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Recovery of Salmonella Gallinarum in the Organs of Experimentally-Inoculated Japanese Quails (Coturnix coturnix)

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ABSTRACT

Salmonellosis is an infection caused by specific or non specific serotypes of the *Salmonella* genus, responsible for losses in the poultry industry. Fowl typhoid, caused by *S.* Gallinarum (SG) is important because it causes elevated mortality in adult birds, leading to economic losses in the poultry industry. This study aimed at quantifying the number of viable SG cells in the liver, spleen, lung, cecum, and reproductive tract (ovary and testicles) of experimentally inoculated Japanese quails (*Coturnix coturnix*), as well as SG shedding in their feces. One hundred and two Japanese quails, with four months of age at the beginning of the experiment, were used. The birds were inoculated with three bacterial cultures containing different concentrations ($6x10^4$ CFU/0.1mL, $2x10^5$ CFU/0.4mL, or $5x10^6$ CFU/0.2mL) of SG resistant to nalidixic acid. On days 1, 4, 7, and 14 after the inoculation (dpi) individual cloacal swabs were collected from six birds per group, which were subsequently sacrificed for organ sampling. The swab samples were streaked directly on plates containing brilliant green agar and nalidixic acid (VBNal). Samples that were negative after 24h, were streaked again. The collected organs were individually macerated and transferred to buffered peptone water at 0.1%. The solutions were immediately diluted serially for CFU counting in VBNal. SG was successfully recovered from one quail, which was inoculated with $2x10^5$ CFU/0.4mL, and from five quails of the group inoculated with $5x10^6$ CFU/0.2mL inoculum. All of the analyzed cloacal swab samples were negative. Therefore, this study demonstrated it was difficult to isolate SG from the analyzed organs and that it was not possible to recover thepathogen in the cloacal swabs collected from inoculated quails. These results may be explained by the absence of flagella in SG, inducing weak intestinal immune response in the beginning of the infection and preventing its isolation in cloacal swab samples. The low positivity rate of the analyzed organs may be due to the immune status of the euthanized birds, since the SG dissemination in the animal organism occurs mostly close to death, which was observed in the birds found dead during the experiment.

INTRODUCTION

Salmonella Gallinarum is a pathogenic serotype of *Salmonella* specific of birds (Setta *et al.,* 2012),and it is characterized by its capacity to grow in systemic locations (Chadfield *et al.*, 2003). This serotype has been isolated from several poultry species, such as chickens, turkeys, pheasants, ducks, guinea fowl, and quails (Shivaprasad, 2000).

Quails have been commercially reared both for egg and meat production (Schmid & Wechsler, 1997), and its production is currently expanding in Brazil. Some researchers have reported both the presence

of *Salmonella* spp. (Bacci *et al.*, 2012) and increasing severity of its infection in quails (Rocha e Silva *et al.*, 2013).

Most avian salmonellosis infections are transmitted through the fecal-oral route. The small intestine is the main site of invasion, which occurs through the gut-associated lymphoid tissues (GALT) and Peyer's patches. The ceca are also colonized, although *Salmonella* Gallinarum and *S*. Pullorum are auxotrophic (Chappell *et al.*, 2009).

The adhesion and/or colonization of the avian intestinal tract is initially associated with LPS (lipopolysaccharides), flagella, fimbriae, and some external membrane proteins present in *Salmonella* spp. (Berndt *et al.*, 2007). Several studies suggest that the presence of flagella is involved in the inflammatory response at the adhesion site and the absence of these structures may avoid the recognition of the pathogenic agent by the immune system. This contributes for the development of systemic infections (Chappell *et al.*, 2009) due to the difficulty in mounting an adequate immune response (Lahiri *et al.*, 2010), culminating in the dissemination of the agent in the environment or in high bird mortality.

The disease caused by SG (fowl typhoid) causes significant economic losses in poultry production (Proux *et al.*, 2002). Due to the expansion of quail rearing in the last few years and the lack of studies on this species, the knowledge of the behavior of the infection of these birds by SG is essential. This study aimed at quantifying the number of viable SG cells in the liver, spleen, lung, cecum, and reproductive tract (ovary or testicle) of experimentally infected Japanese quails (*Coturnix coturnix*), as well as to evaluate its shedding in the feces.

MATERIAL AND METHODS

Birds

In total, 102 (51 female and 51 male) Japanese quails (*Coturnix coturnix*) were used. Birds were 16 weeks old at the beginning of the experiment, and were randomly assigned to three groups (G1, G2 and G3) with 32 birds each. Six birds were kept as controls. Two quails (one male and one female) were housed per cage (22x21x16cm) in batteries placed in a pyramid at the inoculation facilities of the Sector of Ornithological Studies of the State University of Ceará. Water and feed were provided *ad libitum*. The feed did not contain any antibiotics, and birds were not submitted to vaccination or internal parasite control. House temperature (25.5ºC) and photoperiod (16h light/day) were controlled. This project was approved by the local Ethics Committee for the Use of Animals under protocol number 10244779-9/26.

Bacteriological pre-inoculation monitoring

Before the beginning of the experiment, birds were tested for the presence of *Salmonella* spp. in order to ensure that they were free from this pathogen. The procedure was performed according to Zancan *et al.* (2000), with modifications, as follows: individual cloacal swabs impregnated with selenite-cystine and novobiocin (40µg/mL) (SCNov) were directly streaked onto brilliant green agar plates containing nalidixic acid (100µg/mL) (VBNal) and incubated at37ºC for 24h. Negative samples for *Salmonella* spp. were again plated in VBNal and incubated at 37ºC for 24h to confirm the absence of the pathogen in the birds.

The control quails were euthanized and their organs (liver, spleen, lung, cecum, and ovary or testicle) were collected for microbiological examination. The fragments of these organs were macerated, inoculated in 0.1% buffered peptone water, and incubated at 37ºC for 24h. A loopful of each material was then transferred to tubes containing SCNov and incubated at 37ºC for 24h. Subsequently, samples were streaked onto plates containing VBNal and incubated at 37ºC for 24h.

Inoculum preparation

Salmonella Gallinarum strain resistant to nalidixic acid (SGNal^r) isolated from chickens (*Gallus gallus*) was used to prepare the inoculum. This strain was donated by the Department of Veterinary Pathology of UNESP, Jaboticabal, Brazil. The inoculum was prepared according to Rocha e Silva *et al.* (2013) and three distinct concentrations of the inoculum ($6x10^4$CFU/0.1mL, $2x10^5$CFU/0.4mL e $5x10^6$ CFU/0.2mL of *Salmonella* Gallinarum Nal^r/mL) were obtained.

Salmonella Gallinarum inoculation

All birds received 0.1mL of the inoculum via gavage, directly in the crop, with the aid of a cannula linked to a 1mL syringe. Group 1 birds (G1) received $6x10^4$CFU/0.1mL, group 2 (G2) received $2x10^5$CFU/0.4mL, and group 3 (G3) received $5x10^6$ CFU/0.2mL.

Post-inoculation monitoring

During the experimental period, mortality and clinical signs were observed daily. Dead birds were submitted to necropsy for gross evaluation and their organs (liver, spleen, lung, cecum, and reproductive

tract) were collected for microbiological analyses according to Rocha e Silva *et al.*(2013).

Fecal material was collected with the aid of sterile cloacal swabs impregnated in SCNov from euthanized birds on 1, 4, 7, and 14 days post-inoculation (dpi) and directly streaked onto VBNal. Both the broth and the plates were incubated in a bacteriological incubator at 37ºC for 24h, after which samples negative for *Salmonella* spp. were again plated onto VBNal.

On 1, 4, 7, and 14 dpi, six birds per group were randomly selected and euthanized by neck dislocation. At necropsy, the spleen, liver, lung, ceca, and reproductive tract (ovary ortesticle) were submitted to gross examination and any changes were duly recorded. Samples of the examined organs were aseptically collected for CFU counting. The microbiological procedure was performed according to Rocha e Silva *et al.* (2013).

Results

The microbiological analyses showed that the cloacal swabs from all quails, as well as organ samples (spleen, liver, lung, ceca and reproductive tract) of the birds euthanized before the beginning of the experiment were negative for *Salmonella* spp.

It was not possible to countCFU/mL in the organ samples because no colony growth was observed. However, SG was recovered from 4.16% (1/24)of G1 birds and 20.83% (5/24) of G2 and G3 birds on 4 dpi and 7 dpi (G3), respectively, after selective enrichment inselenite-cystine broth (Table 1). The only positive G2 quail on 4 dpi presented SG in liver and lung samples. In theG3 group, on 4 dpi, SG was successfully recovered from the liver, spleen, and lung of two quails, and from testicle of one bird. On 7 dpi, SG was isolated from the ovaries of three quails, and from the liver, spleen, and lung samples of one quail.

Table 1 – Isolation of *Salmonella* Gallinarum fromthe organs of experimentally infected and euthanized Japanese quails *(Coturnix coturnix)*

	Positive birds																					
Group	1st Day						4th Day						7th Day						14th Day					
	Bird	L	S	C	Lg	RT	Bird	L	S	C	Lg	RT	Bird	L	S	C	Lg	RT	Bird	L	S	C	Lg	RT
G1	1	-	-	-	-	-	7	-	-	-	-	-	13	-	-	-	-	-	19	-	-	-	-	-
	2	-	-	-	-	-	8	-	-	-	-	-	14	-	-	-	-	-	20	-	-	-	-	-
	3	-	-	-	-	-	9	-	-	-	-	-	15	-	-	-	-	-	21	-	-	-	-	-
	4	-	-	-	-	-	10	-	-	-	-	-	16	-	-	-	-	-	22	-	-	-	-	-
	5	-	-	-	-	-	11	-	-	-	-	-	17	-	-	-	-	-	23	-	-	-	-	-
	6	-	-	-	-	-	12	-	-	-	-	-	18	-	-	-	-	-	24	-	-	-	-	-
G2	33	-	-	-	-	-	39	-	-	-	-	-	45	-	-	-	-	-	53	-	-	-	-	-
	34	-	-	-	-	-	40	-	-	-	-	-	46	-	-	-	-	-	55	-	-	-	-	-
	35	-	-	-	-	-	41	+	-	-	+	-	47	-	-	-	-	-	56	-	-	-	-	-
	36	-	-	-	-	-	42	-	-	-	-	-	50	-	-	-	-	-	57	-	-	-	-	-
	37	-	-	-	-	-	43	-	-	-	-	-	51	-	-	-	-	-	58	-	-	-	-	-
	38	-	-	-	-	-	44	-	-	-	-	-	52	-	-	-	-	-	60	-	-	-	-	-
G3	65	-	-	-	-	-	71	-	-	-	-	-	78	-	-	-	-	-	89	-	-	-	-	-
	66	-	-	-	-	-	72	-	-	-	-	-	80	-	-	-	-	+	90	-	-	-	-	-
	67	-	-	-	-	-	73	+	+	-	+	+	82	-	-	-	-	-	91	-	-	-	-	-
	68	-	-	-	-	-	74	-	-	-	-	-	83	-	-	-	-	+	93	-	-	-	-	-
	69	-	-	-	-	-	76	+	+	-	+	-	86	+	+	-	+	+	94	-	-	-	-	-
	70	-	-	-	-	-	77	-	-	-	-	-	87	-	-	-	-	-	95	-	-	-	-	-

L: Liver; S: Spleen; C: Ceca; Lg: Lung; RT: Reproductive Tract

The pathogen was also isolated from several organs of birds that died during the experiment (Table 2). In G1, one bird presented SG in the liver, spleen, ceca, lung, and testicle, while the other positive bird presented SG in liver and ovary. In G2 birds found dead, all organs were positive (liver, spleen, ceca, lung, and reproductive tract), with the exception of a single bird that presented negative ceca and liver. All organs of the eight G3 birds that died during the experiment were positive for SG, except for four birds, which presented negative cecum samples.

Table 2 – Isolation of *Salmonella* Gallinarum from the organs of experimentally infected Japanese quails *(Coturnix coturnix)* that were found dead during the 14 days of the experiment

Day	Group	N°Bird	Organs				
			L	S	C	Lg	RT
5th dpi	G1	32	+	+	+	+	+
	G2	48	+	+	+	+	+
		54	+	+	+	+	+
	G3	75	+	+	+	+	+
		81	+	+	+	+	+
6th dpi	G3	79	+	+	-	+	+
		84	+	+	-	+	+
		88	+	+	-	+	+
7th dpi	G3	77	+	+	-	+	+
		92	+	+	+	+	+
9th dpi	G3	85	+	+	+	+	+
10th dpi	G1	26	+	-	-	-	+
	G2	49	+	+	+	+	+
		62	-	+	-	+	+

L: Liver; S: Spleen; C: ceca; Lg: Lung; RT: Reproductive Tract

Birds showed typical fowl typhoid clinical signs, including ruffled feathers, closed eyelids, diarrhea, apathy, typically remained quiet in a cage corner, and were frequently found dead after such signs were observed.

Bird mortality began on 5 dpi and was observed until 10 dpi. The main macroscopic changes observed in the birds found dead were hepatomegaly and splenomegaly, dilated gallbladder, and hemorrhages in the liver, intestines, and deformed ovarian follicles. On the other hand, the main findings in euthanized birds were hemorrhagic or icteric liver with or without petechiae, dilated gallbladder, splenomegaly, hemorrhagic or icteric spleen with or without petechiae, and hemorrhagic ovarian follicles (Table 3).

Birds that remained alive at the end of the experiment (24 dpi) were euthanized and cloacal swab and organ samples were collected. All samples were negative for SG.

DISCUSSION

The percentage of SG isolated from the organs of the euthanized quails was much lower when compared with the quails that naturally died during the experiment. This may be explained by the fact that the infectivity of that microorganism, which is enhanced by its high concentration in the tissues when the bird is near death (Buxton & Davies, 1963).

Salmonella spp. stimulates receptors in the intestinal cells known as toll-like receptor 5 (TLR5). Once activated, these receptors initiate the production of pro-inflammatory interleukins (IL-1, IL-6 and IL-8), which together with macrophages, heterophils, and natural killer cells (NK), are part of the innate immune

Table 3 – Macroscopic changes of the organs of quails experimentally inoculated with *Salmonella* Gallinarum

Organs/ Lesions	Euthanized birds						Number of birds	Birds dead due to the disease													Total of birds
	Experimental groups							Experimental groups													
	G2	G3						G1		G2				G3							
	41	73	75	79	82	86		26	32	48	49	54	62	77	79	81	84	85	88	92	
Liver																					
Hypertrophy						x	01		x			x		x	x	x	x	x			07
Hemorrhage	x		x				02			x						x		x			03
Necrosis							00	x				x		x	x		x	x			06
Icterus		x			x		02														00
Gallbladder								x													01
Spleen																					
Hypertrophy	x	x				x	03	x	x		x		x		x	x	x	x	x	x	10
Pale				x			01														00
Ovary																					
Hemorrhage				x		x	02						x				x			x	03
Atrophy						x	01	x									x		x		03
Uterus																					
Dark						x	01														00

response, an initial line of defense against the invasion of *Salmonella* in the host's body. Innate immunity helps preventing the systemic infection and triggers the humoral and the cell-mediated acquired immune responses (Berchieri Júnior & Freitas Neto, 2009). However, the ineffective immune response caused by SG due to the low or absent IL-6 production, a pro-inflammatory cytokine, may have favored the penetration of the pathogen without any tissue damage and, in the absence of adequate immune response, the systemic infection developed without alerting the host (Kaiser *et al.*, 2000).

Although almost all organs of the quails that were found dead were positive for SG, the ceca was less frequently affected. Evaluating different Salmonella serotypes, Setta *et al.* (2012) found that *S. Enteritidis* and *S. Infantis* colonize the ceca more efficiently than SG and *S. Pullorum*, and Barrow *et al.* (1999) mentioned that it is difficult to isolate SG from the cecum because of its poor intestinal colonization, which explains the high mortality caused by this serotype in this study. Systemic *Salmonella* infections induce both cellular and humoral responses and the persistence of the bacteria in the gastrointestinal tract maintains a more prolonged immune response (Wigley *et al.*, 2005). In chickens, the activity of macrophages may play an important role in systemic resistance to *Salmonella* (Wigley *et al.*, 2002).

All cloacal swab samples analyzed were negative for SG, which was expected, because it is very difficult to isolate SG from cloacal swabs. According to Proux *et al.* (2002), this serotype does not seem to be shed in large numbers in the feces, thereby limiting environmental contamination and consequently reducing the risk of horizontal infection. Nevertheless, Berchieri *et al.* (2000) asserted that the transmission of infection among susceptible birds is essentially horizontal by the ingestion of feces and mucus containing SG or yet by cannibalism.

In the present study, SG shedding did not present a dose-response behavior relative to the three concentrations of the inoculum applied, as occurs with other *Salmonella* trains. When challenged with *S.* Enteritidis, laying hens shed this pathogen for longer periods when high concentrations of inoculum were used, since oral exposure significantly affects important parameters of exposure and infection by *Salmonella*, such as the number of bacteria in internal tissues (Gast *et al.*, 2011).

Several studies with chickens have reported some of the clinical signs that were observed in the present study, such as closed eyes (Freitas Neto *et al.*, 2007), ruffled feathers and diarrhea (Alvarez *et al.*, 2003), and apathy (Garcia *et al.*, 2010). Also, some of the gross changes observed during necropsy were consistent with those found in chickens, including hepatomegaly and hemorrhagic ovarian follicles (Berchieri *et al.,* 2000), as well as deformed and congested liver (Hossain & Islam, 2004).

CONCLUSION

Salmonella Gallinarum was not recovered from any of the evaluated organs of the experimentally infected quails. However, it was successfully quantified in birds that died of the disease during the experimental period. Therefore, we suggest that the immune system of the birds that survived may have cleared the infection, hindering the recovery of the pathogen in birds that did not die of the infection. It is difficult to recover SG in fecal samples of experimentally inoculated quails, which suggests that infected quails that do not present clinical signs do not contribute for the horizontal transmission of the pathogen via feces.

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