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Seroprevalence, Identification, and Pathology of Salmonellosis in Selected Poultry Farms at Barishal District of Bangladesh

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ABSTRACT

Salmonellosis is a critical challenge in commercial poultry farming. This study aimed to calculate seroprevalence, identify *Salmonella spp.*, and its pathological investigation from January to December 2019. One hundred fifty (150) serum samples and fifty (50) cloacal swabs from apparently ill and healthy birds were collected in this study. Seroprevalence was observed by serum plate agglutination (SPA) tests. The tentative diagnosis of salmonellosis was made based on history, clinical signs and bacteriological studies. *Salmonella spp.* was further confirmed using an automated microbiological method (VITEK®2). Post mortem examination was done in apparently sick birds, and gross and microscopic pathological lesions were investigated and recorded in a datasheet. The overall seroprevalence of salmonellosis was 42.67% in commercial chickens. Age wise prevalence of avian salmonellosis showed significantly highest infection rate in adult layers (≥ 45 days old) 65.31%, then 40.74%up to 45 days. In case of broiler, the prevalence rate in 0-10 days, 11-20 days, and 21-35 days were 13.63%, 44.12%, and 16.67%, respectively. Seasonal influence showed significantly highest proportionate prevalence of salmonellosis during summer 66.15%in comparison to winter 25.00% and rainy 24.44% seasons respectively. In bacteriological study, 12 samples were positive for Salmonellae among 50 cloacal samples. Out of 12 positive samples 5 samples were selected for the automated microbiology system VITEK® 2, and only two samples were confirmed as *Salmonella gallinarum*. Gross pathology of representative organs revealed bronze-colored enlarged liver, hemorrhages in the spleen and lungs, and hemorrhages in the ovary with stalk development. Microscopically, multifocal nodule formation and infiltration of inflammatory cells in the liver parenchyma and marked congestion with inflammatory cells in the spleen, hemorrhage and congestion in the lungs and intestine.

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Introduction

Salmonella belongs to the Enterobacteriaceae family of bacteria (Bennasar et al.,2000; Grimont et al., 2000). *Salmonella* is a gram-negative, intracellular, straight rod-shaped bacteria that is non-encapsulated, facultative, non-spore producing, and motile with peritrichous flagella (Gray and Fedorka-Cray, 2002). Most *Salmonella* is motile except for the poultry-specific serotypes of *Salmonella gallinarum (S. gallinarum)* and *Salmonella pullorum (S. pullorum)* (Grimont et al., 2000). Clinical indications of fowl typhoid include yellow or greenish diarrhea in mature birds, which is indicative of a septicemic disease in poultry (Ali, 2012), higher mortality, as well as low-quality chicks born from contaminated eggs (Anemia, sadness, difficult breathing, and diarrhea in older birds cause feces to cling on the vent. In pullorum disease, a baby chick suffers white bacillary diarrhea (Ali, 2012). Epidemiological information, etiology, and pathology of a specific illness are necessary for successful disease preventive and control methods. Most poultry illnesses are diagnosed at the field level in our country based on symptoms and gross lesions, but laboratory diagnosis is required for confirmation (Haider et al., 2004). In recent years, diagnostic laboratories have been concerned about decreasing its time to diagnose *Salmonella* infections (Saha et al., 2007). The current standard laboratory method takes 6 to 7 days to grow and identify *Salmonella* serovars. Even these approaches are inconvenient, time-consuming, and do not assure sensitivity or species specificity. *Salmonella* serovars are also undetectable in some clinical samples containing tiny organisms (Stone et al., 1994). As a result, a faster and more sensitive approach for identifying *Salmonella* serovars from clinical samples is required (Saha et. al., 2007). The slide agglutination test, invented by

Runnels et al. (1927) for use with serum and adapted by Schaffer et al. (1931) for use with whole blood by employing colored antigen, has been the gold standard in the diagnosis of pullorum illness and fowl typhoid for many years (Tuchili et al., 1995). The slide agglutination technique was found to be simple, sensitive, dependable, cost-effective, and time-effective, with just a minimal amount of antigen, sera, and accessories required. This approach may be used to diagnose salmonellosis in commercial poultry farms and to determine anti-*Salmonella* antibody titers in infected and vaccinated hens in farm settings (Parvin et al., 2012).

In the last 20 years, several automated techniques for bacterial identification have been developed, based on automated interpretation of biochemical test findings or the use of microdilution trays after overnight incubation and photometric growth determination (Ferraro, 1995; Thornsberry, 1985). Rapid bacterial identification due to technological advancements is increasingly acknowledged as having therapeutic and economic benefits (Barenfanger et al., 1999). The automated microbiological system VITEK®2 was created in 1970s. When the main inoculum has been developed and standardized, the VITEK®2 system automates all identification operations. This gadget allows for kinetic analysis by reading each test every 15 minutes. The optical system integrates multichannel fluorimeter and photometer measurements to record fluorescence, turbidity, and colorimetric data. The Greater Barishal region (coastal area) of Bangladesh is essential in socio-economic development. Poultry farming is one of the most critical aspects for the people of this region. As a result, disease-free poultry rearing and farming are essential for the country's economic development and the safe production of chicken products.

Avian salmonellosis causes significant economic losses in poultry industry through mortality and reduced production. So, for controlling of the disease in commercial chickens, proper surveillance, pathology and accurate identification are necessary. Serodetection of *Salmonella spp.* using accessible commercial *Salmonella* Antigen (Ag); necropsy and histopathology to study pathological changes in representative organs and isolation and identification of *Salmonella spp.* from swab samples are practiced. The current study was conceptualized to investigate the seroprevalence of salmonellosis in commercial chickens, isolation and identification of *Salmonella spp.* using modern bacteriological procedure and pathological investigation of the disease in the selected area (Crowley et. al., 2012; Pincus et al., 2014).

Materials and Methods

Sampling

The study was conducted at the Department of Pathology and Parasitology, ANSVM faculty of Patuakhali Science and Technology University (Barishal campus) in Bangladesh from January 2019 to December 2019. A total of one hundred fifty (150) serum samples were collected randomly from ten (10) different commercial poultry farms in the Barishal district. Identification of *Salmonella spp.* was performed from fifty (50) cloacal swab samples obtained from Serum Plate Agglutination (SPA) test positive cases. Suspected birds were considered for post mortem examination and representative samples were investigated for histopathological changes. Broiler (1-10 days; 11-20 days; 21-35 days) and layers (up to 45 days and >45 days) were subdivided into several age groups. The study period was divided into three seasons, namely summer (mid April – mid June), rainy (mid June - mid August), and winter (mid December - mid February) seasons.

Serum Plate Agglutination (SPA) Test

Blood samples were collected aseptically from the wing vein of birds. *Salmonella* O Group D (Somatic 9, 12) Antigen for Slide Test manufactured by S & A REAGENTS LAB Ltd., Part (4 SoiLatphrao-wanghin 28, LatPhrao, Bangkok-10230 in Thailand) was used following the standard protocol described by the manufacturer (Figure 1).

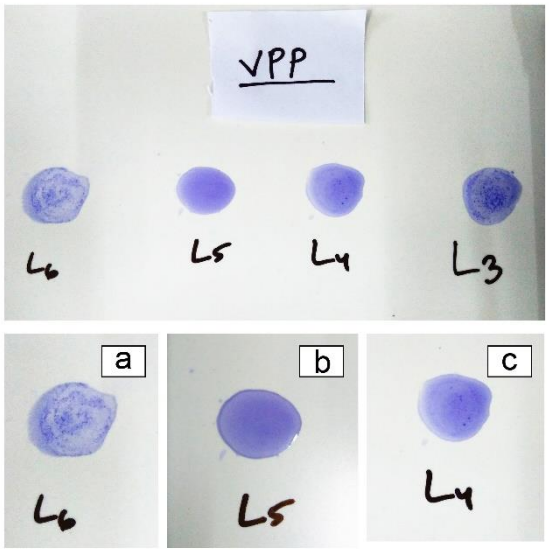

Figure 1. Results of the rapid serum plate agglutination test for Salmonella are positive.
Here, (a) positive (agglutination with fine granules within 1 minute), (b) negative (no agglutination), (c) suspect (slight agglutination after 2 minutes).

Isolation and Identification of Salmonella spp.

Sterile swab sticks were used to collect samples directly from the cloaca, which were then supplemented in nutrient broth and Salmonella Shigella (SS) agar media and incubated at 37°C for 24 hours in a bacteriological incubator. Colorless or transparent colonies or black colonies on SS agar were suspected of being *Salmonella spp.* after 24 hours of incubation. To obtain pure culture, the organisms were subcultured into several *Salmonella* selective media (SS agar, Xylose Lysine Deoxycholate (XLD) agar, MacConkey agar, Eosin Methylene Blue (EMB) agar, Triple Sugar Iron (TSI) agar). Gram stain was used to characterize the Salmonellae colonies morphologically, according to the user's standard bacteriological procedures. The motility test was performed to differentiate motile bacteria from non-motile ones (Hasan et al., 2012; Parvej et al., 2016).

Biochemical tests

For this study, isolated organisms with supporting growth characteristics of *Salmonella spp.* were subjected to sugar (Carbohydrate) fermentation test, TSI agar slant reaction, Methyl red (MR) reaction, Voges-Proskauer (VP) reaction and indole reaction according to the standard procedures (Parvej et al., 2016).

Confirmation of Salmonella through VITEK® 2

The VITEK®2 (bioMérieux) is an optical system that automatically performs all the steps required for identification after a primary inoculum has been prepared and standardized. This system allows kinetic analysis by reading each test every 15 min. The optical system combines multichannel fluorimeter and photometer readings to record fluorescence, turbidity, and colorimetric signals. Standard protocol was followed as described by the manufacturer (Pincus, 2014).

Pathology

Necropsies were performed on dead or sick birds by standard protocol. The gross lesions of apparently infected organs (liver, spleen, lung, heart, trachea, intestine and ovary) were noted in a datasheet. Representative samples were collected and fixed into 10% neutral buffered formalin solution for histopathological study. Formalin-fixed samples of the suspected organs were processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin according to a standard method for histopathological study (Titford, 2009).

Statistical Analysis

The chi-square tests were performed to know the significance of the associations between the prevalence parameters of the diseases in poultry. Two multivariable logistic regression models were fitted for identifying effect of season and age that significantly influences the prevalence of disease in poultry (Islam et al., 2009).

Results and discussion

Seroprevalence of Salmonellosis in Poultry

In this research, the overall seroprevalence of salmonellosis in broiler and layer was 42.67% (Table 1). In broiler, 21 seropositive cases were recorded with the prevalence was 32.31%; and in layer the prevalence was 50.59%. Similar findings also reported by Sikder et al. (2005); Akter et al. (2007); Islam et al. (2008) who reported seroprevalence at 23.46%, 23.11%, 43.4% respectively. aul et al. (2017) and Habib-ur rahman et al. (2003) reported 53.33% and 63.5% respectively which was higher than the findings of present study. On the other hand, Sikder et al. (2005); Akter et al. (2007) reported 23.46%, 23.11% which were less than the findings of present study.

The highest prevalence was found in broilers at 11-20 days (44.12%), followed by 21-35 days (16.67%) and 0-10 days (13.63%) old broiler chickens (Table 2), which was compared with the report of Badruzzaman et al. (2015) who reported expansive prevalent in 8 to 20 days old (2.843%) followed by 0-7 days old (1.706%) and 21-35 days old (1.753%) broiler chickens. In the case of commercial layer birds, the significantly highest ($p<0.05$) outbreak of salmonellosis was recorded in the age group of >45 days (65.31%) than the age group of up to 45 days (40.74%) (Table 2); these findings were in agreement with the report of Rahman (2004) who showed the highest infection rate in adult layers (53.25%).

Salmonellosis in commercial chickens in the study area was considerably higher in the summer season 66.15% than in winter 27.50% and rainy 31.11% respectively (Table 3). Similar findings were reported by Rahman et al., (2004) who found the highest prevalence of *Salmonella* in summer 48.05% in comparison to rainy 28.31% and winter 23.66% seasons. Badruzzaman et al., (2015) concluded that diseases frequently occurred in the summer season 39.85%, which agrees with our result; however, he found a higher prevalence in winter 32.80% instead of the rainy season 27.35%. However, similar results were found by another group of researchers who reported diseases were significantly higher in summer40.5% followed by the rainy season 32.11% and winter 27.2% (Kwon et al., 2010). These variations of salmonellosis prevalence rate in different areas might be due sample size and species variation, lack of biosecurity, egg shell transmission and lower immune status of birds, environmental condition, geographical distribution (Uddin et al.,2010; Talha et al., 2001).

Table 1. Seroprevalence of Salmonellosis

	Broiler		Layer		Total Infected(n)	Prevalence (%)
	Infected/(n)	Prevalence	Infected/(n)	Prevalence		
Serology	21/65	32.31	43/85	50.59	64/150	42.67

n= No. of birds examined

Table 2. Age wise seroprevalence of salmonellosis in broiler and layer chickens

Breed (n)	Total infected	Age Group (Examined)	Age-wise infected	Prevalence (%)	P-value of $\chi 2$ test
Broiler (65)	21	0-10 days (22)	3	13.63	0.043
		11-20 days (34)	15	44.12	
		21-35 days (18)	3	16.67	
Layer (85)	43	Up to 45 days (27)	11	40.74	
		>45 days (49)	32	65.31	

P-value<0.05 indicate significant.

Table 3. Season-wise seroprevalence of salmonellosis in commercial chickens

Season	Total No. of Birds examined	Season wise no of infected birds	Prevalence (%)	P-value of $\chi 2$ test
Summer	65	43	66.15	0.037
Rainy	45	11	24.44	
Winter	40	10	25.00	
Total	150	64	42.67	

P-value<0.05 indicate significant.

Table 4. Results of cultural and morphological characteristics

Colony morphology	
S.S. agar	Opaque, translucent, colorless, smooth, round with black centered colonies
XLD agar	Black centered colony
MacConkey agar	Pale, colorless, smooth, transparent, raised colonies
EMB agar	Pinkish colonies without metallic sheen
TSI agar.	Black-colored colony
Half strength N.A.	Colourless colonies
Staining characteristics	Pink, short rod-shaped, gram-negative bacilli, bacteria arranged in single or paired
Motility test (Hanging drop method)	Negative; (S. pullorum, S. gallinarum)

Table 5. Results of biochemical test

Carbohydrate fermentation test						Dulcitol Test	TSI test	M.R. test	V.P. test	Indole test
Bacteria	Dextrose	Glucose	Sucrose	Lactose	Maltose					
SP	AG	AG	-	-	-	-	+	+	-	-
SG	A	A	-	-	A	AG	-	+	-	-

Here, SP= *Salmonella pullorum*, SG= *Salmonella gallinarum*, TSI = Triple Sugar Iron, M.R. = Methyl Red, V.P. = Voges-Proskauer, AG = Acid and Gas, A = Acid, (+) = positive reaction, (-) = negative reaction.

Bacteriological Identification

Colony morphology and staining characteristics

The colony morphology of *Salmonella spp.* found in this investigation summarized in the table 4. From fifty (50) cloacal samples, twelve (12) samples were culturally positive for *Salmonella spp.* The organisms were presumptively identified by isolating and identifying bacterial colonies with typical culture features. After 24 hours of incubation at 37°C in an incubator, *Salmonella spp.* developed translucent, black smooth, small round colonies on SS agar (Figure 2c), pink color colonies with black centers on XLD agar (Figure 2d), and black color colonies with the formation of hydrogen sulfide gas in TSI agar. Salmonellae generated colorless, smooth, pale, and transparent colonies in MacConkey agar (Figure 2e) and pinkish colonies in EMB agar (Figure 2f). In Gram's staining, the morphology of the isolated bacteria was pink, short rod-shaped, gram-negative bacilli, bacteria arranged in single or paired, the motility test was done by hanging drop method for detecting motile and non-motile *Salmonella* organisms. Here in this study, *S. pullorum* and *S. gallinarum* were non-motile (Chowdhuri et al., 2011; Sikder et al., 2005; Hossain et al., 2006; Parvej et al., 2016).

Biochemical test

In the present study, specific biochemical tests were used to confirm *Salmonella* (Table 5). In the carbohydrate fermentation test, the isolates that fermented glucose and dextrose; produced acid and gas but did not ferment lactose, sucrose those indicated positive for Salmonellae as described previously (Paul *et al.,* 2017, Hossain et al., 2006; *Parvej et al., 2016*). Isolates fermented dulcitol and produced acid and gas, which indicates *S. gallinarum*, and other isolates neither ferment nor produce acid or gas, indicating *S. pullorum*. These observations are strongly correlated with the theme of (Hossain et al., 2006; Parvej et al., 2016). Isolates were positive for the MR test but negative for VP and Indole test, indicating *Salmonella spp.* (Hossain et al., 2006).

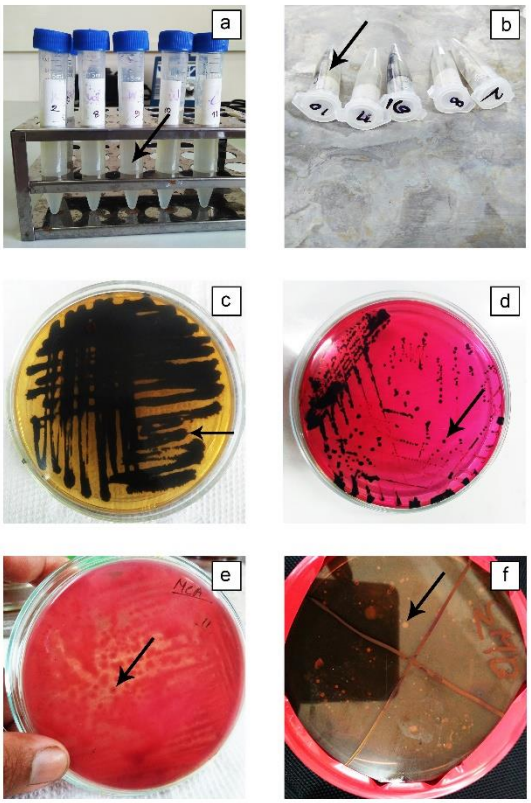

Figure 2. Cultural characteristics of *Salmonella* in different media.

(a) *Salmonellae* showing turbidity (arrow) on the broth after 24 hrs of incubation at 37°C in an incubator, (b) *Salmonellae* showing colourless colonies (arrow) in half strength Nutrient agar after 24hrs of incubation at 37°C in an incubator, (c) *Salmonellae* showing colourless colonies in SS agar with a dark central spot (arrow) reflecting production of hydrogen sulfide on the media, (d) *Salmonellae* showing pink color colony with black centre in XLD agar (arrow) with a production of hydrogen sulfide on the media, (e) *Salmonellae* showing colourless, smooth, pale and transparent colonies (arrow) in MacConkey agar, (f) *Salmonellae* showing pinkish colonies (arrow) in EMB agar.

Confirmation of Salmonella spp. by VITEK® 2 system

From 12 culturally positive isolates five samples (comprising 2 *S. gallinarum* and 3 *S. pullorum*) were selected for the VITEK 2 system. At the time of confirmatory diagnosis by the VITEK 2 system, only selected two isolates of *S. gallinarum* were confirmed by it. Here, remaining 3 isolates of *S. pullorum* showed negative results for VITEK 2, the reason is that the VITEK 2 system which only identify *S. gallinarum*, *Salmonella enterica spp. arizonae*, *Salmonella enterica spp. diarizonae*, *Salmonella* group, *Salmonella* ser. *Paratyphi* A, *Salmonella* ser. *Typhi*, and cannot identify *S. pullorum* (Meteab et al., 2018; Muna et al., 2016; Crowley et. al., 2012).

Pathology of Salmonella spp. Infected Organs

Gross pathology

In this study, necropsy examination was performed in apparently healthy and sick birds. The gross lesions were noted in the datasheet. Results were found to be unabsorbed yolk sac with yellowish liver and soiled vent, livers were friable with bronze discoloration (Figure 3a), dark colored and congested spleen (Figure 3b), congested and pneumonic lung (Figure 3c), heart (Figure 3d), and hemorrhagic trachea (Figure 4a), hemorrhagic intestine (Figure 4b), mildly congested egg follicles (Figure 4c) with misshaped ova and stalk formation (Figure 4d). Similar findings with intensity of the severity of lesions in different organs were described by many investigators (Paul et al., 2015; Hasan et al., 2012; Hossain et al., 2006; Saha et al., 2012; Sujatha et al., 2003)

Microscopic pathology

Microscopically the section of the liver showed congestion, hemorrhages, focal degeneration, multifocal nodule formation and infiltration of inflammatory cells (arrow) (HEx10) (Figure 5a), marked congestion (arrow) (HEx40) and focal degeneration in spleen (Figure 5b),diffuse congestion (Figure 5c) and hemorrhages in lung, and infiltration of inflammatory cells (HEx10), intestine exhibited erosion in the intestinal mucosa and infiltration of mononuclear cells in the submucosa (arrow) (HEx10) (Figure 5d) were commonly observed during histopathology; these lesions were indicative of salmonellosis, which were already mentioned by several authors (Hossain et al., 2006; Habib-ur-Rahman et al., 2003; Haider et al., 2004, Saha et al., 2012).

Conclusion

Salmonellosis is a devastating poultry disease in Bangladesh. In this study, 42.67% cases were found to be positive for salmonellosis in the selected Barishal district of Bangladesh. Highest prevalence was recorded in broiler at 11-20 days aged group and in layer at ≥45days old group compared with aged group of birds. The poultry of this studied area were more prone to salmonellosis in summer season than winter and rainy season. From swab samples 12 *Salmonella spp.* positive isolates were stocked that was culturally and biochemically positive. For further confirmation, two isolates were identified using the VITEK 2 system as *S. gallinarum*. Major pathological changes were in liver, spleen, lung, heart, trachea and egg follicles.

This is the first report on surveillance, identification and pathological investigation of *Salmonella spp.* in some selected coastal regions of Bangladesh. On the basis of pathology and serological test, the results of this study suggest that Salmonellosis that cause major problem for both broiler and layer production and also cause high morbidity and mortality in commercial chickens. The seasonal and age related prevalence suggests special attention for controlling of salmonellosis in this coastal area. So, it may be concluded that this report may help poultry practitioner of this region for taking necessary steps in controlling avian salmonellosis.

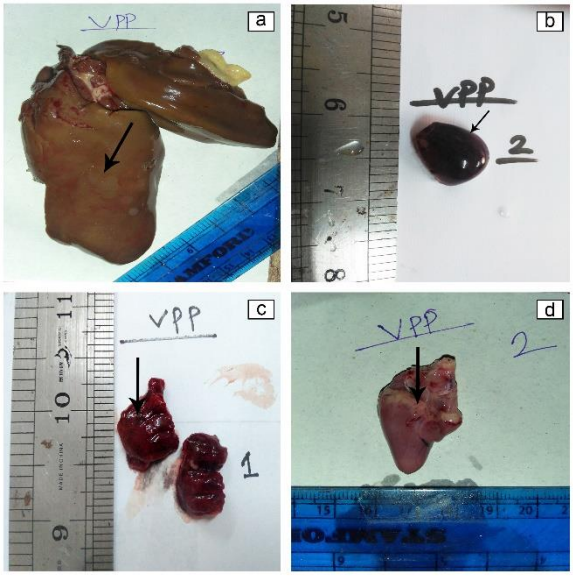

Figure 3. Gross lesions (I)

(a) Friable, swollen with coppery bronze sheen (arrow) of liver, (b)Spleen shows severe congestion (arrow) in 21 days' broiler, (c) Severe congested lung (arrow) of 17 weeks' layer bird, (d) Heart shows hemorrhage and congestion (arrow) in layer bird.

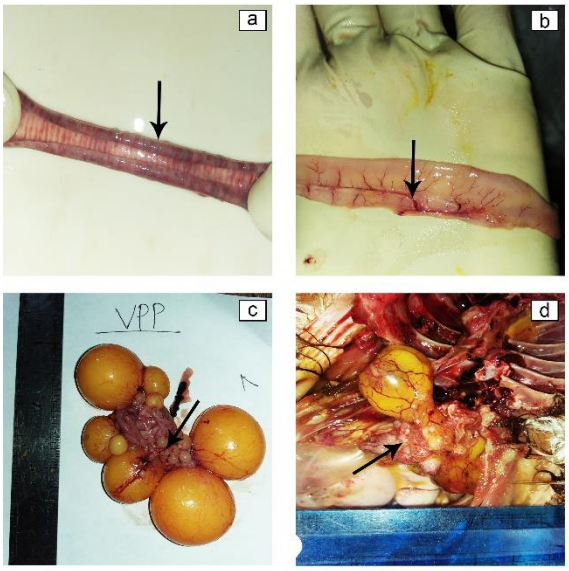

Figure 4. Gross lesions (II)

(a) Hemorrhagic trachea (arrow) in the Salmonellosis affected 17 weeks layer bird, (b) Intestine shows hemorrhage (arrow) in layer bird, (c) Salmonellosis affected egg follicles shows misshaped ova, and (d) hemorrhage(arrow) with stalk formation in layer bird.

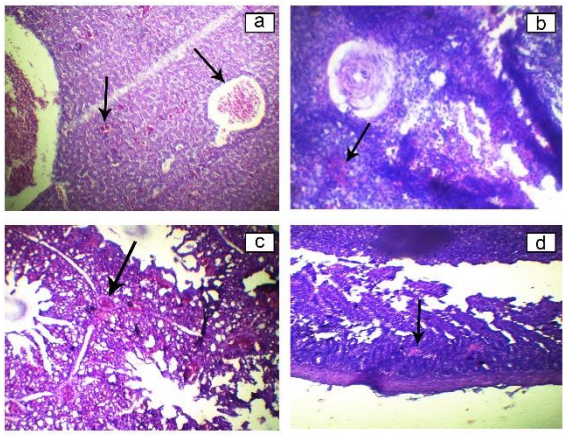

Figure 5. Microscopic lesions

(a) Salmonella infected liver shows multifocal nodule formation and infiltration of inflammatory cells (arrow) (HEx10), (b) Salmonella infected spleen shows marked congestion (arrow) in 21 days broiler (Hex40), (c) Salmonellosis infected lung shows severe congestion,and (d) infiltration of inflammatory cells (arrow) (HEx10), (e) Salmonella affected yolk sac shows severe congestion (arrow) (HEx10),(f) Salmonella affected intestine exhibits erosion in the intestinal mucosa and infiltration of mononuclear cells in the submucosa (arrow) (HEx10).

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