Research Article

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Pathological and Molecular Investigation of Paratyphoid *Salmonella* Infection in Broiler Chicks in Sulaymaniyah Province, Kurdistan/Iraq

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Abstract

Paratyphoid infection is a serious-infectious disease of poultry with economic and public health consequences. We investigated paratyphoid *Salmonella* infections and their associated histopathological changes in broiler chicks in Sulaymaniyah province using molecular, histopathological and immunohistochemical techniques. A total of 130 broiler farms, aged between 1 to 20 days were investigated. The results showed that the prevalence of paratyphoid Salmonella in the region was 28.5%, including *Salmonella enteritidis* (70.3%) and *Salmonella typhimurium* (29.7%). The rate of infection was higher (76%) among 1-10 days-old chicks in comparison to 11-20 days-old chicks (24%). The distribution of the pathogen was varied among the tested organs, including the caecum (97.3%), liver (91.9%) and yolk-sac (86.5%). The rate of infection by *S. enteritidis* in 1–10-day-old chicks in the organs was higher (67.9%) than the rate of infection by *S. typhimurium* in the liver (25%), yolk-sac (25%) and caecum (28.6%). There was a highly significant positive correlation between *S. enteritidis* and *S. typhimurium* infections in the liver (r=0.818, P=0.000, n=37). At the same time, a highly significant strong negative correlation between *S. enteritidis* and *S. typhimurium* infections was found between the inspected organs. The most obvious pathological changes were degeneration and necrosis of the hepatocytes and sloughing of the caecal epithelium. Claudin-1 expression and distribution among

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cellular compartments were mostly affected by Salmonella enteritidis-positive cases. The findings of this study showed that there was a widespread paratyphoid *Salmonella* infection in the region, and associated with severe histopathological and immunohistochemical changes, especially among 1-10 days-old chicks

Keywords: *Salmonella,* Paratyphoid*, Salmonella entritidis, Salmonella typhimurium,* Broilers.

Introduction

Paratyphoid (PT) infection is one of the most common infectious diseases in the poultry industry and has economic and public health importance (1) PT *Salmonella* spp. are Gram-negative caused by non-host adapted motile serotypes belonging to the family *Enterobacteriaceae* (2) More than 2,500 *Salmonella* serotypes have been described in the White-Kauffmann-Le Minor scheme (3, 4)and they can infect a wide range of animal species, including humans, with subclinical and clinical manifestations (5) Among other paratyphoid serovars, *Salmonella* enteritidis
and *Salmonella* typhimurium are the leading causes of *Salmonella* infection in poultry (6).

In addition to the clinical signs and gross lesions, avian Salmonellosis, particularly in young chicks, appears to be associated with several histopathological lesions, including necrosis of mucosal epithelial cells and villi in the cecum with infiltrations of lymphocytes, macrophages and heterophils, congestion of the liver and necrosis of hepatocytes, splenic lymphoid depletion and necrosis are also expected in the severe cases (7, 8).

In spite of its high prevalence and economic and zoonotic importance, there is a limited information regarding paratyphoid *Salmonella* in broiler chicks in Sulaimani province/Iraq. Thus, this study aimed to investigate paratyphoid *salmonella* infection in broiler chicks in the region using molecular, histopathological, and immunohistochemical approaches.

Materials and Methods

Study area and Sample collection

The study was conducted from November 2021 to Aug 2022. A total of 130 broiler farms with suspected paratyphoid *Salmonella* cases, aged between 1 to 20 days were investigated. Three-birds, which had typical signs of *Salmonella* infection were selected from each farm. After being subjected to clinical and post-mortem examination, tissue samples from the localized lesions of the liver and caecum, and unabsorbed yolk sac were aseptically collected from each bird, and transferred in ice boxes to the research center laboratory at the college of veterinary medicine, the University of Sulaimani for further investigation. Each sample, which was taken from each organ, were analyzed separately.

DNA extraction

Total DNA was extracted from 20–35 mg of each tissue sample (liver, caecum, and unabsorbed yolk sac) using Genomic DNA Extraction Kit (AddBio Co., Korea) according to the manufacturer's instructions. The extracted DNA was stored at -20 °C and used as a template for PCR assays.

Molecular confirmation of *Salmonella* serovars

At first a general primer set was used to detect the *salmonella* genus then the positive samples were exposed to serovar specific primers, including *Salmonella typhimurium*, *Salmonella enteritidis,* (9), (Table1). PCR reaction (20 µl) was set using 10 µl of 2x master mix (AddMulti Taq Master Mix kit, AddBio Co., Korea), 3 µl of the DNA template, 1µl of each forward and reverse primers (10 pmol/µl) and the volume was completed by adding 5 µl ultrapure water. Reaction mixtures were subjected to the following thermo-cycler conditions (Prime Thermal Cycler, Bibby Scientific Ltd., UK): Initial denaturation was at 95 °C/5 min: 95 °C/30 sec, 58 °C/35 sec, and at 72 °C/30 sec for 35 cycles with a final elongation step at 72 °C/10 min, followed by holding at 4 °C. Then, the DNA bands were visualized (Figure 1) using agarose gel (1.5%) and UV Transilluminator **(Ingenius, USA).**

Histopathology Examination

The infected chicks' liver and cecum samples were subjected to histopathological examination at Anwar Shexa Medical City Histopathology Lab in Sulaimani Governorate. First, the specimens were fixed for at least 24 h in neutral buffered formalin (10%) before being dehydrated in a graded ethanol series. Then, the tissues were dehydrated, cleaned in xylene, and fixed in paraffin. Thin tissue sections(4µm) were prepared from each sample for histopathological study and stained with hematoxylin and eosin (H&E). The lesions were evaluated using a light microscope (Leica, Germany) and computer-assisted image analysis software (Am ScopeTM, Japan).

Immunohistochemistry

The histopathological sections for immunohistochemistry were prepared according to the methods described by other researchers (10, 11). Briefly, after tissue processing, tissue was fixed on adhesive-coated slides, dewaxed in xylene and rehydrated in series washes of ethanol and distilled water. The antigen retrievaland Claudin-1 rabbit polyclonal primary antibodies (1:100; DAKO, Denmark) was used to target the Claudin-1 protein. Biotinylated goat anti-rabbit secondary antibody (DAKO, Denmark) and Streptavidin peroxidase enzyme (DAKO, Denmark), and 3-amino-9-ethyl benzidine (Dako, Germany) chromogen was used for color development and visualization of the molecules and counterstained with Gill's Haematoxylin. The results were quantified basis on the degree of positively stained cells in IHC staining Claudin-1 into no staining or 0-scale for 5% positive staining, Scale-1 for 6-25 percent positive staining, scale-2 for 26-50 percent positive staining, scale-3 for 51-75 percent positive staining, and scale-4 for >75 percent positive staining. The intensity of Claudin-1 staining was rated on a scale of weak (+1), moderate (+2), moderate-strong (+3), and intense (+4). The positive reactivity extent and level of staining intensity were multiplied to get a total staining score ranging from 0 to 16 (12).

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) was used for statistical analysis

(Frequency, percentage, crosstabulation, correlation, and Chi-square). $P \leq 0.05$ was considered statistically significant.

Results

Identification of *Salmonella* serotypes

The results showed that 37 out of 130 (28.5%) flocks were positive for paratyphoid *Salmonella* spp. The rate of infection was higher (76%; 28/37) among younger aged birds (1-10 days) compared to the older birds (11-21 days) (24%; 9/37). The infection rate with *Salmonella enteritidis* was higher (70.3%; 26/37) compared to *Salmonella typhimurium* (29.7%; 11/37). The caecum was found to be the predominant (36/37; 97.3%) site of *Salmonella* infection, which was followed by the liver (34/37; 91.9%) and yolk sac (32/37; 86.5%). The rate of infection by *Salmonella enteritidis* in 1–10-day-old chicks in the caecum, liver, and yolk was 67.9% (19/28). While, it was 66.7% (6/9) in the liver and caecum and 44.4% (4/9) in the yolk sac in 11-20 days old chicks. The rate of infection by *Salmonella typhimurium* in 1–10-day-old chicks was 28.6% (8/28) in the caecum and 25% (7/28) in the liver and yolk sac. While, 22.2% (2/9) of the liver and yolk sac and 33.3% (3/9) of the caecum were positive for *Salmonella typhimurium* in 11–20 days old chicks (Table2). The results showed a highly significant positive correlation between *S. enteritidis* and *S. typhimurium* infections in the liver (r=0.818, P=0.000, n=37). At the same time, a highly significant strong negative correlation between *S. enteritidis* and *S. typhimurium* infections was found between the inspected organs (Table3).

Histopathological changes in visceral organs

In the caecum, moderate lesions such as sloughing of the caecal epithelium and goblet cells, damaged and atrophied crypts glands, infiltration of chronic inflammatory cells from mucosa to serosa (transmural inflammation) (Figure2a, 2b), and atrophy of the caecal muscularis mucosa and externa was found in association with the *Salmonella typhimurium* infection (Figure2c, 2d). While, *Salmonella enteritidis* was associated with severe cryptitis, crypt abscess, and severe inflammatory reactions (Figure 3a-3d). The liver of the infected chicks with *Salmonella typhimurium* showed moderate changes, including marked congestion of the central vein and sinusoidal capillary. The hepatocytes were swollen and a few were necrotized with pyknotic nuclear changes and eosinophilic cytoplasm (Figure 2f, 2g). A moderate proliferation of kupffer cells and centrilobular infiltration of inflammatory cells with fibrin deposition were also found (Figure 2i). Meanwhile, pronounced hepatocyte degeneration and necrosis with pyknotic features, surrounded by severe chronic inflammatory cells with giant cells (Figure3e, 3f) and kupffer cells proliferation and chronic inflammatory infiltration diffusion within the liver parenchyma were induced by the *Salmonella Enteritidis* (Figure3g-3i).

Immunohistochemistry

Despite the regular expression of Claudin-1 by the normal hepatocyte and caecal mucosa

and their localization within the cell membrane, Claudin-1 protein was not found (score 0) in the liver and caecum of *Salmonella typhimurium* infected chicks (Fugure-4a-4d). However, the expression of Claudin-1 protein by the infected hepatic cells with *Salmonella enteritidis* was increased (Figure 4). The immunostaining pattern of Claudin-1 was weak and localized in the nuclei of the hepatocytes (Figure 4e). The Claudin-1 trapping in the hepatocyte cytoplasm was about 30% (Score-2) of the liver parenchyma (Figure4f).

The caecal epithelium and crypts of the *Salmonella Enteritidis* infected chicks were found to have diffused, moderate to strong brownish nuclear staining (staining score 9) for Claudin-1 immunolabeling in 55% of caecal parenchyma (Figure4g, 4h).

Table 1. Oligonucleotide primers.

Genes	Primer Names	Primer sequence	Amplicon size (bp)	Reference
Salmonella genus specific	*ST 11* ST 15	GCCAACCATTGCTAAATTGGCGCA GGTAGAAATTCCCAGCGGGTACTG G	429	(9)
Salmonella typhimurium *fliC*	*Fli 15* Tym	CGGTGTTGCCCAGGTTGGTAAT ACTCTTGCTGGCGGTGCGACTT	559	
Salmonella enteritidis sefA	Sef 167 Sef 478	AGGTTCAGGCAGCGGTTACT GGGACATTTAGCGTTTCTTG	312	

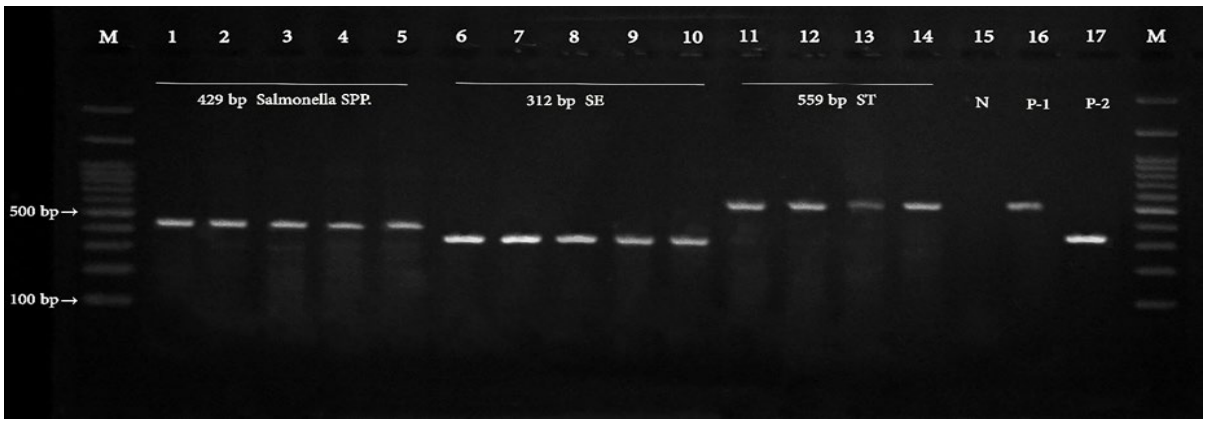

Figure-1: Agarose gel-electrophoresis analysis of Uniplex PCR amplicon of Salmonella genus, S. typhimurium, and S. enteritidis. Lane M: 100-bp DNA ladder. Lane 1, 2, 3, 4, and 5: positive for *Salmonella* genus (429 bp). Lane 6, 7, 8, 9, and 10: positive for *Salmonella enteritidis* (312 bp). Lane 11, 12, 13, and 14: Positive for *Salmonella typhimurium* (559 bp). Lane 15: Negative control. Lane 16: *Salmonell*a typhimurium positive control. Line 17: *Salmonella enteritidi*s positive control.

Table 2: **Frequency and percentage of PT *Salmonella* infection**

Organ	Salmonella types	Age-group	Positive	Negative	Total	X^2
Cecum	*S. enteritidis*; 26 (70.3%) *S. typhimurium;* 11 (29.7%)	1-10 days	28(48.3%) 21.5%	30(51.7%) 23.1%	58(100%) 44.6%	0.000
		11-20 days	9(12.5%) 6.9%	63(87.5%) 48.5%	72(100%) 55.4%	
		Total	37(28.5%)	93(71.5%)	130(100%)	
	S. enteritidis	1-10 days	19(67.9%) 51.4%	9(32.1%) 24.3%	28(100%) 75.7%	0.94
		11-20 days	6(66.7%) 16.2%	3(33.3%) 8.1%	9(100%) 24.3%	
		Total	25(67.6%)	12(32.4%)	37(100%)	
	S. typhimurium	1-10 days	8(28.6%) 21.60%	20(71.4%) 54.10%	28(100%) 75.70%	0.78
		11-20 days	3(33.3%) 8.10%	6(66.7%) 16.20%	9(100%) 24.30%	
		Total	11(29.7%)	26(70.3%)	37(100%)	
Liver	*S. enteritidis*	1-10 days	19(67.9%) 51.4%	9(32.1%) 24.3%	28(100%) 75.7%	0.94
		11-20 days	6(66.7%) 16.2%	3(33.3%) 8.1%	9(100%) 24.3%	
		Total	25(67.6%)	12(32.4%)	37(100%)	
	S. typhimurium	1-10 days	7(25.0%) 18.9%	21(75.0%) 56.8%	28(100%) 75.7%	0.86
		11-20 days	2(22.2%) 5.4%	7(77.8%) 18.9%	9(100%) 24.3%	
		Total	9(24.3%)	28(75.7%)	37(100%)	
Yolk Sac	*S. enteritidis*	1-10 days	19(67.9%) 51.4%	9(32.1%) 24.3%	28(100%) 75.7%	0.21
		11-20 days	4(44.4%) 10.8%	5(55.6%) 13.5%	9(100%) 24.3%	
		total	23(62.2%)	14(37.8)	37(100%)	
	S. typhimurium	1-10 days	7(25.0%) 18.9%	21(75.0%) 56.8%	28(100%) 75.7%	0.86
		11-20 days	2(22.2%) 5.4%	7(77.8%) 18.9%	9(100%) 24.3%	
		Total	9(24.3%)	28(75.7%)	37(100%)	

	Organs		
Salmonella infection	Caecum	Liver	Yolk-sac
	36(97.3%)	34(91.9%)	32(86.5%)

*** The data were analyzed using crosstabulation. P-value less than 0.05 was considered statistically significant.**

Table-3: Correlations between PT Salmonella serovars and site of infections

Quires			*S. Typhimurium*		
			Caecum	**Liver**	**Yolk**
S. Enteritidis	**Caecum**	Correlation Coefficient	-0.939***	-0.818**	-0.818***
		Sig.	0.000	0.000	0.000
	Liver	Correlation Coefficient	-0.939***	0.818***	-0.818***
		Sig.	0.000	0.000	0.000
	Yolk	Correlation Coefficient	-0.834***	-0.727***	-0.727***
		Sig.	0.000	0.000	0.000
	N		37	37	37

*** The data were analyzed using Kendall's tau-b non-parametric correlations. P-value less than 0.05 was considered statistically significant.**

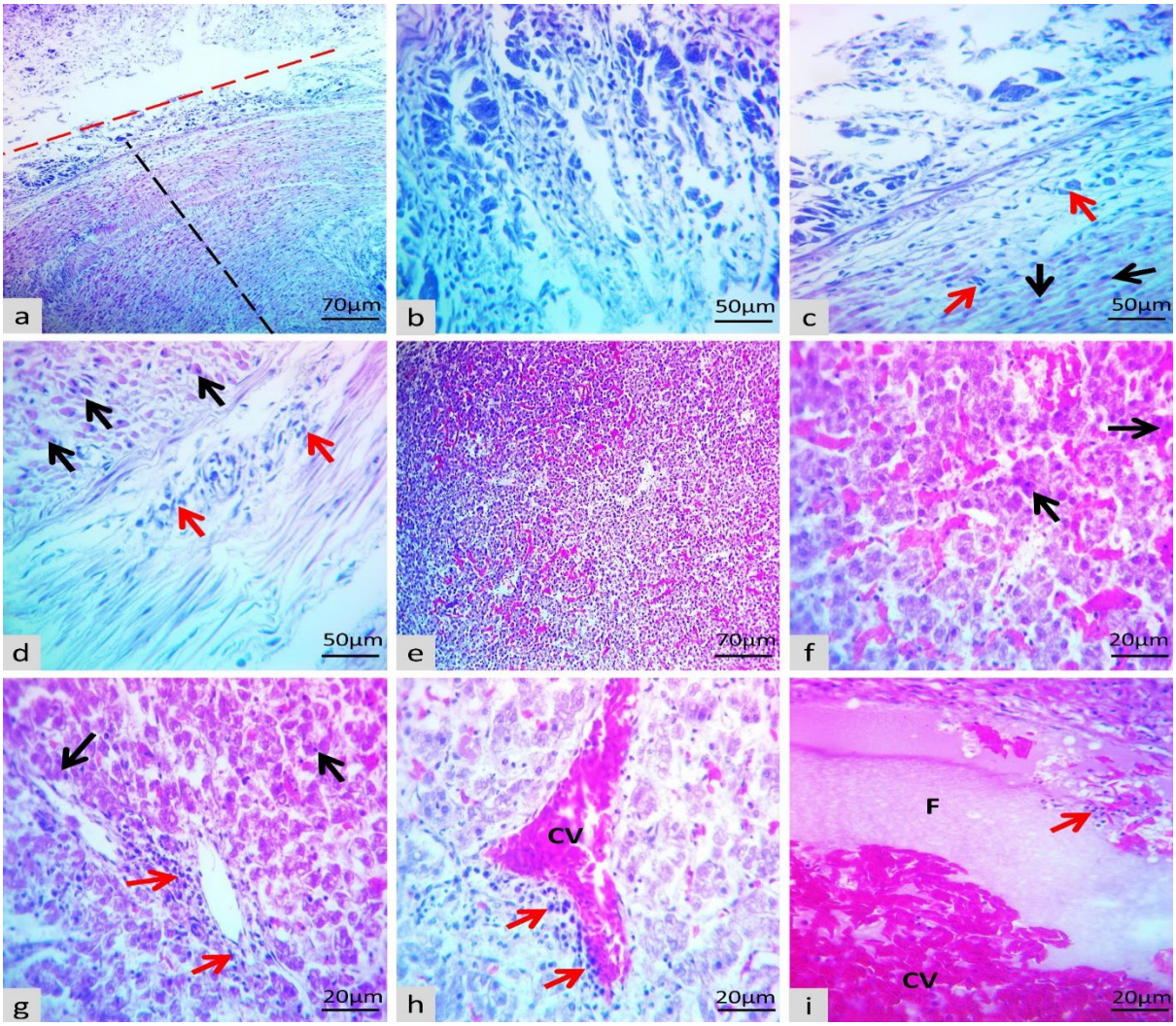

Figure 2: Histopathological sections of the caecum and liver of the infected chicks with *S. typhimurium*. A & B; complete sloughing of the caecum epithelial lining (red dash line), damaged and atrophied crypts, and transmural inflammatory reaction (black dash line). C & D; atrophied caecal muscularis mucosa and externa (black arrows), infiltration of the inflammatory cells in both layers till serosa (red arrows). E-I: Marked congestion of the central vein (CV), congested sinusoidal capillary and swollen hepatocytes, with pyknotic changes of the hepatocytes (black arrows), proliferation of kupffer cells, and centrilobular infiltration of inflammatory cells (red arrows) with fibrin deposition as indicated by letter F (H&E stain).

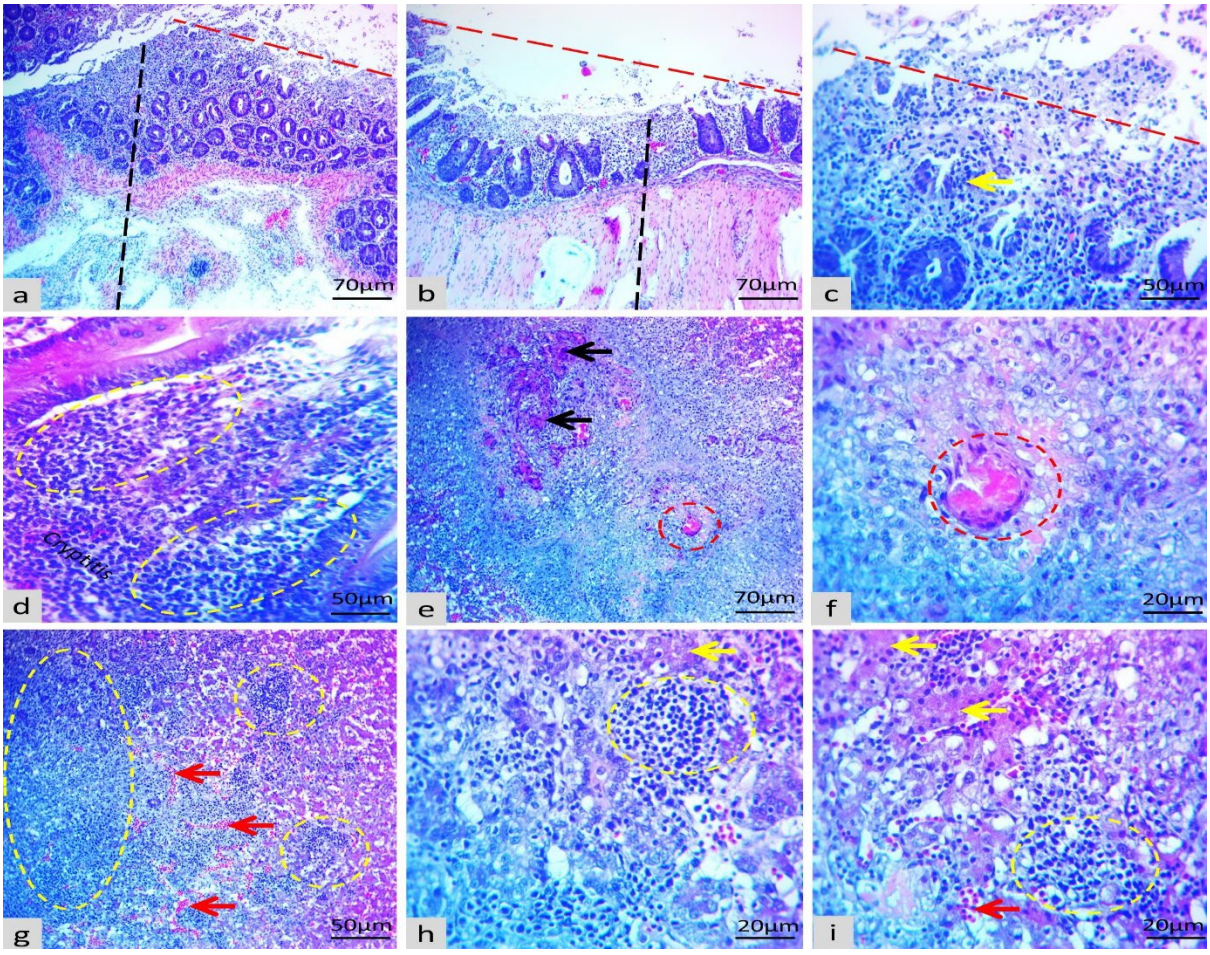

Figure 3: Histopathological sections of the caecum and liver of the infected chicks with *S. enteritidis*. A-D; Sloughing of caecum epithelial lining (red dash line), partial to complete atrophy of the crypts, cryptitis, crypt abscess (yellow arrows), transmural inflammatory reaction (black dash line). E & F; Marked degeneration and necrosis of hepatocytes (black arrows) surrounded by inflammatory cells containing giant cells (red ring) and proliferation of kupffer cells. G-I; Congestion of the central vein and sinusoidal capillaries (red arrows), degeneration of hepatocytes with necrotic features (yellow arrows), inflammatory reaction within the liver parenchyma (yellow rings) (H&E stain).

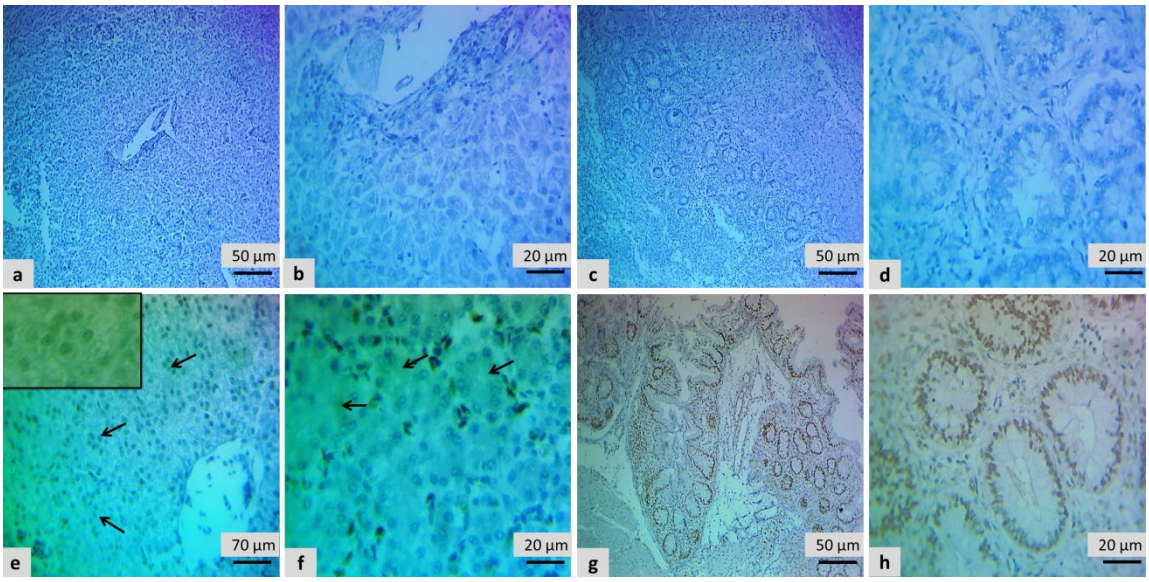

Figure 4: Immunohistochemistry of Claudin-1 protein in the liver and caecum of the infected chicks with *S. typhimurium and S. enteritidis*. A-D; No immunostaining in the hepatocytes (A & B, Score 0) and caecum (C & D, score 0) of the infected chicks with *S. typhimurium*. E & F; Weak nucleo-cytoplasmic immunostaining (score 2) of infected hepatocytes with *S. enteritidis*. G & H; Moderate to strong diffuse nuclear immunostaining (score 9) of the caecal crypts infected with *S. enteritidi*.

Discussion

Paratyphoid *salmonella*, especially *Salmonella* enteritidis and *Salmon ella* typhimurium, are among the most common bacterial diseases in the poultry industry, particularly in young-age chicks in the region. Our study's prevalence of *Salmonella* infection was 28.5% (37/130). Previous studies also reported nearly similar results in Colombia (26.6%) and Pakistan (26.0%) (13, 14). While, other studies in other Iraq provinces, including Basra (9.2%) (15) and Babylon (7.3%) (16). Some other countries, such as Iran (9.23%) (17) Egypt (16%) (18), and China (17.4%) were reported a lower prevalence. Meanwhile, other counties, including Bangladesh (35%), India (45.5%), South Africa (35.4%), and Brazil (51%) were found to have a higher rate of infection (19-21).

The results showed that the caecum was the leading site of infection (97.3%), followed by the liver (91.9%) and yolk sac (86.5%). The same pattern of infection was reported by (22) (caecum 100%, Liver 90%) and (15) (Caecum 12%, Liver 8%). The higher rate of infection of the caecum might be related to the anatomy and structure of the caecum, which have a blind end with a low content flow rate, and the ceca have been shown to be a primary site of colonization for *Salmonella* (15).

The highest rate (76%) of *Salmonella* infection was detected among 1–10 days-old chicks compared to 11–20 days old (24%) chicks. Similar pattern of infections was also reported by (23). This might be attributed to the fact that adult birds are more resistant to

Salmonella than young chicks due to the development of the gut microflora (24). Generally, PT infection remains subclinical, except in very young birds (23). More often, paratyphoid infections in chickens are characterized by asymptomatic colonization of the intestinal tract and internal organs and resulting in contamination of finished carcasses (25). Moreover, *S.* enteritidis can invade the reproductive system and results in transovarian transmission (26).

Our study showed that *Salmonella enteritidis* was the most abundant (70%) serovar compared to *Salmonella typhimurium* (30%). These results concurred with those observed in broiler chickens in Iraq (10%, 4.7%), (27), Türkiye (21.9%, 9.4%) (28), Iran (56.8%, 29.7%) (17), and Egypt (11.4%, 8.6%) (29). In contrast, investigations from India (23.8%, 76.2%) (30), Pakistan (12.6%, 14.7%) (14) and China (6%, 9%) (31) showed that *Salmonella typhimurium* is the most predominant serotype in poultry flocks.

The histopathological changes in the liver and caecum observed in the present study are consistent with findings in avian salmonellosis (21, 22). The observed histopathological changes in the present study, which were associated with the *Salmonella* infection, including sloughing of caecal epithelium, cryptitis and atrophy of crypts gland, necrosis, infiltration of the caecum and liver by inflammatory cells, congestion of the central vein and sinusoidal capillaries were also reported by other researchers (8, 32, 34).

Tight junction (TJ) proteins, particularly Claudin-1, are critical components of the tissue cells and are primarily required for maintaining intestinal barrier functions (35). Their expression appears to be disrupted by *Salmonella* infection, which might increase the chance of pathogens' permeability to the intestinal lumen and increase the rate of endogenous infection and endotoxemia (36, 37). The immunohistochemical data revealed that *Salmonella* typhimurium was associated with a dramatic suppression of Claudin-1 expression in both the caecum and the liver. While, its expression was highly upregulated in the caecum of *Salmonella enteritidis*-infected chicks. In contrast to our finding, a weak word of Claudin-1 was found in the intestinal cells of *Salmonella* typhimurium and *Salmonella* enteritidis-infected broiler chickens (38, 39).

To the best of our knowledge, there was no data about the level of Claudin-1 expression in the liver of *Salmonella*-infected broilers chicks. The suppression of Claudin-1 expression might be related to the presence of effectors, as its expression seems to be affected by *avrA* and *sopB* gene products in mice (40, 41).

In conclusion, PT *Salmonella* is one of the most severe bacterial diseases and has a high prevalence in the region. The reported serovars were caused severe histopathological lesions in broiler chicks. In addition, immunohistochemical results showed a change in claudin-1 expression linked to barrier homeostasis in the liver and caecum sections.

Acknowledgments

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Conflict of interest

The authors have no conflict of interest

Ethical approval

The study was conducted according to the principles of ethics after being approved by the Ethics Committee at the College of Veterinary Medicine, University of Sulaimani.

Availability of data and materials

The datasets generated during and analyzed during the current study are available from the corresponding author when requested.

Authors' contributions

SAA and NHA wrote the manuscript text. NHA conducted the experiments. SH and NHA prepared the histopathological figures. SAA and NHA generated and analyzed the data. All authors reviewed the manuscripts. All authors read and approved the final manuscript.

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دراسة مرضية وجزيئية لعدوى السالمونيلا نظيرة حمى التيفوئيد في افراخ فروج اللحم في محافظة السليمانية، العراق

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الخلاصة

عدوى نظيرة حمى التيفوئيد تسببها السالمونيلا المتحركة غير المتكيفة مع المضيف، وهي مرض خطير – مُعدٍ يصيب الدواجن وله عواقب اقتصادية وصحية عامة. تم دراسة عدوى السالمونيلا نظيرة حمى التيفوئيد (*Salmonella enteritidis* و *Salmonella typhimurium*) والتغيرات النسجية المرضية المصاحبة لها في افراخ فروج اللحم في محافظة السليمانية باستخدام التقنيات الجزيئية والتشريح المرضي والكيميائي المناعي. تم فحص 130 حقل فروج اللحم مشتبه بها فى حالات السالمونيلا نظيرة حمى التيفوئيد تتراوح اعمارها بين 1-20 يوم. أظهرت النتائج أن انتشار السالمونيلا نظيرة حمى التيفوئيد في المنطقة بلغ 28.5٪ والمتضمنة *S. enteritidis* 70.3% (و) *Salmonella typhimurium* 29.7 ٪(. كانت نسبة الإصابة بين الافراخ التي بعمر 1-10 أيام هي أعلى (76٪) مقارنة بالأفراخ التي بعمر 11-20 يوم وهي (24٪). اختلف توزيع المسبب الممرضي بين الأعضاء المفحوصة بما في ذلك الأعور (97.3٪) والكبد (91.9٪) وكيس المح (86.5٪). كان معدل الإصابة بالبكتيريا *S. enteritidis* في الكتاكيت بعمر 1-10 أيام في الأعور والكبد والصفار أعلى (67.9٪) مقارنة بمعدل الإصابة ب *Salmonella typhimurium*. في الكبد (25٪) وكيس المح (25 ٪) والأعور (28.6 ٪). تقريبًا تم العثور على نمط العدوى نفسه في الافراخ بعمر 11-20 يومًا. كانت هناك علاقة ارتباط موجبة عالية بين *enteritidis* و *Salmonella typhimurium* في الكبد (n = 37 ، P = 0.000 ، r = 0.818). في الوقت نفسه تم العثور على علاقة سلبية قوية ذات دلالة إحصائية بين *S. enteritidis* و *Salmonella typhimurium* بين الأعضاء التي تم فحصها. كانت التغيرات المرضية الأكثر وضوحا هي تنكس ونخر خلايا الكبد وانسلاخ ظهارة الأعور. تم الكشف عن تعبير كلودين -1 فقط في الحالات الإيجابية للسالمونيلا المعوية. أظهرت نتائج هذه الدراسة انتشار عدوى السالمونيلا نظيرة حمى التيفوئيد في المنطقة وترتبط بتغيرات نسجية ومرضية مناعية شديدة وخاصة بين الافراخ بعمر 1-10 أيام.

الكلمات المفتاحية: السالمونيلا ، نظيرة حمى التيفوئيد ، *S. enteritidis* ، *Salmonella typhimurium* ، افراخ.