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Experimental infection of commercial layers using a *Salmonella enterica* sorovar Gallinarum strain: blood serum components and histopathological changes

Kleber O. Garcia[1]; André M. Santana[2]; Oliveiro C. Freitas Neto[1]; Kalina M.M.G. Simplício[2]; Antonio C. Alessi[1]; Ângelo Berchieri Júnior[1]; José J. Fagliari[2]

[1] Department of Veterinary Pathology, Faculdade de Ciências Agrárias e Veterinárias - FCAV/UNESP Jaboticabal - SP, Brazil
[2] Department of Clinics and Surgery, FCAV/UNESP Jaboticabal - SP, Brazil
Corresponding author: Kleber O. Garcia - Faculdade de Ciências Agrárias e Veterinárias - FCAV/UNESP/DPVE
Via Paulo Donato Castellane, s/n CEP 14884-900 – Jaboticabal-SP, Brazil E-mail: klebergarcia7@hotmail.com

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Abstract

The purpose of the study was to evaluate the blood serum components and histopathological findings of commercial layers experimentally infected with *Salmonella* Gallinarum (SG), the microorganism responsible for the fowl typhoid. 180 commercial layers were distributed into three groups (G): G1 and G2 received 0.2mL of inoculum containing $3.3x10^8$ and $3.3x10^5$ CFU of resistant SG to the nalidix acid (Nal^r)/mL, respectively, directly into their crops; G3 did not receive the inoculum (control group). The birds were inoculated when they were 5 days old and the euthanasia was performed 24 hours before and after infection and 3, 5, 7 and 10 days after the administration of the inoculum. In each day of collection, blood samples were obtained for biochemical tests of the blood serum besides macroscopic and histopathological examination of the birds. Data were submitted to analysis of variance by the SAS statistical program and the means were compared by Tukey´s test ($P<0,05$). In the serum biochemical profile it was observed that the infection interfered in the values of total protein, albumin, calcium, phosphorus, cholesterol, triglycerides, GGT and ALT in the infected groups. The macroscopic examination showed hepatomegaly, alteration of the hepatic color and hemorrhagic spots in the kidneys of animals from G1. The histopathology showed degeneration of hepatocytes in G1 and G2 although other lesions like multifocal hepatic necrosis and inflammatory infiltrate on the liver and kidneys were restricted to G1. The alterations were more evident on G1 which received a higher concentration of bacteria/mL when compared to G2. The results showed that the correlation between biochemical alterations and macroscopic and histopathological lesions can assist the comprehension of the pathophysiology of fowl typhoid, supplying important information for the diagnosis and prognosis of this disease.

Key Words: fowl typhoid, serum biochemical profile, histopathology, commercial layers

Introduction

Fowl typhoid caused by *Salmonella* Gallinarum (SG) is a disease worldwide distributed that attacks commercial poultry and other galliform species. It is a severe systemic disease with clinical signs in young and adult birds such as diarrhea, dehydration, weakness and loss of appetite as well as macroscopic and microscopic lesions in the liver, spleen, kidneys, heart, bursa of Fabricius and ovary (3, 21), leading to relevant economic losses due to high mortality rates and accentuated egg production reduction (4).

In spite of the adoption of prevention programs, fowl typhoid is still report in Mexico, Central America, South America, Africa, India and South Korea (3, 12, 13, 18). In most countries of Europe, North America and Australia the disease has been eradicated, however in the 90´s a breakdown of classic fowl typhoid was diagnosed involving 18.000 commercial brown layers kept in battery cages in Denmark (8) demonstrating the required attention on this disease.

SG in birds is characterized by septicemia and toxemia. Freitas Neto et al. (9), studying infected birds

with SG observed hepatomegaly with friable consistency and yellow-greenish color of the liver besides multifocal necrosis in the histopathological examination. The authors also observed spleen congestion and inflammatory infiltrate in the heart and kidneys.

Tissue lesions are largely responsible for metabolic alterations of the blood serum in most of the diseases. Increased values of aspartate aminotransferase (AST) are highly suggestive of hepatic or muscular damage in birds (6). A high serum activity level of gamma-glutamyl transferase (GGT) has been reported in birds naturally infected with micotoxins (1). Maciel et al. (15), studying the hepatic function of broilers fed with diets containing aflatoxin, verified a decrease in the serum concentrations of cholesterol which was attributed to a decrease in the hepatic synthesis of this metabolite once the target organ for the action of aflatoxins is the liver (19).

Considering that tissue lesions are directly related to metabolic alterations and studies have demonstrated that SG infections can cause lesions in a variety of organs (3, 5, 21) the use of auxiliary laboratorial analysis is fundamental to better understanding of the pathophysiology of this disease.

' that there are few studies about metabolic alterations of the blood serum in infected birds by *Salmonella* Gallinarum, the aim of this study was to evaluate the metabolic alterations of the blood serum in birds experimentally infected by *Salmonella* Gallinarum, associating these alterations to clinical signs and macroscopic and histopathological examinations to gather auxiliary information for the diagnosis and prognosis of the fowl typhoid.

Material and Methods

Birds and housing

A number of 180 commercial semi-heavy (brown) layers, considered susceptible to the infection by *Salmonella* Gallinarum were used. Blood samples were collected at arrival at the experimental facilities for the serological test (fast serum agglutination test in smear). Fecal swabs were taken from the transport crate for the bacteriological detection of *Salmonella*, in order to ensure that birds were free from pathogens that might compromise the experiment (23). The results showed that all birds were negative for *Salmonella* Gallinarum. Birds were then divided into three groups (G1, G2, and G3) of sixty individuals each, and housed in battery cages, located at the isolation units of the Laboratory of Veterinary Pathology of the Department of Veterinary Pathology, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista (FCAVJ-UNESP), Brazil. Birds received water and food *ad libitum*.

Preparation of the inoculum

A nalidixic-acid resistant *Salmonella* Gallinarum strain (SGNal^r), was used, as recommended by Berchieri Júnior et al. (4). The strain was prepared and kept at the Department of Veterinary Pathology (FCAVJ-UNESP). The bacterium was cultivated in nutrient broth (Difco-244620), incubated in a shaking incubator (100 strokes/min) at 37°C, for 24h. Cultures were prepared in two different dilutions: one with approximately $3.3x10^8$ CFU of S. Gallinarum Nalr/mL and another with $3.3x10^5$ CFU of S. Gallinarum Nal^r/mL.

Experimental procedure

G1 and G2 birds received 0.2mL of an inoculum containing $3.3x10^8$ and $3.3x10^5$ CFU of *Salmonella* Gallinarum, respectively, directly into the crop using a cannula. G3 birds did not receive any inoculum (control group). The birds were challenged at 5 days of age and sacrificed to obtain blood samples. Euthanasia was carried out (ten birds at a time in each group) 24 hours before (1DBI) and after (1DAI) infection, and 3 (DAI), 5 (5 DAI), 7 (7 DAI) and 10 (10 DAI) days after infection.

Clinical evaluation

Birds were submitted to a physical examination twice daily - in the morning and afternoon - and, in the case of death, mortality was recorded, as described by Oliveira et al. (17).

Blood collection and laboratory analysis

Birds were rendered unconscious, and then submitted to euthanasia. Blood samples were collected from the cervical vein. Blood samples for white blood cell counts were collected in tubes containing 10% EDTA. Blood samples for the biochemical exams were collected in tubes with no anticoagulant; after coagulation and retraction of the clot, serum samples were obtained for analysis procedure.

Biochemical exams of blood serum

The laboratorial exams were undertaken in the Laboratory for Research Support of the Clinics and Surgery Department and the Laboratory of Ornitopathology from the Veterinary Pathology Department of FCAV/UNESP/Câmpus de Jaboticabal. Total serum protein (biuret method), albumin (bromocresol green method), triglycerides (Trinder method), cholesterol, total calcium (Labtest method) and phosphorus concentrations (Basques-Lustosa method), as well as the activities of *gamma-glutamyl transferase* (GGT) (Szasz modified method) and *alanin aminotransferase* (ALT) (Reitman-Frankel method) were accomplished with the use of commercial kits (Labtest). Sample readings were made in semi-automatic spectrophotometer (LABQUEST).

Gross and histopathological exams

After blood collection and posterior euthanasia, the aspect, size and color of the organs were examined, using the control group as reference. In each moment of sample collection, fragments of liver, spleen, thymus, bursa of Fabricius and kidneys were obtained for histopathological examination. Fragments of organs were fixed in 10% phosphate buffered formalin, pH 7.2. and processed for paraffin embedding according to routine methods (2). Sections of 5µm thickness were stained with hematoxilin-eosin.

Statistical analysis

A completely randomized experimental design with in a 3x6 factorial arrangement (infection levels and pre and post-infection moments) was used for statistical analysis. Data were submitted to analysis of variance using SAS statistical program, and means were compared by Tukey's test ($P < 0.05$).

Results and Discussion

Clinical Evaluation

Starting at third day after infection (DAI) birds from group 1 (G1) showed typical clinical signs of fowl typhoid such as apathy, prostration, dropped wings, anorexia, dehydration and yellow-greenish bloody diarrhea. This clinical picture is similar to the one observed by Smith (22), in birds, three to four days after the oral inoculation of *S*. Gallinarum, and by Freitas Neto et al. (9), who observed apathy, prostration, anorexia and ruffled feathers. In birds from group 2 (G2), these signs appeared in the fifth DAI, however, in a mild dimension. Twenty four birds from group G1 and eleven from G2 died. This higher mortality rate in G1 is probably due do the higher bacterial concentration of SG used on this group, which was reported by Oliveira et al. (17).

Serum Biochemical Profile

The data presented at the Table 1 shows that there were significant alterations in the serum biochemical parameters in birds from G1 and G2 when compared to those from G3 (control group), with prominence to G1 which received a higher bacterial concentration than G2.

Total protein (TP) serum concentrations decreased in G1 and G2 from fifth and third DAI, respectively, maintaining these decreased levels until the last day of collection when compared to the day before infection (DBI). However, albumin serum concentrations showed relevant alterations only in the fifth DAI of the G1 (Table 1). Albumin, which is synthesized only in the liver, is the main responsible for maintaining the oncotic blood pressure; it may occur hypoalbuminemia in cases of hepatic insufficiency, malnutrition and gastrointestinal disturbances within other conditions (16). This albumin reduction in the fifth DAI at G1 coincided with the day which the histopathological exam revealed hepatocyte degeneration, multifocal necrosis and inflammatory infiltrate in the liver of most birds (Figures 1 and 3). Although albumin half-life is superior to the period studied, the decrease in the levels of this protein might be due to hepatic disturbances, observed in G1, or even because it is considered a negative acute phase protein (11).

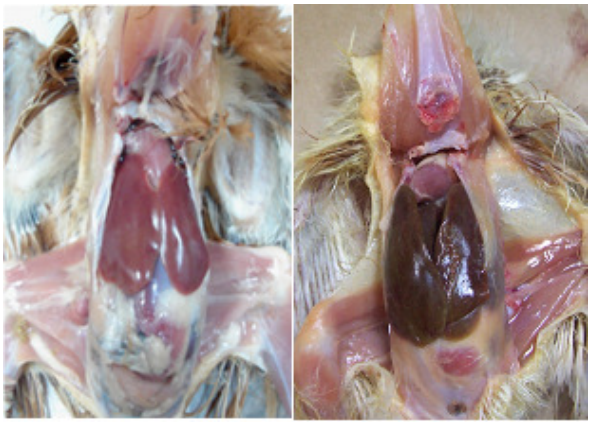

Figure 1. Macroscopic aspect of bird from control group or G3 (left), showing normal liver, compared with G1 bird, at seventh day after inoculation with *Salmonella* Gallinarum (right), presenting hepatomegaly and liver with altered color.

Renal disturbances cause serum calcium decrease due to protein loss, leading to hypoalbuminemia, or by calcium reabsorption decrease (14). Phosphorus is a bone, cellular membrane and cell function major component. Alterations in phosphorus concentration may be due to excess or deficiency of vitamin D and renal disturbances (20). Calcium and phosphorus concentration decreased from seventh and fifth DAI, respectively, in birds from G1 (Table 1). This decrease may be related to renal lesions showed in Figures 2 and 4. Additionally, the decrease in calcaemia and phosphataemia in birds from G1 may be related to a low ingestion of these nutrients, likely because these birds were not feeding properly in reason of the infection by SG.

In G1 and G2 birds it was noticed an accentuated decrease at serum levels of cholesterol starting at first DAI reaching a minimum value in the fifth DAI in G1 when the cholesterolemia had a decrease of four times when compared to the values of the DBI (Table 1). In general, the serum concentration of triglycerides decreased in the birds from G1 and G2, similarly to what was observed with the cholesterol. These results might be due to less food ingestion by the birds or due to the lipid metabolism compromised by hepatic lesions showed in the Figures 1 and 3. Such results are similar to the findings of Aravind et al. (1) and Maciel et al. (15) in broilers with hepatic dysfunction caused by micotoxin.

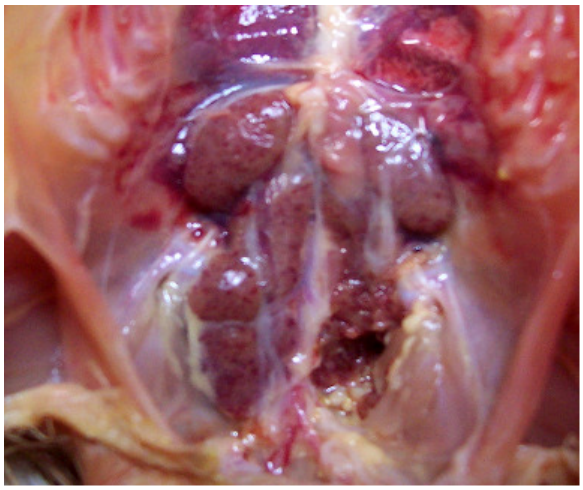

Figure 2. Macroscopic aspect of G1 bird in the fifth day post inoculation with *Salmonella* Gallinarum, presenting kidney with hemorrhagic spots spread on the organ.

Significant increase was verified in serum activities of GGT at birds from G1 and G2 during the infection; the values increased about three times in both groups when compared to DBI, as shown in the Table 1. Serum activity of ALT also increased gradually at G1; in G2 birds there were oscillations. These results proceed because enzymatic activity of these enzymes generally increase when there are hepatic lesions as verified, mainly in G1 birds (Figures 1 and 3). Meyer et al. (16) observed that GGT is a membrane enzyme, therefore its serum activity can be elevated from the beginning of hepatic lesion. Schimidt et al. (20) reported that the activity of hepatic enzymes, as ALT and GGT, increase when there are hepatic lesion due to overflowing of these enzymes current of the compromising of the hepatocyte's membrane.

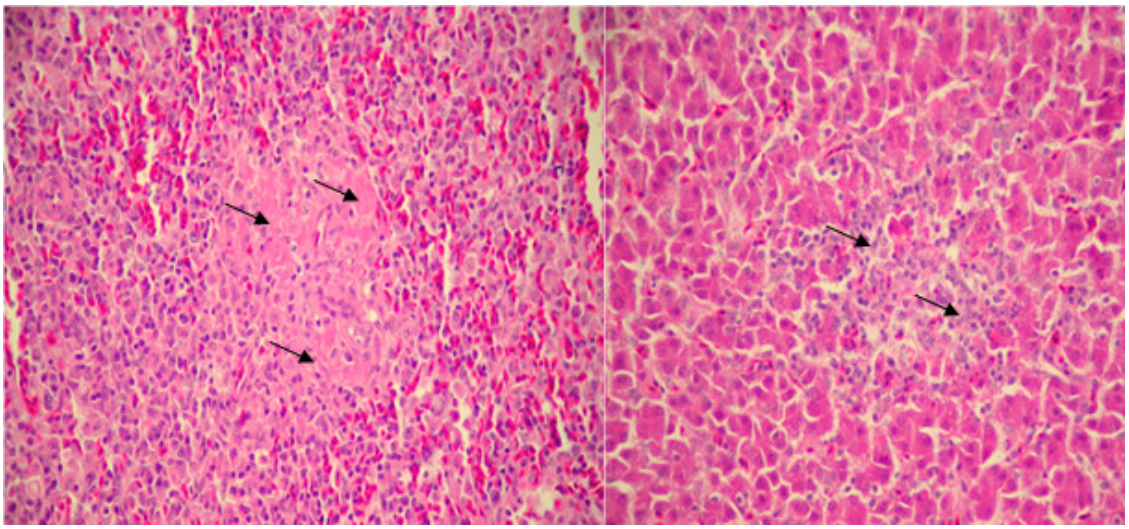

Figure 3. Photomicrography of histological section of the liver of G1 bird, showing multifocal necrosis (left) and inflammatory infiltrate (right), in the center of the images (arrows), at fifth day after inoculation by *Salmonella* Gallinarum (HE, Obj. 40x).

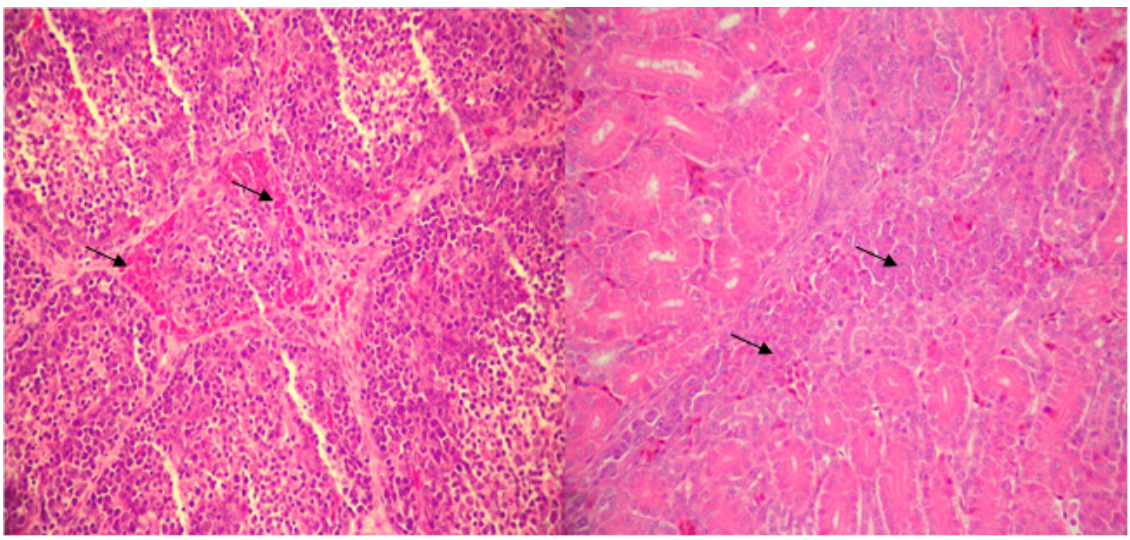

Figure 4. Photomicrography of histological section showing inflammatory infiltrate (arrows) in the bursa of Fabricius (left) and kidney (right) in G1 bird, at fifth day post inoculation by *Salmonella* Gallinarum (HE, Obj. 40x).

Table 1. Means of total protein serum levels, albumin, calcium, phosphorus, cholesterol, triglicerydes, GGT and ALT in brown layers experimentally infected with *Salmonella* Gallinarum Nal^r.

Parameters	Days of sample collection	Groups			CV
		1	2	3	%
Reference values	**1 DBI**	3.44 ^{AB}	4.26 ^{A}	3.83	18.2
Total protein (g/dL)	**1 DAI**	3.67 ^{A}	3.46 ^{AB}	3.77	21.3
	3 DAI	3.80 ^{Aa}	3.19 ^{Bb}	3.50 ^{ab}	25.7
	5 DAI	3.04 ^{B}	3.09 ^{B}	3.39	23.2
	7 DAI	2.95 ^{Bb}	2.89 ^{Bb}	3.47 ^{a}	25.5
	10 DAI	*	3.11	3.25	18.9
CV (%)		20.9	31.5	19.2	
Reference values	**1 DBI**	1.54 ^{Aa}	1.61	1.62	15.6
Albumin (g/dL)	**1 DAI**	1.58 ^{Aa}	1.68	1.51	35.4
	3 DAI	1.51 ^{Aa}	1.64	1.44	27.6
	5 DAI	0.94 ^{Bb}	1.60 ^{a}	1.63 ^{a}	30.8
	7 DAI	1.37 ^{A}	1.44	1.55	30.7
	10 DAI	*	1.51	1.50	21.5
CV (%)		25.8	30.1	28.9	
Reference values	**1 DBI**	10.1 ^{A}	8.00	8.72	11.5
Calcium (mg/dL)	**1 DAI**	8.77 ^{A}	9.00	8.50	24.6
	3 DAI	8.79 ^{A}	7.80	7.92	8.80
	5 DAI	7.83 ^{AB}	8.40	9.15	15.1
	7 DAI	6.14 ^{Bb}	7.66 ^{ab}	8.85 ^{a}	14.4
	10 DAI	*	9.0	9.22	20.0
CV (%)		25.2	16.1	12.4	
Reference values	**1 DBI**	9.60 ^{A}	8.82	8.89	17.5
Phosphorus (mg/dL)	**1 DAI**	8.66 ^{A}	10.1	8.47	27.5
	3 DAI	7.94 ^{AB}	9.01	7.97	22.1
	5 DAI	6.37 ^{Bb}	9.35 ^{a}	8.32 ^{a}	29.5
	7 DAI	7.73 ^{AB}	8.16	7.80	25.9
	10 DAI	*	8.83	8.21	23.2
CV (%)		26.0	27.6	14.5	
Reference values	**1 DBI**	351 ^{A}	358 ^{A}	342	10.2
Cholesterol (mg/dL)	**1 DAI**	170 ^{Bb}	168 ^{Bb}	321 ^{a}	18.1
	3 DAI	156 ^{bBCb}	159 ^{Bb}	354 ^{a}	23.8
	5 DAI	94.0 ^{Dc}	141 ^{Bb}	335 ^{a}	32.4
	7 DAI	133 ^{Cb}	137 ^{Bb}	323 ^{a}	26.1
	10 DAI	*	143 ^{Bb}	331 ^{a}	20.8
CV (%)	%	28.2	30.8	15.5	
Reference values	**1 DBI**	105 ^{Aa}	135 ^{Aa}	109	17.8
Triglicerydes (mg/dL)	**1 DPI**	59.8 ^{Bb}	100 ^{ABa}	111 ^{a}	25.6
	3 DPI	59.9 ^{Bb}	107 ^{ABa}	105 ^{a}	20.1
	5 DPI	119 ^{Aa}	82.3 ^{Bb}	121 ^{a}	12.8
	7 DPI	65.3 ^{Bb}	98.4 ^{ABa}	113 ^{a}	26.6
	10 DPI	*	81.6 ^{B}	99	20.4
CV (%)		28.6	16.6	12.9	
Reference values	**1 DBI**	6.36 ^{C}	6.54 ^{C}	6.36	16.5
GGT (U/L)	**1 DAI**	9.54 ^{Ba}	6.36 ^{Cb}	6.77 ^{b}	33.1
	3 DAI	14.0 ^{ABa}	9.54 ^{Bab}	7.01 ^{b}	24.1
	5 DAI	19.1 ^{Aa}	8.48 ^{BCb}	6.88 ^{b}	28.8
	7 DAI	17.8 ^{Aa}	15.3 ^{Aa}	6.50 ^{b}	25.9
	10 DAI	*	20.4 ^{Aa}	8.10 ^{b}	32.9
CV (%)		37.3	23.9	17.5	
Reference values	**1 DBI**	10.5 ^{B}	10.5 ^{A}	8.98	13.8
ALT (U/L)	**1 DAI**	9.42 ^{Bb}	13.6 ^{Aa}	9.07 ^{b}	27.8
	3 DAI	11.28 ^{Ba}	5.23 ^{Bb}	8.85 ^{ab}	18.7
	5 DAI	15.23 ^{Aa}	6.54 ^{Bb}	10.15 ^{a}	18.6
	7 DAI	16.98 ^{Aa}	10.5 ^{Ab}	9.36 ^{b}	23.0
	10 DAI	*	13.6 ^{Aa}	9.85 ^{b}	18.3
CV (%)		38.7	25.1	15.1	

Means followed by different capital letters in the same column or by different small letters in the same row are significantly different by the Tukey's test (P<0,05).

Group 1= oral inoculation of SGNal^{r}x10^8; Group 2= oral inoculation of SGNal^{r}x10^5; Group 3= did not receive inoculum (control group).

*= Group 1 – all birds were dead at this day; CV= Coefficient of variation; DBI= day before inoculation; DAI= day after inoculation;

Gross and histopathological exams

Macroscopic and microscopic alterations in the organs which showed lesions are presented in Figures 1 to 4. In G1 it was observed hepatic alterations starting at third DAI. The macroscopic exam showed hepatomegaly, alterations in the color of liver besides friable consistency (Figure 1). Renal lesions were verified in G1 birds at fifth DAI (Figure 2). Similar lesions were reported by other authors that

studied experimental infection with SG in birds (3, 5, 7, 9, 10, 21). Histopathological exams in G1 birds showed, starting at third DAI, multifocal hepatic necrosis with heterophilic infiltrate. In the fifth and seventh DAI it was noticed more serious lesions, with hepatocyte degeneration, multifocal degeneration and heterophilic infiltrate in the liver, similar lesions also reported by Hall (10), Shivaprasad (21) and Freitas Neto et al. (9).

In G2 birds it was verified just a discreet hepatic degeneration from the seventh DAI.

The microscopic exam showed considerable lesions in G1 birds and a larger number of affected birds when compared to G2 whose lesions were milder; besides a fewer number of birds showed lesions at G2. The most affected organ in G1 and G2 was the liver; however an inflammatory infiltrate was observed in the kidneys and bursa of Fabricius which were restricted to birds from G1 (Figures 2 and 3); a similar result was reported by Freitas Neto et al. (9) who verified in experimental infection by S. Gallinarum that the main organs affected were the liver and heart, although other organs, as lungs and kidneys, showed discreet lesions.

Overall, it was observed that in serum biochemical profile as in macroscopic and histopathological exams, the alterations were more evident in birds from G1, who received a higher bacterial concentration of SG, reinforcing what was reported by Oliveira et al. (17) that the prevalence and mortality rates of avian typhoid are directly related to the infecting dose.

Regarding the results of this study it was verified that tissue lesions observed mostly in the liver and kidneys from G1 birds were related to the alterations showed in the serum biochemical profile. The albumin decrease, the hepatic enzymes GGT and ALT increase and the cholesterol and triglycerides decrease were related to hepatic alterations; the decrease of the electrolytes calcium and phosphorus were due to renal disturbances. Clinical signs were more evident in the birds during the corresponding days when major alterations were observed in the laboratorial exams.

These facts show the importance of associating results obtained at serum biochemical profile, organ lesions observed through macroscopic and histopathological exams and also by the clinical signs presented by the birds for a better understanding of the pathophysiology of fowl typhoid, supplying information for the study of diagnosis and prognosis of this important avian disease.

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