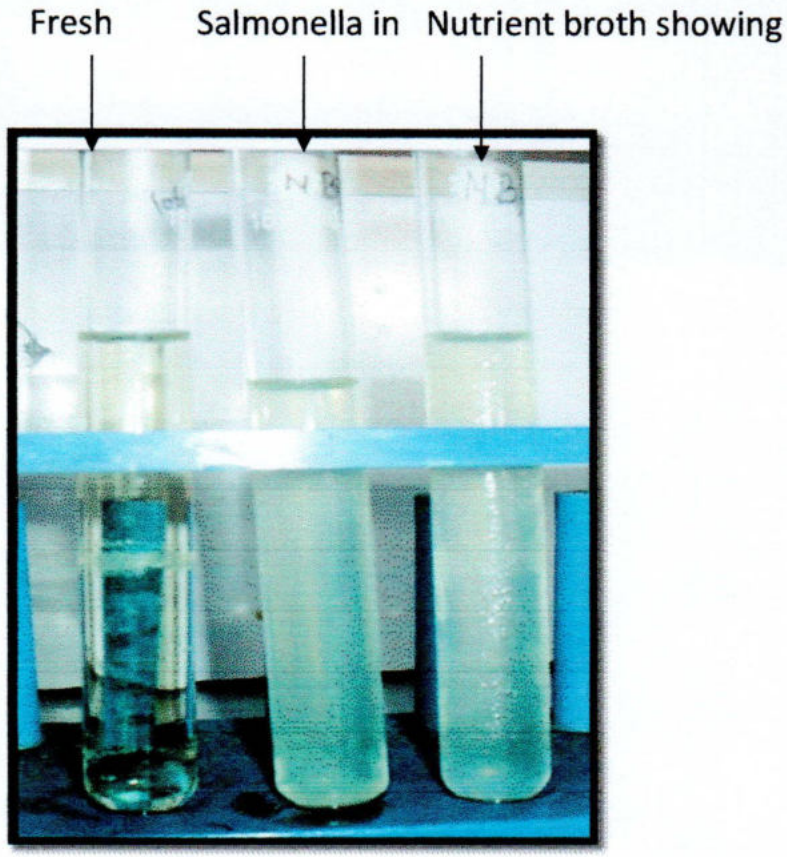

Plate 06: Culture of salmonella in nutrient broth.

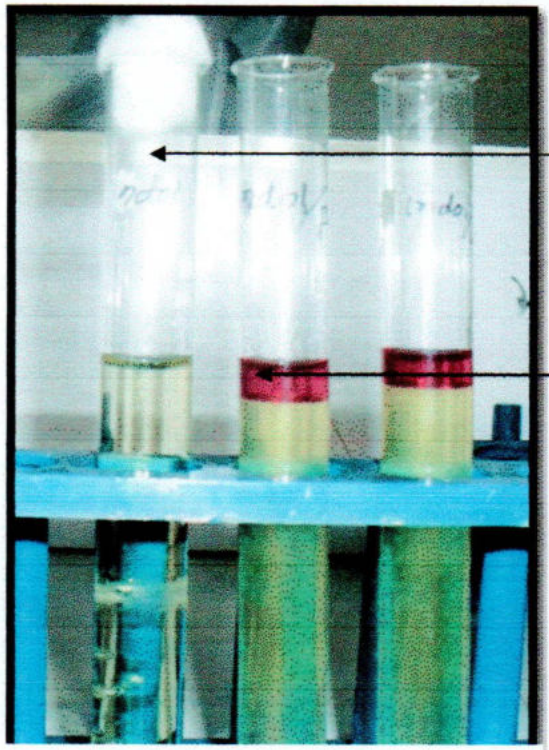

Plate 7: Indole test

Plate 08: Triple Sugar Iron agar **test**

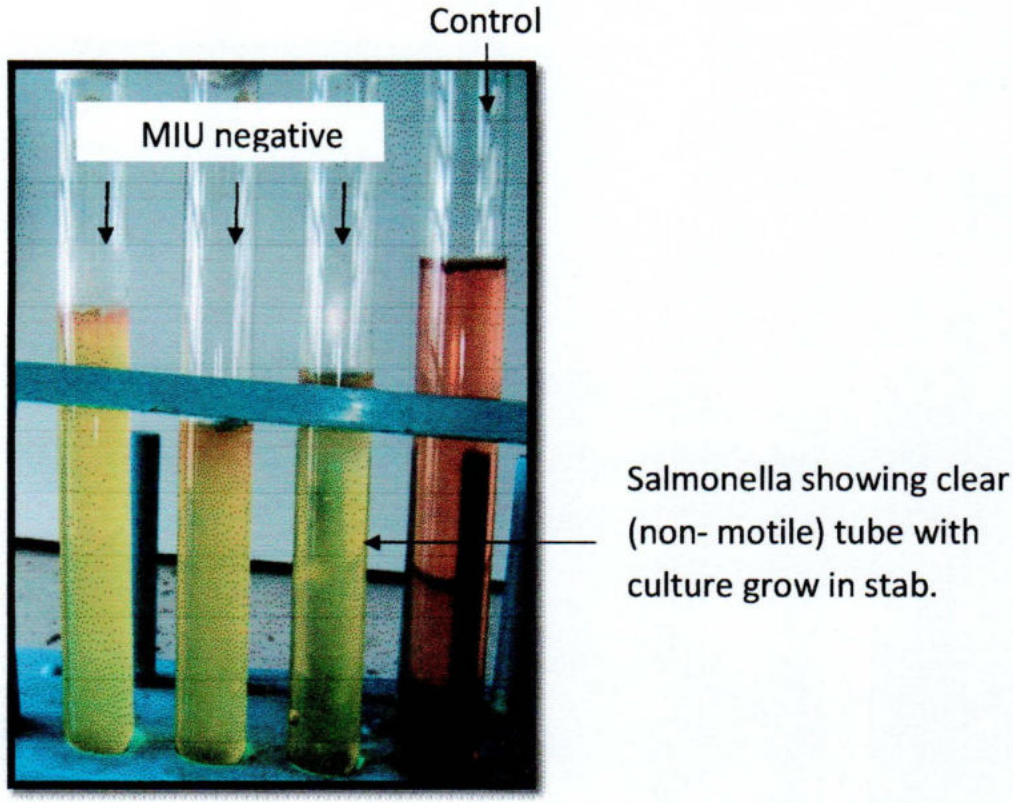

Plate 9: Motility Indole Urease (MIU) test

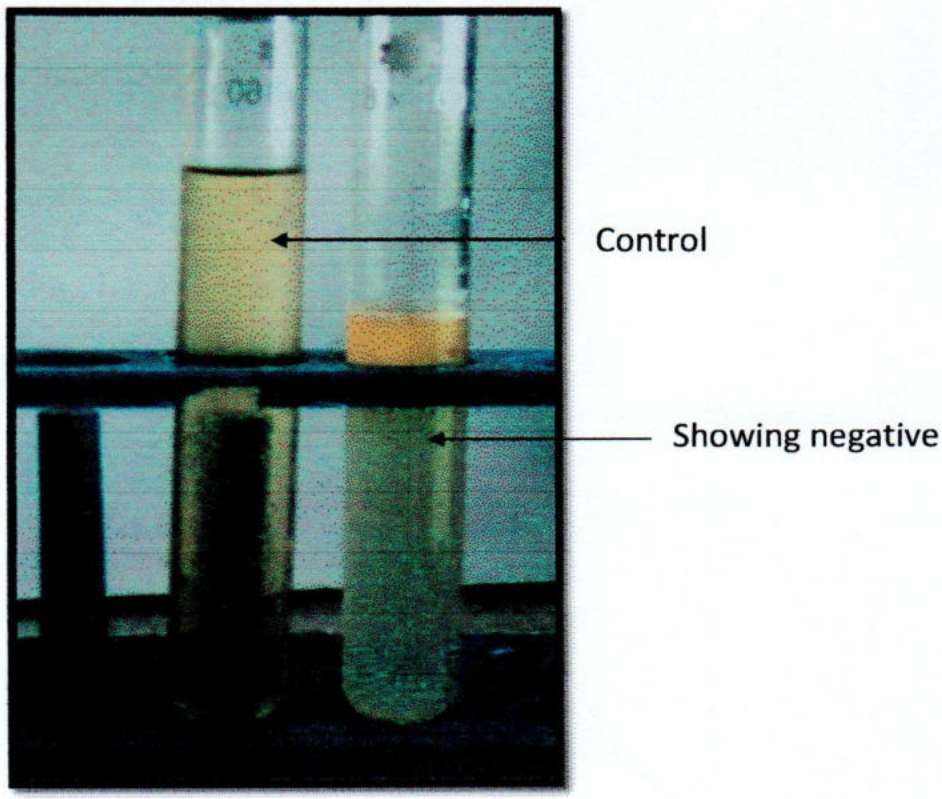

Plate 10: Voges proskaeur test

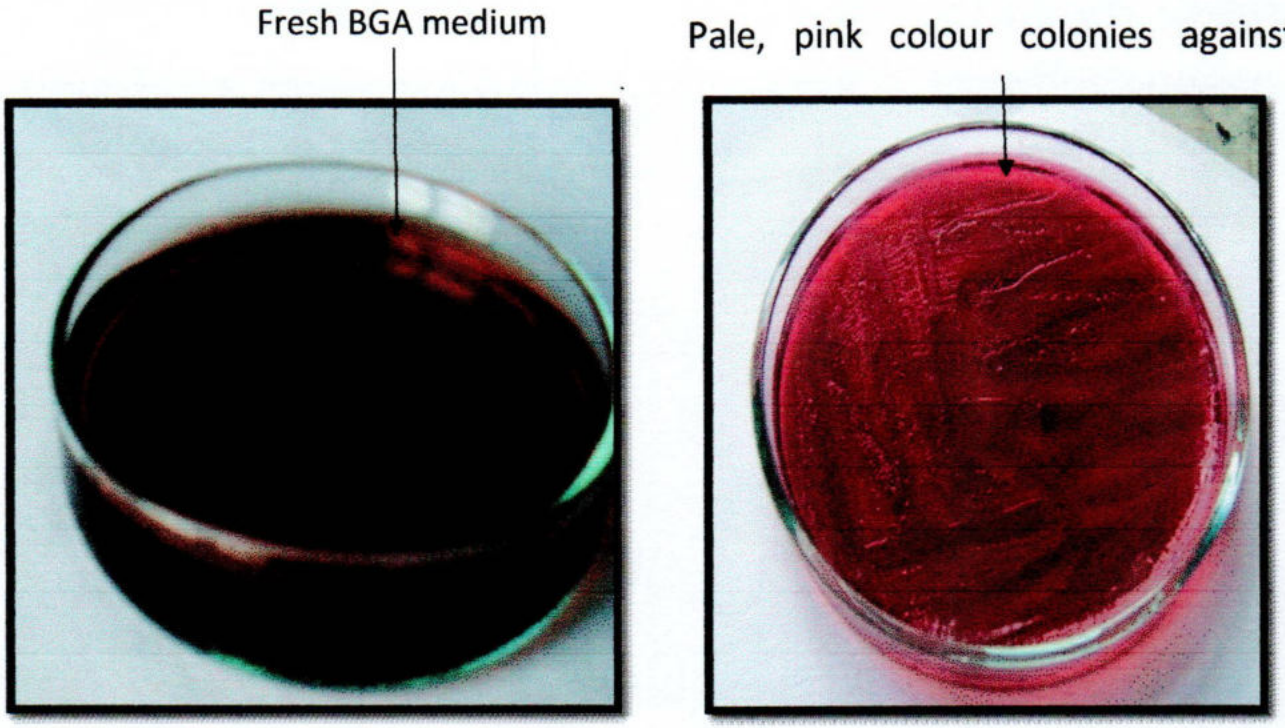

Plate 11: Brilliant green agar media

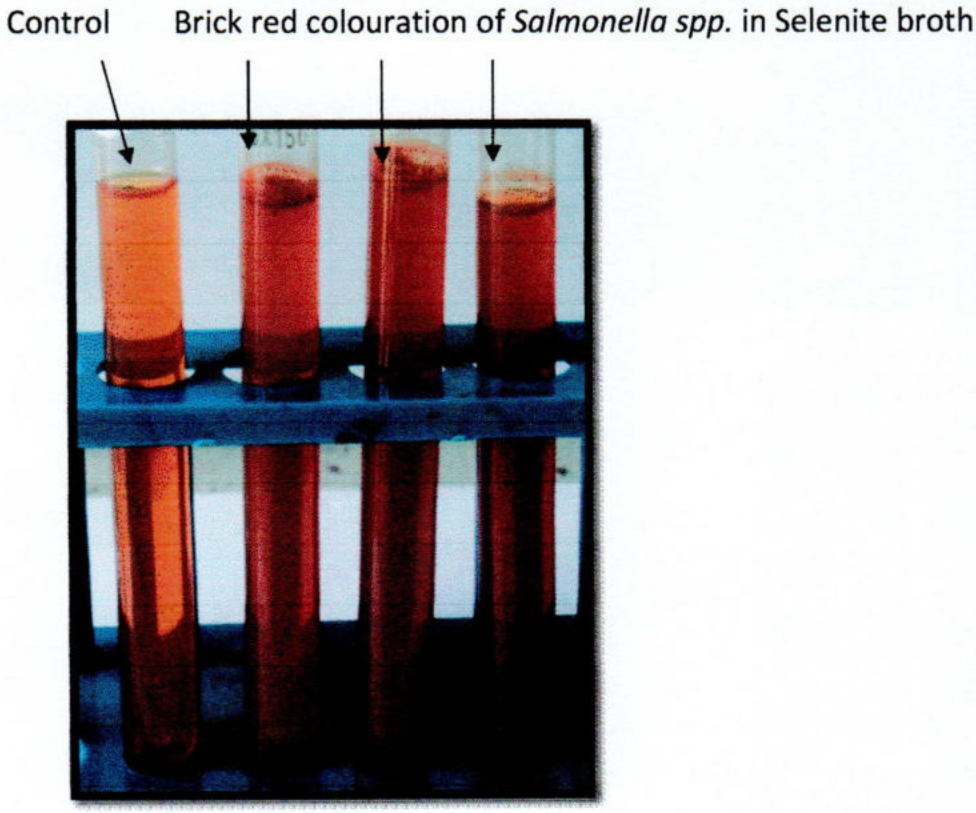

Plate 12: Selinite broth

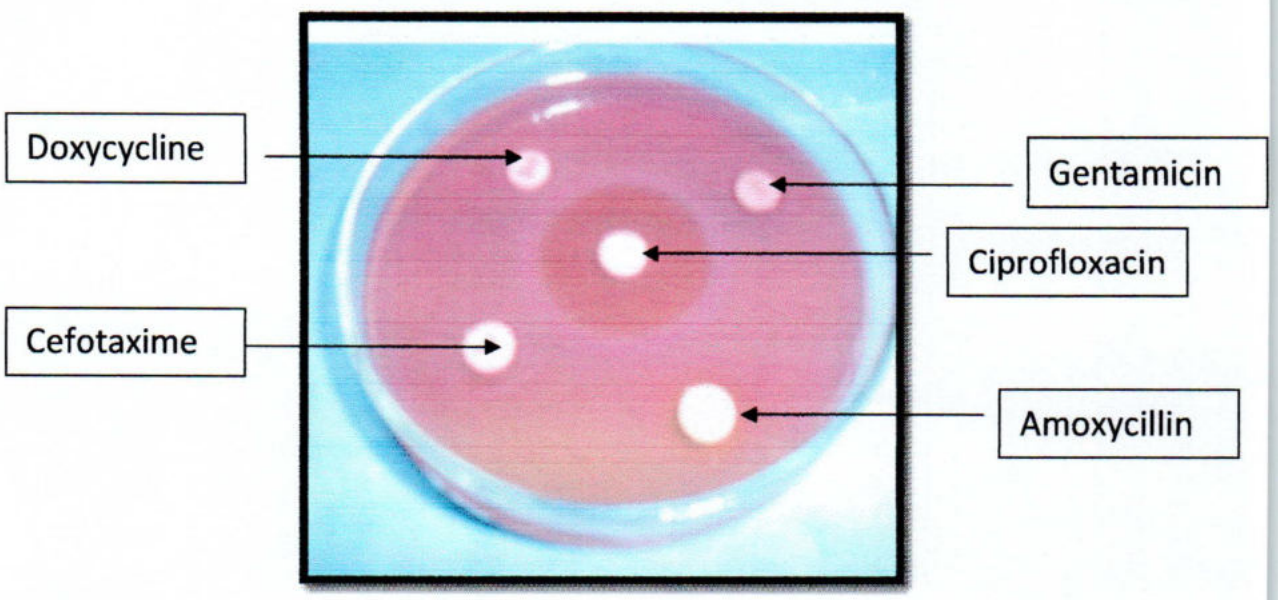

Plate 13: Sensitivity test

Table 6. Antimicrobial agents and their disc concentration

Antimicrobial agent	Effects of antibiotics on *Salmonella*
Amoxycillin	-
Gentamicin	+
Doxycycline	+
Tetracycline	-
Bacitracin	+
Clindamycine	+
Cefotaxime	+
Ciprofloxacin	+++
Levofloxacin	+++
Erythromycin	+
Colistin sulphate	++
Cephradine	+

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

The present research work was conducted to isolate and identify the *Salmonellae spp.* of duck, pigeon and Duck of different farm at Dinajpur district as well as antibiogram study of those isolated species. The isolation and identification of *Salmonella* is a difficult task. There was a great difficulty for the isolation of *Salmonella* in the present study due to the prevalence of the organisms like *E. coli* and other non-identified organisms in the samples. Cultures on specific media, Gram's staining and biochemical tests were performed to confirm the isolates as *Salmonella.*

Salmonella causes infection in all ages of poultry and this is responsible for a considerable loss in poultry industry (Habrun *et al.,* 2006). This infection may be a problem in poultry rearing farm and area where this will surely impair the sound health of these birds and give the poor quality and quantity of meat and egg, which leads to poor market value (Molla *et al.,* 2006; Yadav *et al.,* 2006).

Substantial economic loss results from mortality and poor growth of infected animals and birds. Infection by *Salmonella* is a common cause of food poisoning in humans (Hobbs and Robert, 1993). So, it is of great economic concern and public health significance. Isolation of *Salmonella spp* from duck was reported in many other countries (Rettger et al., 1920) but there is no available report on isolation of *Salmonella spp* from Duck, pigeon and duck in Bangladesh. So, it can be claimed that this is one of the new report of Salmonella isolation in Bangladesh.

Specific enrichment media and biochemical tests were used for the isolation and identification of the *Salmonella spp* which were previously suggested by a number of researchers (Habrun et al., 2006; Dhruba *et al.*, 1999; Sharma and katock 1996; Ruiz *et al.*, 1992; Mallinson *et al.*, 1991, Buxton and Fraser 1977). In this study, colony characteristics of *Salmonella* from different species of birds on Mc agar, SS agar and BG agar (Table 3, Page 51) were similar to the findings of other authors (Buxton and Frascr, 1977; Merchant and Packer, 1967 and Shaffer et al.,1964

In Gram's staining, the morphology of the isolated *Salmonella spp* exhibited Gram negative small rod shaped, single or paired arrangement under microscope which were similar to the observation by other researchers (Gene, 2002 and Freeman, 1985).

Differentiation of *Salmonella* into species level was difficult based on sugar fermentation pattern (Freeman, 1985). In sugar fermentation test, all of the isolates fermented dextrose, maltose, mannitol and no one fermented lactose & sucrose (Table 5, Page 41) which satisfies statement of Buxton and Fraser, 1977. In addition to that, all the isolates were positive to methyl red test and negative to voges-proskauer & indole test.

In this study, the isolated salmonella were highly sensitive to Levofloxacillin, Ciprofloxacin, Azithromycin, Cefotaxime; moderately sensitive to Gentamicin, Azithromycin, Pefloxacin, Colistin sulphate, Cefotaxime, Erythromycin, Carbinicillin and to Ciprofloxacin. They are less sensitive to Tobramycin, Bacitracin, Erythromycin, Colistin sulphate, Doxycycline, Tetracycline, Carbinicillin, Cefotaxime while resistant to Amoxycillin, Tetracycline, Bacitracin, Tobramycin, Doxycycline, Carbinicillin, Erythromycin, Cefotaxime and to Colistin sulphate.

The antibacterial resistance observed in the isolated Salmonellae might be due to indiscriminate use of those antibacterial agents in field condition in the farm of the study areas or rapid chromosomal mutation and the presence of specific plasmid DNA. The sensitivity tests were performed by disc diffusion method using 12 different antibiotic discs (Table 03, Page 39).This will provide guideline to the veterinarian to select appropriate antibiotics to reduce the economic losses through selecting the sensitive antibiotics. This finding satisfy the result of Tania Mondal *et al.* (2008) and Chugh and Suheir (1983); Zhang *et* al.(1998); Banani *et al.* (2003), Batabyal *et al.* (2003) and Kobayashi *et al.* (2007)

In the present study duck, Duck, and pigeon isolates showed fully resistance to Amoxycylin which is closely related with the result of Sultana Parvin (2008); Sung *et al.* (2002) and 78.57% resistance to Gentamicin which is closely related to Stefanov V *et al.*, 1986.

From the present study it could be concluded that the selected farm should be checked periodically to know status of *Salmonella* infection and the positive reactors should be culled and biosecurity plan of the farms should be improved and maintained strictly.

The present study will provide over all idea about the antibiotic sensitivity pattern of the local *Salmonella* isolate which will guide the veterinarians and physicians in selecting the most effective drug against *Salmonellosis* in human, animals and birds.

Different morphological, cultural and biochemical tests were performed to confirm the isolates as *Salmonella spp.* And it can be stated that, the isolates responsible for Salmonellosis in the selected farms might be any one of the agents which is in my selected bird species namely *S. pullorum. S gallinarum, S. anatum, S. enteritidis, S. Newport, S. Oregon, S. saintpaul, S. munhattan or S. manchester.*

Further study in connection with this research work might be

i. Isolation and identification of Salmonella organism from environmental samples of parent stock from which the organisms are transmitted from parent to the offspring's.

ii. Comparative study of Porin proteins of duck, pigeon & Duck Salmonella with other *Salmonella spp.* of various hosts.

iii. Detail genomic analysis of the isolates to obtain the idea about molecular basis of pathogenicity and drug resistance.

iv. Development of vaccines against Salmonellosis from the field isolates.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSSION

The objectives of the present study was to isolate and identify *Salmonella spp.* from sick and dead duck, pigeon and Duck from field cases and to study the sensitivity of these isolates to various antibiotics. Isolation of the organisms and bacteriological examination were carried out in the bacteriology laboratory of Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

For the present research works a total number of 90 spleen, liver and intestinal samples were collected, out of which 31samples were identified as positive of *Salmonella spp* and each positive samples was treated as one isolate. The prevalence of Salmonella in duck, pigeon, Duck were recorded as 39.58%, and 28.57% respectively.

All the Salmonella isolates except two duck samples revealed the same morphological, cultural and biochemical characteristics. Major findings of the morphological characteristics include Gram negative, short rod shaped organism. The cultural characterization revealed opaque, translucent, colourless and smooth – translucent colonies in Salmonella – Shigella (SS) agar, pale pink colour colonies in Brilliant green agar and pale colourless smooth, translucent, raised colonies in MacConkeys agar. In case of biochemical characterization all the isolate showed fermentation of dextrose, maltose, mannitol and no fermentation in lactose & sucrose, negative result in indole and voges- proskaure test but positive result to methyl red test.

Antibiotic sensitivity pattern of isolated Salmonella were performed against 14 antibiotics belonging to different generic groups. It was revealed that all of the isolates were highly sensitive to Ciprofloxacin, Levofloxacillin and to Azithromycin. So these three drugs might be highly effective to control Salmonellosis in study area.

The prevalence of Salmonellosis in poultry in Dinajpur district as found in this study is not insignificant. Therefore, it calls for immediate attention to introducing a continuous monitoring of Salmonella infection by randomized detection of antibody by serological test, culling of infected and carrier bird, maintenance of strict hygienic measures in the farms since the disease has a great public health importance.

From the findings of the study it may be concluded that:

i. Salmonellosis is one of the most hazardous disease in poultry sector in Dinajpur district.

ii. Pigeons may be the carrier of *Salmonella* infection

iii. Ducks are the susceptible hosts for *Salmonella* infection.

iv. Ducks are also the susceptible hosts for *Salmonella* infection.

v. Ciprofloxacin, Levofloxacillin and Azithromycin are the most effective antibiotic in the treatment of Salmonellosis among the antimicrobial agents used in the present study.

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