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Research article

Quantitative distribution and interaction of *Salmonella* Zega with host cells in visceral organs of chickens infected orally, intraperitoneally and per cloaca

Fakilahyel Musa Mshelbwala [a,*], Najume Doguwar Giginya Ibrahim [b], Shehu Naallah Saidu [c], Emmanuel Gbenga Babatunde [a], Amina Kinta Fakilahyel Kadiri [c], Funmilola Clara Thomas [d], Clara Nna Kwanashie [e], Michael Agbaje [f]

[a] *Department of Veterinary Pathology, Federal University of Agriculture, Abeokuta, Nigeria*
[b] *Department of Veterinary Pathology, Ahmadu Bello University, Zaria, Nigeria*
[c] *Department of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria*
[d] *Department of Veterinary Physiology, Pharmacology and Biochemistry, Federal University of Agriculture, Abeokuta, Nigeria*
[e] *Department of Veterinary Microbiology, Ahmadu Bello University, Zaria, Nigeria*
[f] *Department of Veterinary Microbiology, Federal University of Agriculture, Abeokuta, Nigeria*

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ABSTRACT

Immunohistochemical study of the visceral organs of chickens experimentally infected with *Salmonella* Zega by three routes was carried out to compare the quantitative distribution and interaction of the organism with host cells. 100 birds comprising of 2 week-old chickens were divided into 4 groups of 25 each. Group A was inoculated orally, group B intraperitoneally, group C were administered per cloaca and D were not inoculated and served as control. All the infected birds were inoculated with 0.2 ml of 1×10^8 cfu of the bacteria. Two birds from each group were sacrificed every 24 h post infection. Samples of visceral organs were collected for immunohistochemistry. The distribution of *Salmonella* Zega in every organ was taken as Mean ± SD of the number of foci of immunoreactions and Compared using a 2-way ANOVA. The interaction of *Salmonella* Zega with host cells was determined by taking the percentage of the days post infection in which immunoreactions were detected in host cells in each route of infection. The distribution of the organism was highest in the lung of intraperitoneally infected chickens (83.95 ± 27.89) and lowest in the heart (5.21 ± 3.65) of chickens that were infected per cloaca. The highest percentage of interaction of *Salmonella* Zega was recorded in the epithelial (100%) and blood (100%) cells in all the routes of infection. There were variations in the distribution of *Salmonella* Zega in visceral organs of chickens but the level of interactions with host cells were similar even when infected through different routes.

1. Introduction

Salmonella species are tissue invasive and acquire multiple virulence genes for intercellular survival and production of Vi antigen for interaction and penetration of mucous epithelium for systemic infection (Allen-vercoe et al., 1999; Hasen-wester and Hensel, 2001; Hensel, 2006). The molecular basis describing *Salmonella* invasion and pathogenesis in poultry is not well defined. However, it is suggested that systemic *S.* Gallinarum shows trophism to lymphoid tissues, such as payer's patches and caecal tonsils, and can cross the gut during the early stages of fowl typhoid and subsequently enter the intestinal lymphoid tissues (Lowry et al., 1999). For systemic infection to be established, it has been suggested that *Salmonella* is trafficked within macrophages and dendritic cells to systemic organs such as liver and spleen where they multiply and become disseminated to other organs. The *Salmonella* must survive inside the macrophages and dendritic cells for it to establish systemic disease. A number of mechanisms are involved in mediating the survival of *Salmonella* organism within the infected cell, including SPI-2 encoded proteins that inhibit the normal maturation of phagosome to form an inhibitory *Salmonella* containing vacuole. This enables the bacteria to survive, persist, replicate and produce systemic infection (Hensel, 2000; Galan, 2001; Jones et al., 2001, 2007; Wigley et al., 2002; Cheminay

* Corresponding author.
E-mail address: mshelbwalafakilahyel@yahoo.com (F.M. Mshelbwala).

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et al., 2005). The clinical signs, pathological and immunohistochemical findings in visceral organs of chickens experimentally infected with *S.* Zega has been described recently (Mshelbwala et al., 2019), where the severity of pathological findings correlated with that of immunoreactions, but the quantitative distribution of the organism in such organs have not been determined. Exploring its tissue distribution and interactions with host cells in birds will provide information on its pathogenesis and pathogenicity in avian species and will determine the preferred organs to be collected at postmortem for its isolation during outbreaks. Therefore, the present study compared the distribution and interaction of *Salmonella* Zega with host cells in visceral organs of chickens experimentally infected orally, intraperitoneally and per cloaca, using immunohistochemical technique.

2. Materials and methods

2.1. Ethical aproval

Ethical approval regarding the use of experimental animals was obtained from the ethical committee on experimental animals of the Ahmadu Bello University, Zaria. The experiment conforms to the regulatory standard of the Ahmadu Bello University, Zaria; whose guidelines met that of the council for the international organization for Medical Sciences and the international council for laboratory animal science.

2.2. Source of birds and bacteria (Salmonella Zega)

Day-old pullets were purchased from a certified hatchery (Agritet®, Yola, Adamawa State, Nigeria) and raised to 2 weeks using deep litter management system, in the Poultry Pen of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria. They were considered *Salmonella* free, when no growth of the organisms was observed from 3 consecutive cloaca swab culture at 24 h interval.

The *Salmonella Zega* used in the present study was isolated during a previous study (Mshelbwala et al., 2017). The isolates were serotyped at the World Organization for Animal Health/OIE, Reference Laboratory for *Salmonella*, Istuto Zooprofilatico Sperimentale Dellevenzie, Padova Italy using the White-Kauffman-Le Minor Scheme (Grimont and Weill, 2007).

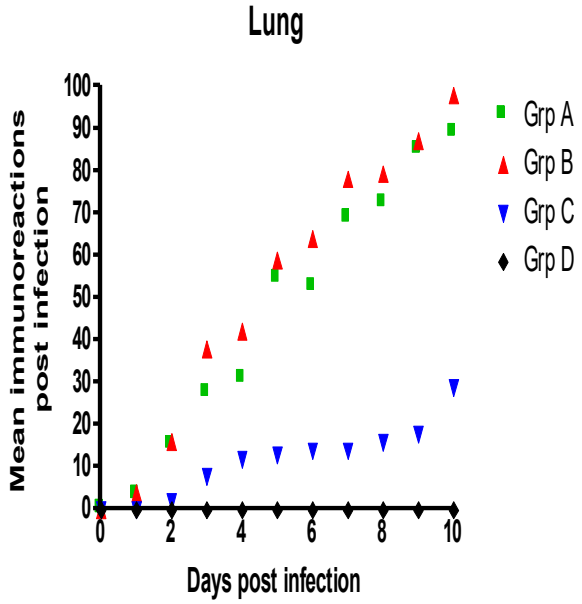

Figure 1. Graph of immunoreactions in the lung according to route of infection and days post infection.

2.3. Standardization of the inoculation dose of Salmonella Zega

Eight hundred million Colony-forming units of the *Salmonella* Zega was determined using sensititre nephelometer (TREK Diagnostic Systems, UK) as prescribed by the Clinical and Laboratory Standard Institution (2006, 2010) and Goldman et al. (2008).

2.4. Experimental design

A total of 100 Isa brown chicks (pullets; 2 week-old) were grouped into four; A, B, C and D. Each group consists of 25 birds each. Groups A, B, and C were administered 0.2 ml of 1 x 10^8 colony forming unit of the *Salmonella* Zega per os, intraperitonealy and per cloaca, respectively. Group D was not inoculated with the organism and served as control. All the groups were fed commercial poultry feed and administered sterile clean water *ad-libitum*.

Two birds from each group were sacrificed on day zero before infection and at 24 h interval post infection (pi) from day 1 to day 10 post infection. Postmortem examination were carried out and tissue samples of the visceral organs were collected and fixed in 10% buffered formalin for immunohistochemical evaluation to determine the distribution and interaction of the organism with host cells in visceral organs.

2.5. Procedure for immunohistochemistry

Tissue sections were processed and stained with *Salmonella* polyclonal antibody using the standard immunohistochemistry method; Avidin Biotin Streptavidin Peroxidase Complex (ABC) method (Shi et al., 1999; Myers, 2004; Rasmo-vera, 2005). The procedure was also described by Mshelbwala et al. (2018).

2.6. Organ-specific distribution of pathogens

Immunoreactions in the visceral organs were examined using light microscope and four micrographs of non-overlapping field of views were randomly taken from two birds that were sacrificed. The number of foci in each field of view, according to the route of infection and days pi were recorded. The amount of immunoreactions in each organ was determined by taking the means of the number of foci of immunoreactions from the four microscopic fields. The severity of the immunoreactions was scored according to the method described by Wild et al. (2002).

2.7. Interaction of pathogens with host cells

The rate of interaction of *Salmonella* Zega with host cells was determined by taking the percentage of the days post infection in which immunoreactions were detected in host cell-type in each route of infection according to days pi.

2.8. Data analysis

The distribution of *Salmonella* Zega in every organ was taken as Mean $\pm$ SD of the number of foci of immunoreactions per 4 microscopic fields. Comparisons of the distribution of the organism in the visceral organs were done by evaluating the difference between means in each route of infection and according to days pi, using 2-way ANOVA.

3. Results

3.1. Distribution of Salmonella Zega in visceral organs

The severity of immunoreactions in each organ increased significantly ($P > 0.05$) with the days post infection (Figures 1, 2, 3, 4, 5, 6, 7, and 8) and were too numerous to count by day 10 of the experiment. The overall mean immunoreactions for the different organs varied with the type of organ and the route of infection. The lungs showed the highest

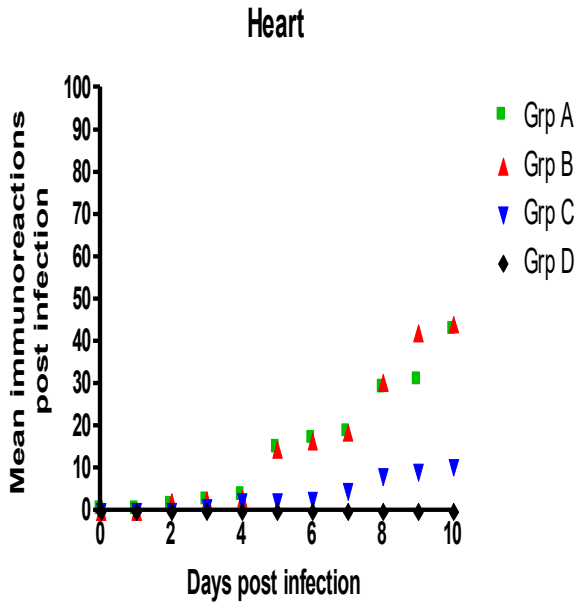

Figure 2. Graph of immunoreactions in the heart according to route of infection and days post infection.

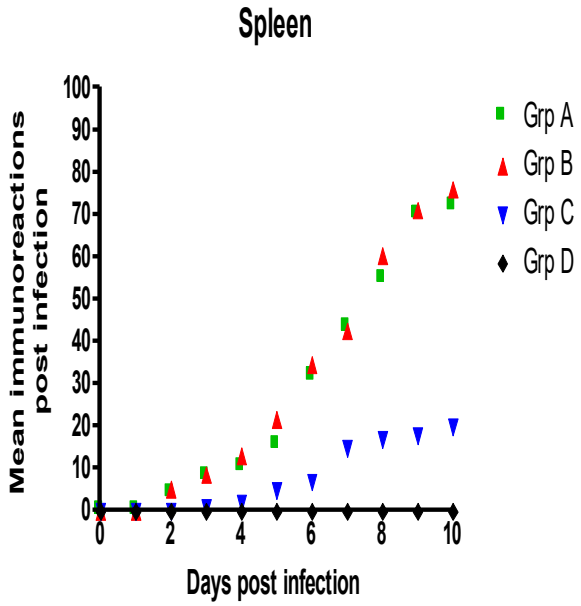

Figure 4. Graph of immunoreactions in the spleen according to route of infection and days post infection.

overall mean immunoreactions amongst the visceral organs in intraperitoneally (83.55 ± 27.89) and orally (77.52 ± 27.67) ($P > 0.05$) infected chicks. The liver, spleen, intestine and caecum showed relatively high mean immunoreactions in chicks infected orally and intraperitoneally while the heart had the lowest ($P > 0.05$) mean immunoreactions in all the routes of infection. In chicks infected per cloaca, mean immunoreactions were highest in the caecum (18.74 ± 11.31) ($P > O.O5$). However, the mean immunoreactions in all the visceral organs among treated groups were lowest in chicks infected per cloaca. There was no significant difference ($P > 0.05$) in the mean immunoreactions in the visceral organs between birds that were infected orally and intraperitoneally, except in the lung; but there were significant difference ($P > 0.05$) between birds infected per cloaca compared to oral or intraperitoneal inoculations (Table 1).

3.2. *Interaction of Salmonella Zega with host cells*

Immunoreactions were observed in different host cells. The rate of interaction varied with the type of cell. The highest percentage of interaction of *Salmonella* Zega was recorded in the epithelial cells of the lung, kidney, proventriculus, small intestine and caecum (100%) and blood cells (red blood cells, monocytes) (100%), 24 h post infection, in oral and intraperitoneal routes of infection. Immunoreactions were observed in the endothelial cells 2 days post infection in oral and intraperitoneal routes in the lung, liver, kidney, small intestine and caecum. In the lung, the reactions were confined to the epithelial cells of the air sacs (pneumocytes), blood cells (red blood cells and monocytes) and endothelial cells 2 days post infection in oral and intraperitoneal routes (Table 2). Immunoreactions spread to the interstitium from day 3 and 4

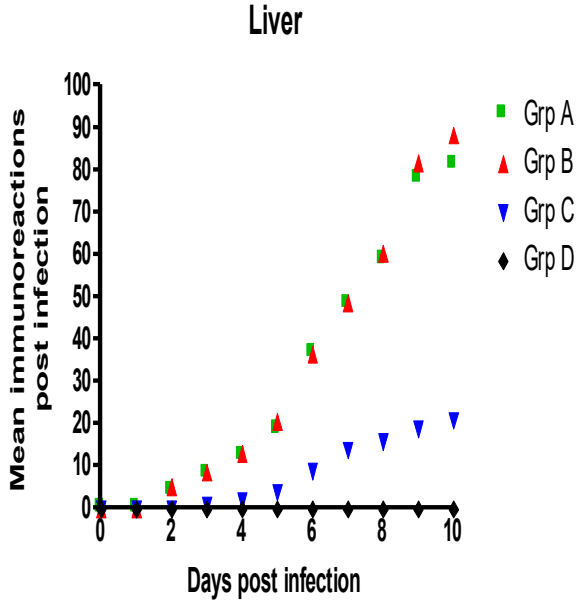

Figure 3. Graph of immunoreactions in the liver according to route of infection and days post infection.

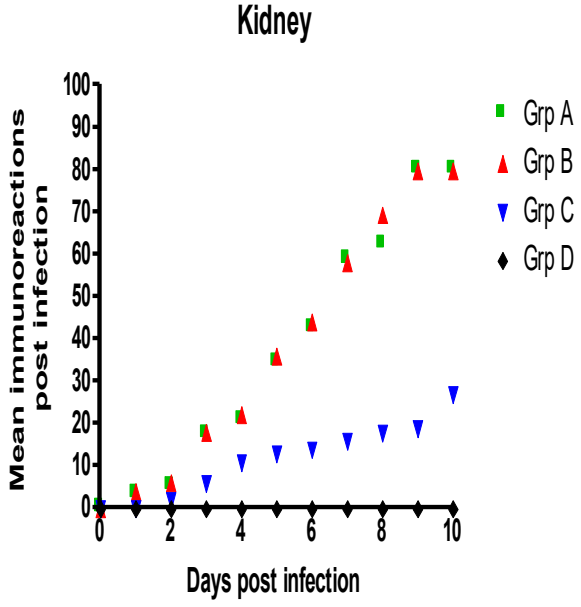

Figure 5. Graph of immunoreactions in the kidney according to route of infection and days post infection.

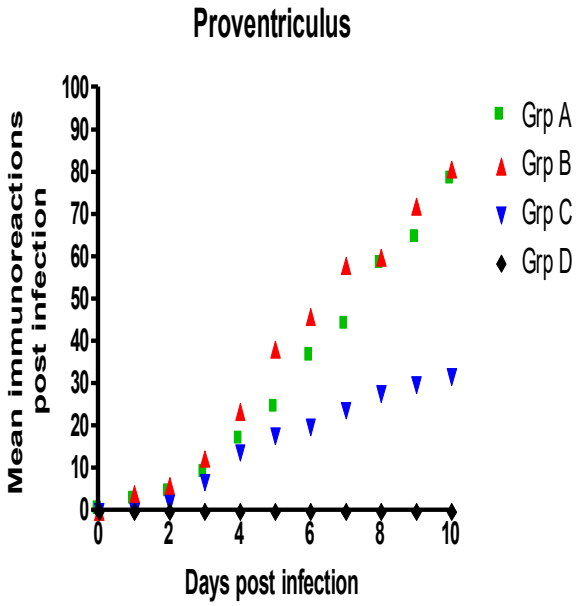

Figure 6. Graph of immunoreactions in the proventriculus according to route of infection and days post infection.

post infection, which were observed in macrophages and lymphocytes. The reactions were severe by 7–10 days post infection, affecting all cell types including inflammatory and resident cells of the lung (Figure 9). Immunoreactions in hepatocytes were observed 3 days post infection in oral and intraperitoneal routes and became severe from 8 to 10 days post infection (Figure 10). Immunoreactions were also observed in macrophages and lymphocytes in the liver and spleen 3 and 4 days post infection respectively and became severe by day 7 till the end of the experiment. Immunoreactions were observed in myocytes from 4 days post infection in chicks infected orally and intraperitoneally and progressed till the end of the experiment. Immunoreactions in heterophils were first observed in the lung and liver 4 days post infection in orally and intraperitoneally infected chicks. In the spleen and kidney,

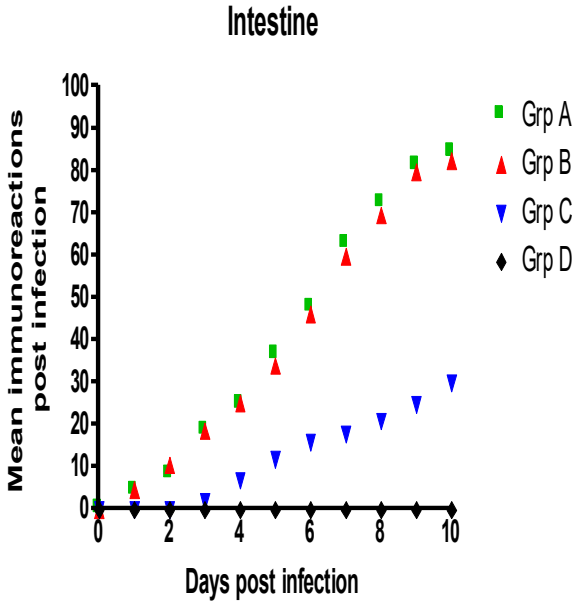

Figure 7. Graph of immunoreactions in the small intestine according to route of infection and days post infection.

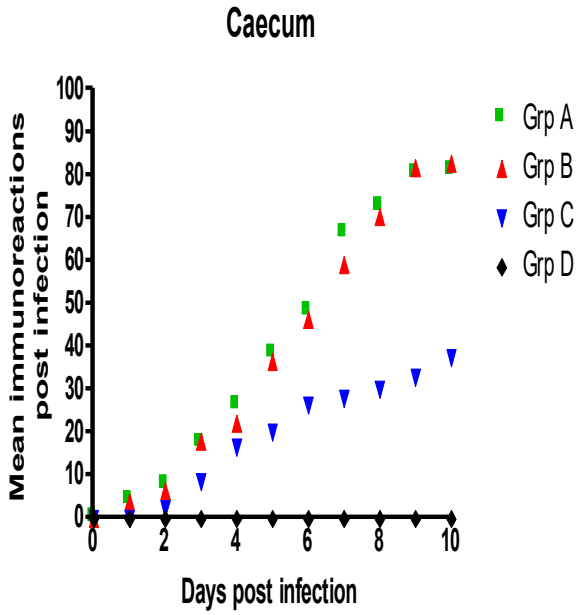

Figure 8. Graph of immunoreactions in the caecum according to route of infection and days post infection.

Table 1. Mean ± Std. Organ-specific Distribution of *Salmonella* Pathogens in experimentally induced *S.* Zega infection in 3 week-old chicks.

S/N	Organ	Mean ± Std According to Group			
		Group A	Group B	Group C	Group D
1.	Lung	77.52 ± 27.67^{b}	83.95 ± 27.89^{a}	14.29 ± 7.49^{c}	–
2.	Heart	17.63 ± 14.31^{e}	19.55 ± 16.30^{e}	5.21 ± 3.65^{g}	–
3.	Liver	66.37 ± 26.52^{c}	67.17 ± 26.24^{c}	11.21 ± 7.92^{f}	–
4.	Spleen	62.04 ± 23.16^{c}	64.14 ± 22.82^{c}	11.08 ± 7.75^{f}	–
5.	Kidney	40.52 ± 28.89^{d}	41.95 ± 29.09^{d}	14.28 ± 7.51^{f}	–
6.	Proventriculus	40.52 ± 28.89^{d}	41.95 ± 29.09^{d}	13.06 ± 5.91^{f}	–
7.	Small intestine	62.82 ± 30.02^{c}	61.75 ± 29.14^{c}	15.92 ± 8.66^{f}	–
8.	Caecum	63.62 ± 30.19^{c}	62.05 ± 29.41^{c}	18.74 ± 11.31^{e}	–

Values with the same superscripts are not significantly different ($P > 0.05$).

immunoreactions were observed in lymphocytes and macrophages from 4 days post infection (Figures 11 and 12).

In the per cloaca route of infection, immunoreactions were observed in the epithelial cells of the caecum 48 h post infection, but reactions in other organs were observed 5 days post infection. The least interaction was recorded in heterophils after 7 days pi (30%) and myocytes 6 days post infection (40%) in birds infected per cloaca.

Table 2. Duration of Interaction of *Salmonella* Zega with host cells according routes of infection and days post infection.

S/N	Host cells	Routes of infection/duration of interaction in days (%)		
		Oral	Intraperitoneal	Per cloaca
1.	Epithelial cells	1-10 (100)	1-10 (100)	1-10 (100)
2.	Myocytes	5-10 (60)	4-10 (70)	8-10 (30)
3.	Hepatocytes	3-10 (80)	3-10 (80)	6-10 (50)
4.	Macrophages	3-10 (80)	3-10 (80)	6-10 (50)
5.	Lymphocytes	4-10 (70)	4-10 (70)	7-10 (40)
6.	Heterophils	4-10 (70)	4-10 (70)	8-10 (30)
7.	Endothelial cells	2-10 (90)	1-10 (90)	4-10 (70)
8.	Blood cells	2-10 (90)	1-10 (100)	5-10 (60)

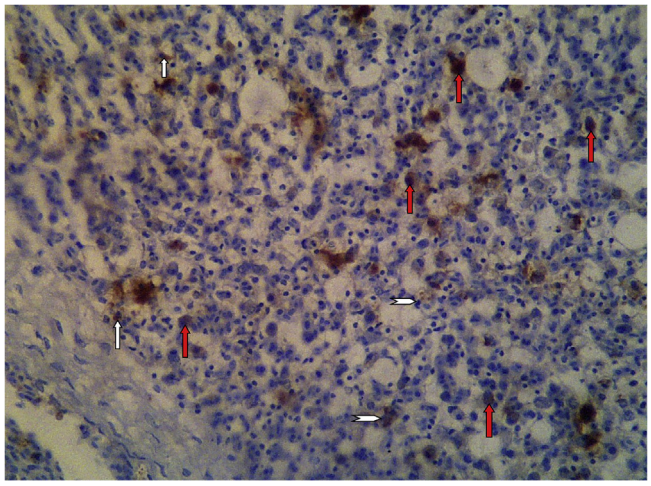

Figure 9. Section of the lung of 3 week-old chick infected intraperitoneally with *Salmonella* Zega showing immunoreactions in heterophils (arrow heads), lymphocytes (white arrows) and macrophages (red arrows) 8 days post infection (x400; Streptavidin peroxidase, counterstained with Haematoxylin).

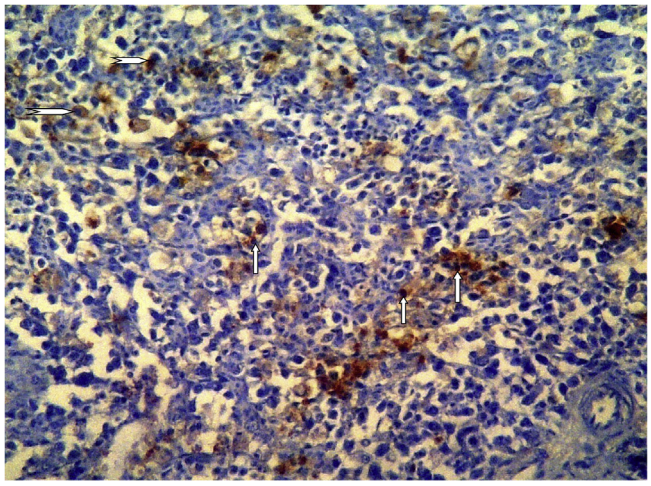

Figure 11. Section of the spleen of 3 week-old chick infected orally with *Salmonella* Zega showing immunoreactions in lymphocytes (arrows)and macrophages (arrow heads) 10 days post infection (x400; Streptavidin peroxidase, counter stained with Haematoxylin).

4. Discussion

The distribution and interaction of *Salmonella* pathogen with host cells in systemic organs in chicken is central in understanding the pathogenesis of *Salmonella* infection of avian species (Ricter-Dahlfors et al., 1997; Gorvel and Meresse, 2001; Okamura et al., 2005). Information in the literature on the quantitative distribution of *Salmonella* Zega in visceral organs of chicken based on different routes of infection is not available. In the present study, the similarities in the distribution of *Salmonella* Zega in visceral organs of chickens infected orally and intraperitoneally suggest a preferential infection of these organs. The large distribution of the organism in the lung in the groups infected orally and intraperitoneally in the present study may be attributed to the systemic nature of the infection in which the large blood supplies and anastomosis of blood vessels in the organ might have accounted for the high immunoreactions. The relatively large number of immunoreactions seen in the liver, spleen, intestine and caecum of chickens infected orally and intraperitoneally might suggest that *Salmonella* Zega shows organ specific target for infection and has tropism for these organs irrespective of the route of infection. Shu-Xuan et al. (2008) reported that quantitative studies of the regular distribution pattern for *Salmonella* Enteritidis in the internal organs of mice after oral challenge by a specific real-time polymerase chain reaction showed higher distribution in the liver and spleen. *Salmonella* organisms are known to replicate in the reticuloendothelial systems, while lymphoid tissues are targets for *Salmonella* invasion (Carter and Collins, 1974a and b; Henderson et al., 1999). It has been reported that *Salmonella* infection of lymphoid tissue can account for the depletion and necrosis of lymphoid follicles of the spleen seen in most *Salmonella* infections (Henderson et al., 1999). In a separate study, Chengdu et al. (2018) reported that the highest copy numbers of S. Enteritidis in internal organs were recorded in the heart and liver, with about 2 x 10^2 to 6 x 10^6 copies of DNA target sequences in chickens infected orally. However, unlike the report of Chengdu et al. (2018), who infected chickens with *S*. Enteritidis, the heart showed less distribution with *Salmonella* Zega in the present study, from day 5–7 post infection. But immunoreactions became very severe as the disease progressed between day 8–10 post infection. The heart might have been tolerant to

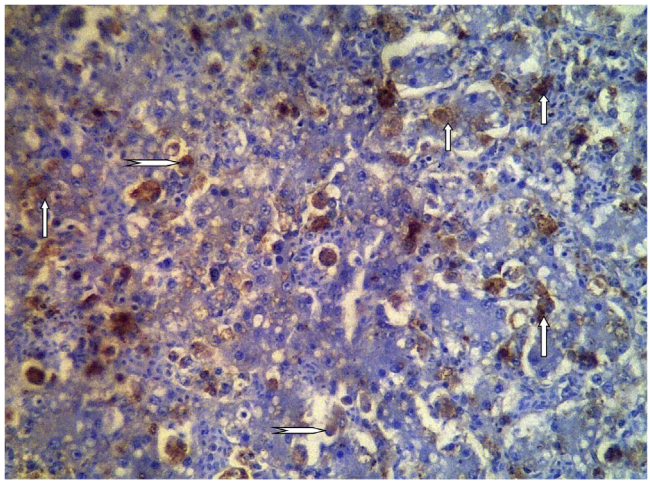

Figure 10. Section of the liver of 3 week-old chick infected orally with *Salmonella* Zega showing immunoreactions in hepatocytes (arrows), heterophils, lymphocytes and macrophages (arrow heads) 10 days post infection (x400; Streptavidin peroxidase, counter stained with Haematoxylin).

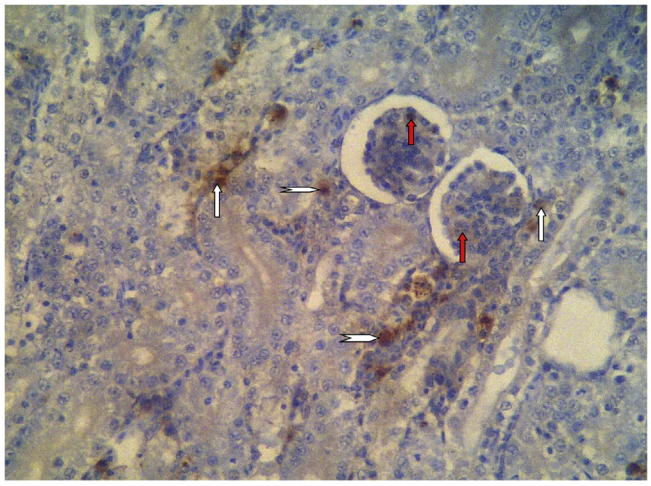

Figure 12. Section of the kidney of 3 week-old chick infected orally with *Salmonella* Zega showing immunoreactions in tubular (white arrow) and glomerular (red arrows) epithelial cells (arrow) lymphocytes and macrophages (arrow heads) 5 days post infection (x400; Streptavidin peroxidase, counter stained with Haematoxylin).

Salmonella Zega in the beginning but became susceptible when the bird's immune system had become compromised at the later stages of the disease. It could also be due to serotype specific preference for a particular organ. It was suggested that systemic *S.* Gallinarum shows trophism to lymphoid tissues, such as payer's patches and caecal tonsils, and can cross the gut during the early stages of fowl typhoid to enter the intestinal lymphoid tissues (Lowry et al., 1999). In birds infected per cloaca, the high distribution of immunoreactions in the caecum compared other organs in the early stage of the disease point to a localized infection of the caecum by *S*. Zega through this route.

In the present study, the interaction of S. Zega with epithelial cells of the small intestine, caecum and blood cells were observed within 24 h post infection in oral and intraperitoneal routes of infection and in hepatocytes, lymphocytes and macrophages 3 days post infection. It has been reported that *Salmonella* Pullorum bacteria were present in the cytoplasm of hepatocytes, epithelia of caecum and in epithelia of crop following a sequential pathological and immunohistochemical study of pullorum disease in experimentally infected chicks (Shahinuzzaman et al., 2011). They appeared as rod shaped reddish brown colored organisms in the cytoplasm of hepatocytes and epithelium of the crop and caecum (Henderson et al., 1999, Shahinuzzaman). Also, in an experiment to study the adhesion and invasion of the caecum by *S.* Enteritidis*,* phage 4, using immunohistochemistry, in one day old specific pathogen free leghorn chicks, positive staining bacilli were associated with the epithelial surface and were present in the lumen of the caecal crypt. They were also observed in the interstitial tissue and in the cytoplasm of macrophage-like cells in the lamina propria. The granulomatous nodules containing positive staining bacilli were present in the submucosa of the caecum of one bird at 14 day after inoculation (Desmidt et al., 1998). The interaction of *Salmonella* Zega with epithelial and blood cells 24 h after infection may suggests epithelial cell involvement and haematogenous spread of the organism to other organs early in the pathogenesis of avian salmonellosis. Epithelial cells of the intestine are known to be infected during the early events of the pathogenesis of the disease when chickens were infected orally (Carter and Collins, 1974a and b; Henderson et al., 1999; Hensel, 2000). Central to the pathogenesis of *Salmonella* infection is its ability to infect and survive inside host cells, including lymphoid tissues, epithelial cells and macrophages (Ricter-Dahlfors et al., 1997; Gorvel and Meresse, 2001; Okamura et al., 2005). Immunoreactions in macrophages and lymphocytes in oral and intraperitoneally infected chicks observed on days 3 and 4 respectively indicate a significant role played by these immune cells in the pathogenesis of avian salmonellosis. It was reported that interaction of *Salmonella* with microphages is crucial to the progression of systemic infection in both birds and mammals (Barrow et al., 1994). However, the presence of immunoreactions in epithelial and blood cells 24 h post infection in both oral and intraperitoneal route suggest that *Salmonella* Zega have preference for these cells irrespective of the route of infection and may not require macrophage trafficking to other organs before establishment of systemic infection. *Salmonella* showed less interaction with heterophils. This may be due to the ability of the heterophils to kill the *Salmonella* pathogens (Carter and Collins, 1974a) and also agrees with the report of Henderson et al. (1999) that avian polymorphonuclear cells (PMNs) are competent killers of *Salmonella* species but macrophages are not as efficient at killing *Salmonella* Pathogen. Moreso, Macrophages are known to serve as a vehicle for dissemination of *Salmonella* organism to reticuloendothelial system (Buchmeier and Hefferon, 1991; Abshire and Neidhart, 1993). *Salmonella* organisms are obligate intracellular pathogens and require host cells to survive, replicate and produce local or systemic disease. *Salmonella* Gallinarum and S. Enteritidis have been demonstrated in the cytoplasm of the epithelial cells of the crop, intestine, caecum and cloaca; and in the cytoplasm of hepatocytes by various workers (Desmidt et al., 1998; Henderson et al., 1999; Shivaprasad and Barrow, 2008; Beyaz et al., 2010; Shahlinuzzaman *et al.*, 2011; Tunca et al., 2012). However, our present study determined the quantitative distribution and interaction of *Salmonella* Zega with host cells in the visceral organs.

In conclusion, *Salmonella* Zega showed highest distribution in the lung, intestines, caecum, followed by reticuloendothelial systems and least distribution in the heart when chickens were infected orally or intraperitoneally, but showed highest distribution in the caecum when inoculated per cloaca. The organism interacted with wide range of host cells but showed tropism for epithelial and blood cells irrespective of the route of infection.

Declarations

Author contribution statement

F. Mshelbwala: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

N. Ibrahim and S. Saidu: Conceived and designed the experiments; Performed the experiments.

A. Kadiri: Performed the experiments; Analyzed and interpreted the data.

C. Kwanashie, F. Thomas and M. Agbaje: Analyzed and interpreted the data.

E. Babatunde: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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