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Salmonellosis in layer chickens: Molecular detection and histopathological features of *Salmonella* spp. from laying hens

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Abstract
Salmonellosis caused by *Salmonella* spp. is a notorious organism in the commercial poultry industry. In the present study, *Salmonella* organisms were isolated and identified from ovaries of dead layer birds and from inner content of laid eggs of different poultry farms located in Nilphamari district of Bangladesh. A total of 66 ovarian swabs for bacteriological & visceral organs (liver, lung, spleen, egg follicles and intestine) of 66 dead birds for pathological(gross &histopathology) study from 12layer farms and 24 laid eggs (2 eggs/farm) from reported 9 *Salmonella* infected farms constituted samples of the study. Molecular detection of *Salmonella* spp. was done by PCR using *Salmonella* genus specific primers. The overall prevalence of *Salmonella* spp. was 15.6% (n=14/90). Grossly livers of *Salmonella* affected birds were enlarged, congested, friable and bronze colored with white necrotic foci. Egg follicles were congested, hemorrhagic, discolored with stalk formation. Intestines showed hemorrhagic to catarrhal enteritis. At histopathology, livers were congested with formation of multifocal nodules and egg follicles were congested with huge leukocytic infiltration. Infiltration of heterophils in intestinal mucosa was found. Three *Salmonella* isolates were found from 24 laid egg samples and isolation rate was 12.5% reporting transovarian transmission in poultry Salmonellosis. All 14 isolates were confirmed as *Salmonella* spp. by amplification of 16SrRNA gene. The study indicates that Salmonellosis has emerged as one of the most serious problem in poultry industry.

Keywords: Salmonellosis, molecular detection, histopathology, laying hens

1. Introduction
In poultry salmonellosis causes heavy economic loss due to mortality and low production [1] Salmonellosis has become a great problem with expansion of poultry farming in Bangladesh [2]. It is most significant due to the causal agents of the disease are transmitted vertically from parent to offspring. *Salmonella* are Gram negative, short plump shaped rods, non-spore forming, non- capsulated, aerobic and facultative anaerobic organisms [3]. Chickens are the natural hosts for both *S. pullorum and S. gallinarum* [1]. Pullorum disease is usually occurs 2-3 weeks of age and sometimes occurs in adults [5]. Fowl typhoid is frequently referred to as a disease of adult birds and there are also reports of high mortality in young chicks [6]. Risk factors associated with salmonellosis infection in laying hens are flock size, housing system, and farms with hens of different ages [7].
Conventional bacteriological technique is usually recommended for the detection of *Salmonella* spp. from different sources [8]. Molecular techniques, such as PCR have been developed for the monitoring of *Salmonella* spp [8].
It is a major issue of external and internal contamination of egg by *Salmonella* spp. during rearing of laying hens. So, it is very difficult to take proper control measures against salmonellosis [9]. Contamination of egg may occur by vertical or horizontal routes. Vertical transmission is a result of bacterial agent colonization in the ovary and oviduct before egg shell formation and horizontal transmission causes due to external egg shell contamination and may penetrate when crack the shell [10, 11].
The Salmonellosis in chicks and layer chickens must be evaluated to take effective control measures against the diseases [12]. Considering these facts, a study was undertaken for the molecular detection of *Salmonella* spp. and the pathology of different organs of *Salmonella* infected layer birds. The present investigation may play an important role for the study of *Salmonella* infection from layer birds.

2. Materials and Methods

A total of 12-layer farms of Nilphamari district which contained 22,300 birds were considered as experimental birds. The breed was Isa brown layer chicken. Sample birds were collected from the previously selected farms and having no history of *Salmonella* vaccine. The laboratory work was performed at the Department of Pathology and Parasitology, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

2.1 Clinical Examination

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

2.2 Pathological Studies

During study period, postmortem examinations of 90 dead birds were performed from the representative selected 12-layer farms. Postmortem examination was done according to the previously published technique [13]. Gross pathological changes at necropsy were carefully observed and recorded. Histopathological studies were carried out on representative tissues of different organs that were collected and immediately fixed in the 10% formalin for few days. The tissue samples were processed through standard technique. The paraffin blocks were cut at 6 µm thickness using microtome machine (Mu509, Euromex, Japan). The slices were floated on warm water in a water bath at 45ºC for stretching. The sliced tissue was placed on grease free clean glass slide using adhesive like gelatin. The glass slides were then dried at 37º C temperature for 24 hours in an incubator. The slides with tissue sections were stained with routine Hematoxylin and Eosin (H & E) stain method and observed under microscope.

2.3 Isolation and molecular detection of *salmonella* organism

A total of 66 ovarian swab samples were collected. The ovarian swabs were collected in the test tubes. Each test tube containing 10 ml of tetrathionate broth (TTB) with 200µl of iodine-iodide solution [14, 15].

A total of 24 eggs were collected from 12 farms (2 eggs/farms) aseptically. During collection the sample eggs were washed and disinfected by 70% ethanol [16, 17].

The egg contents were homogenized by using electric stirrer. Samples were aseptically cultured into non selective broth with pre-enrichment media (BPW) at 37ºC temperature for 24 hours, then incubation was done in selective enrichment media (SRV) at 37ºC temperature for 24 hours. Then plating was performed onto the following selective agar media: Brilliant green agar (BGA), Salmonella-Shigella (SS) agar, Triple sugar iron (TSI) agar, Eosine methylene blue (EMB) agar [15, 16].

The isolated bacteria were stained by Gram's stain to determine their staining characteristics and purity of the isolates.

Five basic sugars such as dextrose, sucrose, lactose, mannitol and maltose were used for sugar fermentation test. MR test, VP test, indole test ornithine and dulcitol fermentation test [15, 16].

The isolates were confirmed as genus *Salmonella* by Gram stain, biochemical test, and molecular detection by PCR using *Salmonella* genus specific primers.

Amplification of 16 S rRNA gene: For the amplification of 16S rRNA gene of *Salmonella*, template DNA was extracted by boiling method [18]. The primers used in this research are presented in Table 1.

The reaction mixture of the PCR was prepared in a total volume of 25 µL containing 12.5 µL PCR master mix (Promega, USA), 20 pmol/µL of each primer 1 µL, 5 µL of template DNA and 5 µL deionized water. The conditions for amplification of 16SrRNA gene were- 35 cycles with initial incubation at 94 °C for 5 min, denaturation at 94 °C for 60 Sec, annealing at 50 °C for 30 Sec, elongation at 72 °C for 30 Sec and final extension for 5 min. at 72 °C. A 1.5% agarose (Sigma) gel was used for electrophoresis of the PCR products followed by staining with ethidium bromide and visualized by UV trans-illuminator (Biometra, Jena, Germany).

3. Results

A total of 90 different samples of layer were analyzed for the presence of *Salmonella*. The present study showed the overall prevalence of *Salmonella* spp. as 15.6% (n=14/90). The highest and lowest incidence of *Salmonella* was 20% and 10% found in the layer farms in Farm-10 and Farm-3 respectively. (Table 2)

In present study, the gross lesions of 14*Salmonella* infected layer birds were variable. During necropsy, 57% livers were friable, bronze discoloration with white focal necrosis. A total of 43% livers were congested and enlarged with necrotic foci (Fig:1). About 57% egg follicles were congested, hemorrhagic and discolored with stalk formation while 43% egg follicles were mildly congested and hemorrhagic(Fig:2). A total of 71% intestines were hemorrhagic to catarrhal enteritis while 57% only hemorrhagic and congested. About 57% lungs were severely congested and pneumonic while 71% lung showed mild congestion. About 43% spleens were enlarged and discolored. (Table 3)

Only 14*Salmonella* positive dead bird tissues of different organs were selected for histopathology. Table 4 describes the histopathological findings of different organs. In histopathological investigation, all the tissues of different organs of 14 layer birds did not show similar kinds of lesions. A total of 71% livers were congested and formed multifocal nodules with coagulation necrosis (Fig.3) while remaining 28.6% liver showed hepatitis. Besides, 71% lungs were severely congested and hemorrhagic and 28.6% lung showed inflammatory cells in alveoli and bronchi. Infiltration of heterophils and lymphocytes in the mucosa of intestines were found in 43% cases. Severe lymphocytic depletion and focal necrosis in the spleen was found in 57% birds (Fig.4). In the same way57% egg follicles was markedly congested and showed huge leukocytic infiltration (Fig.5).

In the present investigation, all 14 isolates of *Salmonella* organisms showed different cultural characteristics in different media. In TTB broth turbidity was formed. All the isolates showed slightly yellowish white color colonies in BGA and slightly grayish color colonies in SS agar. Formation of black color colony in TSI agar, gray white colony in nutrient agar, pinkish in EMB agar and pale color colonies in McConkey's agar.

In Gram's staining, all the isolates in the present investigation

revealed gram-negative, rod shaped appearance and arranged in single. Chains of more than two bacilli were normally absent. A total 24 laid eggs samples were collected from *Salmonella* infected 12 layer farms. Out of 24 laid eggs 3 samples were positive and isolation rate was 12.5%.

A total of 14 isolates were confirmed as *Salmonella* spp. by amplification of 16SrRNA gene (Figure 6) using genus specific primers.

4. Discussion

Chickens are playing an important role in national economy and reducing poverty by providing egg and other by-products in Bangladesh [19, 20]. Salmonellosis is one of the most important problems for poultry in developing countries. Especially in Bangladesh due to favorable environmental condition for the organism, day by day the incidence of salmonellosis increases. So, it is important to know the source of Salmonellosis in poultry.

In the present analysis, all 14 isolates of *Salmonella* organisms showed different cultural characteristics in different media. These findings of present investigation corresponded with the results of the following studies [21-27].

In Gram's staining, all the isolates in the present investigation revealed gram-negative, rod shaped appearance and arranged in single. Chains of more than two bacilli were normally absent. The study [28] described the morphology of the isolated *Salmonella* bacteria as small rod shaped, gram negative, single arrangement by Gram's staining which supported the findings of the present investigation.

In present investigation, the gross lesions of 14*Salmonella* infected layer birds were variable. These findings were similar with the study of many investigators [29, 1, 30, 31, 32, 12, 33].

Only 14 *Salmonella* positive dead bird tissues of different organs were selected for histopathology. Table 4 describes the histopathological findings of different organs. In histopathological investigation, all the tissues of different organs of 14 layers birds did not provide similar kinds of lesions. The microscopic lesions recorded in the present investigation were almost similar to the lesions described by other authors [29, 1, 34-36].

A total 24 laid eggs samples were collected from *Salmonella* infected 12 layer farms. Out of 9 farms 3 were positive and isolation rate was 12.5%. The results corresponded with the findings of Haider (2009) while the author reported 95% isolation rate of *Salmonella* organism 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/and contamination with the droppings of *Salmonella* infected layer birds.

All the isolates were confirmed by *Salmonella* genus specific primers to detect *Salmonella* was rapid and accurate and was similar with the previous study [37].

However, for useful application of the present research findings further studies should be conducted on serotyping and confirmation of the isolated *Salmonella* from different feed and environmental sample.

Fig 1: Fragile liver with necrotic foci

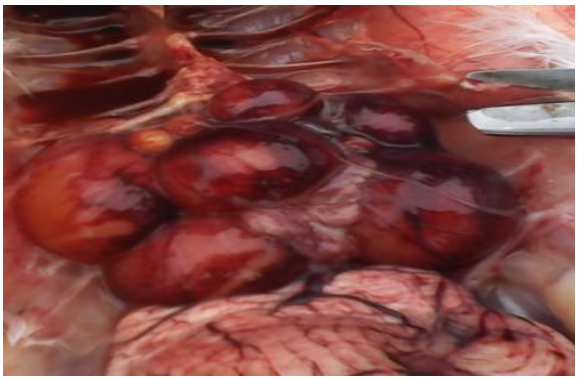

Fig 2: *Salmonella* affected egg follicles become congested and hemorrhagic

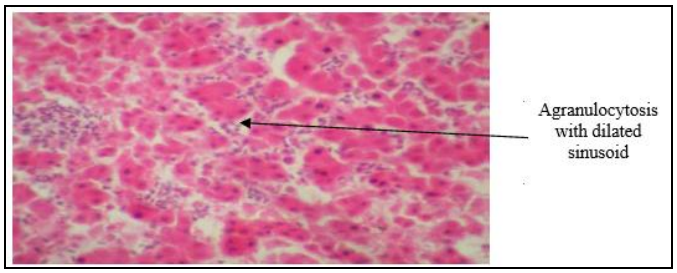

Fig 3: *Salmonella* infected liver shows multifocal nodule formation and infiltration of inflammatory cells (H & E staining X 10)

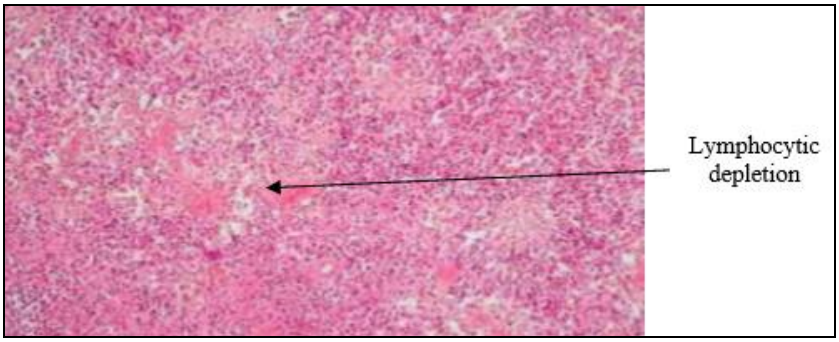

Fig 4: *Salmonella* infected spleen shows severe lymphocytic depletion and marked reticulo endothelial cell hyperplasia (H &E staining X 10)

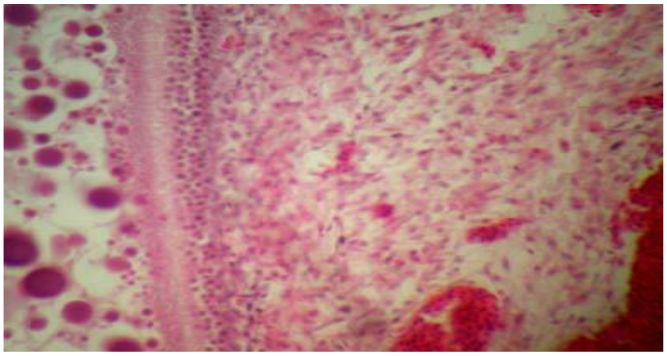

Fig 5: *Salmonella* infected egg follicles shows marked congestion and leukocyticinfiltration (H &E staining X 10)

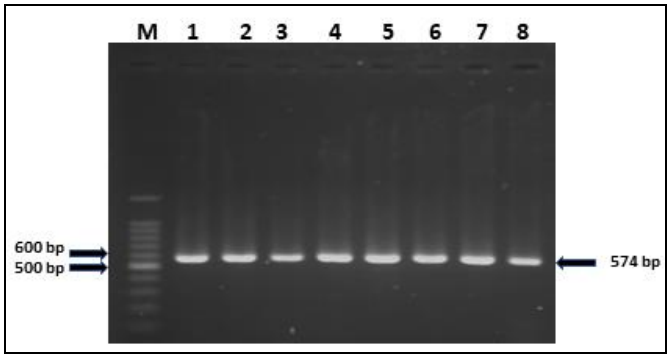

Fig 6: Amplification of 16SrRNA gene of *Salmonella* spp. (574-bp) (Lane M 100 bp ladder, lane 1 positive control, and lane 2-8*Salmonella* spp. positive samples

Table 1: List of primers used for the detection of *Salmonella*

Primers name	Primer sequence (5'-3')	Size of amplicon	Reference
Sal 16SrRNA-F	TGTTGTGGTTAACCGCA	574-bp	Lin and Tsen (1996) [38]
Sal 16SrRNA-R	CACAAATCCATCTCTGGA		

Table 2: Morbidity and mortality of different layer farms

Farms	No. of sample tested	No. of positive sample	Prevalence of *Salmonella* spp. (%)
F-1	12	2	16.7
F-3	10	1	10
F-6	15	2	13.3
F-8	11	2	18.2
F-10	15	3	20
F-11	9	1	11.1
F-12	18	3	16.7
Over all prevalence			15.6

Table 3: Gross pathological findings of *Salmonella* affected birds of different layer farms

Lesions	Infected farm no.								
	F-1 (n=2)	F-3 (n=1)	F-6 (n=2)	F-8 (n=2)	F-10 (n=3)	F-11 (n=1)	F-12 (n=3)	Total N=14	%
Friable, bronze discoloration liver with white focal necrosis	+	-	-	+	+	-	+	4	57
Congested and enlarged liver	-	+	+	-	-	+	-	3	43
Congested hemorrhagic, and discolored egg follicles with stalk formation	+	-	+	+	+	-	-	4	57
Mild congested and hemorrhagic egg follicles	-	+	-	-	-	+	+	3	43
Hemorrhagic to catarrhal enteritis	+	+	-	+	+	-	+	5	71
Congested and hemorrhagic intestine	-	-	+	-	+	+	+	4	57
Severely congested and pneumonic lung	+	+	-	-	+	-	+	4	57
Mild congested lung	-	-	+	+	+	+	+	5	71
Enlarged with discolored spleen	+	-	-	-	+	-	+	3	43

"+"present, "-"absent and "n"= No. of positive bird / farm and F= Farm

Table 4: Histopathological findings of *Salmonella* affected tissues from different farm

Lesions	Infected farm no.							Total N=14	%
	F-1 (n=2)	F-3 (n=1)	F-6 (n=2)	F-8 (n=2)	F-10 (n=3)	F-11 (n=1)	F-12 (n=3)		
Congestion and multifocal nodule formation in liver	+	+	+	-	+	-	+	5	71
Hepatitis and infiltration of inflammatory cells	-	-	-	+	-	+	-	2	28.6
Marked congestion and leukocytic infiltration in egg follicles	+	+	+	-	+	-	+	4	57
Infiltration of heterophils and lymphocyte in the mucosa of intestine	+	-	-	+	-	-	+	3	43
Severely congested and hemorrhagic lung	+	-	+	+	-	+	+	5	71
Inflammatory cells in the alveoli and bronchus		+	-		+			2	28.6
Severely lymphocytic depletion and focal necrosis in the spleen	+	+	+	+	-	-	-	4	57

"+"present, "-"absent and "n"= No. of positive bird / farm and F= Farm

5. Conclusions

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. In future for the control of *Salmonella* infection in poultry prevalence and gene level study need to be performed in Bangladesh to save the poultry industry.

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