UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY OF *SALMONELLA* TYPHIMURIUM AND *SALMONELLA* STANLEY ISOLATES IN CHICKENS

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PATHOGENICITY OF *SALMONELLA* TYPHIMURIUM AND *SALMONELLA* STANLEY ISOLATES IN CHICKENS

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A project paper submitted to the
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It is hereby certified that I have read this project paper entitled "Pathogenicity of *Salmonella* Typhimurium and *Salmonella* Stanley Isolates in Chickens", by Balqis binti Razali and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Project.

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ABBREVIATIONS

cfu	colony forming unit
pi	post inoculation
ELISA	Enzyme-linked immunoabsorbent assay
HE	Haematoxylin and Eosin
BPW	buffered peptone water
RV	Rappaport-Vassiliadis
XLD	xylose lysine deoxycholate agar
TSA	trypticase soy agar
TSI	triple sugar iron agar

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Putra Malaysia untuk memenuhi sebahagian daripada keperluan kursus VPD4999 – Projek.

PATOGENISITI *SALMONELLA* TYPHIMURIUM DAN *SALMONELLA* STANLEY ISOLAT DALAM AYAM

oleh

Balqis binti Razali

Mac 2018

Penyelia: Profesor Dr. Mohd Hair bin Bejo

Objektif kajian ini adalah untuk menentukan patogenisiti isolat *Salmonella* dalam ayam. Tujuh puluh enam anak ayam dibahagikan kepada tiga kumpulan iaitu A, B dan C. Ayam dari kumpulan A (24 ekor) diinokulasi dengan *S.* Typhimurium dan ayam dari kumpulan B (24 ekor) diinokulasi dengan *S.* Stanley dengan $0.1x10^8$ cfu melalui intraperitoneal. Ayam kumpulan C (28 ekor) kekal tidak diinokulasi dan dijadikan kumpulan kawalan. Lapan ekor ayam daripada setiap kumpulan diletakkan dalam kumpulan kematian. Sebelum inokulasi, 4 ekor anak ayam dari kumpulan kawalan dikorbankan. Pada hari 1, 4, 7, dan 14 selepas inokulasi (pi) 4 ekor anak ayam dari setiap kumpulan dikorbankan sebagai pensampelan. Sebelum necropsi, berat badan dan sampel darah diambil untuk pengesanan *Salmonella* antibodi dengan teknik ELISA. Lesi mata kasar direkodkan semasa necropsi. Sampel hati, limpa, tonsil usus dan swab kloaka diambil untuk pemencilan *Salmonella.* Sampel hati diawet dalam 10% bufer formalin untuk pemeriksaan histologi. Kajian menunjukkan berat badan meningkat dalam semua kumpulan sepanjang ujian. Perbezaan ketara ($p < 0.05$) dalam

berat badan antara kumpulan pada hari 1 pi namun tidak terdapat perbezaan yang signifikan dalam berat badan ($p > 0.05$) pada hari 4, 7 dan 14 pi antara kumpulan. Tanda klinikal dilihat dari anak ayam dari kumpulan A pada hari 8 pi adalah kematian secara tiba-tiba manakala anak ayam dari kumpulan B pada hari 6 pi adalah lemah yang mati pada hari berikutnya. Namun, kumpulan C tidak menunjukkan sebarang tanda klinikal. Kadar kematian bagi kumpulan A dan kumpulan B adalah 12.5% setiap satu dan tiada kematian untuk kumpulan C. Nekropsi mendedahkan splenomegali pada anak ayam dalam kumpulan B pada hari 4, 7 dan 14 pi. Akan tetapi, tiada lesi mata kasar dilihat dalam kumpulan lain dan anak ayam yang mati. Lesi histologi pada hati menunjukkan tiada penemuan penting dalam semua kumpulan. *Salmonella* dipencilkan dari kumpulan A, pada hari 1 pi dari limpa (25%) dan hari 7 pi dari hati (25%), tetapi tidak pada hari 4 pi dan 14 pi. Manakala, untuk kumpulan B pada hari 1 pi daripada hati (25%) dan tonsil usus (25%); hari 4 pi dari hati (50%), tonsil usus (25%) dan juga limpa (25%); hari 7 pi dari hati (50%), limpa (50%), tonsil usus (50%), swab kloaka (50%); tetapi hari 14 pi tiada. Namun, tiada pemencilan dari kumpulan C. *Salmonella* dipencilkan dari hati, limpa, tonsil usus dan swab kloaka dari anak ayam mati dari kumpulan A. Sementara itu kumpulan B dari hati, limpa dan tonsil usus. Titer antibodi *Salmonella* menurun dalam semua kumpulan sepanjang ujian. Tiada perbezaan yang signifikan ($p > 0.05$) dalam titer antibodi antara kumpulan pada hari 1 pi dan 7 pi. Walau bagaimanapun, perbezaan yang signifikan ($p < 0.05$) dilihat pada hari 4 pi dan 14 pi. Kesimpulannya, *S.* Typhimurium dan *S.* Stanley isolat yang digunakan dalam kajian ini adalah patogenik kepada ayam kerana ia menyebabkan kematian dan lesi mata kasar, dan dapat diasingkan dari organ. *Salmonella* Stanley isolat lebih patogenik pada ayam berbanding *S.* Typhimurium.

Kata kunci: *Salmonella* Stanley, *Salmonella* Typhimurium, ayam, patogenisiti

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in partial fulfilment of the course VPD 4999 – Project.

PATHOGENICITY OF *SALMONELLA* TYPHIMURIUM AND *SALMONELLA* STANLEY ISOLATES IN CHICKENS

by

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2018

Supervisor: Professor Dr. Mohd Hair bin Bejo

The objectives of this study were to determine pathogenicity of *Salmonella* isolates in chickens and to isolate the agent from the organs. Seventy-six-day-old chicks were divided into three groups namely; group A, inoculated with *S.* Typhimurium (24 chicks); group B, inoculated with *S.* Stanley (24 chicks); and group C, was left uninoculated, and acted as the control group (28 chicks). The chicks in groups A and B were inoculated intraperitoneally with 0.1 ml of $1x10^8$ colony forming unit (cfu) of *S.* Typhimurium and *S.* Stanley, respectively at day old. Eight chicks from all groups were separated and monitored for mortality. Chicks were provided with feed and water *ad libitum* throughout the trial, and monitored for abnormal clinical signs and mortality at least twice daily. Prior to bacterial inoculation, four chicks from group C were sacrificed. On days 1, 4, 7 and 14 post inoculation (pi), four chicks were sacrificed from each group. Body weights and blood samples were collected for detection of *Salmonella* antibody using ELISA technique prior necropsy. On necropsy, gross lesions were recorded and samples of liver were collected and fixed in 10% buffered formalin for histological examination. Samples of liver, spleen, caecal tonsils

and cloacal swabs were collected for bacterial isolation and identification. The study showed that body weight of chickens in all groups increased throughout the trials. There was significant difference ($p<0.05$) in body weight between groups on day 1 pi, although no significant difference in body weight ($p>0.05$) on days 4, 7 and 14 pi between groups. Clinical signs seen from a chick in group A on day 8 pi was sudden death meanwhile a chick from group B on day 6 pi was weakness that was found dead on the next day. However, group C showed no abnormal clinical signs throughout the trial. Mortality rate for groups A and B were 12.5% each and none for group C. Necropsy revealed splenomegaly on chicks in group B on days 4, 7 and 14 pi. Nevertheless, no lesion was seen in other groups and the dead chicks. Histology of liver revealed no significant findings in all groups. *Salmonella* was isolated from group A, on day 1 pi from spleen (25%) and on day 7 pi from liver (25%), but none on days 4 and 14 pi. However, for group B on day 1 pi *Salmonella* was isolated from liver (25%) and caecal tonsil (25%); on day 4 pi from liver (50%), caecal tonsil (25%) and also spleen (25%); on day 7 pi from liver (50%), spleen (50%), caecal tonsil (50%), and cloacal swab (50%); but day 14 pi nil. *Salmonella* was not isolated from group C. *Salmonella* isolation from dead chicks from group A was from liver, spleen, caecal tonsil and cloacal swab meanwhile, group B was from liver, spleen and caecal tonsil. *Salmonella* antibody titre declined in all groups throughout the trials. There was no significant difference ($p>0.05$) in antibody titre between groups on days 1 and 7 pi. However, significant difference ($p<0.05$) was seen on days 4 and 14 pi. In conclusion, *S.* Typhimurium and *S.* Stanley isolates used in the study were pathogenic to chickens as it caused death and gross lesion, and were able to be isolated from the organs.

Salmonella Stanley isolate is more pathogenic in chickens when compared to *S.* Typhimurium.

Keywords: *Salmonella* Typhimurium, *Salmonella* Stanley, commercial broiler chicken, pathogenicity

1.0 INTRODUCTION

1.1 Background of the Study

Salmonella Typhimurium and *Salmonella* Stanley are also known as *S. enterica* subspecies *enterica* serovar Typhimurium and *S. enterica* subspecies *enterica* serovar Stanley, respectively. It is gram negative rods of the Enterobacteriaceae family. *Salmonella* consist of two major species which are *S. enterica* and *S. bongori* (WHO, 2017). *Salmonella enterica* is further divided to six subspecies comprise of over 2500 serovar (OIE, 2008).

Common cause of human foodborne disease is *S. enterica* with variety of animals being identified as reservoirs (Hendriksen et al., 2012). *Salmonella* Typhimurium can be found in a large number of different animals as reservoir without specific source, whereas duck is the only reservoir where *S.* Stanley is found in high frequency (Bangtrakulnonth et al., 2004). Besides, the potential risk for exposure to *Salmonella* via contaminated food rises with growth in consumption of meat and poultry product (Foley et al., 2011).

Geographical distribution is a factor contributing to variation of *Salmonella* serovar between country. In developed countries, *S.* Typhimurium is second most common cause of human salmonellosis (Hendriksen et al., 2012), and its importance is rising in Southeast Asia (Bangtrakulnonth et al., 2004). Bangtrakulnonth et al. (2004) also added that in 1995, *S.* Stanley infections were among the 15 most common serovars in 12 out of 104 countries. A recent study in Thailand showed that from 2002 to 2007, the second most common serovar causing human salmonellosis cases is due to *S.*

Stanley. In 2008, *S.* Stanley was among the most prevalent cause of human Salmonellosis in a few other countries (Hendriksen et al., 2012).

Salmonella Typhimurium often presence in poultry asymptomatically (Bjerrum et al., 2003) either subclinical infection or as a healthy carrier (Antunes et al., 2016) although Al-Abadi & Al-Mayah (2013) found clinical signs shown by infected chickens such as depression, anorexia, huddling together and mild to severe diarrhoea with mortality rate of 10% and lesions of enlargement of liver and spleen and enteritis. However, not much is known regarding *S.* Stanley infection in poultry.

Salmonellosis is suggested to simply transmit to humans through poultry meat through healthy animals as the disease is not detected earlier during processing (Antunes et al., 2016). As less is known about *Salmonella* infection in poultry in Malaysia, thus a study is needed to learn more about *Salmonella* infection for the prevention and control of the infection in chicken farms and prevention of the agent to enter the food chain, and transmission to human.

1.2 Hypotheses

The hypotheses of the study were:

1. H_0**:** There is no significant difference of pathogenicity and *Salmonella* antibody titre between *S.* Typhimurium and *S.* Stanley inoculated chickens and the control group

H_A**:** There is significant difference of pathogenicity and *Salmonella* antibody titre between *S.* Typhimurium and *S.* Stanley inoculated chickens and the control group

2. H_0: There is no significant difference in the bacteria isolation in organs of chickens between *S.* Typhimurium and *S.* Stanley inoculated chickens and the control group

   H_A: There is significant difference in the bacteria isolation in organs of chickens between *S.* Typhimurium and *S.* Stanley inoculated chickens and the control group

1.3 Objectives

The objectives of this study were:

1. to determine the clinical signs and mortality percentage of chickens inoculated either with *S.* Typhimurium or *S.* Stanley isolate
2. to determine the gross and histological lesions, and *Salmonella* antibody titre of chickens inoculated with *S.* Typhimurium or *S.* Stanley isolate
3. to isolate and identify *S.* Typhimurium or *S.* Stanley in organs of the chickens

2.0 LITERATURE REVIEW

2.1 Salmonellosis in Human

Salmonellosis is zoonosis and usually is foodborne with food animals such as chickens, turkeys and pigs being the most common reservoirs. *Salmonella* infection in human can cause gastroenteritis usually with incubation period of 6 hours to 48 hours after ingestion. Gastroenteritis is characterized by nausea, vomiting, cramping abdominal pain and diarrhoea. In some cases, headache, fever, chills and myalgia may be observed. Diarrhoea and fever usually last for duration of 2 to 7 days (Giannella, 1996). Symptoms usually resolved spontaneously in several days to a week.

Infectious dose for healthy human is 10^6 to 10^8 (Antunes et al., 2016). It is life threatening although uncommon except in a very young, very old and debilitated or immunocompromised persons (Antunes et al., 2016; Leedom-Larson & Spickler, 2013).

2.2 Salmonellosis in Poultry

2.2.1 Clinical Signs and Pathological Changes

2.2.1.1 *Salmonella* Typhimurium Infection

Salmonella Typhimurium infection in broiler has been characterized with anorexia, depression, ruffled feathers, huddling together, reluctance to move, somnolence, pasted vents and mild to severe diarrhoea with significant decrease in weight gain (Al-Abadi & Al-Mayah, 2013). However, no clinical sign was noted in infected chicken by Bjerrum et al. (2003).

With high morbidity and mortality during first two weeks of life (Gast, 2013) mortality rate varies from less than 2% (Bjerrum et al., 2003) to 10% (Al-Abadi & Al-Mayah, 2013) with mortality peak level at 3- 7 days of age in natural infection (Gast, 2013).

Upon necropsy, lesions that can be seen are catarrhal to mild haemorrhagic enteritis, severe congestion of liver and enlargement of liver and spleen (Al-Abadi & Al-Mayah, 2013).

2.2.1.2 *Salmonella* Stanley Infection

Less is known regarding this isolate in chickens.

2.2.2 Transmission

There are two routes of transmission for common *Salmonella* infection in poultry either vertical or horizontal. Vertical transmission is an infection acquired from infected parents either through internal or external contamination of eggs. Egg shells are contaminated with faeces during oviposition. Infection can directly transmit to developing embryos or allow exposure of chicks to infection when the shell structure is disrupted during hatching (Gast, 2013).

Horizontal transmission is spread through bird-to-bird contact or ingestion of contaminated feed, water, litter or faeces. This can occur both within and between flocks. Feed may be contaminated with *Salmonella* and act as a source of infection. Moreover, only low levels of paratyphoid salmonellae in feed is needed to make animal susceptible. Besides, stress from environment or stress induced by moulting is known to increase *Salmonella* shedding.

Newly hatched chicks are susceptible to colonization from the gut as they have lack intestinal colonization by salmonellae. In the hatchery, when chicks from *Salmonella*-free eggs are hatched along with surface contaminated eggs they became infected with salmonellae (Gast, 2013; Shivaprasad et al., 2013).

2.2.3 Pathogenesis

Three stages of infection commonly involved in salmonellae infection. When introduced orally, salmonellae first establish intestinal colonization, usually cause persistent faecal shedding. Next, *Salmonella* may cause systemic disease (Al-Abadi & Al-Mayah, 2013) as it multiplies in the macrophage-phagocyte system of the liver and spleen as it invades beyond the gastrointestinal tract, and subsequently spread to other organs as the animals become ill (Gast, 2013; Shivaprasad et al., 2013). Third, high mortality occurs as a result of extensive bacteraemia (Gast, 2013). Death also happen as consequence of dehydration and anorexia resulting from diarrhoea and general malaise (Shivaprasad et al., 2013).

Nevertheless, age of chickens and dose of orally administered salmonellae are strongly associated with occurrence of both intestinal colonization and mortality in chicks. Variation of infectious dose depends on chicken breed and *Salmonella* strains invasiveness (Gast, 2013; Shivaprasad et al., 2013). Shivaprasad et al. (2013) also said no clinical sign seen in infected chicken more than 3- 4 days old although it may be secreted in the faeces. Increasing maturity in cells of the macrophage-monocyte series is said to attribute in increase in resistance. Other than that, route of infection also contribute in infection as chicks are more vulnerable to *Salmonella* infection from inhalation and parenteral routes rather than oral route (Shivaprasad et al., 2013).

2.3 Diagnosis

Salmonella detection can be done through isolation of the agent or serological test.

As lesion is rarely pathognomonic, thus diagnosis without isolation of causative agent can be misleading (Shivaprasad et al., 2013). According to Gast (2013), isolation and identification of causative agent is often the final diagnosis. Various organs can be used for culturing as many *Salmonella* serotypes are highly invasive and can spread systemically to numerous internal tissue. Suitable organs to be taken include liver, spleen, heart, heart blood and kidney. Nevertheless, multiple different organs should be cultured from each bird as lesions do not consistently indicate infected tissue. Portion of intestine such as caudal ileum, caeca, caecal tonsils and caecal contents are suggested for *Salmonella* recovery as it often involved intestinal colonization; and also, cloacal swabs or faecal samples as it is sensitive indicators for persistent intestinal colonization. However, intermittent shedding may affect the latter samples.

Serology is another diagnostic tool used in *Salmonella* detection such as agglutination assays and Enzyme-linked immunosorbent assay (ELISA). However, development of a titre depends on the occurrence of a systemic infection and can only be detected on 3–6 days pi (Shivaprasad et al., 2013). ELISA is more sensitive and detects specific IgG in serum or IgG in egg yolk (Barrow, 1992). This can overcome problem related with intermittent shedding (Skov et al., 2002) as high titres of systemic infection can be detected for many months after infection (Shivaprasad et al., 2013).

Skov et al., (2002) mentioned bacteriologic sampling methods may not be sufficiently sensitive to identify *Salmonella* infected flocks due to low prevalence of infected animals, or the presence of carrier animals showing intermittent or no excretion of the

bacteria. However, it is still needed to detect initial phase of infection in a flock and in relation to infection with serotypes that cause only a weak or no systemic antibody (Gast, 2013; Shivaprasad et al., 2013). Nevertheless, there is no clear sign of current infection in flocks as serology yields positive results much later after infection than bacteriologic culturing (Gast, 2013).

2.4 Treatment and Prevention

Treatment using antibiotics a used for chicken with clinical salmonellosis. Antibiotics used are gentamicin and enrofloxacin. However, usage of enrofloxacin is seen to eliminate *Salmonella* from faeces for a short duration that was again excreted in the faeces although in small number. Besides, with increased in antibiotic resistance it was less used nowadays (Gast, 2013; Shivaprasad et al., 2013).

Intervention can be done by controlling gastrointestinal colonization in newly hatched chicken through competitive exclusion. Oral administration of caecal bacterial flora from mature birds has been shown to increase chick's resistance to *Salmonella* infection by reducing incidence of salmonellosis in poultry (Gast, 2013; Shivaprasad et al., 2013). It is said to be most effective when administered prior exposure to pathogens (Gast, 2013). According to Bjerrum et al., (2003) development of a microflora is sooner as a result of administration of competitive exclusion. Besides, salmonellae-infected chickens when treated with protective microflora has shown to fasten recovery as it enhanced the clearance of concurrent or pre-existing infection. In contrast, Gast & Beard, (1989) mentioned efficacy of competitive exclusion treatments, declined when birds are challenged with constant or severe salmonellae challenge or when birds are exposed to physiological stress.

Other than that, vaccination with killed or live vaccine has been relate to protection against salmonellae as it reduces susceptibility to infection (Gast, 2013; Shivaprasad et al., 2013).

3.0 MATERIALS AND METHODS

3.1 *Salmonella* Isolates

Isolates were obtained from broiler chickens during Standard Operating Procedure of *Salmonella* monitoring programme in Perak, Malaysia. *Salmonella* Typhimurium Strain UPM260 was isolated from 38 days old healthy broiler chicken while *S.* Stanley Strain UPM517 was isolated from 21 days old healthy broiler chicken.

3.2 Inoculum preparation

The inoculum for both strains were prepared as regard to serial dilution methods. Briefly, this method involved serial dilution of a bacterial suspension in sterile water blanks, which served as a diluent of known volume. Once diluted, the suspensions were placed on suitable nutrient media. Before the dilution took place, the isolates were sub cultured twice from storage for 16-18 hours at 37°C. Eight sets of tubes were prepared with 9 ml sterile distilled water and labelled according to dilution number from 10^{-1} to 10^{-8}. One ml of broth culture was added into tube of 10^{-1} and subsequent dilution up to 10^{-8} were made to contain an approximate 10-100 cfu/ml. Further enumeration using spread plate method ensued. A 0.1 ml of last four dilutions (10^{-5} to 10^{-8}) was inoculated and spread into respective plates prior to incubation. Colonies formed the next day were counted and dilution with 10-100 cfu/0.1 ml was selected to be used as inoculum.

3.3 Broiler Chicks

Day-old-chicks of commercial broiler were obtained from Linggi Poultry Farm (M) Sdn. Bhd. Rembau, Negeri Sembilan.

3.4 Experimental Design

A total of 76 day-old chicks were used in this experiment. The chicks were divided into three groups namely; group A, inoculated with *S.* Typhimurium (24 chicks), group B, inoculated with *S.* Stanley (24 chicks) and group C, was left uninoculated, and acts as the control group (28 chicks). The chicks in groups A and B were inoculated intraperitoneally with 0.1 ml of $1x10^8$ colony forming unit (cfu) of *S.* Typhimurium and *S.* Stanley, respectively at day old using 1 ml syringe and 25 G needle. The chicks in each group were further divided into two groups by separating 8 chicks from each group in mortality group and the rest of the chicks were placed in sacrifice group. The chicks were provided with feed and water *ad libitum* throughout the trial, and monitored for abnormal clinical signs and mortality at least twice daily.

At the beginning of experiment prior to bacterial inoculation, four chicks from group C were sacrificed by cervical dislocation. Four chicks from each groups A, B and C were sacrificed on days 1, 4, 7 and 14 post inoculation (pi). Prior to necropsy body weights were recorded and blood samples were collected for detection of *Salmonella* antibody using ELISA technique. On necropsy, gross lesions were recorded and samples of liver were fixed in 10% buffered formalin for histological examination. Samples of liver, spleen, caecal tonsils and cloacal swabs were collected for bacterial isolation and identification.

3.5 Clinical Signs

Clinical signs were observed and recorded for any abnormalities twice a day.

3.6 Gross Lesions

Gross lesions were observed and recorded upon necropsy.

3.7 Bacterial Isolation and Identification

Samples of liver, spleen, caecal tonsils and cloacal swabs were collected for bacterial isolation and identification. Samples were incubated in buffered peptone water (BPW) at 37 °C for 20 hours. On the next day, 1 ml of cultured BPW was inoculated into Rappaport-Vassiliadis (RV) broth followed by 18-24 hours incubation at 42ºC. A loopful of overnight RV selective enrichment culture was streaked onto xylose lysine deoxycholate agar (XLD) and was incubated at 37ºC for 18-24 hours. After incubation, characteristic of colonies on the agars were observed. Typical colonies of *Salmonella* on XLD were red with black centre. Suspected colony was cultured onto trypticase soy agar (TSA) and incubated at 37ºC for 24 hours. It was further confirm based on agglutination reaction with polyvalent antisera and proceed with biochemical confirmation with negative oxidase; urea test negative; triple sugar iron agar (TSI) showed acid butt and alkaline slant with or without presence of gas and presence of sulphide; indole negative, motility positive and positive citrate (ISO 6579, 2002).

3.8 Histopathology

Samples of liver from the chickens were fixed in 10% buffered formalin for at least 24 hours. It was cut to thickness of 3 mm prior inserting to a cassette. Next, it was processed overnight to dehydrate and clearing in varying concentrations of alcohol, xylene, followed by infiltration in paraffin wax. The sample was embedded in paraffin wax using automated tissue processor and sectioned about 4 µm-thick with

microtome. It was then floated onto purified water at 45ºC and mounted on the glass slide and allowed to dry. It was dewaxed with xylene and 70%, 90% and 100% alcohol followed by staining using Hematoxylin and Eosin stain (HE) (Bancroft & Layton, 2013).

3.9 Enzyme-linked Immunosorbent Assay

Salmonella antibody titre was detected using Enzyme-linked immunosorbent assay (ELISA) technique (MVP Sdn. Bhd.).

3.10 Statistical Analysis

Data collected was analysed using SPSS version 22. One way ANOVA was used to determine the differences of body weight of chicks and antibody titer between groups with significant value of ($p<0.05$).

4.0 RESULTS

4.1 Body Weights

At day 0 pi, the body weight was 51.8±0.75g and it rose to 503.3±15.42g on day 14 pi in group A. Similar pattern of increment were observed in groups B and C, although, there was significant different ($p<0.05$) in body weight between groups at day 1 pi. However, there was no significant different ($p>0.05$) between groups at days 4, 7 and 14 pi (Figure, 1; Appendix 2).

4.2 Clinical Signs

Clinical signs seen in group A from mortality group at day 8 pi was sudden death of a chick. However, a chick from group B showed weakness at day 6 pi and died on the next day while the other chicks remained with no clinical sign. In contrast, no clinical sign was observed for group C (Figure, 2).

4.3 Mortality Rate

Mortality rate for group A is 12.5% (1/8). Similar finding for group B that showed 12.5% (1/8) mortality rate. In contrast, no mortality was recorded for group C.

4.4 Gross Lesions

No gross lesion was recorded in the liver. At days 1 and 4 pi chicks' livers were yellowish. The liver at day 14 pi was dark red, without no enlargement (Figure, 3). However, in group B at days 4, 7 and 14 pi splenomegaly were seen from one chick (1/4) on each day while other organs remained normal. On the other hand, group A and group C showed no gross lesion on all days of sampling (Figure, 4).

Both dead chicks from groups A and B showed no abnormal gross lesion.

4.5 Histopathology

On day 1 pi, vacuole can be seen within the cytoplasm of hepatocyte in all groups due to presence of fat and it is normal in newly hatched chicks (Figure, 5). On days 4, 7 and 14 pi, the hepatocyte parenchyma remained normal in all groups (Figure, 6; Figure, 7; Figure, 8).

4.6 *Salmonella* Antibody Titre

Generally, *Salmonella* antibody titre decreased in all groups throughout the trials. It was seen to decline from 401.3±208.16 on day 0 pi to 1.5±0.50 on day 14 pi for group A. There was no significant difference ($p>0.05$) in antibody titre between groups on days 1 and 7 pi. However, significant difference ($p<0.05$) was seen on days 4 and 14 pi. Besides, induction of *Salmonella* antibody titre was observed on day 14 pi for group B (Figure, 9; Appendix 3).

4.7 Bacterial Isolation and Identification

4.7.1 Sacrifice Group

On XLD agar suspected *Salmonella* colony showed colony with black centre (Figure, 10). In group A, on day 1 pi *Salmonella* was isolated from spleen (25%). Meanwhile, on day 7 pi *Salmonella* was isolated from liver (25%). Nevertheless, on days 4 and 14 nil (Figure 11, Appendix 4).

On the other hand, for group B on day 1 pi *Salmonella* was recovered from liver (25%) and caecal tonsil (25%) samples. It was isolated from liver (50%), caecal tonsil (25%)

and also spleen (25%) on day 4 pi. On day 7 pi it was isolated from all organs (50% for each organ). However, *Salmonella* was not isolated on any sample of day 14 pi (Figure 12, Appendix 4).

Salmonella remained negative for group C on all days pi (Appendix 4).

4.7.2 Mortality Group

Dead chick from group A on day 8 pi, showed positive *Salmonella* isolation from all organs (liver, spleen, ceacal tonsils and cloacal swab). However, chick from group B that died on day 7 pi showed positive for *Salmonella* isolation in liver, spleen, caecal tonsils.

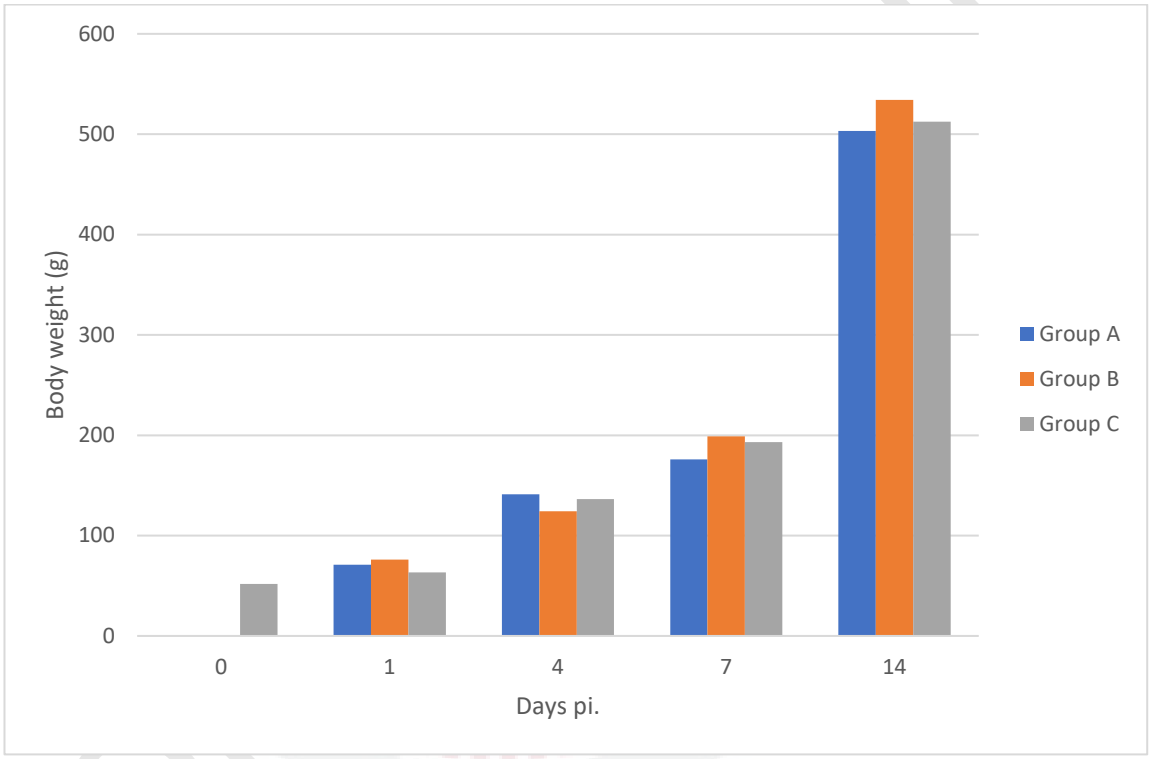

Figure 1: Body weight of chickens throughout the trial.

Figure 2: Normal condition of chicks on; (a) day 1, (b) day 6, and (c) day 12 pi for all groups. (d) Weakness of chicken from group B on day 6 pi.

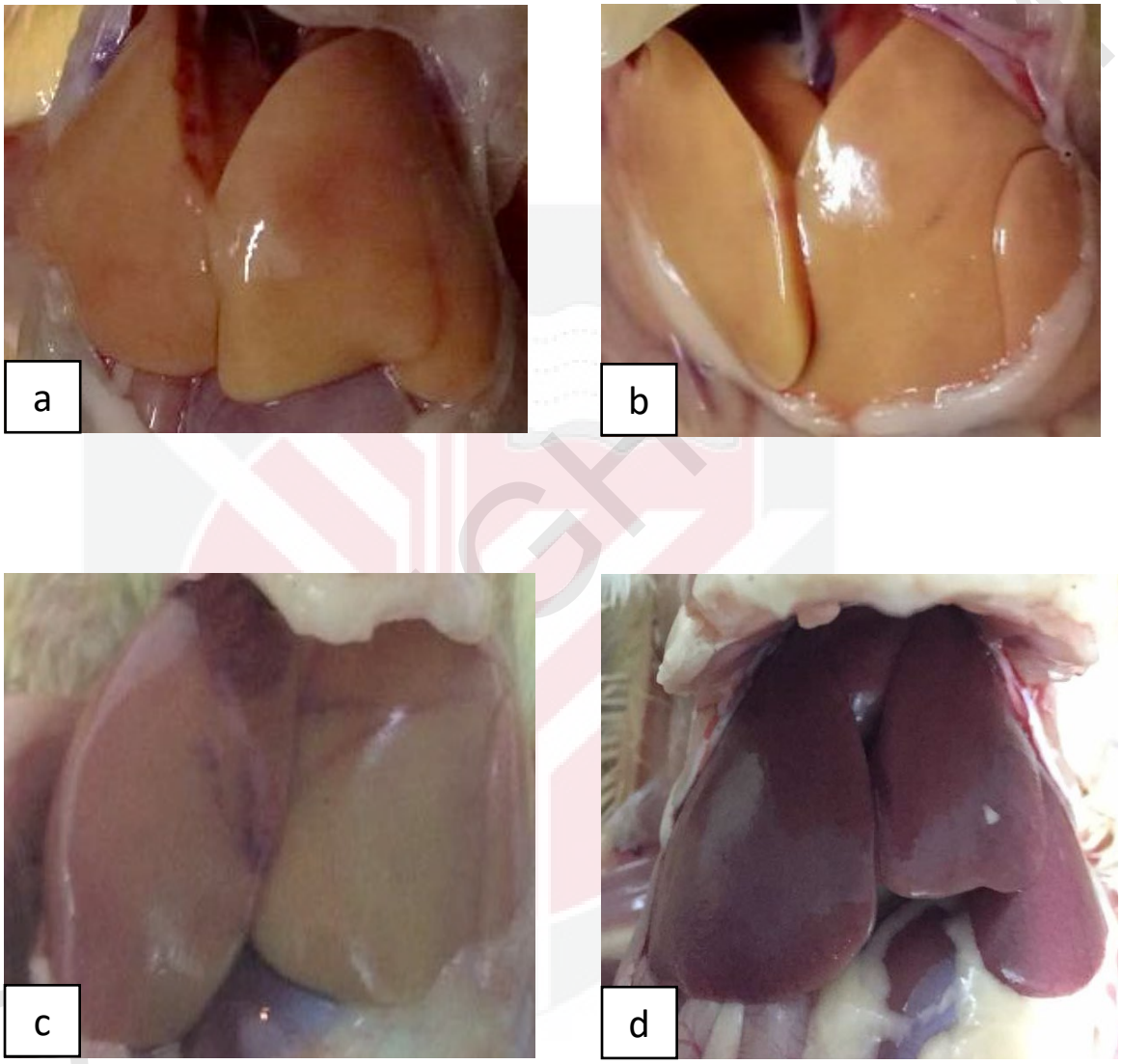

Figure 3: Normal appearance of liver in all groups on; (a) day 1, (b) day 4, (c) day 7, and (d) day 14 pi.

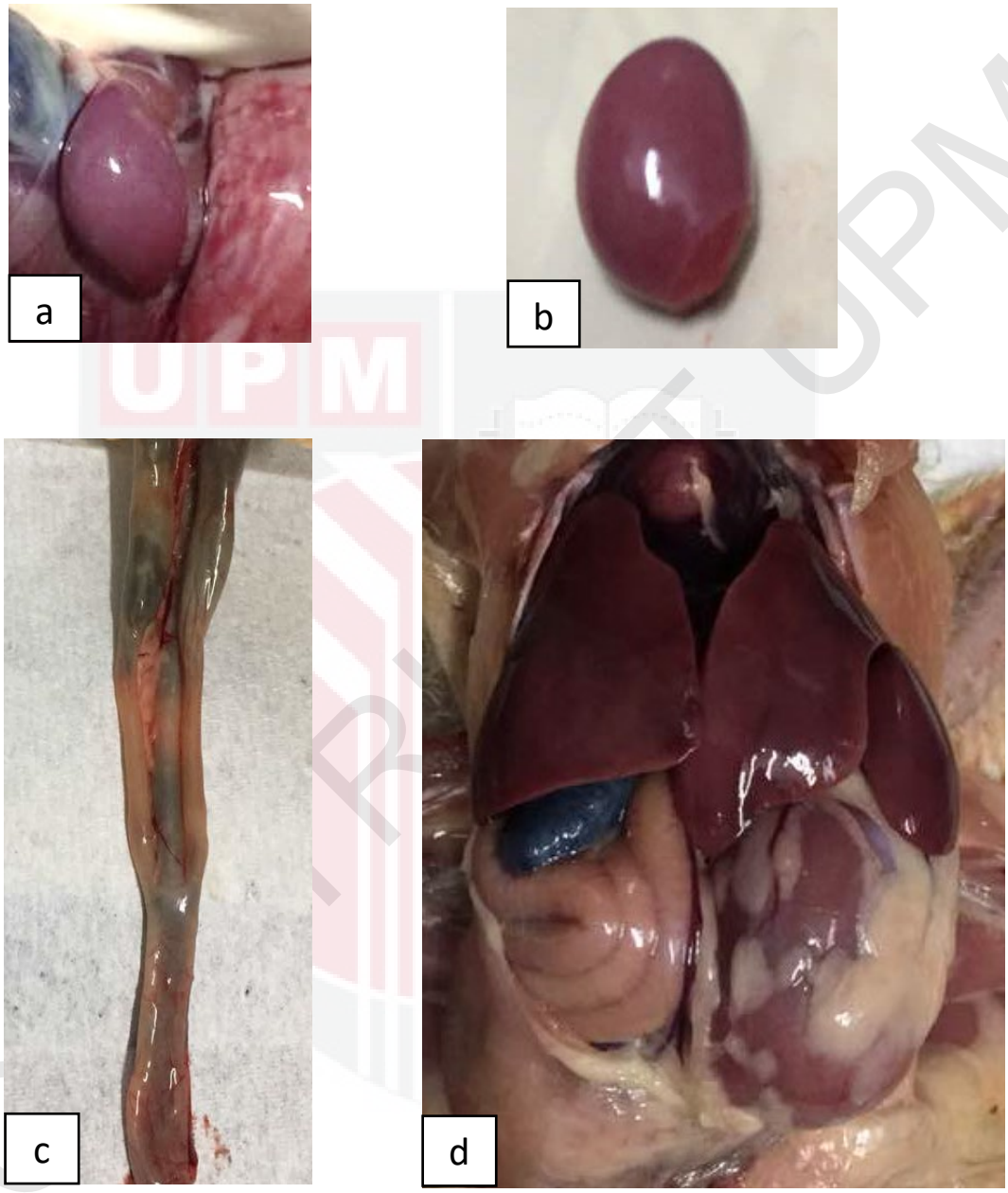

Figure 4: (a) Normal spleen. (b) Splenomegaly of a chick from group B. (c) Normal intestine. (d) Normal visceral organs.

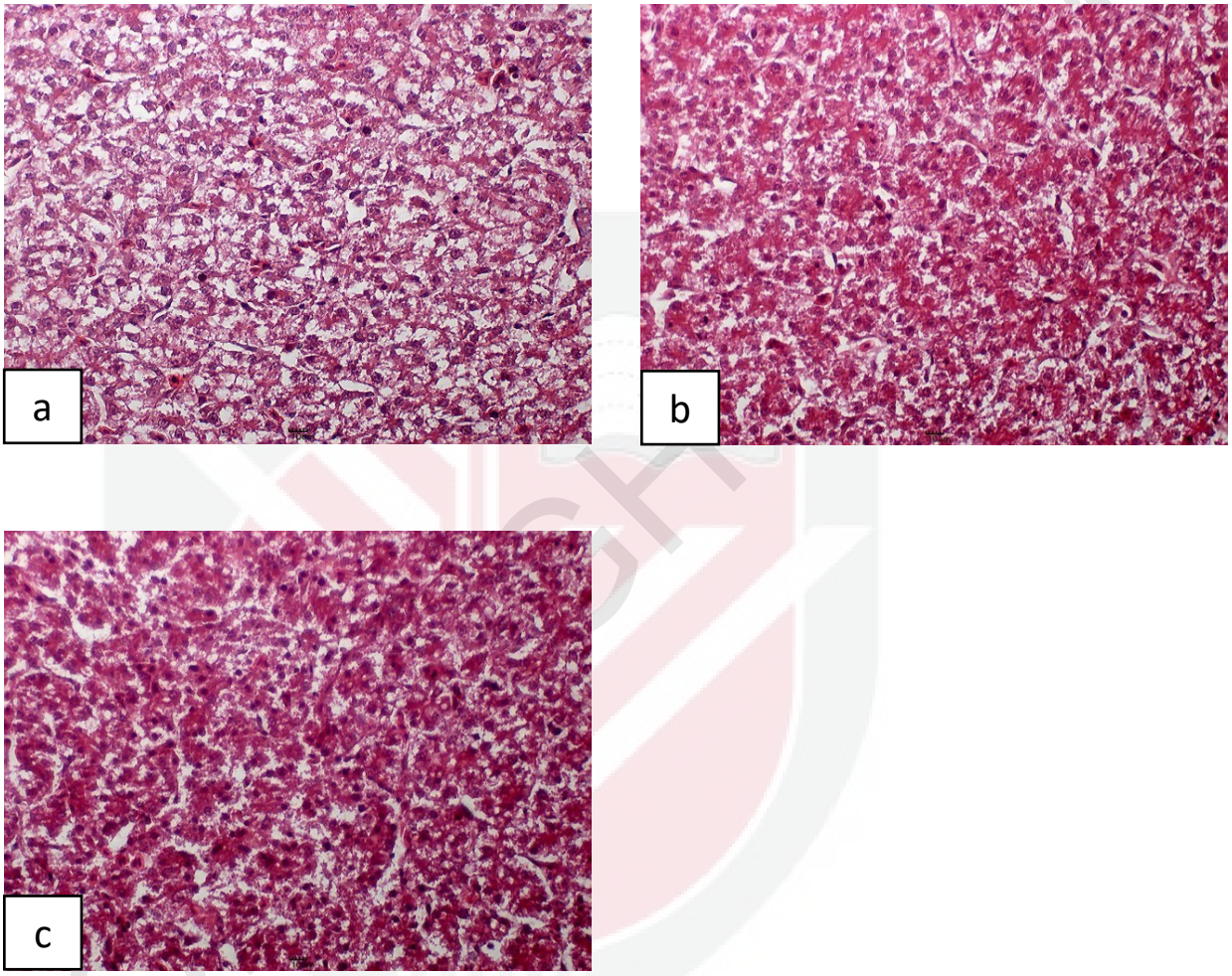

Figure 5: Livers of chicken from groups A, B and C on day 1 pi. Vacuole can be seen due to presence of fat within the cytoplasm of hepatocyte. HE, 40X. Bar= 10µm.

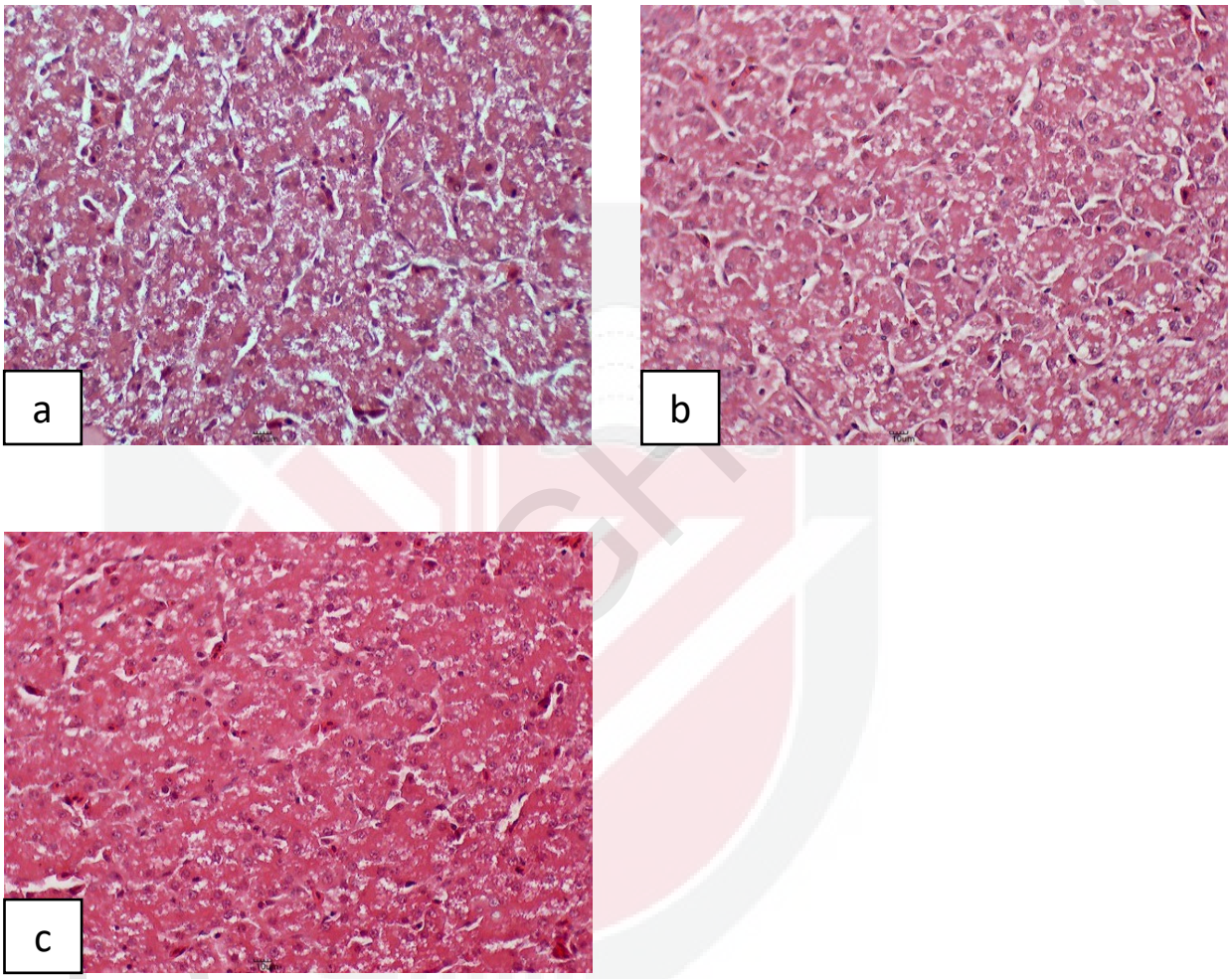

Figure 6: Livers of chicken from groups A, B and C on day 4 pi. No histological lesion was observed. HE, 40X. Bar= 10µm.

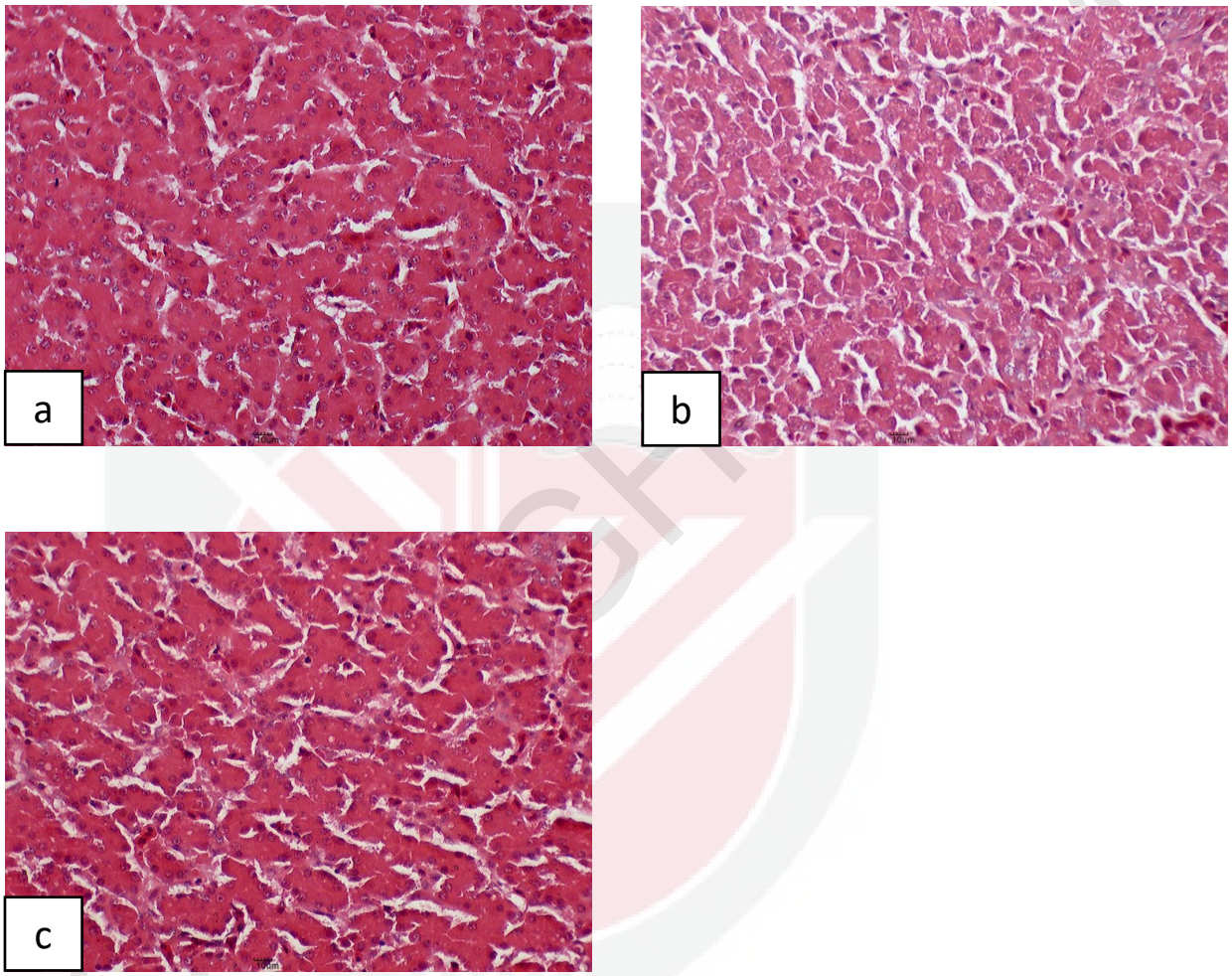

Figure 7: Livers of chicken from groups A, B and C on day 7 pi. No histological lesion was observed. HE, 40X. Bar= 10µm.

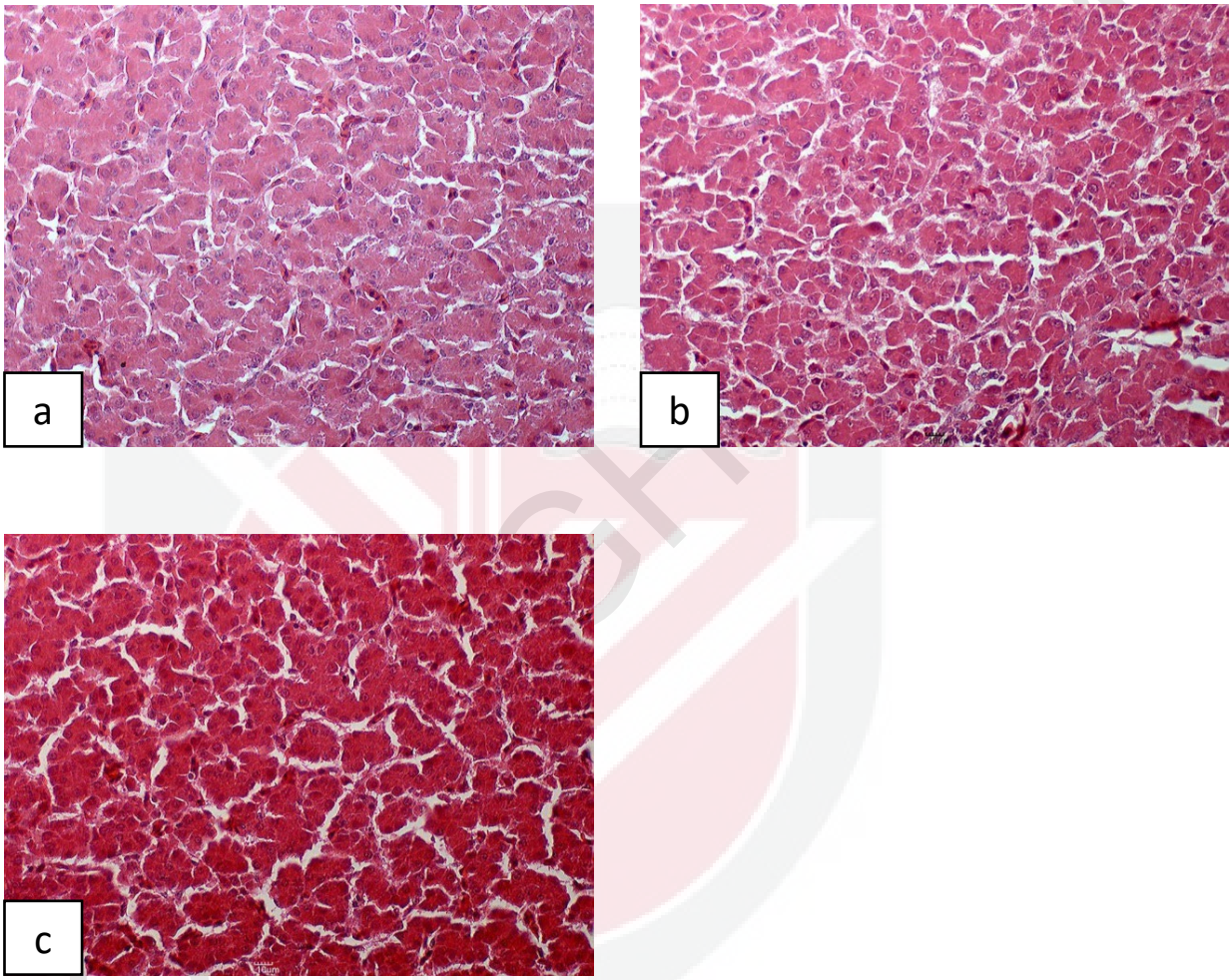

Figure 8: Livers of chicken from groups A, B and C on day 14 pi. No histological lesion was observed. HE, 40X. Bar= 10µm.

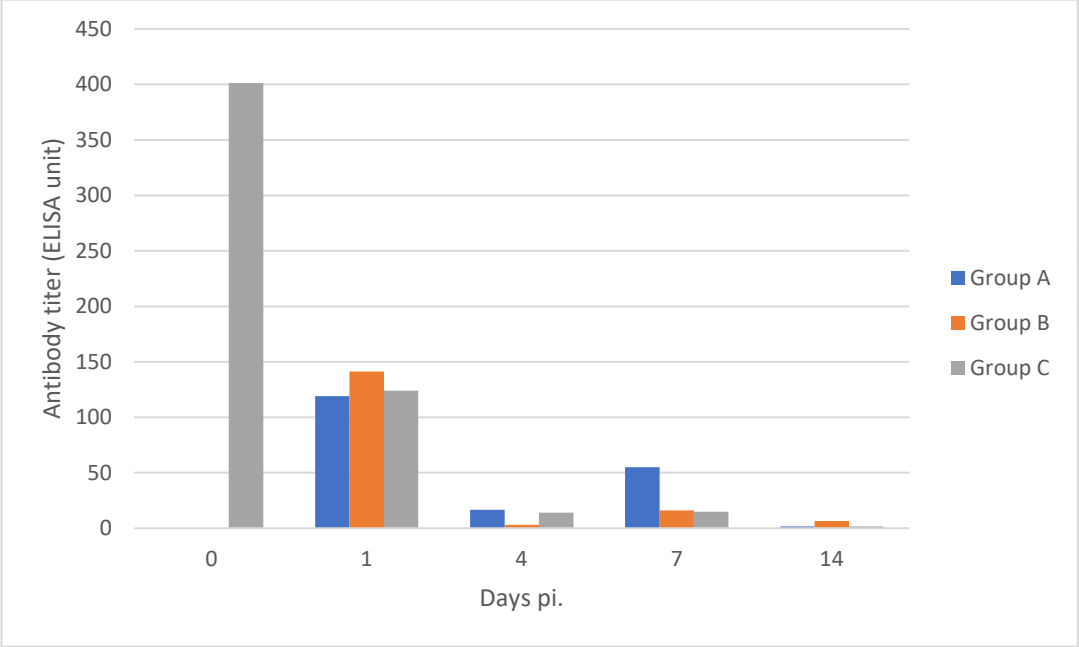

Figure 9: *Salmonella* antibody titre of chickens throughout the trial.

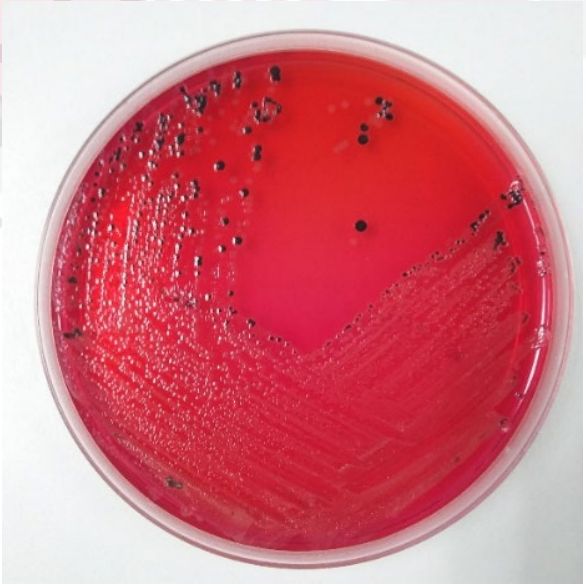

Figure 10: Suspected *Salmonella* colonies with black centre on XLD agar.

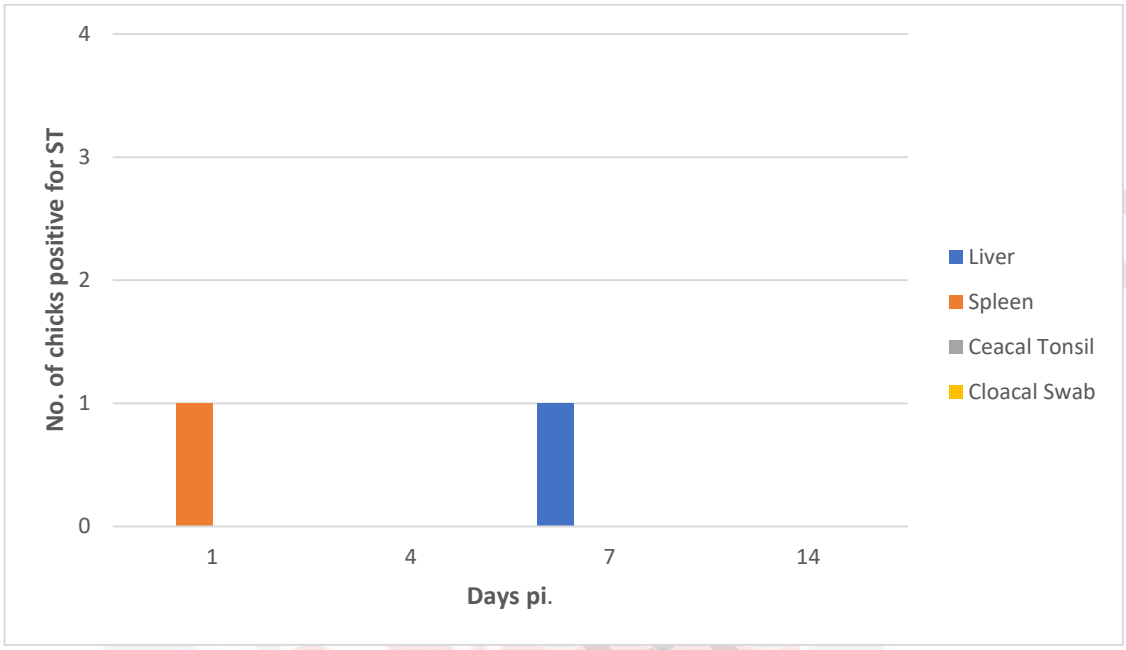

Figure 11: *Salmonella* isolation from chickens in Group A throughout the trial.

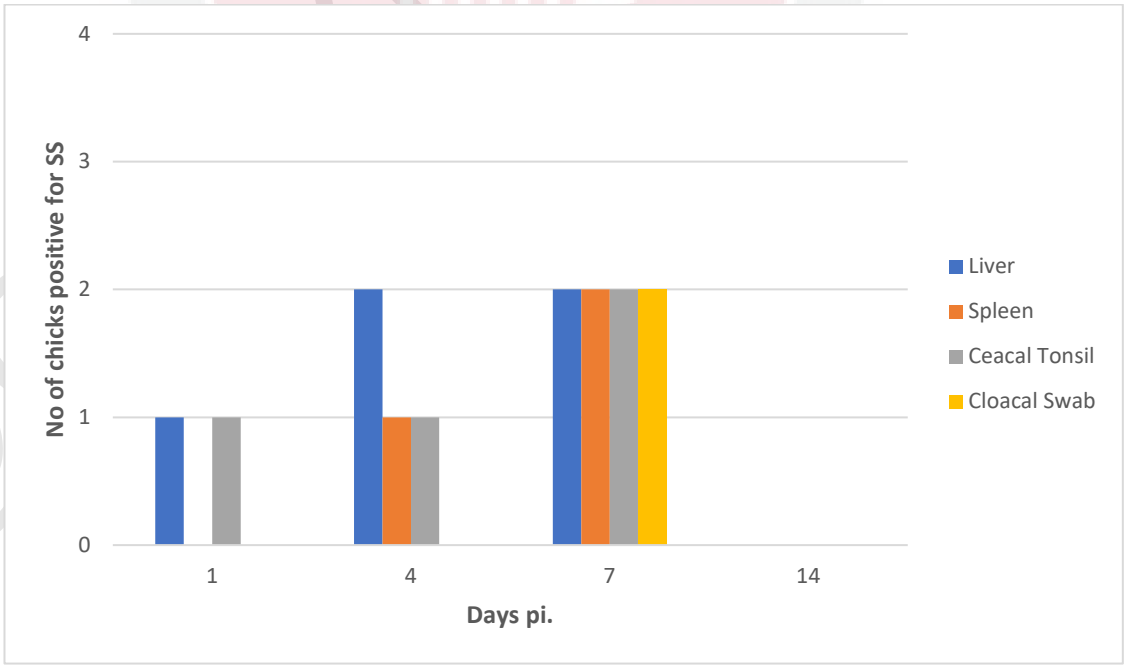

Figure 12: *Salmonella* isolation from chickens in Group B throughout the trial.

5.0 DISCUSSION

In this study, clinical signs seen on *S*. Typhimurium infected chicken was sudden death of 12.5% at day 8 pi. There was no clinical sign shown as reported by Al-Abadi & Al-Mayah (2013) that found chicken to be depressed, anorexia, reluctance to move and recumbent before death and signs of diarrhoea. Mortality rate in present study is supported by Al-Abadi & Al-Mayah (2013) that found mortality rate of 10%. However, Bjerrum et al. (2003) reported mortality rate of less than 2% and asymptomatic chicken that indicate sub clinical infection. On the other hand, clinical signs shown by *S.* Stanley infected chickens was weakness and 12.5% death. However, lack of research can support these findings. Hence, *S*. Typhimurium and *S.* Stanley strain used in this study are able to cause death to infected chickens.

There was no gross lesions recorded for *S*. Typhimurium infected chickens. Lesions such as enteritis, congestion and necrotic foci of liver and enlargement of liver and spleen as reported by Al-Abadi & Al-Mayah (2013) were absent in this study. Nevertheless, gross lesions shown by *S.* Stanley chickens were splenomegaly. This can be explained by intracellular *Salmonella* bacteria that survived despite being phagocytosed by macrophages. It will then multiply and disseminate to the internal organs such as spleen. Acceleration of organ cell proliferation occurred as an instant response to invasion of pathogen, resulting in enlarged organs (Al-Abadi & Al-Mayah, 2013). Thus, gross lesion seen was a result of proliferating infection.

Salmonella Typhimurium was isolated from spleen and liver of chickens at days 1 and 7 pi, respectively, and at day 8 from liver, spleen, caecal tonsil and cloacal swab of dead chicken. This coincides with Bjerrum et al., (2003) that recovered *Salmonella* on

day 1 after exposure to infection. In contrast, *Salmonella* was recovered from liver and caeca from day 3 pi by Al-Abadi & Al-Mayah (2013) and reach peak value at day 7 for all organs, which were liver, spleen and caeca (Bjerrum et al., 2003). Thus, based on this result, it has spread systemically as early as day 1 pi. Systemic invasion, intestine colonization and shedding of organism was seen at day 8 pi as *Salmonella* was isolated from all organs which were liver, spleen, caecal tonsils and cloacal swab at day 8 pi. Meanwhile, *S.* Stanley was isolated from liver and caecal tonsils at days 1, 4 and 7 pi, from spleen at days 4 and 7 pi and from faecal at day 7 pi, while at day 7 was recovered from liver, spleen and caecal tonsils of a dead chicken. Hence, infection has gone systemic and colonized the intestine as early as day 1 based on invasion of liver and caecal tonsils and started to be shed on day 7 pi to the environment through faecal. In addition, death of chicks from both groups can be explained by systemic spread of organism resulting septicaemia (Henderson et al., 1999) and due to burden with organism cause multiple organ failure. This can be observed in this study as the bacteria was isolated from liver, spleen and caecal tonsils. Other than that, low level of systemic infection that resolved through cellular immunity within two-to-three weeks (Wigley, 2014) is the result of clearance of bacteria at day 14 pi shown by negative bacteria isolation. Besides, presence of carrier or sub clinically infected chicken may be explained by bacteria recovery from asymptomatic chickens. Hence, both of these isolates of *S.* Typhimurium and *S.* Stanley are invasive as dissemination to multiple organs and shedding to the environment was recorded.

In this study, *Salmonella* antibody titre was seen to decline throughout the trials. However, previous study reported serum antibody produced by most birds by 7 days

pi (Gast, 2013; Ishola & Holt, 2008). Gast (2013) also added it will reach peak value at 2 week pi although Skov et al., (2002) reported increased in antibody response dramatically after 3 weeks. Antibody titre seen in this study can be explained by maternal antibody received by the chicks from the immunized hens through transfer in egg yolk as a result of vaccination or previous exposure of salmonellosis (Gast, 2013; McMullin, 2004). Other than that, maternal immunity is among the difficulties encountered in infecting young chicks (Marcq et al., 2011). Marcq et al., (2011) also mentioned chicks do not elicit antibody responses until after 2 to 3 weeks of age. In this study, induction of *S.* Stanley antibody titre at day 14 pi was seen but was not recorded for *S.* Typhimurium. This can be due to more pathogenic bacteria that are able to cause immune reaction to the host. Therefore, presence of maternal antibody might interfere with the ongoing infection.

Salmonella serotype, strains and dose of infection can differ greatly in pathological characteristic effects in poultry as it influences invasiveness and mortality in chickens (Bjerrum et al., 2003; Gast, 2013). Based on the clinical signs, gross lesions, bacteria isolation and antibody titre, both isolates of *S.* Typhimurium and *S.* Stanley are pathogenic in broiler chickens following inoculation at day old of age. However, *S.* Stanley is more pathogenic in chicken than *S.* Typhimurium with clinical sign of weakness and earlier death, gross lesion of splenomegaly, bacteria isolation from multiple organs and induction of *Salmonella* antibody titre.

6.0 CONCLUSIONS

Salmonella Typhimurium and *S*. Stanley are pathogenic in broiler chicken because of the clinical signs and invasiveness shown. Clinical signs for *S*. Typhimurium was sudden death while *S*. Stanley cause weakness and death. *Salmonella* Typhimurium and *S*. Stanley can be isolated from liver, spleen, caecal tonsils and cloacal swab of chicken with high frequency of *S*. Stanley when compare with *S*. Typhimurium. Besides, *S*. Stanley isolate is more pathogenic in chickens than *S*. Typhimurium.

7.0 RECOMMENDATIONS

Specific pathogen free chicken can be used to avoid the interference of maternal antibody in order to obtain more precise result on the pathogenicity. Next, is to prolonged duration of study as *Salmonella* antibody titre might increase after two weeks of infection. Besides, different various level of colony forming unit should be consider as the dose of infection can differ in pathological characteristics.

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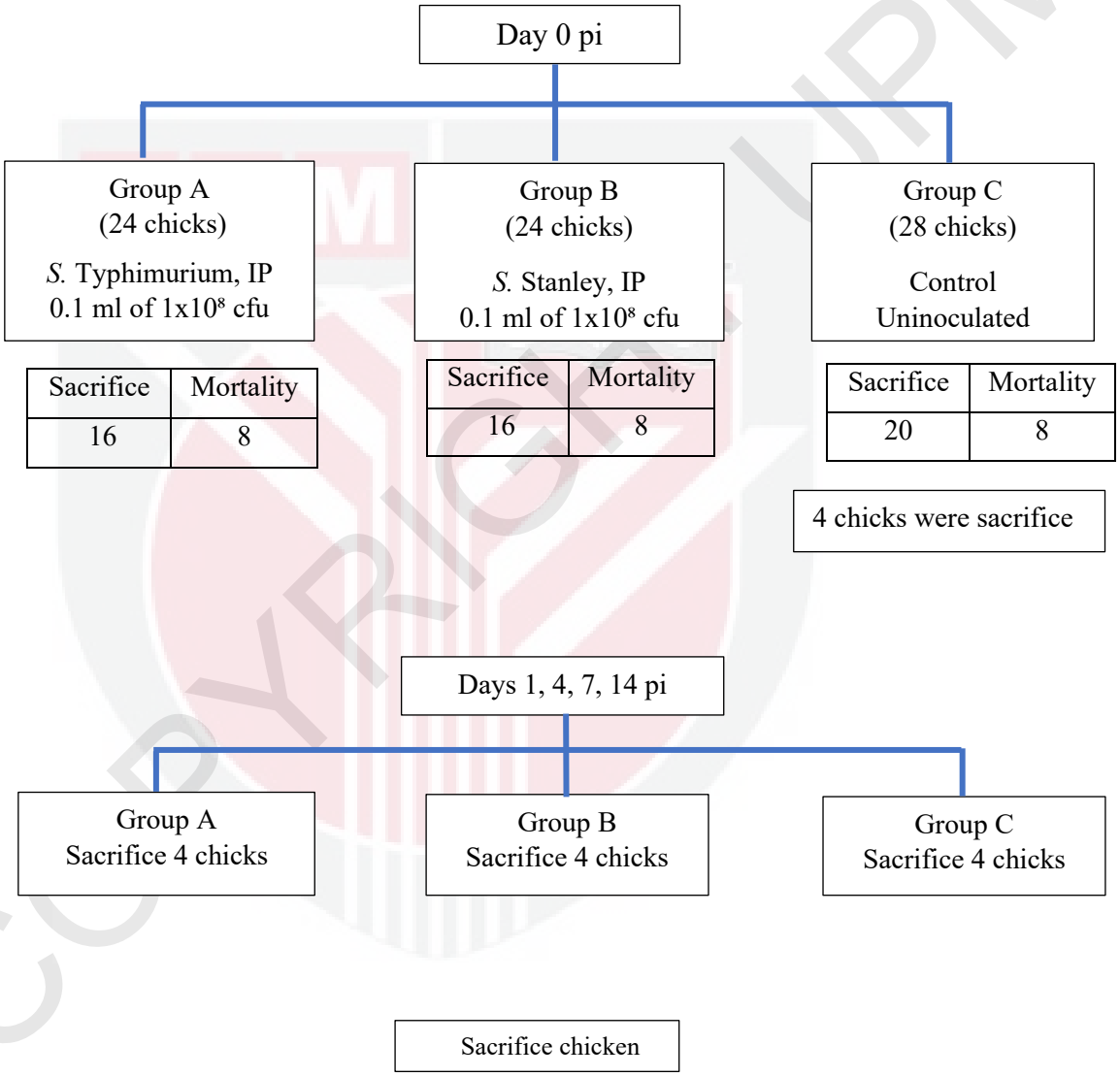
APPENDIX 1

Experimental design for pathogenicity of *Salmonella* Typhimurium and *Salmonella* Stanley Isolates in Chickens



- Record body weights
- Blood samples collection to collect serum for ELISA

On necropsy,

- Gross lesion was recorded
- Samples of liver was fixed in 10% buffered formalin for histological examination
- Samples of liver, spleen, caecal tonsils and cloacal swabs were collected for bacterial isolation and identification

APPENDIX 2

Body weight of the chickens throughout the trial.

Day p.i.	Body weight mean (g) ± SEM		
	Group A	Group B	Group C
0	51.8±0.75		
1	71.0±3.08 [a,b]	76.0±2.48 [a]	63.3±2.66 [b]
4	141.0±6.28 [a]	124.5±11.71 [a]	136.3±7.12 [a]
7	176.0±8.44 [a]	198.8±18.98 [a]	193.3±25.03 [a]
14	503.3±15.42 [a]	534.3±20.91 [a]	512.5±7.71 [a]

Each value is the body weight mean±SEM of 4 chickens from each group.

[a,b] Different superscripts indicate significant difference ($p<0.05$).

APPENDIX 3

Antibody titre of chickens throughout the trial.

Day p.i.	Antibody titre mean ± SEM		
	Group A	Group B	Group C
0	401.3±208.16		
1	119.0±67.34 [a]	141.3±119.63 [a]	124±66.25 [a]
4	16.8±2.95 [a]	3.0±1.15 [b]	14±1.41 [a]
7	55.0±39.50 [a]	16.0±7.88 [a]	15±8.72 [a]
14	1.5±0.50 [a]	6.5±1.94 [b]	1.5±0.50 [a]

Each value is the antibody titre mean±SEM of 4 chickens from each group.

[a,b] Different superscripts indicate significant difference ($p<0.05$).

APPENDIX 4

Bacteria isolation from sacrificed chickens on days 1, 4, 7 and 14 pi.

4.1 Group A (*S.* Typhimurium)

Day pi	Chick ID	Liver	Spleen	Caecal Tonsil	Cloacal Swab
1	1	Negative	Negative	Negative	Negative
	2	Negative	Negative	Negative	Negative
	3	Negative	Positive	Negative	Negative
	4	Negative	Negative	Negative	Negative
4	5	Negative	Negative	Negative	Negative
	6	Negative	Negative	Negative	Negative
	7	Negative	Negative	Negative	Negative
	8	Negative	Negative	Negative	Negative
7	9	Negative	Negative	Negative	Negative
	10	Negative	Negative	Negative	Negative
	11	Positive	Negative	Negative	Negative
	12	Negative	Negative	Negative	Negative
14	13	Negative	Negative	Negative	Negative
	14	Negative	Negative	Negative	Negative
	15	Negative	Negative	Negative	Negative
	16	Negative	Negative	Negative	Negative

4.2 Group B (*S.* Stanley)

Day pi	Chick ID	Liver	Spleen	Caecal Tonsil	Cloacal Swab
1	25	Negative	Negative	Negative	Negative
	26	Negative	Negative	Negative	Negative
	27	Negative	Negative	Negative	Negative
	28	Positive	Negative	Positive	Negative
4	29	Positive	Negative	Negative	Negative
	30	Negative	Negative	Negative	Negative
	31	Positive	Positive	Positive	Negative
	32	Negative	Negative	Negative	Negative
7	33	Negative	Negative	Positive	Negative
	34	Negative	Negative	Negative	Negative
	35	Positive	Positive	Negative	Positive
	36	Positive	Positive	Positive	Positive
14	37	Negative	Negative	Negative	Negative
	38	Negative	Negative	Negative	Negative
	39	Negative	Negative	Negative	Negative
	40	Negative	Negative	Negative	Negative

4.3 Group C (Control)

Day pi	Chick ID	Liver	Spleen	Caecal Tonsil	Cloacal Swab
0	49	Negative	Negative	Negative	Negative
	50	Negative	Negative	Negative	Negative
	51	Negative	Negative	Negative	Negative
	52	Negative	Negative	Negative	Negative
1	53	Negative	Negative	Negative	Negative
	54	Negative	Negative	Negative	Negative
	55	Negative	Negative	Negative	Negative
	56	Negative	Negative	Negative	Negative
4	57	Negative	Negative	Negative	Negative
	58	Negative	Negative	Negative	Negative
	59	Negative	Negative	Negative	Negative
	60	Negative	Negative	Negative	Negative
7	61	Negative	Negative	Negative	Negative
	62	Negative	Negative	Negative	Negative
	63	Negative	Negative	Negative	Negative
	64	Negative	Negative	Negative	Negative
14	65	Negative	Negative	Negative	Negative
	66	Negative	Negative	Negative	Negative
	67	Negative	Negative	Negative	Negative
	68	Negative	Negative	Negative	Negative