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# *Salmonella* in free-range chickens: pathology of subclinical persistent infection

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**Abstract** Testing of apparently healthy free-range chickens revealed the detection of *Salmonella* in 2.11% (5/237) and 8.04% (16/199) of the samples by culture method and PCR, respectively, with an overall detection rate of 8.86% (21/237). Primary histopathological lesions consistent with *Salmonella* infection were observed in the liver and spleen at Days 10 through 150 and in the intestines at Days 120 through 150. Sinusoidal congestion (83.3%) and lymphoid hyperplasia (66.7%) were the most predominant lesions in the spleen persisting from Day 10 until Day 150. Cloudy swelling (40%) with cytoplasmic granulation and typhoid nodules (26.7%) were observed in the liver beginning Day 10. Cecal tonsil activation was observed at Day 10, while structural changes and infiltration of inflammatory cells in the submucosa were the significant histopathological changes in the intestines throughout Day 150. *Salmonella* is a silent threat to public health in subclinical infections. Active surveillance and monitoring of this pathogen should be carried out continuously to improve detection and diagnosis. Sustainable mitigating strategies should be designed for free-range poultry to control *Salmonella* and achieve food security and safety.

**Keywords:** Culture method, Detection, Histopathology, PCR

## Introduction

*Salmonella* of the family *Enterobacteriaceae* is a facultative anaerobe, gram-negative, rod-shaped, non-spore-forming flagellated bacterium surviving a wide-range of environmental conditions (Hsieh *et al.,* 2014; Kurtz *et al.,* 2017). The genus encompasses a large number of serotypes that are genetically very similar but biologically quite different, especially in pathogenic properties and host specificity (Zou *et al.,* 2016). There are more than 2,600 serotypes that can reside and cause foodborne infection in humans (Luo *et al.,* 2018; Pashazadeh *et al.,* 2017; Zhang *et al.,* 2018) typically causing uncomplicated

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gastroenteritis that does not need treatment. The disease, however, is shown to be severe in the young, elderly, and immunosuppressed patients (WHO, 2018).

Infected animals are crucial in the transmission of *Salmonella* to humans. In most cases of animal salmonellosis, the bacterial agent is present in the host for only a short period of time and the *Salmonella* responsible for the disease may go undetected (Chart, 1995). Diagnosis of salmonellosis is invariably dependent on clinical signs and the isolation of the pathogen from feces, blood, or tissues of affected animals. Unfortunately, diagnosis is often misinterpreted as clinical features are quite non-specific and mimic many other diseases. In poultry, infection with many of the *S. enterica* serovars such as Typhimurium and Enterica do not produce clinical manifestation implicating poultry as the largest reservoir for human infections (La Ragione *et al.*, 2013; Hesse *et al.,* 2017). *Salmonella* is transferred to humans either by direct contact with the animal or via ingestion of contaminated poultry and poultry products (Tohidi *et al.,* 2018).

The ability of *Salmonella* to tolerate environmental stress, widespread distribution, multiple drug resistance, and the adaptability to environment has made detection of pathogen stringent and difficult (Pashazadeh *et al*., 2017). Greater risk is expected in free-range chickens that acquire ubiquitous pathogens from their environment. Early exposure of the chicks to the pathogen leads to infection with high mortality and persistent infection for the surviving chickens (Hu *et al*., 1997). In later development, the healthy appearing infected chickens become carriers and shedders of *Salmonella* for a variable period of time (Gast and Beard, 1992). Surveillance and monitoring strategies should therefore depend on reliable and efficient detection methods to ensure food safety. This study aimed to investigate the shedding of *Salmonella* in apparently healthy native chickens and describe the pathology of the lesions present in the internal organs. The endeavor elucidates the nature of *Salmonella* infection in free-range poultry.

## Materials and methods

### *Study site and experimental animal*

The study involved a longitudinal observation of *Salmonella* shedding conducted in January to June 2019. Fifty, day-old Banaba x Paroakan Philippine native chickens were purchased from the Institute of Animal Science and raised at the Native Poultry Farm facility of the College of Agriculture and Food Science, University of the Philippines Los Baños (UPLB), College, Laguna, Philippines. The chicks were raised in a constructed pen and provided

eight hours outdoor access in a controlled environment secured with fences. On their third week, they were allowed extended ranging time and kept in pen at night. Chickens were provided with antibiotic-free diet suited to their age and *ad libitum* water. Biosecurity measures were instituted to prohibit entry of animals and unauthorized people in the study area.

A preliminary analysis for *Salmonella* contamination in the environment and flock was performed before commencing the experiment. This was done to ensure that the site and animals used in the study were *Salmonella*-free. Sample processing and analysis were done at the Molecular Biology and Pathology Laboratory of the College of Veterinary Medicine, UPLB, College, Laguna, Philippines.

### C*ollection, bacterial isolation and identification*

Cloacal swabs, field, feeds and water samples were collected bi-weekly for 150 days and analyzed for *Salmonella* detection. Cloacal swabs were directly inoculated in selective media (BGA and XLD) and the tip of another swab was cut off and deposited into Rappaport Vassiliadis (RV) broth for enrichment and isolation. Field samples were obtained using boot socks following a zigzag pattern of walking in the farm. The boot socks were then immersed in 100 mL of sterile phosphate-buffered saline (PBS) for 10 min to disperse the soil. An aliquot of the sample was used for subsequent enrichment and plating techniques. Feeds (25 g) and water (20 mL) were collected from the feeding and drinking trough of the chickens and pre-enriched in buffered peptone water (BPW) at 1:10 dilution. Growth was then enriched in selenite broth and streaked onto selective media. Bacterial cultures were incubated aerobically at 37$^{o}$C for 24 to 48h. Candidate isolates were identified based on morphological and colonial characteristics and confirmed through their biochemical reactions in Mac Conkey agar, triple sugar iron agar, lysine decarboxylase broth, and urea broth. Further confirmation was done through slide agglutination test using polyvalent O *Salmonella* antisera.

### *DNA extraction and PCR amplification*

Extraction of DNA from the samples and isolates was carried out using QIAamp Fast DNA Stool Mini Kit (Qiagen®, USA) following manufacturer's protocol. About 2 µl of the extracted DNA was suspended in a PCR mixture containing 12.5 µl of GoTaq® Green Master Mix (Promega, USA), 10 µl of nuclease-free water, and 0.25 µl each of the invA primer (10 µM) with the following sequences: invA-F, GTG AAA TTA TCG CCA CGT TCG GGC AA

and invA-R, TCA TCG CAC CGT CAA AGG AAC C. The reaction conditions were set at 94℃ for 7 minutes for initial denaturation, 94℃ for 1 minute for denaturation, 53℃ for 2 minutes for annealing, 72℃ for 3 minutes for extension, and another 72℃ for 7 minutes for a final extension (Rahn *et al*. 1992). Amplicons were demonstrated in UV transilluminator (Vilber, Marne La Vallee, Ile-de-France, France) and considered *inv*A-positive upon production of 284 bp band.

### *Histopathological analysis of tissues*

Five chickens were randomly selected from the flock and euthanized at 10, 30, 120 days, and 150. Representative tissue samples from the liver, spleen, cecum, and ileum were aseptically cut and placed in 10% buffered formalin for histopathological examination. The standard method of Hematoxylin and Eosin (H and E) staining described by Luna (1968) as stated by Gupta *et al.* (2008) was followed with some modifications. The tissues were processed, embedded in paraffin wax, sectioned at 4 to 5 mm and mounted on clean glass slides. All sections stained with H and E were examined under the microscope.

### *Ethical considerations*

The experimental protocol was reviewed and found compliant with the requirements of the Animal Care and Use Committee of UPLB with approval number CVM-2019-008.

### *Statistical analysis*

Descriptive statistics was used to determine the frequency of histopathological lesions using Microsoft Excel. The detection rate was computed by dividing the number of samples positive to *Salmonella* by the total number of samples positive to *Salmonella*. Data with $p$ value $< 0.05$ were considered significant.

## Results

### *Detection of Salmonella*

The preliminary culture and PCR assay of cloacal swabs and field samples were negative of *Salmonella* suggesting that the experiment used an initially *Salmonella*-free environment and flock. Using culture method,

*Salmonella* was detected from the samples at Day 10, 44, and 150 with a detection rate of 2.11% (5/237). Detection by PCR was observed at Day 30 and Day 150 at the rate of 8.04% (16/199). Feed and water sample analysis gave negative results indicating absence of *Salmonella* from these samples throughout the duration of the study. Combining data from both methods, the overall detection rate of *Salmonella* in the experimental free-range farm was 8.86% (21/237).

### *Histopathological findings*

The pathology of *Salmonella* infection in the Philippine native chickens has not been studied and clearly understood. The present study documented the histopathological changes in the liver, spleen and intestine of native chickens infected with *Salmonella* to describe the corresponding tissue damage of *Salmonella* colonization in the intestines and their replication in the liver and spleen during systemic infection.

The frequency of discernible lesions in different organs is presented in Table 1. The most predominant lesion in the liver was cloudy swelling with cytoplasmic granulation (43.3%) accompanied by typhoid nodules (26.7%) and vascular congestion (23.3%). Sinusoidal congestion (83.3%) and lymphoid hyperplasia (66.7%) predominated the lesions in the spleen persisting from Day 30 until Day 150. Microscopic lesions in the intestines were already discernible at Day 10 primarily indicated by activation of the cecal tonsil and lymphoid nodule development (Figure 4a). Remarkedly, the ileum was not largely affected by the colonization during the early stages of infection. Relevant lesions such as active nodule (Figure 4b), thickening of the tunica muscularis (Figure 5c), thinning of the mucosa with fused villi were only observed at Day 120 and 150.

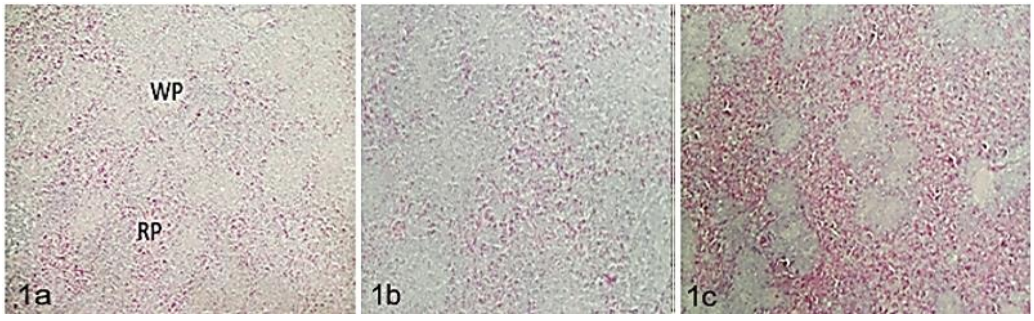


**Figure 1.** Histopathological changes in spleen. (a) Normal spleen. (b) Moderate sinusoidal congestion. (c) Severe sinusoidal congestion with lymphoid hyperplasia

**Table 1**. Frequency of discernible lesions in the liver, spleen, and intestines

| Discernible lesions | Number of discernible lesions in the tissues at different sampling period (n=30) | | | | Total | Occurrence frequency (%) |
|---|---|---|---|---|---|---|
| | 10 days | 30 days | 120 days | 150 days | | |
| Liver | | | | | | |
| Cloudy swelling with cytoplasmic granulation | 3/5 | 4/5 | 0 | 6/15 | 13 | 43.3 |
| Severe swelling almost completely unidentifiable hepatocytes | 0 | 0 | 0 | 3/15 | 3 | 10.0 |
| Typhoid nodules | 1/5 | 2/5 | 0 | 5/15 | 8 | 26.7 |
| Vascular congestion | 0 | 0 | 1/5 | 6/15 | 7 | 23.3 |
| Spleen | | | | | | |
| Mild to severe sinusoidal congestion in spleen | 0 | 5/5 | 5/5 | 15/15 | 25 | 83.3 |
| Lymphoid hyperplasia in spleen | 0 | 4/5 | 3/5 | 13/15 | 20 | 66.7 |
| Cecum | | | | | | |
| Active cecal tonsil | 2/5 | 2/5 | 0 | 1/15 | 5 | 16.7 |
| Lymphoid nodule in cecum | 1/5 | 0 | 0 | 0 | 1 | 3.3 |
| Very thick cecal tunica muscularis with thick long villi. | 4/5 | 0 | 0 | 0 | 4 | 13.3 |
| Thinning of mucosal lining with short mucosal villi fusion | 0 | 0 | 5/5 | 4/15 | 9 | 30.0 |
| Short, blunted and fused villi with mononuclear hyperplasia in the submucousa | 0 | 0 | 1/5 | 7/15 | 8 | 26.7 |
| Ileum | | | | | | |
| Full intact mucosal epithelium; no apparent lesion | 5/5 | 5/5 | 5/5 | 2/15 | 17 | 56.7 |
| Very thick tunica muscularis | 0 | 0 | 0 | 3 | 3 | 10.0 |
| Active lymphoid nodule | 0 | 0 | 0 | 1/15 | 1 | 3.3 |
| Thin mucosa, medium length to fused villi | 0 | 0 | 0 | 3/15 | 3 | 10.0 |

Results showed the histopathological changes occurring in the organs. Many of these changes, including typhoid nodules, lymphoid hyperplasia and influx of inflammatory cells, were significantly associated with *Salmonella* colonization in various studies (Figure 1-5).

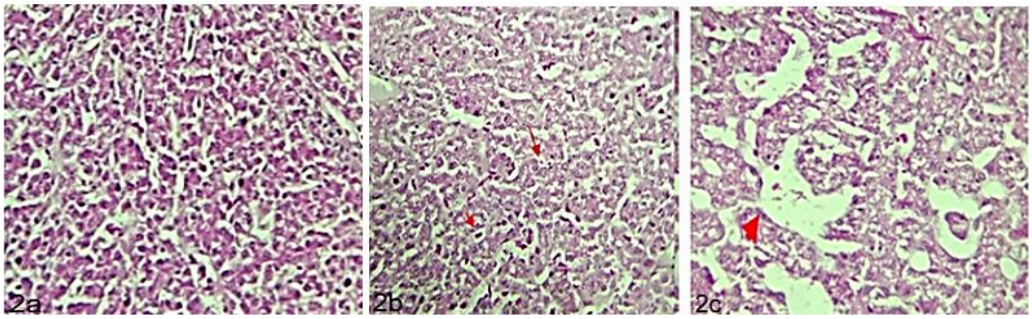


**Figure 2**. Liver. (a) Normal liver, (b) Cloudy swelling of the liver with cytoplasmic granulation and vacuolation (arrow) at 30-day. (c) Loss of hepatic cord (arrowhead) due to severe cloudy swelling at day 120

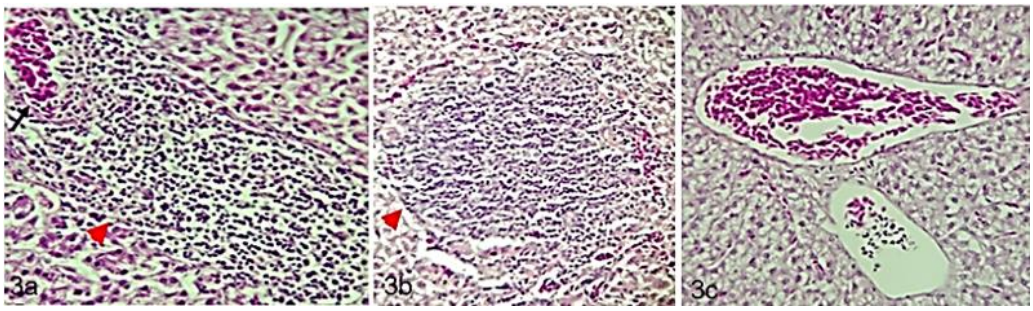


**Figure 3.** Liver. (a-b) Typhoid nodules (arrowhead) with vascular congestion (arrow) at Day 150. (c) Hepatic lipidosis with vacuolation and vascular congestion at Day 120

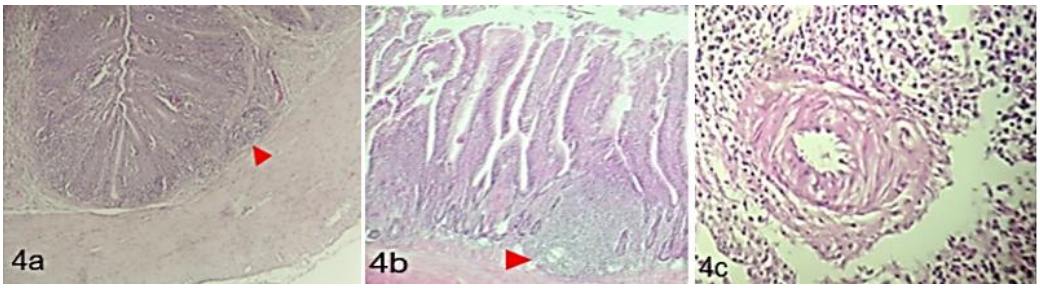


**Figure 4**. Intestines. (a) Lymphoid nodule formation (arrowhead) in the cecum at Day 30. (b) Lymphoid nodule in ileum. (c) Infiltration of inflammatory cells in the mucosa of the cecum

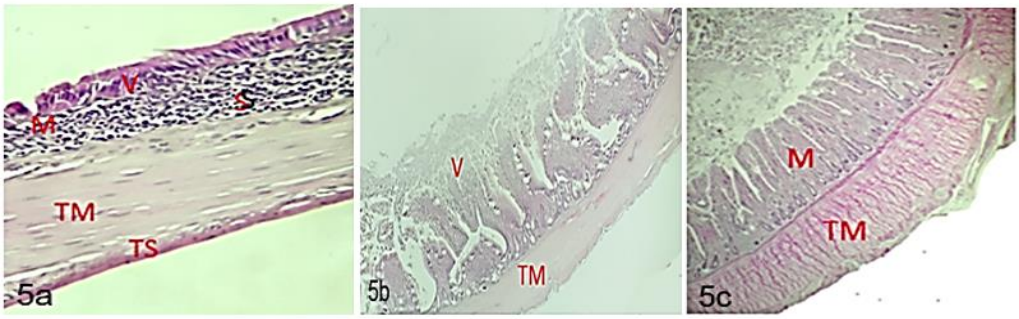


**Figure 5.** Intestines. (a) Thinning of the mucosal layer, fusion of villi, thickening of the tunica muscularis and infiltration of mononuclear cells in the submucosal layer of cecum at Day 120-150. (b) Fusion and blunting of cecal villi at Day 120-150. (c) Thickening of the tunica muscularis in the ileum at Day 150. Hematoxylin-Eosin, 400x. WP-white pulp, RP-red pulp, V-villi, M-mucosa, S-submucosa, TM-tunica muscularis, TS-tunica serosa

## Discussion

Detection of *Salmonella* by culture method followed an intermittent pattern where the bacterium was detected from the cloacal swab and field samples at Day 10, 44 and 150, but undetected at Day 30 and 120. Similarly, *Salmonella* was detected by PCR at Day 30 and 150 but undetected during other sampling periods. This intermittence is a hallmark of persistent salmonellosis characterized by intermittent shedding of very few bacteria (Kranker *et al*., 2003) that could be missed during sampling.

Entry of *Salmonella* into the farm follows different pathways the most common of which is through amplifying vectors such as birds, flies or rodents that are attracted to the feeds and fecal waste of chickens. Ubiquitous *Salmonella* is acquired by these vectors from their environment and spread the organism to another place through their droppings and contaminated body surfaces (Craven *et al*., 2000; Lapuz *et al*., 2007; Leibana *et al*., 2003; Wales *et al*., 2010). Horizontal infection is inherent in a flock with infection developing at Day 10 or earlier in this study.

The histopathologic findings in this study corroborated in establishing a state of infection caused by *Salmonella* colonization of the internal organs. Cloudy swelling with cytoplasmic granulation in the liver (Figure 2b) was observed beginning Day 10 when infection was established through the isolation of *Salmonella* from the native chickens. Concomitant typhoid nodules (Figure 3 a-b) were observed from Day 10 to 150 and vascular congestion (Figure 3c) at Day 120. These histological changes are positively correlated

elsewhere as liver's response to injury caused by infection. The invasion by *Salmonella* causes hepatocytes to round up resulting to membrane damage and consequent influx of fluid into the cell or damage to the cytoskeleton resulting to the loss of cell shape and vascular congestion (Wallig and Janovitz, 2013). The typhoid nodules, which contain an aggregation of histiocytes admixed with lymphocytes and plasma cells, mark the aggressive response of the liver to bacterial invasion (Bharadwaj *et al*., 2009). Hepatic lipidosis and vacuolation (Figure 3c) were demonstrated in the liver of some chickens. These lesions accompanied with leukocyte infiltration in the hepatocytes were also observed in the liver of backyard chickens with chronic infection of *S.* Kentucky (Najmin *et al*., 2018) and commercial chickens infected with *S.* Gallinarum (Freitas Neto *et al*., 2007).

In the spleen, sinusoidal congestion (Figure 1c) and lymphoid hyperplasia (Figure 1b) were the most profound lesions observed persisting from Day 30 until Day 150. The development of these lesions supports the ability of *Salmonella* to traverse systemic routes to reach the spleen after acute infection. *Salmonella* breaches the epithelium, reach the underlying gut-associated lymphoid tissue and invade the phagocytes that enter the lymphatic system and bloodstream (Jones *et al*., 1994). After reaching the liver and spleen, it replicates within the macrophages and causes tissue damage (Salcedo *et al*., 2001). In response, the spleen undergoes lymphoid hyperplasia to increase the number of lymphocytic cells to suppress bacterial infection. Consequently, the increase in the number of cells increases the demand for localized blood supply resulting to venous congestion of the splenic vein.

Microscopic lesions in the liver and spleen, predominantly typhoid nodules and lymphoid hyperplasia, are considered the hallmark of systemic infection (Wigley, 2013). Besides the liver and spleen, the bone marrow and bursa of Fabricius are also major replication sites for *Salmonella* revealing the invasiveness of this pathogen (Henderson *et al.,* 1999). As systemic infection precedes persistent infection in chickens leading to bacterial shedding and environmental contamination, horizontal transmission is expected to follow involving the uninfected chickens in the flock. This is demonstrated by the consequent detection of *Salmonella* from cloacal swabs and environmental samples by either bacterial culture or PCR, and the increasing detection rate and persistence of pathological lesions towards Day 150.

Lesser invasiveness was noted in the ileum than in the cecum. This is because of the slow movement of ingesta in the cecum (Gabriel *et al*., 2006) predisposing it as an ideal organ for *Salmonella* colonization (Dhillon *et al*., 2001). The earliest evidence of intestinal involvement associated with the infection was the activation of cecal tonsil initially observed at Day 10 and

persisted throughout Day 150. Major histopathological changes in the intestines became more prominent at Day 120 where inflammatory cells consisting primarily of heterophils and mononuclear cells infiltrated the submucosa of the ileum and cecum. Consequently, intestinal damaged occurred and exhibited as villous thinning, blunting and fusion of the villi in the cecum accompanied with thinning of the mucosal lining; villi fusion and thickening of the tunica muscularis in the ileum were also observed.

The microscopic lesions in the intestine suggested inflammation with massive infiltration of leukocytes at the mucosal and submucosal layer of the intestines resulting to the thickening of the lamina propia and tunica muscularis. The influx of inflammatory cells to the intestinal mucosa demonstrates the effort of the mucosal immunity to clear the bacteria. Activation of lymphoid tissues evident as early as Day 10 indicated immune response to the presence of foreign body such as bacteria. Villous shortening, blunting and fusion reflected the damaging invasiveness of *Salmonella* to the villi of the intestines.

The progressive histological changes in the tissues were not mirrored in the gross appearance of chickens and the frequency of fecal shedding of the pathogen posing a big challenge in terms of detection. The existence of persistent, subclinical infection in native chickens cannot be discounted as it does not only affect productive performance, but also serves as threat to food safety. Timely, rapid and reliable diagnostic tools detecting subclinical infections should therefore be made available for free-range farms. Moreover, control measures reducing *Salmonella* contamination in the ranging environment should be sustained while alternatives ways to prime the chickens' immunity to enteric pathogens should also be explored.

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