BACTERIOPATHOLOGICAL STUDY OF SALMONELLA INFECTION IN LAYER BIRDS OF SELECTED FARMS IN TANGAIL DISTRICT

A THESIS

BY

MD. RAJU AHMED

Registration No. 1305077
Semester: January- June, 2014
Session: 2013-2014

MASTER OF SCIENCE (M.S.)
IN
PATHOLOGY

DEPARTMENT OF PATHOLOGY AND PARASITOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Submitted to the
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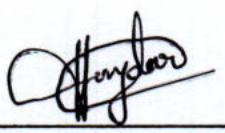

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JUNE, 2014

DEDICATED

TO
MY

BELOVED PARENTS

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The Author
June, 2014

ABSTRACT

A bacterio-pathological study of Avian Salmonellosis was conducted in some selected layer farms at Sadar Upazila of Tangail district, Bangladesh during January-June 2014 to identify and isolate Salmonella organisms by using 39 liver sample and other visceral organs (lung, spleen, egg follicles and intestine) from dead birds of 15 layer farms and 75 laid eggs (5 eggs/farm) from respective 15 *Salmonella* suspected farms. Samples were subjected to isolation and identification of the causal agent followed by necropsy and histopathological study of the affected visceral organs. The used methods were culture in different media, Gram's staining, different biochemical tests. Out of 39 liver samples 17 (43.59%) were found *Salmonella* positive. Whereas, *Salmonella gallinarum*, *Salmonella pullorum* and paratyphoid causing *Salmonella* sp were 70.58%, 17.64% & 11.77% respectively. During necropsy, the variable lesions were found in *Salmonella* affected organs of all 17 birds. About 64.70% livers of *Salmonella* affected birds were found enlarged, congested, friable and bronze coloured with white necrotic foci. Around 52.94% egg follicles were congested, hemorrhagic, discoloured with stalk formation and 70.58% intestines showed hemorrhagic to catarrhal enteritis. At histopathology, 82.35% livers were congested with formation of multifocal nodules characterized by huge infiltration of leukocyte. Infiltration of heterophils in intestinal mucosa was found in 52.94% cases. Four *Salmonella* isolates were found from 15 laid egg samples (5 eggs content comprised as 1 sample) and isolation rate was 26.66% reporting transovarian transmission in poultry Salmonellosis.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
&	and
µm	Micrometer
B	Black
BGA	Brilliant green agar
BPW	Buffered Peptone Water
CC	Cubic centimeter
CV	Co-variance
EMB	Eosine methylene blue
et al.	And this associates
FAO	Food and Agricultural Organization
Fig.	Figure
Gm	Gram
H&E	Hematoxylin and Eosin
i.e.	that is
LSD	Least standard deviation
MIU	Motility, Indole, Urea
mm	Millimeter
MR	Methyline Red test
NO.	Number
°C	Degree of celsius
R	Red
SS	Salmonella-Shigella
TSI	Triple sugar iron
TTB	Tetrathionate broth
VP	Voges Proskauer Test
Y	Yellow
µl	Micro litter

CHAPTER 1

INTRODUCTION

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INTRODUCTION

Poultry rearing may play a very important role for income generation and poverty reduction particularly for the distressed women, unemployed youths in Bangladesh by means of self-employment. For this purpose, a model of semi scavenging poultry rearing system known as Model Breeder Poultry Farm (MBPF) has been developed under the Poultry Management Technology Improvement Projects (PMTIP), Participatory Livestock Development Project (PLDP) and Small Holder Livestock Development Project (SLDP-2). In model farming, Sonali breed of chickens has been reared instead of indigenous local chicken because of their high productivity and increased resistant to diseases (Rahman *et al.*, 1997).

In Bangladesh chickens are playing a significant role in national economy and reducing poverty by supplying meat, egg and other by-products. Several constraints such as the diseases, poor husbandry, low productivity and shortage of feed affect the optimal performance of this industry in Bangladesh (Haque *et al.,* 1991). Salmonellosis in poultry causes heavy economic loss through mortality and reduced production (Khan *et al.*, 1998). With great expansion of poultry rearing and farming, pullorum disease and fowl typhoid have become wide spread problem in Bangladesh (Rahman *et al.*, 1997). Age wise prevalence of avian Salmonellosis showed highest infection rate in adult layers (53.25%) in comparison to brooding (14.55%), growing (16.10%) and pullet (16.10%) (Rahman *et al.*, 2004).

Salmonellae are Gram negative, short plump shaped rods, non-sporeforming, non-capsulated, aerobic and facultative anaerobic organisms and classified under the family *Enterobacteriaceae* (OIE Manual, 2006). Village chickens can act as a reservoir of Salmonellosis and therefore, during prophylactic campaign, that must be taken into account (Bouzoubaa *et al.,* 1992). The birds that survive from clinical disease when infected at a young stage may show few signs of infection but can become carriers (Berchieri *et al.*, 2001). Although more than 2300 serotypes of *Salmonella* have been identified, only about 10% of these have been isolated from poultry (Gast *et al.*, 1997).

Chickens are the natural hosts for both *S. pullorum* and *S. gallinarum* (Snoeyenbos *et al.*, 1991). Pullorum disease is usually confined to the first 2-3 weeks of age and occasionally occurs in adults (Shivaprashad, 1997). Fowl typhoid is frequently referred to as a disease of adult birds and there are also reports of high mortality in young chicks (Christensen *et al.*, 1992). *S. gallinarum* can produce lesions in chicks, which are indistinguishable from those associated with pullorum disease (Threlfall and Frost, 1990). The epidemiology of fowl typhoid and pullorum disease in poultry, particularly with regard to transmission from one generation to the next are known to be closely associated with infected eggs (Wigley *et al.*, 2001).

Eggs are widely used in the catering industry, and contribute to a healthy diet providing protein and essential vitamins and minerals. Salmonellosis is the most common avian diseases that are communicable to humans. However, some eggs can contain *Salmonella* bacteria which are a major contributor to food poisoning cases world wide. It is difficult to estimate exact numbers of *Salmonella* cases as only a small proportion of cases are officially reported and many cases are never diagnosed. For most people, it is an unpleasant illness but in certain groups such as very young, elderly or pregnant women it can be very serious (Cudjoe *et al.*, 1994).

Eggs may become contaminated with *Salmonella* in two main ways: (i) *Salmonella* may silently infect the ovaries of apparently healthy hens and contaminate the eggs before the shells are formed. (ii) *Salmonella* infected bird droppings contain *Salmonella* that can contaminate the outer egg shells and may penetrate when crack the shell (Deryck and Pattron, 2004).

Contaminated eggs produced by infected laying hens are thought to be one of the main sources of human infection with *Salmonella* sp throughout the world. This serovar has a predilection for colonization of the hen's reproductive tract, which may lead to internal contamination of the eggs prior to being laid (Humphrey *et al.*, 1989). Also, when a high level of environmental contamination is present in the hen houses, there is a possibility that *Salmonella enteritidis* or ganisms deposited on the shell due to external soiling, which may subsequently penetrate the egg shell (Guan *et al.*, 2006).

From the above discussion, it is evident that Salmonellosis is growing problem in all over the world. Still now Salmonellosis is great economic losing disease in the small and

large scale poultry farm in Bangladesh. *Salmonella* is an important zoonotic pathogen and its prevalence in the poultry act as a continuous threat to humans. For this reasons, infection pattern of disease and nature of the organism should be studied well.

Therefore, the present study was designed to investigate the pathology of *Salmonella* infection from layer birds of different poultry farms at Tangail district as well as to study the pathology of different organs of *Salmonella* infected layer birds.

In view of above consideration, the present research program was designed with following objectives-

- Clinical study of selected Salmonellosis affected flocks at Tangail district.
- Necropsy and histopathological study of organs of affected birds.
- Bacteriological study of causative organisms (*Salmonella* sp.) collected from affected viscera.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

The review of literature is presented in order to collect the information related to the experiment by other researches in home and abroad in different times. It is a path of demonstration for advancement of the recent study.

The purpose of this chapter is to provide a selective review of the previous research conducted so far in relation to the present study, the number of works directly related to the present study were scanty. Detailed Similar findings with intensity of the severity of lesions in different organs were described by many investigators (Chishti *et al.,* 1985; Khan *et al.,* 1998; Hafeji *et al.,* 2001; Habib-ur-Rehman *et al.,* 2003; Beyaz and Kutsal, 2003; Goswami *et al.,* 2003; Hossain *et al.,* 2003; Prasanna and Paliwal, 2003; Islam *et al.,* 2006 and Deshmukh *et al.,* 2007).

2.1 General information of poultry in Bangladesh

The rural poultry constitute about 80% of the total chicken population (Barua and Yoshimura, 1997) and the rural poultry production system in Bangladesh is typically a smallholder free range scavenging operation (Haque *et al.,* 1991). The government of the people's republic of Bangladesh has conducted some experiments through the Directorate of Livestock Services (DLS) concerning the introduction of exotic, high yielding poultry breeds and their crosses into the scavenging and semi-scavenging condition (Reza and Dhali, 1984; Saleque and Mustafa, 1997). They showed that exotic breeds produce higher numbers of eggs than indigenous hens under both scavenging and semi-scavenging conditions, but this was accompanied by a high mortality rate in the scavenging situation (Howlider and Ahmed, 1982 and Amin *et al.,* 1992).

The Directorate of Livestock Services (DLS) of Bangladesh maintains the breeds of Rhode Island Red (RIR) and Fayoumi at the government poultry farms (Anisuzzaman, *et al.,* 1990). The most successful parent breeds are RIR male and Fayoumi females and the name of the cross of these two breeds is "Sonali" which means golden in Bengali, referring to its brown to red color. A model of semi-scavenging poultry rearing has been developed in Bangladesh comprising small units of rice husk incubators, model breeders,

key rearers and egg producers with a small flock (Saleque and Mustafa, 1997). This model for semi-scavenging poultry has already been applied with a considerable success to the benefit of rural poor women (Anisuzzaman *et al.*, 1990 Saleque and Mustafa, 1997). The landless and mostly illiterate women are the main beneficiary group in this operation (Saleque and Mustafa, 1997). The director of Livestock Services (DLS) of Bangladesh is working currently together with the Participatory Livestock Development Project (PLDP) and Smallholder Livestock Development Project (SLDP) funded by DANIDA.

2.2 History of *Salmonella*

Salmonella was one of the first contagious bacterial agents recognized to cause disease and was first cultured by Georg Gaffky, a German scientist, in 1884 (Maskell, 2006). American scientists Salmon and Smith isolated Salmonella enterica serovar Typhi from infected pigs during the same period. This bacterial genus was later named Salmonella in honor of Salmon's great discovery. Since this time, *Salmonella* has earned itself the title of one of the leading causative agents for food borne disease.

In case of poultry, *Salmonella pullorum* and *Salmonella gallinarum* is the most common species that cause diseases. Fowl typhoid, is a disease that is very closely related to pullorum disease and was recognized by Klein, in 1888, even before pullorum disease. Initially the causal agent was called *Bacillus gallinarum*, and the name fowl typhoid was applied in 1902 (Shivaprashad, 1997). Rettger first described the etilogical agent of pullorum disease in 1899, and the disease was called bacillary white diarrhea or fatal septecimia of young chicks. In 1909 the organism was named as *Bacterium pullorum* (Shivaprashad, 1997).

Salmonella is a facultative anaerobic, Gram-negative bacteria belonging to the Enterobacteriaceae family. Bacterial taxonomists have identified two major *Salmonella* species, *S. enterica and S. bongori* and a total of six subspecies belonging to the first species. The most common subspecies, *Salmonella enterica* sub sp. enterica, includes as many as 1,400 pathogenic serovars typically associated with foods, such as *Typhimurium, Agona, Enteritidis, Montevideo*, and *Tennessee*, among others. In addition to those serovars almost 1,000 more antigenically distinct *Salmonella* belong to the other five subspecies (Ray, 2004).

The widespread occurrence of *Salmonella* in nature is a result of its ability to adapt to a variety of less than ideal conditions. *Salmonella* has a unique capability to grow or survive in many extreme environments. For example, *Salmonella* sp. is known to grow at extreme high and low temperatures, modified atmospheric environments, and high and low pH environments (Abushelaibi *et al.,* 2003 and Doyle *et al.,* 2007). *Salmonella* can survive in many hostile environments, but their ability to persist under dry conditions is of particular relevance to this study.

2.3 Isolation and identification of *Salmonella* organism by culture method

Numerous techniques and methods were described for isolation of *Salmonella* from different types of specimens. An isolation technique for *Salmonella* in one type of sample may not be suitable for the isolation from other sample types (Fricker, 1987). Therefore, it was not possible to recommend a single technique for isolation of *Salmonella.* For the special interest of epidemiological investigations with a view to eradicating, controlling or preventing the disease outbreak, detailed strain identification was essential (Threlfall *et al.,* 1994). The isolates under the family of *Enterobactericae* grow well on artificial media (O.I.E. Manual, 1996). These Gram negative rod shape *Salmonella* are oxydase negative and catalase positive (Douglas and Alice, 1993). Triple sugar iron agar provides a presumptive test for characterization of Salmonella (Doughlas *et al.,* 1998). Additional differentiation of *Salmonella* from other organisms was accomplished by biochemical analysis (Cox *et al.,* 1976).

Selective enrichment media was used for culture of samples, which were likely to be contaminated, egg; feces, cloacal swabs, environmental samples and for subcultures from pre-enrichment media (Blackburn, 1993). The aim of using enrichment media was to increase the ratio of *Salmonella* to competitive organisms (Doughlas *et al.,* 1998 and Blackburn, 1993). Selective enrichment media was used to allow additional expansion of the Salmonella population while suppressing the growth of other organisms (Doughlas *et al.,* 1998 and Harvey *et al.,* 1979). Tetrathionate broth, rappaport vassiliadis broth were commonly used as selective liquid media (Threlfall and Frost, 1990). Selective plating media were subsequently used for isolation (Reeves *et al.,* 1989). Brilliant green agar was commonly used as a selective plating medium (Bourhy *et al.,* 1990). Brilliant green agar remains the most widely used medium for *Salmonella* isolation from poultry sources like environment, eggs, feed, feces etc. (Doughlas *et al.,* 1998).

S. pullorum and *S. gallinarum* grow readily on agar or broth. *S. gallinarum* and *S. pullorum* can be isolated by the use of selective and non-selective media (Shivaprasad, 2000). The nonselective pre-enrichment was used to encourage the growth of very small numbers of *Salmonella* or to allow the recovery of injured Salmonella cells (Fricker, 1987). The use of nonselective media was important when *S. pullorum* or *S. gallinarum* were suspected, as some bacteria may be inhibited by selective media (Doughlas *et al.*, 1998). Commonly used nonselective media was seal infusion broth, brain heart infusion broth, nutrient broth, blood agar, nutrient agar, brain heart infusion agar. MaConkey agar was mildly selective. These media were preferred for isolation from uncontaminated tissues from clinical cases (Doughlas *et al.*, 1998).

There was a current ISO horizontal method, (ISO 6579: 2002) for the detection *Salmonella* spp. in food and animal feed. The method was amended in to include testing of animal faeces and environmental samples from primary production. Similar standard methods have been published elsewhere by other bodies, notably in the USFDA Bacteriological Analytical Manual (BAM). The first stage in traditional detection methods for most food samples is usually a pre-enrichment culture in a non-selective liquid medium such as buffered peptone water, incubated at 37°C for 18 hours. Modified pre-enrichment methods may be necessary for samples containing inhibitory compounds. The pre-enrichment culture is then typically subcultured into two different selective enrichment media, such as Rappaport vasiliadis soy broth (RVS) and Muller kauffmann tetrathionate novobiocin (MKTTn) broth, and incubated for a further 24 hours at 41.5°C Rappaport vasiliadis soy broth or 37°C Muller kauffmann tetrathionate novobiocin broth.

The selective enrichment culture is usually inoculated on to at least two selective agar media and incubated at 37°C for 24 hours. The ISO method specifies the XLD agar and one optional selective medium. A variety of alternatives are available, including bismuth sulphite agar, brilliant green agar and hektoen enteric agar. A number of selective chromogenic agar media specifically designed for the differentiation of *Salmonella* colonies are commercially available. Typical *Salmonella* colonies on selective agar are subcultured onto non-selective media prior to confirmatory testing.

Isolated *Salmonella* organisms showed different cultural characteristics in different media. These were turbidity in tetrathionate broth, pink white color colonies in brilliant green agar, whitish or slightly grayish color colonies in Salmonella-Shigella agar, black

color colony in triple sugar iron agar agar, pale color colonies in Mac Conkey's agar, well defined glistening colonies in blood agar and pinkish colonies in eosin methylene blue agar (Rybolt *et al.,* 2005 and Perez *et al.,* 2004).

Conventional cultural method for the isolation of Salmonella, incubation at 37°C for 18 hr about 0.1 ml of the pre-enriched culture was inoculated into 10 ml of RV-10 (Rappaort vassiliadis broth) and incubated at 43°C for 18-24 hr. Thereafter, a loop full of culture was streaked on Brilliant Green Agar (BGA) and McConkey Lactose Agar (MLA) for the selective isolation of *Salmonella.*

Clinical samples were typically cultured directly onto selective agar media, such as Xylose Lysine Desoxycholate (XLD) agar, and incubated at 37°C for 18-24 hours. In addition, stool samples were usually inoculated into a selective enrichment broth, such as selenite cystine broth and incubated at 37°C for 18-24 hours, before plating out onto selective agar.

Determined the seroprevalence of salmonellosis in layer flocks and antibiogram study following isolation & identification of *Salmonellae*. This study was conducted during the period from January to May 2006 at Gobindapur of Dinajpur district. A total of 225 Star cross 579 brown chickens were studied with rapid serum plate agglutination test. Liver of 200 dead birds was studied for isolation and identification of *Salmonellae*. In vitro antibiotic sensitivity test of isolated *Salmonellae* was performed with commercial sensitivity discs. The overall seroprevalence was recorded 23.11%. The prevalence was varied from age to age. The highest rate was 28% in above 20 weeks of age. The antibiogram study revealed that the isolates were sensitive to ciprofloxacin (80%), nitrofurantoin (100%), sulphamethoxazole/ trimeoprim and amoxycillin (50%), tetracycline (60%) but resistant to penicillin-G and erythromycin. Further studies should be conducted on serotyping of the isolated *Salmonellae*, isolation and identification of *Salmonellae* from different feed and environmental sample.,(Akter *et al.,*2007).

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

Lestari *et al.* (2009) isolated and characterized 126 *Salmonella* isolates ,from conventionally raised (n:141) and organically rose (n:53) chicken carcasses obtained

from 27 retail stores in Baton Rouge, Louisiana. *Salmonella* was isolated from 22% of conventional and from 20.8% of organic chicken samples. Eight Salmonella serovars were identified.

Isolated Salmonella from 400 imported chicken carcasses in Bhutan and from 178 pig carcasses in Vietnam for antibiotic resistance analyzed on a random basis against 14 antimicrobial agents. Among the poultry samples tested, 13% were positive for *Salmonella.*, (Ellerbroek *et al.*, 2010)

Poultry salmonellosis, one of the most prevalent diseases and major source of food-borne infections to humans due to consumption of poultry products is worldwide in distribution. The study was conducted from November 2008 to March 2009 with the aim of isolating *Salmonella* species by conventional culture method and their confirmation by Polymerase chain reaction (PCR). A total of 220 poultry tissue samples and 40 egg samples were processed during the study period for the isolation of Salmonella and an overall prevalence of 7/260 (12.7%) was found. The isolation of *Salmonella* from liver and intestine accounted for the highest among tissue samples processed. The remaining isolates were from spleen, pooled tissue samples and egg sample. According to serotyping result, three of the *Salmonella* isolates belong to *S. Heidelberg* which was the most predominant serotype in the present study. Other serotypes isolated include *S. typhimurium, S. ayinde, S. Essen and S. kastrup*. The PCR amplification of suspected Salmonella isolates produced a product of approximate molecular size 550 bp and proved for the efficient utilization of this tool for the rapid detection of *Salmonella* organisms (Habtamu Taddele Menghistuf *et al.*, 2011)

2.3.1 Staining characters

Freeman, 1985 and Khan *et al.* (1988) showed that Gram's stain from a pure culture of *Salmonella* was reveled the rod shaped, Gram-negative and single or paired in arrangement. *Salmonella* organism is a Gram-negative, long, slender and slightly rounded ends. Goswami *et al.* (2003) identified that the isolates are rod shaped, Gram-negative and non motile. Haider *et al.* (2003) isolated *Salmonella* which were rod shaped, formed short and long chains and were gram negative.

2.3.2 Biochemical characterization

Few biochemical characters were different between *S. pullorum* and *S. gallinarum* (Christensen *et al.,* 1992). Both organisms ferment arabinose, dextrose, galactose, mannitol, mannose, rhamnose and xylose to produce acid, with or without gas. *S. gallinarum* ferments dulcitol, whereas *S. pullorum* does not. *S. pullorum* decarboxylates ornithine, whereas *S. gallinarum* does not (Shivaprashad, 1997 and Shivaprasad, 2000). Decarboxylation of ornithine by *S. pullorum* was considered the single most dependable test for differentiating maltose fermenting *S. pullorum* from *S. gallinarum* (Shivaprasad, 2000).

Representative colonies were tested by using a battery of biochemical tests such as TSI, urease, nitrate reduction, oxidase, indole, methyl red, Voges Proskauer, citrate and sugar (adonitol, dulcitol, glucose, inositol, lactose, maltose, mannitol, salicin, sorbitol and sucrose) fermentation tests. The putative isolates were confirmed by slide agglutination test using polyclonal "O" antiserum specific for genus *Salmonella* (procured from Indian Veterinary Research Institute, Izatnagar).

2.3.3 Identification of Salmonella isolate

Salmonella species are Gram-negative, motile, intracellular bacteria of the family enterobacteriaceae. There are two species in the genus *Salmonella*, *S. Enterica* and *S. Bongori. S. Enterica* is further divided into six subspecies: *S. enterica* subspecies enterica (I), *S. enterica* subspecies salamae (II), *S. enterica* subspecies arizonae (IIIa), *S. enterica* subspecies Diarizonae (IIIb), *S. enterica* subspecies houtenae (IV), and *S. enterica* subspecies indica (VI). About 2,541 serovars of *Salmonella*, over half of which belong to *S. Enterica* subspecies *Enterica*. Approximately 99% of all human and animal infections were caused by serovars in subspecies while systemic illness in humans were caused by host adapted serovars *S. typhi* and *S. paratyphi*, many nontyphoidal serovars such as *S. typhimurium* cause enteric disease in a wide rage of species including humans, cattle, swine and poultry. The ability of *S. typhimurium* and other nontyphoidal species to cause enteric disease lies in it's ability to invade the intestinal epithelial cells of the host. Interaction between *Salmonella* and the host epithelium leads to a neutrophilic inflammatory response.

Under the family of *Enterobactiacae*, the genus *Salmonella* is a facultative intracellular pathogen causing localized or systemic infections; as well as a chronic asymptomatic carrier state (Shivaprashad, 1997). The name *Salmonella enterica* serovar *Gallinarum* has been used for both *S. gallinarum* and *S. pullorum* in the latest edition of Bergey's Manual of Systemic Bacteriology (Le Minor, 1984). Serovar *Gallinarum* can be divided into two biovars: *S. gallinarum* and *S. pullorum*, causing fowl typhoid and pullorum disease, respectively (Le Minor, 1984). *Salmonella* enterica serovar *Gallinarum* biovar Gallinarum and biovar Pullorum are Gram negative, rod shaped, nonsporogenic, non-motile and highly host adapted pathogenic avian bacteria (Douglas *et al.,* 1998 and Kwon *et al.,* 2000).

In Assam, Arunachal Pradesh and Meghalaya state of India there was an experiment to determine the presence of *Salmonella* spp. In the apparently healthy breeder flock of organization poultry farms. A total of 832 cloacal swabs from poultry were examined for the isolation of *Salmonella.* Among the isolate, 4.21% samples were positive for *Salmonella*; 42.9% *S. Enteritidis*.17.1% *S. gallinarum* and 40.0% *S. typhimurium* (Bhattacharya *et al.,* 2004). Goswami *et al.* (2003) conducted an experiment for isolation and identification of Salmonella from 46 freshly dead carcasses and 106 ailing birds. They isolated approximately 22 strains of *Salmonella.*

From different processing stages in a sausage plant that uses mechanically deboned chicken meat (MDCM), and tested for the presence of *Salmonella.* A total of 185 samples were collected in 5 monthly batches. Live *Salmonella* bacteria were isolated from 6 samples of the raw meat and from the emulsion in batches 1 or 2. The 6 isolated strains were all classified as *Salmonella* Albany, which as not previously been reported in MDCM (Luiz *et al.,* 2004). A study was conducted from April 1996 to August 1999; Wherein 102 samples were analyzed for the presences of *Salmonella* spp. 6 samples (5.9%) were positive for Salmonella among the isolates. *S. enteritidis* was the most frequently detected (66.7%), followed by *S. saintpaul* (16.7%). Although the results showed a contamination rate smaller than that found in other research, the greatest frequency of isolate for *S. enteritidis* showed a behavior that was similar to the other recent studies, thus, strengthening this serotype trend to become a public health problem in Bauru (Matheus *et al.,* 2003).

2.4 pathology of Salmonella infected layer bird

2.4.1 Clinical findings of avian Salmonellosis

Fowl typhoid is frequently occur in growing & adult birds characterized by whitish materials to the vent, ruffled feathers, depression &anorexia, loss of egg production. Pullorum disease principally occur in chicks under 3 weeks characterized by adherence of chalky white material to the vent, droopy wings and gasping. Paratyphoid infection usually occur in chicks, pullets characterized by profuse watery diarroea, pasting of the vent area, reluctant to move, ruffled feathers (Habib-ur-Rehman *et al.,* 2003).

2.4.2 Gross pathology

Salem *et al.* (1992) performed an experiment in Delaware State of USA. The pathological lesions of pullorum disease observed in 4-week-old roasters from a completely integrated poultry farm. The livers showed multiple small white foci, petechial hemorrhages, and swelling.

Gorham *et al.* (1994) observed the gross lesions of *Salmonella* were more frequent and severe in chickens infected at 1 day of age and in chickens the lesions were characterized by mild to severe enlarged, firm yolk sacs.

Shivaprasad (2000) reported enlargement of the spleen and liver. Omphalitis was often present in chick. In chronic cases abscessation of the viscera (heart, internal serosa, lungs, liver) and typhlitis characterized by grey casts in the ceca. Gross enlargement of the spleen and liver were observed in affected cases. Oophoritis (inflammation of the ovary) followed by ovarian regression was noted in mature stock. Other lesions observed white nodules in liver; enlarged, dark and swollen spleen and kidneys; creamy or cheesy consistency and white nodules in the wall of the yolk sac; yellow creamy or cheesy material inside the wall of ceca; white nodules and yellow-gray pneumonic lung; white nodules in heart muscle and white material cover the surface (pericarditis) of heart; swollen hock and/or wing joints also sticky. In case of Adults, Misshapen, discolored ova, peritonitis, pericarditis, white foci or nodules in infected male testes, which can be confused with tumors.

Grossly, in all the infected cases the liver was enlarged and congested and in few cases, liver discoloration with focal necrosis. Old raised hemorrhages in the caecal tonsil and

congested deformed ova were other important findings. There was catarrhal inflammation in the intestine.

Shivaprasad (2000); Hafeji *et al.* (2001); and Prasanna and Paliwal (2003) reported that myocarditis , ventriculitis with nodular growths or whitish gray rice grain size nodules on the epicardial surface in the heart and pericardial adhesions were also observed in some cases of Salmonellosis. Hepatomegaly, bronze discoloration, mottling, congestion, whitish necrotic foci on hepatic parenchyma observed in another experiment.

Sujatha *et al.* (2003) isolated and characterized *S. gallinarum* from poultry, where liver was found to be the most suitable organ for isolation of *S. gallinarum*. On postmortem, broiler chickens showed enlarged liver with typical greenish bronze color and uniformly distributed necrotic foci.

Habib-ur-Rehman *et al.,* (2003) reported that the gross lesions of 14 *Salmonella* infected layer birds were variable. During necropsy, 63% livers were friable, bronze discoloration with white focal necrosis. A total of 37.1% livers were congested and enlarged. About 49% egg follicles were congested, hemorrhagic and discolored with stalk formation while 51.2% egg follicles were mildly congested and hemorrhagic. A total of 60.6% intestines were hemorrhagic to catarrhal enteritis while 39.4% only hemorrhagic and congested. About 54.7% lungs were severely congested and pneumonic while 45.3% lung showed mild congestion. About 47.1% spleens were enlarged and discolored.

Beyaz and Kutsal (2003) studied that respiratory involvement of *Salmonella* infection in chicken included nodular growth or grey white foci in the lungs and also observed that the spleen was blackish in some birds and light-colored in others. Grey-white miliary foci also observed in the liver.

Gross pathological alteration produced by *S. gallinarum* in and surrounding areas of Hyderabad City. For this purpose, all the affected organs were collected and brought to the Laboratory for detailed study. The total numbers of birds in different farms were 14900 in which the sick birds were 965 (6.47%) the negative birds 765 (5.13%) and a positive bird was 200 (1.34%). In all affected organs, the rate of incidence of *S. gallinarum* was recorded 36.5%. The organs which showed positive reaction towards the *S. gallinarum* infection were 27 in Ovaries (13.5%), Livers 21 (10.5%), Spleens 21 (10.5%) and Kidneys only 4 (2%). The gross pathological changes observed in overies

due to *S. gallinarum* were discoloration 62.3%, enlargement 45.5%, mottling 49.3%, haemorrhages 38.9% nodulating abscesses 19.4% and necrotic foci 25.9%. Liver showed discoloration 62.3%, enlargement 28.9%, mottling 43.4%, heamorrhages 46.3% nodulating abscesses 5.7% and nectrotic foci 10.1%. In similar way, the frequency of gross pathological alteration, which were observed in spleen comprised of enlargement 25.6%, mottling 41.1% hemorrhages 25.2% nodulating absccesses 15.3% and nectrotic foci 43.5% in affected birds. The affected kidneys showed discolouration 20.0%, enlargement 6.6% haemorrhages 13.3%, nodulating abscesses 6.6% and necrotic foci 20.0% (Syed Habib-ur-Rehman, 2004).

0rji *et al.* (2005) studied the gross lesions due to *Salmonella* infection were airsacculitis, peritonitis with fibrinous exudates.

Salem *et al.* (1992); Msoffe *et al.* (2006) and Deshmukh *et al.* (2007) recorded that the gross lesion in heart of poultry affected by *Salmonella* organism mostly included the flabby texture of heart, hydropericardium, and ascites. Myocardium was sometime enlarged.

Mamun *et al.,* (2011) reported that the lesions in the liver showed enlargement, congestion, hemorrhages, congestion, friable with necrotic foci.

It is showed that the lesions in chicks affected with fowl typhoid were indistinguishable from those associated with pullorum disease. In typical cases of fowl typhoid, appearance of bronze coloured liver was characteristic and prominent lesion. The bronze discolouration of the liver was observed more frequently at 7 to 15 days of age. Livers of affected chicks of this age group also revealed numerous greyish necrotic foci or necrotic patches, reddish haemorrhagic foci which were distributed uniformly on their surfaces. The chicks which died in early age group also showed pale discolouration, enlargement and congestion of liver along with mild distension of gall bladder. In few of these cases diffused areas of necrosis were also observed. The proportionate distribution of gross lesions in fowl typhoid, pullorum disease and paratyphoid observed in naturally infected broiler chicken is given.

The heart generally revealed congestion and thickening of pericardium (4.6%) along with few indistinct necrotic foci in some cases. In a few birds of over 2 weeks of age, small elevated greyish white nodular areas were observed on the myocardium, which

were more predominant on the ventricular region. Spleen revealed congestion and enlargement in chicks at early age (1 to 7 days). Presence of hemorrhagic or whitish diffused or multiple necrotic foci were the consistent lesions in the spleen of birds older than this age group. The lungs in general were congested in 11 per cent of affected birds. At early age, there was mild congestion in lungs whereas chicks older than 1 week, revealed moderate congestion and in a few birds focal areas of consolidation were also observed. The kidneys in general were congested and slightly swollen in 19.8 per cent affected birds. Grossly, intestines appeared congested with hemorrhages mostly on the mucosal side in 24.2 per cent of the affected birds. Dropping were thin and occasionally blood stained. Bursa showed mild to moderate atrophy in 8 per cent of affected birds. In acute cases of Pullorum disease in birds having history of diarrhoea, pasting of vent with loose whitish faecal material, impaction of claoca was observed in 3.1 per cent of affected birds. In addition to above changes in Pullorum disease, unabsorbed yolk and yolk sac infection were observed in 5.4 and 6.2 per cent of affected birds respectively during first week of early age. The grossly observed lesions in Paratyphoid infection included necrotic foci on liver (26.4%), pericarditis with the presence of fibrinous exudates in pericardial sac (14.8%), perihepatitis (16.4%), presence of greyish white nodules on ventricular region of heart (very rare), congestion of intestines (21.4%) and kidneys (14.4%), yolk sac infection (18.3%). The lesions in caeca were more prominent and severe in cases of paratyphoid where in the caeca were inflamed and swollen and in chronic cases revealed presence of cheesy, dry, necrotic material in their lumen (4.8%).,(Nazir *et al.,* 2012)

Similar findings with intensity of the severity of lesions in different organs were described by many investigators (Chishti *et al.,* 1985; Khan *et al.,* 1998; Hafeji *et al.,* 2001; Beyaz and Kutsal, 2003; Goswami *et al.,* 2003; Hossain *et al.,* 2003; Prasanna and Paliwal, 2003; Islam *et al.,* 2006 and Deshmukh *et al.,* 2007).

2.4.3 Histopathology

The histopathology of *Salmonella* infected layer was mostly prominent in liver. They showed thickening of liver capsule, congestion, degeneration and necrotic changes in the parenchyma in pullorum diseases along with this, fatty degeneration was also obserbed in fowl typhoid (prasanna and Paliwal 2003 and Chishti *et al.,* 1985). Khan *et al.,* (1998) observed the focal infiltration in the liver. Multifocal coagulative necrosis of hepatocytes

and inflammation reported by Kinde *et al.,* (2000). Congestion of hepatic parenchyma with occasional mononuclear cell infiltration, diffuse and coagulation necrosis of hepatic cells and occasional septic emboli with per vascular lymphoid cells in liver were common (Hafeji *et al.,* 2001).

In spleen, there was lymphoid depletion in spleenic pulp; degenaritive and necrotic changes and marked reticuloendothelial cell hyperplasia and along with this, fatty degeneration also occurred in *S. gallinarum* infection (Chishti *et al.,* 1985; Khan *et al.,* 1998; Hafeji *et al.,* 2001; prasanna and Paliwal 2003 and Deshmukh *et al.,* 2007). Fibrinous exudation in vascular sinuses of the spleen was reported by Kinde *et al.,* (2000).

In heart, Prasanna and Paliwal (2003) studied the histopathology in experimental fowl typhoid and pullorum diseases in chicken were degenerative to necrotic changes. Hafeji *et al.* (2001) observed the microscopic lesions of *Salmonella* infection in poultry detected mild vascular congestion; sever mononuclear infiltration in myocardium, vascular congestion and hemorrhage, heterophilic infiltration in between cardiac muscles fibers were determined. Thinning of cardiomyocytes along with pericarditis and myocarditis also found (Alisantosa *et al.,* 2000 and Deshmukh *et al.,* 2007 and). Lymphocytic infiltration in the heart also reported by Khan *et al.,* (1998). Deshmukh *et al.,* (2007) found that renal tubular nephrosis with characteristic pathological changes and focal intestinal nephritis in kidney. Degenerative to necrotic changes observed in fowl typhoid and pullorum diseases in chicken (Prasanna and Paliwal 2003). Lymphocytic infiltration in kidney was also stated by Khan *et al.,* (1998). Respiratory involvement of salmonella infection in chicken studied by Prasanna and Paliwal (2003) who observed the degenerative to necrotic changes in lung in experimental fowl typhoid and pullorum diseases. Khan *et al.* (1998) observed lymphocytic infiltration in lung.

In intestine, there was catarrhal to hemorrhagic enteritis in fowl typhoid and pullorum diseases in chicken (Prasanna and Paliwal, 2003). Holt *et al.,* (2006) studied about the comparison of *Salmonella enterica* serovar Enteritidis level in crop. They revealed mild to moderate heterophlic infiltration within the crop lamina propria (LP) or LP and epithelium. Multifocal areas of tissue infiltration, as indicated by marked heterophilic infiltration, with necrosis and sloughing of epithelium, were also observed in crops.

Microscopic lesions of *Salmonella* infection in poultry detected in ceca were sever congestion, mild hemorrhage and mononuclear cell infiltration in mucosa and submucosa with degeneration and desquamation of lining epithelium (Hafeji *et al.,* 2001). Khan *et al.* (1998) observed the non exudative serositis in the intestine with infiltration of RE cells. Histological examination of the intestinal tract of *salmonella enteridis* infected molted hens showed increased inflammation in epithelium and lamina propria of colons and ceca, compared with unmolted infected hens (Holt and Poter 1992). In the ovarian folicales, fibrinosupporative inflammation has been observed by Kinde *et al.,* (2000). The proportion of the reproductive organ infection (ovary and oviduct) was relatively higher. Alisantosa *et al.* (2000) observed at 7 days post infection in several birds, especially these was inoculated with *salmonella enteritidis* PT4, which showed retained yolk sacs containing coagulation materials.

Microscopically, the section of livers showed congestion, hemorrhages, focal necrosis with infiltration of mononuclear cells. The pulmonary lesions consisted of sero-fibrinous exudation with mononuclear cell infiltration. The intestinal mucosa exhibited congestion, hemorrhages and infiltration of plasma cells, heterophils and macrophages.

Brito *et al.* (1995) inoculated day old chicks with *Salmonella* either via feed or by direct inoculation. The microscopic examination revealed presence of organism in macrophage. Colonization of organism in alimentary tract and visceral organs occurred more quickly in birds that inoculated orally. Kinde *et al.* (2000) observed fibrinosuppurative inflammation of the peritoneum. Other histopathological lesions of *Salmonella* infection in chicken included congestion, perivascular cuffing, endotheliosis and meningitis in the brain (Prasanna and Paliwal, 2003).

Microscopically, liver showed congestion, hemorrhages, focal degeneration and necrosis. There was accumulation of leukocytes which replaced the degenerated or necrotic liver cells. In intestine there were inflammatory cells in the mucosa and submucosa, and the infiltrated reactive cells were heterophils and lymphocytes. *Salmonella* affected lungs of chickens showed pneumonia and congestion. Egg follicles showed marked congestion with leukocytic infiltration in the yolk. Yolk sacs showed infiltration of leukocytes and marked congestion.

2.5 Isolation of *Salmonella* sp in inner content of laid eggs

Isolated and identified *Salmonella* organisms from ovaries of dead layer birds and from inner content of laid eggs of different poultry farms. The thirty eight ovarian swabs for bacteriology, visceral organs (liver, lung, spleen, egg follicles and intestine) of 38 dead birds for pathological study from 15 layer farms and 45 laid eggs (5 eggs/farm) from reported 9 *Salmonella* infected farms constituted samples of the study. Samples were subjected to isolation and identification of the causal agent followed by gross and histopathological study of the affected visceral organs.

The main challenge concerning the detection of *S. enteritidis* in eggs is the typically very low rate of egg contamination, even among eggs originating from *S. enteritidis* infected flocks (Humphrey *et al.,* 1989 and Humphrey, 2000). In addition, only very few *S. enteritidis* organisms are deposited within contaminated eggs. In many countries flocks of commercial laying hens are vaccinated against SE, which further reduces the rate of contamination in eggs (Davies *et al.,* 2004). These factors make the detection of *S. enteritidis* in raw eggs a challenging task. In many countries, such as the United Kingdom (UK), *S. enteritidis* infected flocks represent a small proportion of all flocks, which translates into a very low overall prevalence of contaminated eggs at the retail level. Recent survey of retail eggs in the UK indicated a prevalence of infection of 0.34% (none in egg contents) in boxes of six eggs on shells only, which represents an even lower prevalence of infection of individual eggs (Food Standards Agency, United Kingdom. 2004). In such situations of very low prevalence, if a sufficient precision of the prevalence estimate is desired, the required sample size approaches the size of the population, which is clearly uneconomical (Morales *et al.,* 1999).

The detection of *Salmonella enterica* serovar enteritidis (SE) in eggs is hampered by a typically low prevalence of contaminated eggs, the low number of SE organisms in such eggs, and the presence of inhibitory substances in the egg albumin. For these reasons, the analysis of large pools of eggs is normally necessary, which presents logistic and microbiological challenges associated with a low number of target organisms from a large volume of sample matrix. In some studies using artificially inoculated eggs the standard procedure for *Salmonella* culture consisting of pre-enrichment, followed by selective enrichment and plating has been replaced by incubation of the egg pools at 25°C to 37°C followed by direct plating. However, in most cases using pools of naturally

contaminated eggs, it may be necessary to enhance the traditional three-step method by addition of antibiotics or iron supplements (Carrique-Mas *et al.*, 2008).

Seventeen of 38 ovarian swabs (44.7%) were *Salmonella* positive. The percentage of *Salmonella gallinarum*, *Salmonella pullorum* and paratyphoid causing *Salmonella* were 70.6%, 17.6% & 11.8%, respectively. About 53% livers of *Salmonella* affected birds were enlarged, congested, friable and bronze coloured with white necrotic foci. About 59% egg follicles were congested, hemorrhagic, discoloured with stalk formation and 70.6% intestines showed hemorrhagic to catarrhal enteritis. At histopathology, 76.5% livers were congested with formation of multifocal nodules and 82.4% egg follicles were congested with huge leukocytic infiltration. Infiltration of heterophils in intestinal mucosa was found in 47.1% cases. Four *Salmonella* isolates were found from 9 laid egg samples (5 eggs content comprised as 1 sample) and isolation rate was 44.4% reporting transovarian transmission in poultry Salmonellosis.(A. K. Saha *et al.*, 2012).

In most situations, in order to avoid overwhelming laboratory resources, the contents of between 5 and 40 eggs are pooled and cultured together. However, there might be some reduction in the test sensitivity due to a dilution effect derived from pooling eggs (Humphrey *et al.*, 1992), although the precise magnitude of this effect is unknown. The culture of large pools of eggs, especially if pre-enrichment is used, constitutes an additional limitation for some laboratories where there may not be sufficient incubator space. Because direct plating would not be able to detect fewer than 105 CFU/ml of *Salmonella* (Gast *et al.*, 1995), additional steps or enhancements to the culture procedures are necessary. Pools of egg contents are either incubated or pre-enriched (or both), with or without supplementation of additives that promote the growth of *Salmonella*. More recently, molecular techniques have been developed in order to speed up testing times, although most of them still use a pre-enrichment or an incubation step. Typically low prevalence of contaminated eggs, the low numbers of *S. enteritidis* organisms in such eggs, and the bacteriostatic effect of the albumen, multiplication of these relatively few organisms are necessary to reach detectable levels in culture.

This can be achieved using traditional three-step *Salmonella* culture methods. These methods proceed as follows:

- Incubation of the sample in non-selective pre enrichment media to resuscitate sub-lethally damaged SE organisms

- Incubation in selective enrichment media that contain inhibitory substances to suppress competing organisms
- Plating onto selective agar media to allow the differentiation of SE organisms from other Enterobacteria.

Three-step culture methods have been used in several studies (Anonymous, 1994 and De Louvois, 1994), and thought to be considerably more sensitive than other methods involving fewer steps (Valentin-Bon *et al.,* 2003), and although the pre-enrichment step was not always carried out (Ebel E.D *et al.,* 1993). The main disadvantage of three-step culture methods is that they take more time, and use more media and incubation space. Therefore abbreviated methods and/or modifications have been attempted.

The most common sources of *S. enteriditis* infections are from undercooked and raw eggs (Centers for Disease Control 2003). Internally contaminating the intact egg (Stephenson *et al.,* 1991; Gast *et al.,* 2003 and Egg Safety Center, 2007). *S. enteriditis* tends to grow favorably in the nutrient-rich egg yolk, but growth in egg white is virtually inhibited by the presence of ovotransferrin (Baron *et al.,* 2004). Ovotransferrin has an antibiotic effect by binding free iron, therefore depriving bacteria of its use (Valenti *et al.,* 1982). The use of an enrichment broth with supplemental iron can help overcome the effects of ovotransferrin and allow the *S. enteriditis* to grow to detectable levels (Cudjoe *et al.,* 1994; Gast and Holt 1998 and Chen *et al.,* 2001). An incubation step of 12 or more hours is usually sufficient for *S. enteriditis* inoculated at10 cfu/mL to reach detectable levels of 105 cfu/mL at 37°C in liquid egg pools supplemented with enrichment broth (Gast and Holt, 2003).

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

The research work was divided into 3 parts: (1) Isolation and identification of *Salmonella* sp. from liver in dead layer birds by different culture method (2) Pathological study of poultry Salmonellosis from infected layer birds (3) Bacteriological detection of *Salmonella* sp. in inner content of eggs from infected layer farms by traditional three-step method.

3.1 Experimental Animal/Birds

Layer birds

ISA brown: ISA brown is actually of French origin with Institute de Selection Animale. ISA brown chickens are prolific egg layer that can lay up to 300 eggs in a year of egg production. ISA brown was created by crossing Rhode Island Red (RIR) rooster and Rhode Island White hen. Feather color brown, shank light brown, comb red & egg shell brown in color.

3.1.1 Experimental area

The study was conducted during the period from January to June 2014, at Tangail district. There were 15 selected layer farms which contained 28,020 birds where there was no history of using Salmonella vaccine. The histopathological studies were performed at the pathology laboratory of Hajee Mohammad Danesh Science and Technology University, Dinajpur and Bacteriological test was performed at the Microbiology laboratory of Hajee Mohammad Danesh Science and Technology University, Dinajpur.

3.2 Flow chart of the experimental design:

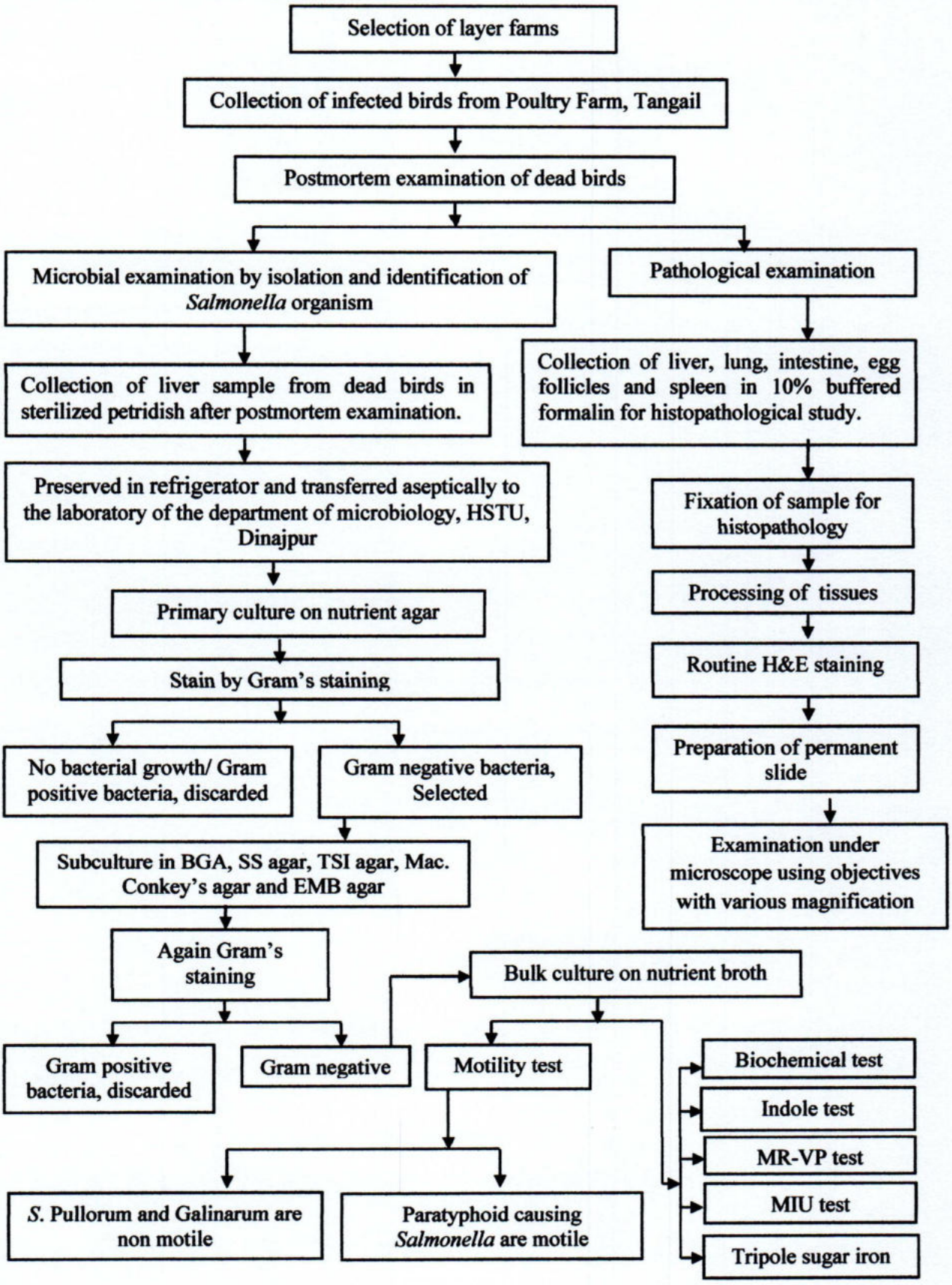

Fig: Schematic representation of the experimental layout

3.3 Isolation and identification of *Salmonella* organism by culture method

3.3.1 Materials for Isolation and identification of *Salmonella* organism by culture method

3.3.1.1 Sample collection

Liver of a total of 39 dead birds were collected from suspected same flock along with the necropsy study. During the experimental period, postmortem examinations of 39 dead birds were performed from 15 poultry farms. For isolation and identification of *Salmonella* organisms, the samples were stored in icepack with sunlight protected black colored box and brought to the bacteriology laboratory, Department of Microbiology, Hajee Mohammad Danesh Science & Technology University, Dinajpur . Isolation and identification of *Salmonella*e were performed as per procedure described by OIE (2000), Merchant and Packer (1967) and Cowan (1985). *Salmonella*e sample were isolated from the collected liver samples by sterilized inoculation loop. Primary culture was performed in nutrient agar. Subcultures were performed in blood agar, MacConkey (MC) agar and Salmonella-Shigella (SS) agar to get pure culture and cultural characteristics.

3.3.1.2 Glassware and other appliances

During the experimental period sterilized Glass wares and appliances were used as follows:

Test tubes (with or without Durham's fermentation tube and stopper), petridishes, conical flask, pipette, slides and coverslips, hanging drop slide, incubator, freeze, thermometer, water bath, iat, microscope, sterilizing instruments, electronic balance, sterilized cotton, immersion oil, beaker, hand gloves, spirit lamps, match lighter, bacteriological loop, glass spreader and forceps, scissors and autoclave.

3.3.1.3 Media for culture

3.3.1.3.1 Solid Media

3.3.1.3.1.1 Nutrient agar

Nutrient agar (NA) medium was used to grow the Salmonella organisms from the collected samples (Cheesebrough, 1984).

2.3 gms of Bacto-Nutrient agar (Difco) was suspended in 100 ml cold distilled water taken in a conical flask and heated up to boiling to dissolve the Medium completely. After sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass Petri dishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein to accomplish the surface be quite dry, the medium was allowed to solidiff for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged by incubating overnight at 37^0C and used for cultural characterization or stored at 4^0C in refrigerator for future use (Carter, 1979).

3.3.1.3.1.2 MacConkey agar

MacConkey agar (MC) medium was used for culturing the organisms under the Enterobacteriaceae (Cheesebrough. 1 984).

5gms of dehydrated Bacto-MacConkey agar (Difco) was suspended in 100 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein. To accomplish the surface be quite dry. The medium was allowed to solidifi, for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used for cultural characterization or stored at 4^0C in refrigerator for future use (Carter, 1979).

3.3.1.3.1.3 Eosin methylene blue agar

Eosin methylene blue (EMB) agar medium used for the purpose of observing differential growth of Salmonella spp. and Escherichia coli (Cheesebrough, 1984).

38 gms of EMB agar base (Himedia, India) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidis, for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged and used or stored at 4^0C in- refrigerator for future use (Carter, 1979).

3.3.1.3.1.4 Salmonella-Shigella agar

Salmonella-Shigella (SS) agar medium was used as a selective medium for Salmonella organism which causes enhancement of the growth of Salmonella while inhibiting the growth of other contaminating organisms and shows typical colony characters (Cheesebrough, 1984).

6 gms of dehydrated Salmonella-Shigella agar (Himedia, India) was suspended in 100 ml of cold distilled water taken in a conical flask and heated upto boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass petridishes (medium sized) and in 15 ml quantities in sterile glass petridishes (large sized) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidifl, for about 2 hours with the covers of the petridishes partially removed. The sterility of the medium was judged by keeping the petridishes in the incubator at 37^0C for overnight and then used for cultural characterization or stored at 4^0C in refrigerator for future use (Carter, 1979).

3.3.1.3.1.5 Brilliant green agar

Brilliant green agar (BGA) medium was used as a selective medium for the isolation and identification of Salmonella organisms (Cheesebrough, 1984).

According to the direction of manufacturer 52 gm of BGA powder (Oxoid, England) was suspended in 1000 ml of distilled water in a conical flask. It was then gently heated with gentle agitation and brought just to the boil to dissolve the medium completely. After sterilization by autoclaving, the medium was cooled to 50^0C, mixed properly and poured into sterile petridishes (10 ml in each petridish) and allowed to solidify. Then the petridishes were incubated at 37^0C for overnight to check their sterility and used to culture the organism or stored at 4^0C in refrigerator for future use Carter, 1979).

3.3.1.3.1.6 Triple sugar iron (TSI) agar

This medium is used as a selective medium for *Salmonella* organisms and also for slant culture of the *Salmonella* organisms.

65 gram of TSI agar was suspended in 1000 ml distilled water and boiled to dissolve the medium completely, then autoclaved, poured on petri dishes, sterility checked by incubation and stored at 4^0C refrigerator until use.

3.3.1.3.2 Liquid Media

3.3.1.3.2.1 Nutrient broth

Nutrient broth (NB) was used to grow the Salmonella organisms from the samples collected from the study areas before performing biochemical test and disinfectant efficacy test (Cheesebrough, 1984).

2 grarns of Bacto-Nutrient broth was dissolved in 250 ml of cold distilled water and heated upto boiling to dissolve it completely. The solution was then distributed in tubes, stoppered with cotton plugs and sterilized in the Autoclave machine at 121°C and 15 pounds pressure per square inch for 15 minutes. The sterility of the medium was judged by incubating overnight at 37^0C and used for cultural characterization or stored at 4^0C in refrigerator for future use Carter, 1979).

3.3.1.4 Media used for biochemical test

In order to identify Salmonella the following media were used for biochemical tests:

3.3.1.5 Chemicals, reagents and solutions

The following Chemical, reagents and solutions were used for conducting staining and biochemical tests: Crystal violate, acetone alcohol, 70% alcohol, gram's iodine, MR-VP solution, 50% buffered glycerin, safranin, methyl red, l0% KOH, phosphate buffered saline (PBS), kovac's reagent, peptone water, glycerin.

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

A quantity of 3.4 gm of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the tubes were autoclaved at 121°C maintaining a pressure of 15 lb/sq. inch for 15 minutes. After autoclaving, the tubes containing medium were incubated at 37^0C for overnight to check their sterility and then stored in a refrigerator for future use (Cheesbrough, 1985).

3.3.1.5.2 Methyl-Red solution

The indicator methyl-red (MR) solution was prepared by adding 0.1 gm of Methyl -red powder (Difco, USA) in 300 ml of 95%alcohol and diluting this to 500 ml with the adding of 200 ml of distilled water.

3.3.1.5.3 Voges-Proskauer solution

3.3.1.5.3.1 Alpha- naphthol solution

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

3.3.1.5.3.2 Potassium hydroxide solution

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

3.3.1.5.4 Kovac's reagent

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

3.3.1.5.5 Normal saline solution

Normal saline solution was prepared by adding 0.85 gms of crystalline sodium chloride in 100 ml of distilled water in a sterilized flask and autoclave for 15 minutes at 15 lb/sq inch at 121°C.

3.3.1.5.6 Phosphate buffered saline solution

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

3.3.2 Methods for isolation and identification of *Salmonella* organisms

3.3.2.1 Collection and transportation of samples

Samples were collected from selected poultry farm of sadar upazilla of Tangail district. Death chicken were immediately brought to Upazilla livestock office, sadar, Tangail. After that, postmortem was performed in Upazilla livestock office Laboratory and samples (liver, lung, spleen, heart) were collected. Sterile instruments and aseptic measures were followed during collection of samples. Samples were kept in sterile petri dishes. The samples were incised with sterile scissors.

3.3.2.2 Primary culture on nutrient agar

With the help of sterile inoculating loop the collected samples were directly inoculated into nutrient agar and incubated at 37^0C for 24 hours. The incubated media were then examined for growth of bacteria. Smooth, glistening and opalescent colony were found on nutrient agar.

3.3.2.3 Morphological study (Gram's staining)

- A loopful of sterile distilled water was placed in the center of a clean sterile slide.
- A small colony was picked up with a bacteriological loop and was mixed with distilled water in a slide.
- The colony was made to thin smear on a slide.
- The smears were fixed by heat then air dry.
- 0.5% crystal violet solution was then applied on the smear for one minute.
- Lugol's iodine was then added to act as mordant for one minute.
- Acetone alcohol was then added to decolorize for 1-2 seconds.
- Then the slide was washed with water.
- Safranine was added as counter stain and allowed for one minute.
- The slide was then washed with water.
- Then the slide was blotted with blot paper and was allowed to dry.
- The slide was examined under microscope with high power objectives with immersion oil.

Gram positive (violate colour) organisms are discarded and gram negative (pink coloured), small rod shaped, single or paired arranged organisms were selected.

3.3.2.4 Secondary culture on differential media

The gram negative organisms were inoculated into MacConkey agar, EMB agar and incubated at 37° C for 24 hours. The incubated media were then examined for growth of bacteria.

3.3.2.5 Culture on selective media

In case of EMB agar non-metalic sheen colony was sub cultured. In case of MC agar-colorless, translucent colony was sub cultured on selective media (SS agar, BG agar, Selenite broth). In case of SS agar colorless, translucent sometimes black colony was sub cultured. In case of BG agar, light pink colony against a rose pink background was sub cultured. Thus single pure colony was obtained. Thus single pure colony was obtained.

3.3.2.6 Biochemical characterization

Isolated organisms with supporting growth characteristics of *Salmonella* on various media were maintained on SS agar and BG agar and were subjected to biochemical tests (sugar fermentation test, TSI agar slant reaction, MR-VP reaction and indole reaction, MIU test).

3.3.2.6.1 Methyl Red (MR) test

The test was performed by inoculating a colony of the test organism into 0.5 ml sterile glucose phosphate peptone broth. After overnight incubation at 37^0 C, a drop of MR solution was added and mixed thoroughly. A red coloration was considered as positive and indicated an acid PH of 4.5 or less resulting from the fermentation of glucose while a yellow coloration was considered negative (Cheesbrough, 1985).

3.3.2.6.2 Voges-Proskaure test

2ml of sterile glucose phosphate peptone water were inoculated with the 5ml of test organisms. It was incubated at 37°C aerobically for 48 hours. A very small amount (knifepoint) of creative was added and mixed. 3 ml of potassium hydroxide were added and snaked well. The bottle cap was removed and left for an hour at room temperature. It

was observed closely for the slow development of pink color for positive cases. In negative cases, there was no development of pink color (Cheesbrough, 1985).

3.3.2.6.3 Indole test

Two ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) was added and mixed thoroughly. The tube was then allowed to stand for a while. The appearance of red color on the whole medium was considered as a positive test for the production of indole by the organisms (Cheesbrough, 1985).

3.3.2.6.4 Triple sugar iron agar slant reaction

TSI agar was used to detect the non-lactose fermenters and the dextrose fermenters. The medium also helped to determine the ability of the organisms to produce hydrogen sulfide (H_2S). The organisms under study were heavily seeded with a platinum needle over the surface of the slant and stabbed into the butt of the tubes of TSI agar. After an incubation period of 24 hours at 37^0C, aerobically the tubes were examined for all changes in the slant or in the butt. In TSI agar slant the presence of yellow color and gas bubbles in the media were considered as production of acid and gas respectively in slants or in butt as the case may be. The red or dark pink coloration of the media in slant or in butt was considered as alkaline reaction. The black coloration in any part of media was considered as the production of H_2S (hydrogen sulfide).

3.3.2.6.5 MIU (Motility, Indole, Urea) medium

Suspected colony was inoculated into the tube containing MIU medium. Then the medium was incubated at 37^0C for overnight. Absence of turbidity throughout the medium was indicated non-motile *Salmonella* organisms

3.3.2.7 Motility test

The motility test was performed to differentiate motile bacteria from non-motile one (Cheesbrough, 1985). Before performing the test, a pure culture of the organism was allowed to grow in NB. One drop of cultured broth was placed on the clean cover-slip and was placed invertedly over the concave depression of the hanging drop slide to make hanging drop preparation. Vaseline was used around the edge of 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 100 power objective of a compound light microscope using immersion oil. The motile and non-motile organisms were identified by observing motility in contrasting with swinging movement of bacteria.

3.3.2.8 Maintenance of stock culture

During the experiment it was necessary to preserve the isolated Salmonella organisms for longer period. For this purpose pure culture of isolated Salmonella organisms were kept in stock culture and this was performed in two methods as stated below.

3.3.2.8.1 Agar slant

The organisms isolated and identified as *Salmone*lla were inoculated into the SS and TSI agar slants and incubated at 37^0 C for 24 hours in bacteriological incubator and then examined for luxuriant growth. Then the sterile mineral oil was poured into the tubes having luxuriant growth until the colonies were covered completely. The tubes were sealed off with paraffin wax and kept at room temperature for future use. By this method, bacteria can be preserved with no deviation of their original characters for few months.

3.3.2.8.2 50% Sterile buffered glycerin

Fifty percent sterile buffered glycerin was made by mixing 50 parts pure glycerin and 50 parts PBS. Then the pellet bacteria obtained by centrifugation at 2500 rpm for 20 minutes were mixed with 50% sterile buffered glycerin in small cryovials and were preserved at -20^0C. This method is more appropriate for preserving bacteria with no deviation of their original characters for several years.

3.4 Clinical examination

3.4.1 History

Under this point the following data were collected from the farmers: Name of farmer, name of area, total number of birds in farm, total number of affected birds, daily mortality and total mortality. During the period present and previous history was taken from each farmer.

3.4.2 General examination of clinical findings

At first the external appearance of the bird was observed then general condition of the chicken, condition of vent, feathers. Presence or absence of diarrhea was also marked.

3.4.3 Postmortem examination

It was conducted with the help of rubber gloves, a pair of shears, scissors, knife, scalpel and forceps.

3.4.3.1 Technique

At first the chicken was laid on its back and each leg to expose the internal organs, in turn drawn outward away from the body while the skin was incised between the leg and abdomen on each side. Then the both legs were grasped firmly in the area of the femur and sent forward, downward and outward, until the head of both femurs were broken free of the acetabular attachment so that both legs lied flat on the table. The skin was cut between the two previous incisions at a point midway between keel and vent. The cut edge was then forcible reflected forward, cutting was necessary, until the entire ventral aspect of the body including the neck, was exposed. For exposing of the viscera, knife was used to cut through the abdominal wall transversely mid-way between the keel and vent, then through the breast muscle on each side. Positioning shears were used to cut first the rib cage, the coracoids and clavicle on both side. With some care this was done without severing the large blood vessels.

3.5 Histopathology

During the whole period of study, postmortem examinations of 39 dead birds were performed from the representative selected 15 layer farms. Gross pathological changes at necropsy were carefully observed and recorded.

For histopathology, the tissues were preserved in 10% neutral buffered formalin. 10 % formalin was prepared as follows-

40 % formaldehyde	10 ml
Distilled water	90 ml

Then trimming of the tissues was done with 1.5 X 1 cm size. Tissues were then kept in running tap water for overnight to wash out formalin. Dehydration was done in ascending grades of alcohol using 50%, 70%, 80%, 95% and three changes of absolute alcohol for one hour in each. Cleaning of sections was done in chloroform by two changes, one and half an hour for each. Paraffin embedding with melted paraffin wax at 56°C by two changes, one and half an hour for each. Preparation of paraffin block was done. Sectioning of the block was done with a microtome (Mu509, Euromex, Japan) at 5µm thickness. The sections were allowed to spread on warm water bath (45°C) and taken on oil and grease free glass slides. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The slides containing sections were air dried and kept in cool place until staining (Luna, 1968).

3.5.1 Preparation of different stains

3.5.1.1 Harris' hematoxylin solution

Hematoxylin crystals	5.0 gm
Alcohol, 100%	50.0 ml
Ammonium alum	100.0 gm
Distilled water	1000.0 ml
Mercuric oxide (red)	2.5 gm

The hematoxylin was dissolved in alcohol and the alum in water by the aid of heat. The two solutions were removed from heat and thoroughly mixed and boiled rapidly. After removal from heat, mercuric oxide was added slowly. The mixture was reheated to a similar way until it became dark purple and removed immediately and plunged the vessel into a basin of cold water and kept until cooled. Just before use, 2-4 ml of glacial acetic acid was added per 100 ml of solution to increase the precision of the nuclear stain. Before use, the prepared solution was filtered.

3.5.1.2 Eosin solution

3.5.1.2.1 Stalk eosin solution

Eosin Y, water soluble	1.0 gm
Distilled water	20.0 ml
Alcohol (95%)	80.0 ml

The ingredients were mixed properly and stored for use.

3.5.1.2.2 Working eosin solution

Eosin stock solution	1 part
Alcohol, 80%	3 part

Just before use 0.5 ml of glacial acetic acid was added to each 100 ml of stain and stirred.

3.5.2 Routine hematoxylin and eosin staining procedure

The sectioned tissues were deparaffinized in three changes of xyline for three minutes in each change. Then the tissues were rehydrated through descending grades of alcohol. Three changes in absolute alcohol for three minutes in each (95% alcohol for two minutes; 80% alcohol for two minutes; 70% alcohol for two minutes). It was washed with distilled water for five minutes. It was stained with Harris' hematoxylin for fifteen minutes and washed in running tap water for 10-15 minutes. The tissues were then differentiated in acid alcohol (1 part HCl and 99 parts of 70% alcohol) by 2 to 4 dips. Then it was washed in tap water for five minutes followed by 2-4 dips in ammonia water until sections were bright blue and stained with eosin for one minute. The sections were then differentiated and dehydrated in alcohol (95% alcohol: three changes, 2-4 dips in each; absolute alcohol: three changes, 2-3 minutes in each). Then the sections were cleaned in xyline for three changes for five minutes in each. Finally the sections were mounted with cover slip using DPX and dried (Luna, 1968).

3.5.3 Histopathological studies and photomicrography

The microphotographs made from the selected areas of tissues section (with the help of digital camera connected with microscope) under 10x and 40x microscopic objectives were placed in this thesis for better illustration of the result.

3.6 Isolation of *Salmonella* sp in inner content of laid eggs

This test was performed by following the method described by Poppe *et al.* (1992) and Haider (2009). Total 75 eggs were collected from 15 farms (5 eggs/15 farms) followed by washing and disinfection of egg surface by 70% ethanol. Manually homogenization of pooled egg contents was performed by using stirrer. Incubation of homogenized egg contents was done with pre-enrichment media (BPW) at 37°C temperature as a standard

proportion of 1:10 fold dilution for 48 hours followed by incubation in selective enrichment media (SRV) at 37°C temperature as a proportion of 1:10 fold dilution for 24 hours. Then plating was performed onto selective agar media (BGA, SS, TSI & EMB agar).

3.6.1 Collection of laid eggs

A total of 75 laid eggs (5 eggs/farm) samples were collected from *Salmonella* suspected 15 layer farms of Tangail region during this study period. The *Salmonella* infected poultry farms were detected by isolation of *Salmonella* sp from Liver of dead layer birds.

3.6.2 Washing and disinfected of egg surface

The egg shells were cleaned and disinfected prior to exclude any potential *Salmonella* contamination on the external surface. Cleaning of egg shells were achieved by brushing and surface disinfection was performed by soaking in 70% ethanol.

3.6.3 Homogenization / pooled of egg contents

Separate homogenization was performed manually by using stirrer together with 5 laid eggs from each infected layer farm for detection of *Salmonella* sp. Homogenization was thought to be done because; the yolk is high in iron content, making it an excellent medium for bacterial growth compared with the albumen, which is iron deficient.

3.6.4 Culture methods

This was achieved using traditional three-step *Salmonella* culture methods. These methods proceed as follows:

3.6.4.1 Preparation of pre-enrichment media (Buffer Peptone Water)

Peptone	:	10 g
Sodium chloride	:	5 g
Disodium phosphate	:	3.5 g
Monopotassium phosphate	:	1.5 g
Final pH	:	7.2 ± 0.2 at 25°C

20 g of the medium were dissolved in one liter of purified water and heated with frequent agitation to complete dissolve the medium and then autoclaved at 121°C for 15 minutes.

3.6.4.1.1 Addition of antibiotic (Novobiocin) in pre-enrichment media

0.5ml novobiocin was added (2% filtered sterilized aqueous solution) in 500ml of pre-enrichment media (Buffer peptone water) which inhibit other bacterial growth that significantly enhanced the isolation of *Salmonella* sp in a small proportion of egg contents.

3.6.4.1.2 Use of additives in pre-enrichment media

0.1mg cysteine was demonstrated as additives in 500ml pre-enrichment media for significantly increase the growth rate of (*Salmonella* sp) bacteria.

3.6.4.2 Incubation of homogenized egg contents with pre-enrichment media (BPW)

20ml homogenized / pooled eggs content and 180ml pre-enrichment media (Buffer peptone water) were incubated at 37^0C temperature as a standard proportion of 1:10 fold dilution for 48 hours.

3.6.4.3 Preparation of selective enrichment media (Semisolid Rappaort- Vassiliadis media)

Composition of Semisolid Rappaort- Vassiliadis media (Formula / Liter)

Enzymatic digest of casein	:	4.59 g
Casein acid hydrolysate	:	4.59 g
Sodium chloride	:	7.34 g
Potassium dihydrogen phosphate	:	1.47 g
Magnesium chloride, anhydrous	:	10.93 g
Malachite green oxalate	:	0.037 g
Agar	:	2.7 g
Final pH	:	5.6 ± 0.2 at 25°C

31.6 g of the medium was suspended in one liter of purified water and heated with frequent agitation and boiled for one minute to dissolve the medium completely.

3.6.4.4 Incubation with selective enrichment media (SRV)

After incubation of pooled eggs content with pre-enrichment media, 20ml component was taken and again incubated with 180ml selective enrichment media (Semisolid

Rappaort-Vassiliadis media) at 37^0C temperature as a proportion of 1:10 fold dilution for 24 hours.

3.6.4.5 Preparation of selective agar

BGA, SS agar, TSI agar and EMB agar were used as selective media for detection of *Salmonella* organisms in laid eggs content. Preparation of selective agar procedure was described previously.

3.6.4.6 Plating onto selective agar media

After incubation of the component (Pooled eggs content and BPW) with selective enrichment media (SRV), that was plated onto selective agar by inoculating loop.

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

In this research experiment, first part consisted of isolation and identification of *Salmonella* organism collected from different layer farms of Tangail district. By necropsy, 39 liver samples were taken for determining the cultural prevalence of *Salmonella* from dead birds. Second portion comprised of gross lesions in different organs as well as histopathological study and third portion consisted of isolation of *Salmonella* sp. from inner content of laid eggs. All experiment were performed in the Pathology Laboratory, Department of Pathology and Parasitology and Bacteriology laboratory, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

4.1 Isolation and identification of *Salmonella* from chicken

4.1.1 Cultural characteristics

4.1.1.1 On Nutrient agar (NA)

On Nutrient agar (NA) isolates produced translucent, opaque and smooth colonies. (Fig2)

4.1.1.1.2 On Nutrient broth (NB)

Salmonella isolates produced turbidity in nutrient broth (Fig 6).

4.1.1.1.3 S-S agar

On S-S agar suspected isolates produced translucent, slightly grayish color, smooth, small round colonies (Fig 4).

4.1.1.1.4 Brilliant green agar

Samples inoculated onto BG agar plates produced pale pink color colonies against a pinkish background, which was rose-pink colour before growth.

4.1.1.1.5 MacConkey agar

Samples inoculated onto MacConkey agar plates produced colorless, smooth, transparent and raised colonies (Fig 3).

The overall cultural characteristics in different culture media are summarized in Table 1.

Table.1 Cultural characters of isolated *Salmonella* in different media

Samples	Name of the media*	Colony characters	No. of isolate	Positive in culture
39 Liver sample cultured in Selective media	Nutrient agar	*Salmonella* organism showed translucent, opaque, smooth colonies in Nutrient agar.	17	43.59%
	Brilliant green agar (BGA)	*Salmonella* organism showed slightly yellowish white color colonies in BGA.		
	Salmonella-Shigella (SS) agar	Slightly grayish color colonies showed in SS agar.		
	Mac Conkey agar	Colorless, smooth, transparent and raised colonies in McConkey agar.		
	Eosin methylene blue (EMB) agar	*Salmonella* organism showed pinkish colony in EMB agar.		
	Triple sugar iron (TSI) agar	Black color colony showed in TSI agar.		
	Nutrient broth	Turbidity in the broth.		

Note: * All 17 isolates of *Salmonella* were produced specific colony characters in 7 different media.

4.1.2 Identification (Gram's staining)

From pure culture in different media, Gram's staining was performed to observe *Salmonella* organism. Among 39 liver samples, 17 isolates revealed light microscopically gram-negative, pink coloured, rod shaped organism and arranged in single or paired pattern (Fig.6) under the microscopic examination.

Table 2. Characteristics of *Salmonella* chicken isolates by Gram's staining method and motility test

Isolate	Motility		Morphology	Staining
	By MIU	By Hanging drop		
Salmonella spp.	Non motile and motile	Non motile(15) and motile(2)	Small rod, single or paired, pink in color	Gram-negative

4.1.3 Carbohydrate fermentation and different biochemical tests

4.1.3.1 Indole test

Salmonella isolates from chicken was indole negative. They did not produce any red color.

4.1.3.2 MIU test

Absence of turbidity trough out the medium was indicated non-motile *Salmonella* organisms (Fig. 8) and presence of turbidity was indicated motile *Salmonella* organisms.

4.1.3.3 Methyl red (MR) test

In the MR test the appearance of the red colour in the media after the addition of 3 ml of methyl red with the cultural growth was observed and thus indicating the isolated *Salmonella* were positive for MR test (Fig. 10).

4.1.3.4 Voges-Proskauer (V-P) test

In the Voges-Proskauer (V-P) test, no change of colour of the media was observed after the addition of 3 ml of 3% KOH to 3 ml V-P broth media with the cultural growth of the isolated *Salmonella* and thus indicated that the isolated *Salmonella* from chicken was negative for V-P test.

4.1.3.5 Triple Sugar Iron (TSI) agar slant reaction

On TSI agar slant, *Salmonella* isolates from chicken produced acid (yellow) and gas in the butt, hydrogen sulfide gas in both butt and slant and the alkaline reaction in the slant which revealed red color (Fig. 9).

Table 3. Carbohydrate fermentation tests and biochemical tests of isolated *Salmonella* from dead bird.

Among 39 samples 17 isolates were supported as a Salmonella organism on the basis of culture and Gram's staining method. Finally these 17 *Salmonella* isolates were reconfirmed by different biochemical tests.

No. of sample	MR	VP	Indole	Motility	Isolate	TSI			MIU	No.of isolate
						Butt	Slant	H_2S		
39	+	-	-	-	*S. pullurum*	Y	R	+	Absence of turbidity	3
	+	-	-	-	*S. gallinarum*	B	R	+	Absence of turbidity	12
	+	-	-	+	Paratyphoid causing *Salmonella*	B	B	+	Presence of turbidity	2

Y= Yellow, B= Black, R= Red, MR= Methyl Red, VP= Voges Proskauer, TSI= Triple sugar iron, MIU= Motility Indole Urea.

All the isolates of *Salmonella* were MR positive; VP and indole test are negative.

4.1.3 Results of motility test

Isolates was found to be 15 *Salmonella* non motile and 2 *Salmonella* were identified as motile when examined using hanging drop slide under microscope (Table-3). The characteristics of chicken *Salmonella* isolates by Gram's staining method and motility test are presented in (Table -2).

4.1.4 Cultural prevalence of *Salmonella* isolate from dead birds

Total 39 samples (Liver sample) were taken from dead birds. In liver samples, out of 39 samples 17 were positive and the cultural prevalence was 43.59% (table.4).

Table 4. Cultural prevalence of *Salmonella* isolates from liver samples of dead birds

Farm	No. of dead birds	Liver sample		Cultural prevalence
		tested	Positive in culture	
F-01	39	5	3	43.59%
F-02		2	0	
F-03		2	2	
F-04		1	0	
F-05		4	3	
F-06		3	2	
F-07		2	1	
F-08		2	0	
F-09		1	1	
F-10		3	0	
F-11		3	2	
F-12		4	0	
F-13		2	1	
F-14		3	2	
F-15		2	0	
Total		39	17	

F=Layer farm

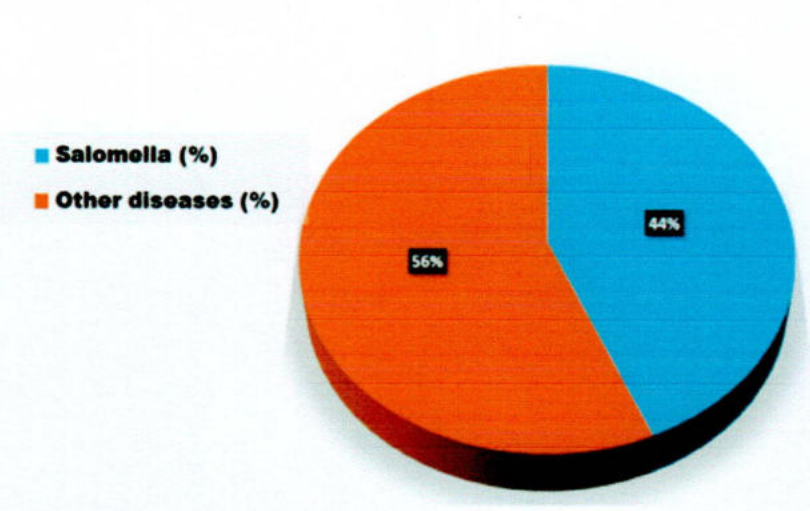

Fig. 1: Cultural prevalence of avian salmonella.

4.1.5 Isolation and distribution of *Salmonella* from dead birds

Total 17 *Salmonella* were isolated from 39 Liver samples of dead birds. In dead birds, the cultural prevalence was 43.59%. Among the 17 isolates, 70.58% (12) were *Salmonella gallinarum*, 17.64% (03) were *Salmonella pullorum* and 11.77% (02) isolates were identified as paratyphoid causing *Salmonella* (Table.5)

Table 5. Observation of prevalence of *Salmonella* from dead birds

No. of sample	No. of isolates	Prevalence (%)	Distribution	Species	(%)
39	17	43.59	12	*S. gallinarum*	70.58
			3	*S. pullurum*	17.64
			2	Paratyphoid causing *Salmonella*	11.77

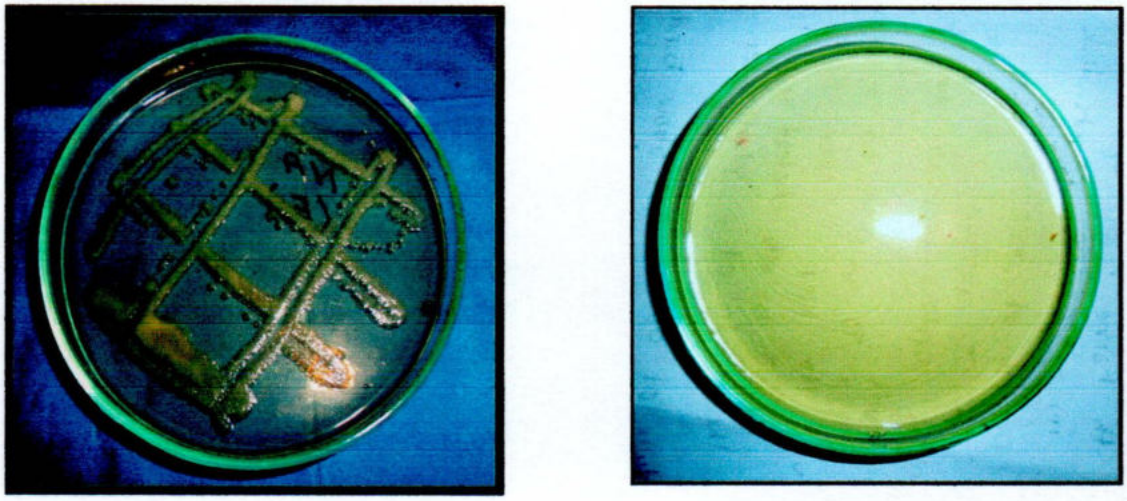

Fig. 2: Growth of *Salmonella* in Nutrient agar showing circular, smooth, opaque, translucent colonies (left) and control (right).

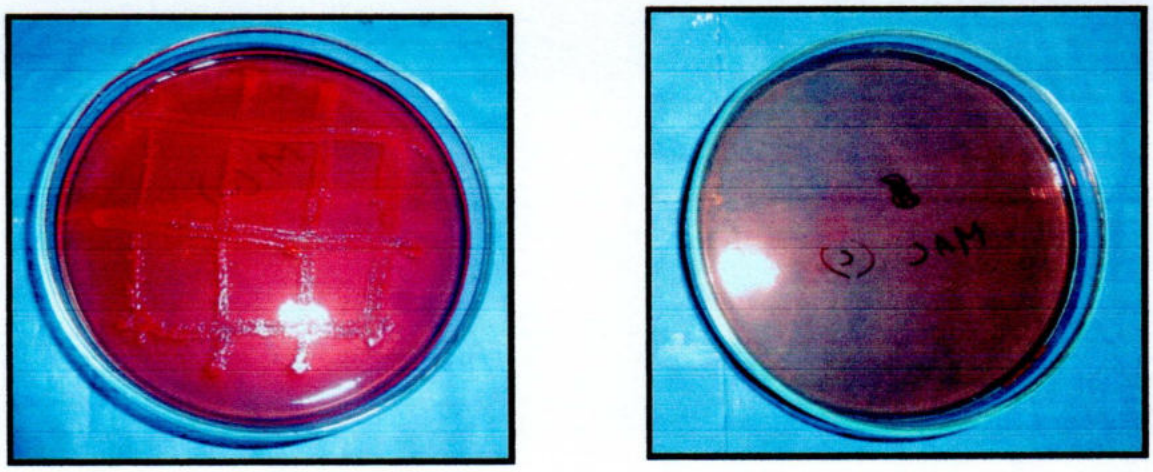

Fig. 3: Growth of *Salmonella* in MacConkey agar showing colorless, smooth, transparent and raised colonies (left) and control (right).

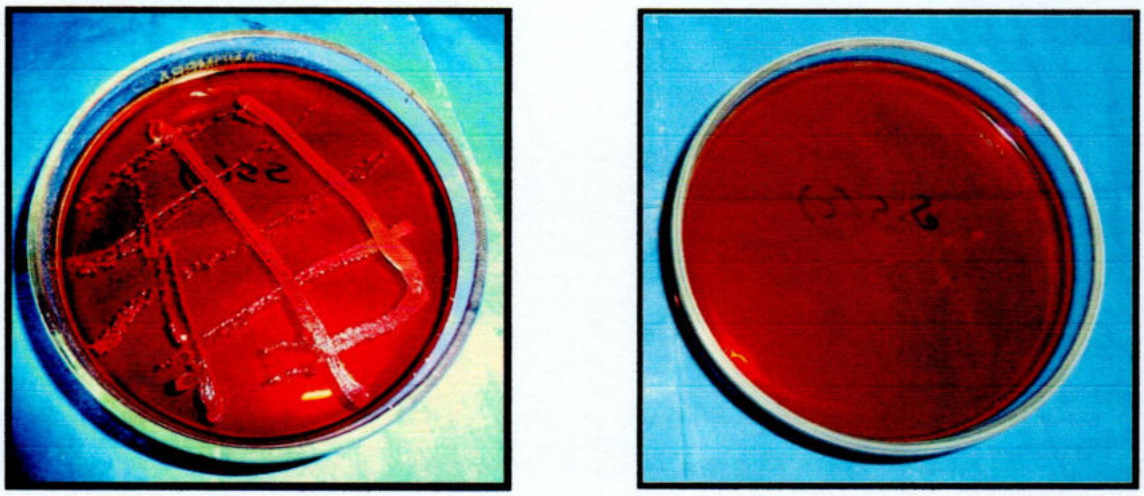

Fig. 4: Growth of *Salmonella* in Salmonella-Shigella (SS) agar showing Slightly grayish color colonies (left) and control (right).

Fig. 5: Growth of *Salmonella* in EMB agar showing pinkish colonies (left) and control (right).

Fig. 6: Growth of *Salmonella* in Nutrient broth showing turbidity (right) and control (left).

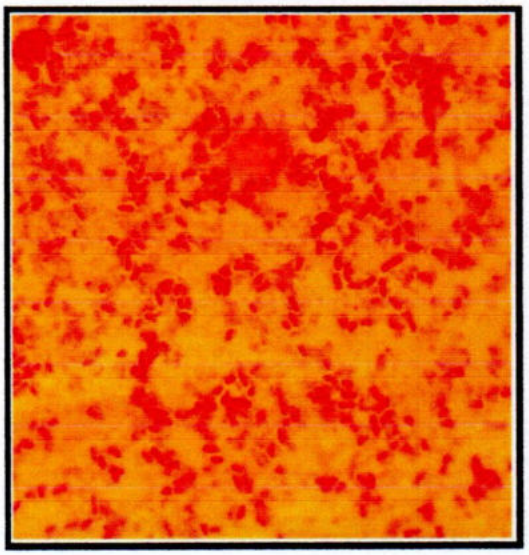

Fig. 7: Gram negative small rod shaped organism arranged in singl and pairs.

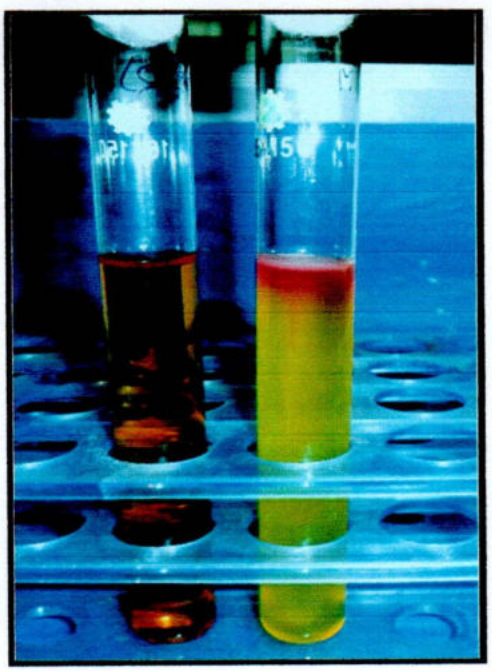

Fig. 8: MIU test showing absence of turbidity (right) and control (left).

Fig. 9: In TSI test showing yellow slant and black butt (right) and control (left).

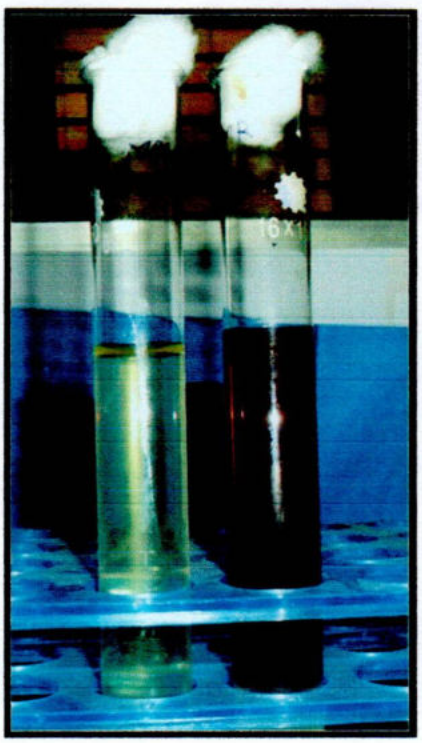

Fig. 10: MR test showing bright red colour of the medium (right) and control (left).

4.2 Clinical findings of avian Salmonellosis

In the present study the following clinical findings were observed; fowl typhoid is characterized by whitish materials to the vent, ruffeled feathers, depression, anorexia, loss of egg production. Pullorum disease is characterized by adherence of chalky white material to the vent (Fig. 11), droopy wings, gasping. Paratyphoid infection is characterized by profuse watery diarrhea, pasting of the vent area, reluctant to move, ruffled feathers and finally death of affected bird.

4.2.1 Pathological studies

4.2.2 Gross pathology

Total 39 dead birds were collected for necropsy from 15 different farms and gross pathological findings recorded carefully. In present study, the gross lesions of 17 *Salmonella* infected layer birds were variable. During necropsy, 64.70% livers were found friable and bronze discoloration with white focal necrosis (Fig. 13 and Fig. 14). A total of 47.05 %livers were congested and enlarged with necrotic foci (Fig. 13). About 52.94% egg follicles were congested, hemorrhagic and discolored with stalk formation while 47.05% egg follicles were mildly congested and hemorrhagic (Fig. 12). A total of 70.58% intestines were hemorrhagic to catarrhal enteritis while 38.88% only hemorrhagic and congested. About 64.70% lungs were severely congested and pneumonic while 35.29% lung showed mild congestion. About 38.88% spleens were enlarged and discolored.

4.2.3 Histopathology

Only 17 *Salmonella* positive samples were selected for histopathology. Table 7 describes the histopathological findings of different organs. In histopathological investigation, all the samples of 17 layer birds did not evoke similar kinds of lesions. A total of 82.35% livers were congested and formed multifocal nodules with coagulation necrosis while remaining 29.41% liver showed hepatitis (Fig. 17). Besides, 82.35% lungs were severely congested and hemorrhagic and 23.53% lung showed inflammatory cells in alveoli and bronchi (Fig. 19). Infiltration of heterophils and lymphocytes in the mucosa of intestines were found in 52.94% cases (Fig.16). Sever lymphocytic depletion and focal necrosis in the spleen was found in 47.05% birds.

Table 6. Gross pathological findings of *Salmonella* affected birds of different layer farms

Lesions	Infected farm no.									Total N=17	%
	F-1 (n=3)	F-3 (n=2)	F-5 (n=3)	F-6 (n=2)	F-7 (n=1)	F-9 (n=1)	F-11 (n=2)	F-13 (n=1)	F-14 (n=2)		
Friable, bronze discoloration liver with white focal necrosis	+	+	-	+	+	-	+	+	-	11	64.70
Congested and enlarged liver	-	-	+	+	-	-	+	+	-	8	47.05
Congested haemorrhagic, and discolored egg follicles with stalk formation	+	+	-	+	-	+	-	+	-	9	52.94
Mild congested and haemorrhagic egg follicles	-	-	+	-	-	+	+	-	+	8	47.05
Haemorrhagic to catarrhal enteritis	+	+	+	-	+	+	-	-	+	12	70.58
Congested and haemorrhagic intestine	-	-	+	+	-	+	-	+	-	7	38.88
Severely congested and pneumonic lung	+	+	+	-	-	+	-	-	+	11	64.70
Mild congested lung	-	-	-	+	+	-	+	+	-	6	35.29
Enlarged with discolored spleen	+	+	-	-	-	+	-	+	-	7	38.88

"+"present, "-"absent "n"=No. of positive birds / farm, F= farm

Table 7. Histopathological findings of *Salmonella* affected tissues of different organs

Lesions	Infected farm no.									Total N=17	%
	F-1 (n=3)	F-3 (n=2)	F-5 (n=3)	F-6 (n=2)	F-7 (n=1)	F-9 (n=1)	F-11 (n=2)	F-13 (n=1)	F-14 (n=2)		
Congestion and multifocal nodule formation in liver	+	+	+	+	-	+	-	-	+	13	76.47
Hepatitis and infiltration of inflammatory cells	-	-	-	-	+	+	+	+	-	5	29.41
Marked congestion and leucocytic infiltration in egg follicles	+	+	+	+	-	-	+	-	+	14	82.35
Infiltration of heterophils and lymphocyte in the mucosa of intestine	+	-	-	+	+	-	-	+	+	9	52.94
Severely congested and hemorrhagic lung	+	+	-	+	+	-	+	+	+	14	82.35
Inflammatory cells in the alveoli and bronchus			+			+				4	23.53
Severely lymphocytic depletion and focal necrosis in the spleen	+	+	-	-	+	-	+	-	-	8	47.05

"+"present, "-"absent "n"=No. of positive birds / farm, F= farm

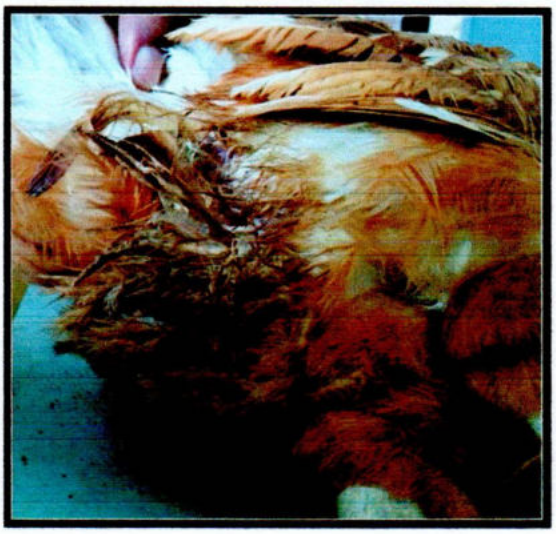

Fig. 11: Salmonella affected bird shows Chalky white material in vent

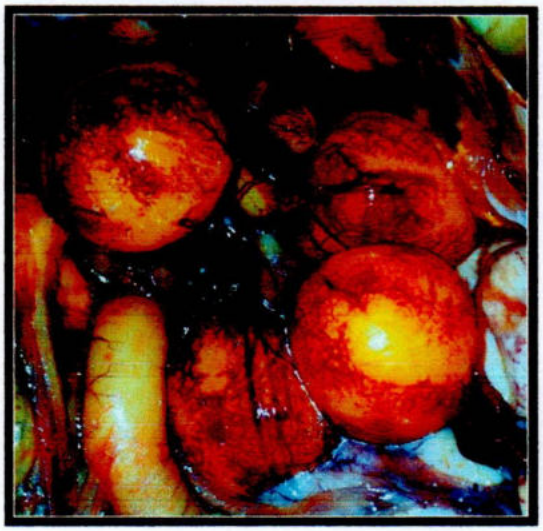

Fig. 12: Salmonella affected egg follicles shows haemorrhagic, congested and discolored with stalk formation

Fig. 13: Salmonella affected liver shows fragile with necrotic foci

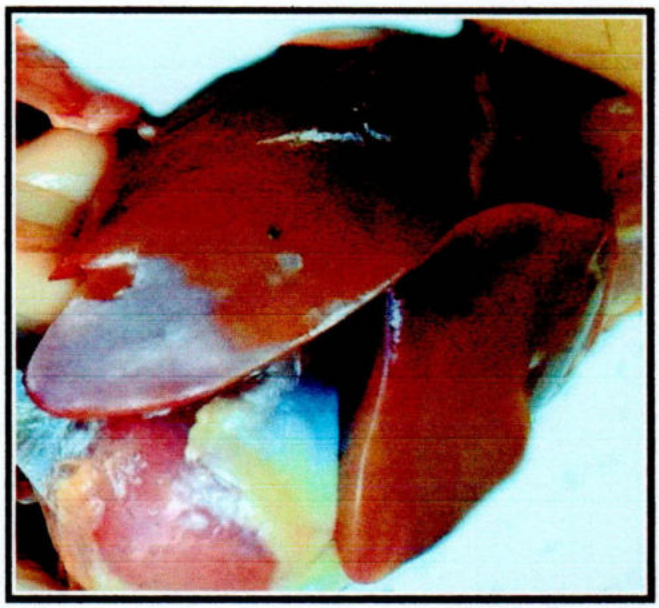

Fig. 14: Salmonella affected liver shows bronzed discoloration.

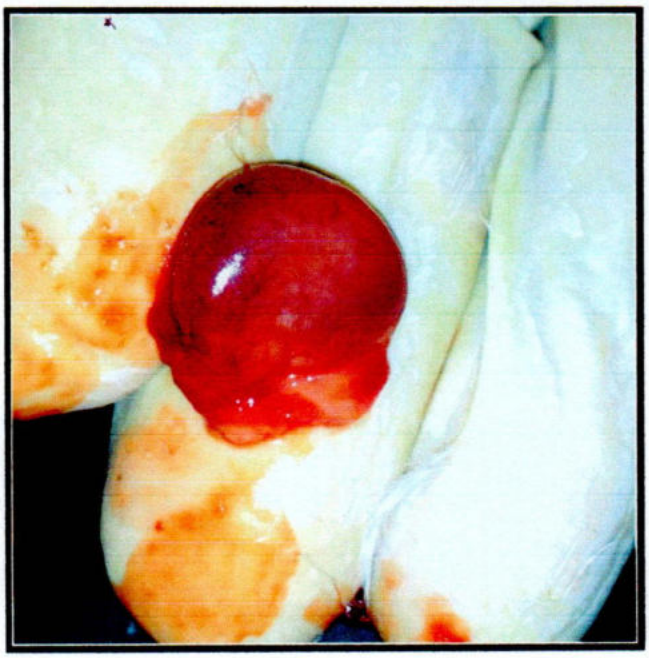

Fig. 15: Salmonella affected Enlarged and discolored spleen

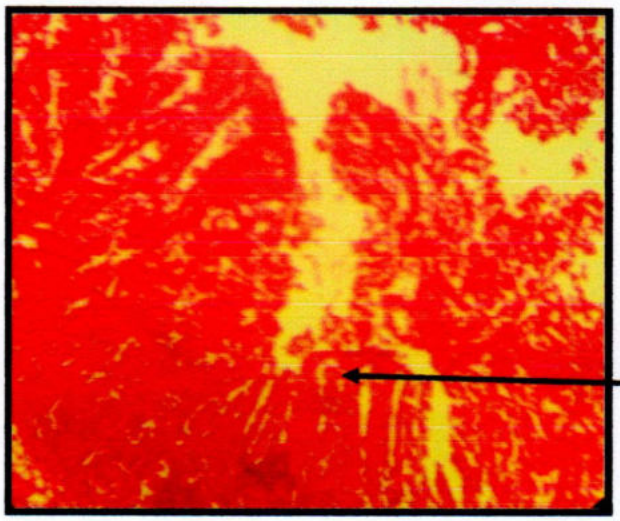

Fig. 16: Salmonella affected intestine shows epithelial destruction, reactive cells infiltration (H & E staining X 40)

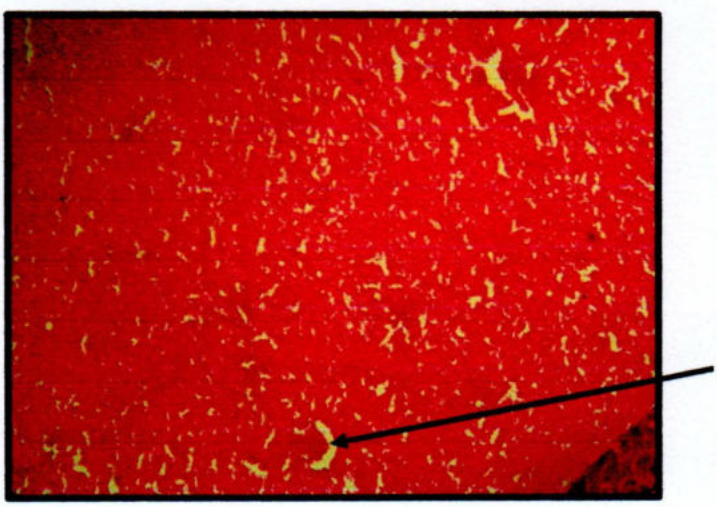

Fig. 17 : Salmonella affected liver shows severly congestive liver paranchyma (H & E staining X 40)

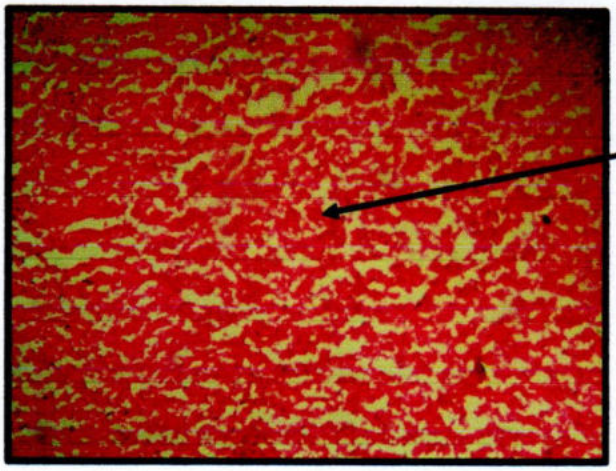

Fig. 18 : Salmonella affected liver destruction of hepatic cords and more acidophilic cytoplasm (H & E staining X 40)

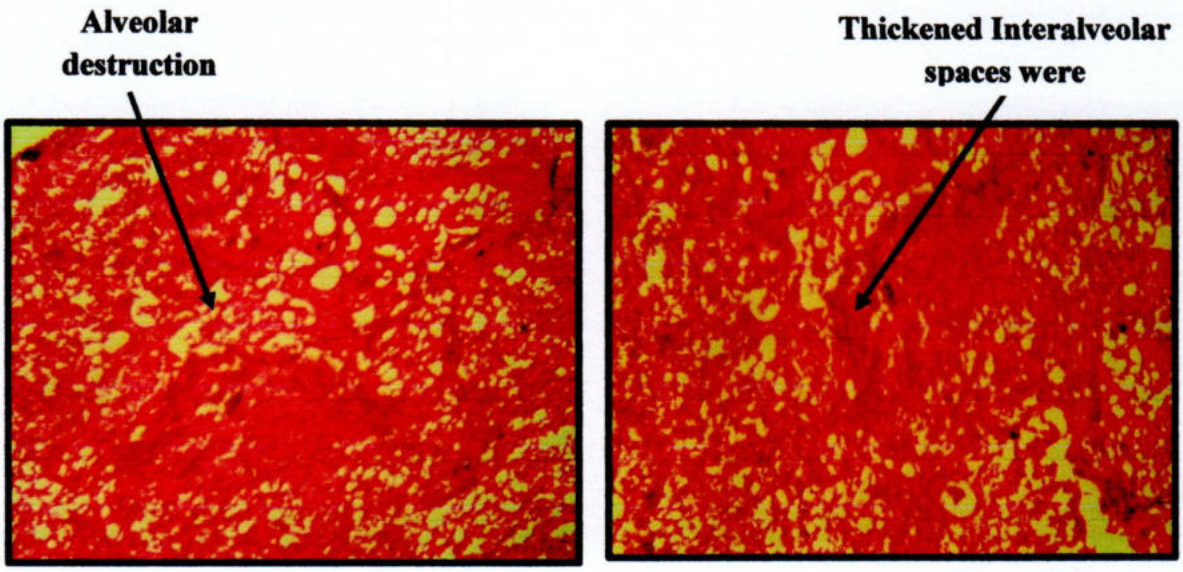

Fig. 19 : Salmonella affected lungs shows interalveolar spaces were thickened, alveolar destruction, congestion and huge leukocytic infiltration in lungs (H & E staining X 40)

4.3 Isolation of *Salmonella* sp in inner content of laid eggs

4.3.1 Isolation and cultural characters of *Salmonella* sp in inner content of laid eggs on selective media

A total 75 laid eggs (5 eggs/farm) samples were collected from *Salmonella* suspected 15 layer farms. Out of 15 farms 4 were positive and isolation rate was 26.66% (Table. 8).

Table 8. Isolation rate of *Salmonella* sp of inner content of laid eggs from *Salmonella* infected farms

No. of infected farms	Total no. of eggs	No. of eggs in each farm	Sample sizes	No. of isolates*	Isolation rate (%)
15	75	5eggs/farm	15	4	26.66 %

Note: * 5 eggs content comprised as 1 sample

Isolated *Salmonella* organisms showed different cultural characteristics in selective media. These were yellowish white color colonies in BGA, slightly grayish color colonies in SS agar, black color colony in TSI agar and pinkish in EMB agar .

4.3.2 Identification (Gram's staining)

From pure culture in different media, Gram's staining was performed to observe *Salmonella* organism. Among 15 samples of laid eggs content, 4 isolates revealed light microscopically gram-negative, rod shaped organism and arranged in single pattern.

4.3.3 Cultural prevalence of *Salmonella* sp in inner content of laid eggs from infected farms.

The inner content of laid eggs of four farms was *Salmonella* positive in culture media among the fifteen infected farm and the cultural prevalence was 26.66% (table. 9).

Table 9. Cultural prevalence of *Salmonella* sp in inner content of laid eggs

Infected farm No.	No. of eggs in each farm	Positive on culture media	Cultural prevalence of *Salmonella*
F-01	5	√	
F-02	5	×	
F-03	5	×	
F-04	5	×	
F-05	5	√	
F-06	5	×	
F-07	5	×	
F-08	5	×	
F-09	5	√	**26.66%**
F-10	5	×	
F-11	5	×	
F-12	5	×	
F-13	5	√	
F-14	5	×	
F-15	5	×	
Total	**75**	**4**	

F =layer farm, √= Positive in culture, ×= Negative in culture

Table 10. Overall isolation rate of *Salmonella* sp from livers of dead birds and inner content of laid eggs from live birds

Isolation rate of Liver samples from dead birds				**Isolation rate of laid egg's content from infected farms**			
Source of samples	**Sample size**	**No. of isolates**	**Isolation rate**	**Source of samples**	**Sample size**	**No. of isolates**	**Isolation rate**
Liver from dead birds	39	17	**43.59%**	laid Egg content 5 eggs/farm	15	4	**26.66%**

CHAPTER 5

DISCUSSION

Poultry is an emerging and important sector that has been contributing progressively to our economy for the past decade. Poultry is one of the fastest growing and most promising industries with the brightest of futures for our country. In spite of such potentiality, poultry farming is confronted with acute problem of diseases. Among these, Salmonellosis is one of the ailments that cause heavy economic losses.

The present study was aimed principally to determine the cultural prevalence by isolation and identification of *Salmonella* organism from dead birds using different types of culture media, Gram's staining, biochemical tests. Necropsy was done for observing gross pathological changes and routine histopathological methods used for the detection of tissue level alterations in *Salmonella* infected cases. In addition to traditional three-step culture method was used for isolation of *Salmonella* sp from laid eggs content.

In the present study, all 17 isolates of *Salmonella* organisms showed different cultural characteristics in different media. In this study simultaneously several different selective media were used to culture the organism because all of them are not equally suitable for all the serovers of *Salmonell*a (OIE manual, 2006). The isolates showed slightly yellowish white color colonies in BGA, slightly grayish color colonies in SS agar, black color colony in TSI agar, gray white colony in nutrient agar, pinkish in EMB agar and pale color colonies in McConkey agar. These findings of present study corresponded with the results of Old (1990); Yuno *et al.* (1995); Sharma and Katok (1996); Sujatha *et al.,* (2003); Perez *et al* .,(2004); Rybolt *et al.,* (2005) and Ahmed *et al.,* (2008).

In the present study, total 39 liver samples were taken from dead birds of which 17 were *Salmonella* positive and the cultural prevalence was 43.59%. Most of the investigators isolated *Salmonella* organism in cloacal swabs from live birds or in liver swabs from dead birds. The comparison of incidence of Salmonellosis from liver may compatible with incidence of other organs. However, Lee *et al.* (2001) found 47.60% cultural prevalence from liver swabs of dead birds while Habib-ur-Rehman *et al.* (2003) described 34.5% cultural prevalence of *Salmonella* infected liver swab. On the other

hand, Ahmed *et al.* (2008) found 64% prevalence of *Salmonella* in liver of dead birds while in cloacal swab the prevalence was 57%.

From the above findings it is revealed that liver is the rich source for *Salmonella* organisms, which is in close agreement with Sujatha *et al.* (2003).

In Gram's staining, the morphology of the isolated Salmonella from chicken Gram negative, small rod shaped, single or paired in arrangement under microscope which was supported by other researchers (Cheesbrough, 1985 and Freeman, 1985).

All the isolates were positive to methyl red test and negative to indole test. Reaction in TSI agar slant with the chicken isolate produced red slant, yellow butt with the production of H_2S gas which strongly supports the observation of Merchant and Packer (1967).which was very much similar to present study.

In motility test, the isolates show swinging and non swinging movement which differentiates the motile bacteria from non-motile, so the isolated Salmonella organisms were motile and non-motile. Motility test was fundamental basis for the detection of motile and non-motile *Salmonella* organisms (Buxton and Fraser, 1977; Freeman, 1985 andHossain, 2002). In motility test, 15 *Salmonella* isolates were identified as non-motile and 2 *Salmonella* were identified as motile and percentage of motile *Salmonella* was 11.77%. These results were similar to Christensen *et al.,* 1996 and they found 11.69% motile *Salmonella*. On the other hand, there was small variation with the results of Buxton and Fraser, 1977 which may be due to the difference in managemental condition. The results of the present study were also supported by other investigators (Shane, 1989; Pomeroy and Nagaraja, 1991; Islam *et al.,* 2006; Ahmed *et al.,* 2008; who found 13.5%, 12.82%, 13.5%, and 13.71% motile *Salmonella*, respectively).

In the present investigation, among 17 isolates, 70.58% (12) were *Salmonella gallinarum*, 17.64% (03) were *Salmonella pullorum* and 11.77% (02) isolates were identified as paratyphoid causing *Salmonella*. The findings of present study corresponded with (Buxton and Fraser, 1977; Shane, 1989; Pomeroy and Nagaraja, 1991; Christensen *et al.,* 1996.)

In the present study the following clinical findings were observed; fowl typhoid is characterized by whitish materials to the vent, ruffled feathers, depression, anorexia, loss of egg production. Pullorum disease is characterized by adherence of white chalcky

material to the vent, droopy wings, gasping. Paratyphoid infection is characterized by profuse watery diarrohea, pasting of the vent area, reluctant to move, ruffled feathers and finally death of affected bird. The percentage of morbidity was highest in the farm no. 14 (40%) and the lowest was farm 1 (17%). The percentage of mortality was highest in the farm no. 14 (30%) and the lowest was farm 1 (7%). Similar findings were described by many investigators, Chishti *et al.,* (1985); Khan *et al.,* (2001); Habib-ur-Rehman *et al.,* (2003); Beyaz and Kustal *et al.,* (2003); Goswami *et al.,* (2003); Hossain *et al.,* (2003).

In present study, the gross lesions of 17 *Salmonella* infected layer birds were variable. During necropsy, 64.70% livers were friable, bronze discoloration with white focal necrosis. 47.05% livers were congested. 52.94% egg follicles were congested, haemorrhagic and discolored with stalk formation while 47.05% egg follicles were mildly congestion and haemorrhagic. 70.58% intestines were haemorrhagic to catarrhal enteritis while 38.88% only haemorrhagic and congested. 64.70% lungs were severe congested and pneumonic while 35.29% lung was mild congested. 38.88% spleen was enlarged and discolored. Similar findings with intensity of the severity of lesions in different organs were described by many investigators (Chisshti *et al.,* 1985; Khan *et al.,* 1998; Hafeji *et al.,* 2001; Habib-ur-Rehman *et al.,* 2003; Beyaz and Kutsal, 2003; Goswami *et al.,* 2003; Hossain *et al.,* 2003; Prasanna and Paliwal, 2003 Syed-Habib-ur-Rehman *et al.,* 2004; Islam *et al.,* 2006 and Deshmukh *et al.,* 2007).

For histopathological investigation only 17 *Salmonella* positive dead bird tissues of different organs were selected for histopathology. Table 7 describes the histopathological findings of different organs. In histopathological investigation, all the tissues of different organs of 17 layer birds did not evoke similar kinds of lesions. A total of 76.47% livers were congested and formed multifocal nodules with coagulation necrosis while remaining 29.41% liver showed hepatitis. Besides, 82.35% lungs were severely congested and hemorrhagic and 23.53% lung showed inflammatory cells in alveoli and bronchi. Infiltration of heterophils and lymphocytes in the mucosa of intestines were found in 52.94% cases. Sever lymphocytic depletion and focal necrosis in the spleen was found in 47.05% birds. Whereas, about 82.35% egg follicles was markedly congested and showed huge leukocytic infiltration. The microscopic lesions recorded in the present investigation were almost similar to the lesions described by other authors (Chishti *et al.,*

1985; Calnek *et al.,* 1991; Khan *et al.,* 1998; Kinde *et al.,* 2000; Hafeji *et al.,* 2001; Prasanna and Paliwal, 2003; Holt *et al.,* 2006 and Msoffe *et al.,* 2006).

In the present study, 4 *Salmonella* isolates were found from laid eggs (5eggs/farm) content from 15 *Salmonella* suspected farms and isolation rate was 26.66%. The results were slightly lower than the findings of Haider (2009) while the author reported, isolation rate of *Salmonella* organism 95% from outer shell, 45% from inner shell, 35% from egg albumin and 50% from egg yolk. It can be concluded that, laid eggs content received contamination by *Salmonella* organism as a vertical transmission of Salmonellosis or as well as contamination with the droppings of *Salmonella* infected layer birds.

CHAPTER 6

SUMMARY AND CONCLUSION

CHAPTER 6

SUMMARY AND CONCLUSION

In present study, total 39 samples (Liver sample) were taken from dead bird. In liver samples, out of 39 samples 17 were positive and the cultural prevalence was 43.59%. Among the 17 isolates, 70.58% (12) were *Salmonella gallinarum*, 17.64% (03) were *Salmonella pullorum* and 11.77% (02) isolates were identified as paratyphoid causing *Salmonella.*

The gross lesions of 17 *Salmonella* infected layer birds were variable. During necropsy, 64.70% livers were friable, bronze discoloration with white focal necrosis .A total of 47.05 %livers were congested and enlarged with necrotic foci. About 52.94% egg follicles were congested, hemorrhagic and discolored with stalk formation while 47.05% egg follicles were mildly congested and hemorrhagic . A total of 70.58% intestines were hemorrhagic to catarrhal enteritis while 38.88% only hemorrhagic and congested. About 64.70% lungs were severely congested and pneumonic while 35.29% lung showed mild congestion. About 38.88% spleens were enlarged and discolored. A total of 47% liver, 53% egg follicles and 70.6% intestine were affected in this study.

A total 75 laid eggs (5 eggs/farm) samples were collected from *Salmonella* suspected 15 layer farms. Out of 15 farms 4 were positive and isolation rate was 26.66%.About 43.59% *Salmonella* positive cases were recorded from liver samples.

Histologically, 82.35% liver and 52.94% intestines revealed tissue changes with variation in birds. *Salmonella* isolation rate was 26.66% in laid eggs of *Salmonella*-affected farms indicating transovarian transmission in poultry Salmonellosis.

From the above findings, it may be concluded that Salmonellosis has emerged as one of the most serious problems having adverse effects on poultry and human being (due to zoonotic impact). So the farmer should conscious during buying the day old chick, so that they should collect day old chick from those breeder farm in where Salmonella vaccination schedule properly followed and maintained. Strong bio-security, hygienic management both feed and water, proper vaccination, medication and finally the awareness of farmer can control avian Salmpnellosis. In future for the control of *Salmonella* infection in poultry, vaccine production and gene level study would be performed in Bangladesh to save the poultry industry.

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