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Salmonella Gallinarum Virulence in Experimentally-Infected Japanese Quails (Coturnix japonica)

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ABSTRACT

Salmonella Gallinarum is the agent of fowl typhoid in poultry and infects mainly adult galliforms, causing significant economic losses in poultry production. Because quails are susceptible to this disease and quail production is becoming increasingly important in Brazil, this study was carried out to evaluate the virulence of *Salmonella* Gallinarum strain to quails. The inoculum was prepared from *S.* Gallinarum strain resistant to nalidixic acid. Forty eight 16-week-old Japanese quails were randomly distributed in three groups. Before the experiment, cloacal swabs were collected from all birds in order to confirm they were free from *Salmonella* spp. Cloacal swabs and fecal samples were collected on days 03, 06, 09, 12, and 15 post-inoculation. Birds that died during the experiment were submitted to post-mortem examination, and had their organs aseptically collected for bacteriological examination. All eggs produced during the experiment were also examined. The mortality rate recorded during the experiment was 43.75% (21/48). *S.* Gallinarum was recovered from the organs of the birds that naturally died during the experiment, but the agent was not isolated from the organs of sacrificed birds. No egg sample was positive for *Salmonella* Gallinarum. It was concluded that *S*. Gallinarum may be recovered from the organs of experimentally-infected Japanese quails.

INTRODUCTION

Salmonella spp. genetics allows these bacteria to adapt to many environments, fomites and both mammal and non-mammal hosts (Sanchez-Vargas *et al*., 2011). The consequences of *Salmonella* infections depend on inherent pathogen factors, such as virulence, and on the host's capacity of building an adequate immune response to fight the pathogenic agent (Lahiri *et al*., 2010).

The nature of pathogenicity of *Salmonella* Gallinarum is multifactorial (Kokosharov, 2003); however, its endotoxin plays an essential role and it is directly linked to the virulence of this microorganism (Kokosharov, 2002). *S.* Gallinarum naturally infects avian species, which body temperature is 5ºC higher than that of mammals. It is possible that different environmental stimuli during natural infection influence the host specificity of this bacterium (Pascopella *et al*., 1995).

Specific *Salmonella* Gallinarum infection is commonly systemic (Evans, 2011), causing sepsis independently of age, but usually affecting birds older than 3 months. Mortally is usually high and may reach 100% in some infected flocks (Uzzau *et al*., 2000). Common symptoms are depression, weakness, ruffled feathers (Freitas Neto *et al*., 2007), weight loss, 50-70% drop in egg production (Ezema *et al*., 2009), prostration, apathy, drooped wings, loss of appetite, dehydration, and greenish-yellow to bloody diarrhea (Garcia *et al*., 2010). Fowl typhoid affects

primarily chickens and turkeys, but pheasants, quails, and guinea-fowl are also susceptible (Shivaprasad, 2000).

Japanese quail production for meat and eggs has expanded in many countries, including Brazil (Minvielle, 2004). Hence, knowledge on the diseases that may affect Japanese quails, including salmonellosis, is essential (Minvielle, 2004). There are literature reports on the isolation of *S*. Typhimurium, *S*. Virchow, *S*. Meleagridis and Group B and C_1 *Salmonella* from the cloaca, liver and intestines of Japanese quails (Al-Nakhli, 2005), *S*. Typhimurium variant Copenhagen, *S*. Typhimurium, and *S.* Hadar from live commercial quails and carcasses, and *S*. Paratyphi from the environment (Sander *et al*., 2001).

This study was carried out to evaluate the virulence of *Salmonella* Gallinarum in Japanese quails because there is little information on this subject.

MATERIALS AND METHODS

Birds

Forty eight 16-wk-old Japanese quails (24 females and 24 males) were used in the present experiment. Birds were randomly distributed in three groups (G1, G2 and G3) with 16 birds each. Birds were housed per sex in cages, measuring 22x21x16cm and organized in pyramid batteries, located at the inoculation facilities of the Center of Ornithological Studies of the State University of Ceará (UECE). Water and feed were supplied *ad libitum*. Feed did not contain any antibiotics. Birds were not submitted to any vaccination or chemical treatment against parasites. Room temperature (25°C) was controlled, and a 16h light lighting program was applied. The present study was approved by the Committee of Ethics of Animal Use of UECE under protocol number 10244779-9/26.

Bacteriological monitoring before inoculation

Before the trial started, all birds were checked to ensure they were free from *Salmonella* spp. The test performed according to Wigley *et al*. (2001), with some changes, as follows: individual cloacal swabs were collected, immersed in selenite-cystine broth (M025; HIMEDIA®) containing novobiocin (40µg/mL, SIGMA®) and directly plated (0h) on brilliant green agar (M016; HIMEDIA®) containing nalidixic acid (100 µg). Both cloacal swabs and plates were incubated in a bacteriological oven at 37°C for 24h. After incubation, cloacal swab samples that were negative for *Salmonella* spp. were again plated in brilliant green agar containing nalidixic acid (100µg) and incubated t 37°C for 24h to confirm the absence of that bacterium in the experimental birds. In addition, six birds were sacrificed for the collection of liver, spleen, cecum, lung, and reproductive tract (testicle or ovarian follicle) samples. Tissue samples were individually ground and placed in test tubes containing peptone water at 0,1% at 37°C for 24h, after which an aliquot was transferred to a tube containing selenite-cystine broth with novobiocin (40µg/mL) and incubated for 24h at 37°C. Samples were then plated in brilliant greed agar with nalidixic acid (100µg) and incubated at 37°C for 24h.

Inoculum preparation

The inoculum was prepared using a nalidixic acid resistant *Salmonella* Gallinarum strain (SGNalr) isolated from chicken (*Gallus gallus domesticus*) and provide by the Department of Veterinary Pathology of UNESP, Jaboticabal, SP, Brazil. The test performed according to Berchieri *et al*. (2001), with some modifications, as follows: the bacterial culture was prepared in 5mL buffered peptone water at 0.1% and statically incubated in a bacteriological oven at 37°C for 24h, and The culture was then serially diluted in order to determine the number of colony-forming units (CFU; Miles *et al*., 1938), after which three different inocula (10^4, 10^5, and 10^6CFU de *Salmonella* Gallinarum Nalr/mL) were obtained.

Inoculation

All birds received via gavage directly in the crop, with the aid of a cannula coupled to a 1mL syringe, 0.1mL of the inoculum. Group 1 (G1) birds received $5x10^5$CFU/mL; group 2 (G2), $1x10^6$CFU/mL; and group 3 (G3), $2x10^7$CFU/mL .

Post-inoculation monitoring

During the trial, clinical signs and mortality were daily recorded. Dead birds were submitted to gross examination, and their organs collected for microbiological tests for the presence of *Salmonella* Gallinarum.

Fragments of the liver, spleen, cecum, lungs, and reproductive tract were aseptically collected, individually ground and placed in tubes with peptone water at 0.1% (7365A; Acumedia®). Samples were then plated on brilliant green agar with nalidixic acid (100µg) and incubated at 37°C for 24h. Plates were read, and the

samples negative for *Salmonella* Gallinarum were transferred to selenite-cystine broth with novobiocin (40µg/mL), incubated in bacteriological oven at 37°C for 24h, and then plated on brilliant green agar with nalidixic acid (100µg).

Samples that did not present colonies with typical morphological profile of *Salmonella* Gallinarum, which are small colonies (2-4mm) and have smooth edges and circular shape (Bergey *et al.*, 1994; Gast, 1997), were submitted to an agglutination test using polyvalent O antiserum poly (Difco®).

Cloacal swab samples

Individual cloacal swabs were collected of all birds (pool of two birds) on 03, 06, 09, 12, and 15 days post-inoculation (dpi) and processed as described by Zancan *et al*. (2000) with some modifications. Individual cloacal swabs were immersed in selenite-cystine broth (M025; HIMEDIA®) containing novobiocin (40µg/mL, SIGMA®), plated on brilliant green agar containing nalidixic acid (100µg) and then incubated in selenite-cystine broth. Both broth and plates were incubated at 37°C for 24h. Cloacal swab samples negative for *Salmonella* Gallinarum were re-plated on brilliant green agar with nalidixic acid (100µg).

Fecal samples

Fresh feces from all birds were collected in plastic bags placed under each cage on 03, 06, 09, 12, and 15 dpi. Samples were weighed and placed in tubes containing 5mL peptone water (1g feces: 10mL) and directly plated on brilliant green agar containing nalidixic acid (100µg) and incubated at 37°C for 24h. Samples negative for *Salmonella* Gallinarum were then transferred to selenite-cystine broth with novobiocin (40µg/mL) and incubated at 37°C for 24h, after which samples were plated on brilliant green agar with nalidixic acid (100µg) and incubated at 37°C for 24h.

Organ sampling

At the end of the experimental period, birds that were still alive were sacrificed by neck dislocation and liver, spleen, cecum, lungs, and reproductive tract samples were aseptically collected and submitted to bacteriological testing for the presence of *Salmonella* Gallinarum. Organs were ground, weighed, and individually placed in tubes containing 5mL peptone water at 0.1% (1g:10mL) and subsequently plated on brilliant green agar containing nalidixic acid (100µg). Samples (tubes and plates) were then incubated at 37°C for 24h. Samples that did not produce colonies with the typical morphological profile of *Salmonella* Gallinarum were transferred to tubes containing selenite-cystine broth with novobiocin (40µg/mL) to try to increase bacterium recovery. Samples were then incubated at 37°C for 24h and plated on brilliant green agar containing nalidixic acid (100µg) and incubated under the same previously described conditions.

Procedure for the isolation of *Salmonella* Gallinarum in eggs

The eggs produced during the entire experimental period were daily collected, separately placed in quail-egg boxes and stored in a cold room at a temperature of approximately 7°C. At the end of the experiment, all eggs were separated according to production date and experimental group. Eggs were not previously disinfected and were then broken in a sterile beaker (pool of eggs produced during two consecutive days by each group), homogenized (entire egg, including both eggshell and internal content), and placed in a bacteriological oven at 37°C for 24h, as described by Berchieri *et al*. (2001), with some modifications. After this period, samples were homogenized, collected using sterile swabs, and transferred to selenite-cystine broth with novobiocin (40µg/mL) and incubated at 37°C for 24h, after which they were plated on brilliant green agar with nalidixic acid (100µg) and incubated at 37°C for 24h.

STATISTICAL ANALYSIS

Total mortality was compared using non-parametric tests – Chi-square test with Yates correction or Fisher's exact test, as indicated – at 5% significance level.

RESULTS

None of the birds evaluated in the pre-inoculation period were positive for *Salmonella* spp., according to the applied microbiological tests.

Mortality started to be observed after 5 dpi and continued for further six days (Figure 1). Total mortality during the experiment was 43.75% (21/48 birds), divided as follows: 8.3% (4/48), 4.16% (2/48), and 31.25% (21/48) in G1, G2 e G3, respectively.

Within groups G1, G2, and G3, mortality rates were 25% (4:16), 12.5% (2:16) e 94.7% (15:16), respectively, with significant difference ($p>0.05$) between G3 and the two other groups.

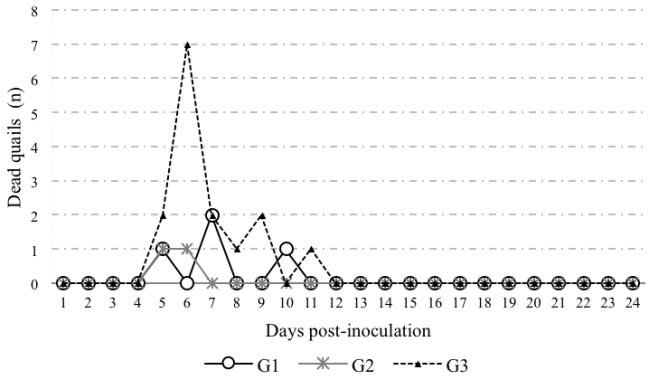

Figure 1 – Daily mortality curve of Japanese quails (*Coturnix coturnix japonica*) orally infected with *Salmonella* Gallinarum.

All fecal and cloacal swab samples collected on 03, 06, 09, 12, and 15 dpi were negative for *Salmonella* Gallinarum. Almost all organs collected from birds that naturally died during the experiment were positive for the bacterium, except for four birds that presented negative cecal samples and one bird that had both negative cecal and reproductive tract samples (Tables 1, 2, and 3). All evaluated organs from quails sacrificed at the end of the experimental period (24 dpi) were negative for the agent.

Table 1 – Recovery of *Salmonella* Gallinarum from the organs of Japanese quails orally inoculated with 5x105CFU/mL (G1) and that naturally died during the experiment.

Birds	Spleen	Liver	Lung	Cecum	Reproductive tract
02	+	+	+	+	+
06	+	+	+	+	+
07	NR	+	+	+	+
11	+	+	+	-	-
Positive (%)	100%	100%	100%	75%	75%

* Birds identified as positive in the plating phase

NR: No result available

Table 2 – Recovery of *Salmonella* Gallinarum from the organs of Japanese quails orally inoculated with $1x10^6$CFU/mL (G2) and that naturally died during the experiment.

Birds	Spleen	Liver	Lung	Cecum	Reproductive tract
40	-	-	-	-	-
43	NR	+	+	+	+
Positive (%)	100%	50%	50%	50%	50%

* Birds identified as positive in the plating phase

NR: No result available

Table 3 – Recovery of *Salmonella* Gallinarum from the organs of Japanese quails orally inoculated with $2x10^7$CFU/mL (G3) and that naturally died during the experiment.

Birds	Spleen	Liver	Lung	Cecum	Reproductive tract
17	+ *	+ *	+	+	+
18	+	+	+	+	+
19	+	+	+	+	+
20	+	+	+	-	+
21	+	+ *	+	+	+
22	+	+	+	+ *	+
23	+	+	+	-	+
24	+	+	+	+	+
25	+	+	+	+	+
26	NR	+	+	+	+
27	+	+	+ *	+	+
28	+	+	+	-	+
29	NR	+	+	-	+
31	+	+	+	+	+
32	+	-	-	-	-
Positive (%)	100%	93.3%	93.3%	62.5%	93.3%

* Birds identified as positive in the plating phase

NR: No result available

The main clinical sign was apathy. Birds remained quiet in the corner of the cage, with their eyes closed, ruffled feathers, and presented greenish-yellow watery feces, which sometimes contained blood. The main findings at gross examination were enlarged and hemorrhagic liver, enlarged gall bladder, enlarged spleen, hemorrhagic ovarian follicles, and petechial hemorrhages in the intestinal tract.

During the experiment, 170 eggs (G1 = 65, G2 = 80; G3 = 25) were collected, and all were negative for *S.* Gallinarum.

DISCUSSION

The high mortality recorded in the present experiment indicates that Japanese quails are sensitive to *Salmonella* Gallinarum (SG). This result was expected, because scientific literature mentions that SG produces a severe disease, with significant mortality rates in chickens (Compra *et al*., 2008). Mortality caused by SG may be higher than 80% in broilers (Paiva *et al*. 2009), and may reach almost 100% in inoculated young brown layers (Oliveira *et al*., 2005). Accordingly, 94.7% mortality was recorded in G3 in the present experiment.

Although all birds were shown to be sensitive to the pathogen, the administration of inocula with different concentrations caused different mortality rates, in agreement with studies with semi-heavy layers receiving inocula with different CFU levels (Berchieri Jr. *et al* .,

2001; Garcia *et al.*, 2009). Despite receiving a more concentrated inoculum than G1, mortality in G2 was lower, albeit not statistically significant, demonstrating that increasing SG concentration from $5x10^5$CFU to $1x10^6$CFU did not influence mortality rate. On the other hand, the administration of the inoculum with $2x10^7$CFU resulted in significantly higher mortality in G3 birds compared with G1 and G3. This was expected due to the high pathogen concentration in that inoculum. Buchholz & Fairbrother (1992) recorded mortality on 6 and 7 dpi in quails orally inoculated with 10^5, 10^6 and 10^7CFU/mL of *S*. Pullorum, which concentrations are similar to those used in the present study. Those authors also found that the intravenous administration of inocula at 10^6 and 10^7 CFU resulted in mortality on 3 dpi. Therefore, the period birds start dying does not depend only on the concentration of the inoculum, but also on the inoculation route.

There are few reports on the evaluation of SG virulence in quails, but studies with chickens are consistent with the results obtained in the present study, particularly relative to the time mortality was recorded, which was between 5 and 11 dpi. Shivaprasad (2000) suggested that birds infected with SG may die within four days after exposure, but they usually start dying five days later and mortality commonly persists for 10 days. Chadfield *et al*. (2003) orally inoculated young broilers with SG and found that mortality started five days after inoculation. Assoku *et al*. (1970) also recorded the same time for the beginning of mortality, with a persistence of six further days, as observed in the present study.

Although the results of the present study are consistent with other reports, it must be noted that mortality rates caused by fowl typhoid may considerably vary, and many factors may influence the severity of this disease, such as host's susceptibility (Sato *et al*., 1997). Freitas Neto *et al*. (2007) found that semi-heavy commercial layers orally inoculated with approximately $3.3x10^5$CFU/mL and that were sensitive to SG started to die on 4 dpi, whereas the SG-resistant birds started dying on 6 dpi, demonstrating the bird sensitivity to the microorganism may determine the beginning of mortality. The high mortality recorded in this experiment indicates that quails may be more susceptible to SG than chickens, as the mortality rate related with the concentration of the inoculum was relatively higher than those presented in studies with chickens. Oliveira *et al*. (2005), working with layers, recorded 94% mortality, close to that recorded in the present experiment in G3 birds, when using a SG inoculum at 8.5 x 10^8CFU/mL. Other studies administered higher concentrations than those used in this trial. Barrow *et al*. (1987) reported 50% mortality in 2-wk-old broilers orally inoculated with $1.8x10^9$CFU/mL and Rychlik *et al*. (1998) observed 60% mortality in 3-wk-old layers inoculated with $1.3x10^9$CFU/mL.

Relative to the presence of SG in fecal and cloacal swab samples, the behavior of this microorganism in inoculated quails was different than that of chickens inoculated with different *Salmonella* strains. Research studies demonstrated that, when orally inoculated with SG, layers presented positive cloacal swab samples for more than 40 dpi (Oliveira *et al*., 2005), and those inoculated with *Salmonella* Entetitidis were positive both in fecal (Nakamura *et al*., 2004; Ishola *et al*., 2009), and cloacal swab (Maddadi *et al*., 2010) samples. Other serotypes, such as *S*. Pullorum (Berchieri *et al*., 2001) and *S*. Typhimurium (Beal *et al*., 2004), have also been recovered from cloacal swabs of laying chickens submitted to oral inoculation. *S*. Typhimurium was isolated in cloacal swab samples up to seven weeks post-infection (Beal *et al*., 2004).

Oliveira *et al*. (2005) reported that the pathogen may be isolated from feces from dying chickens, and that those with no clinical symptoms that survive the infection may excrete *Salmonella* for 35 days. Freitas Neto *et al*. (2007) observed that the agent may be isolated from fecal samples on 7 dpi in semi-heavy layers. In the present experiment, only the organs of the quails that died were positive for SG; according to Beer (1998), the microorganisms present in the animal may significantly replicate after death, which may have allowed SG isolation in the present experiment. On the other hand, SG was not recovered from the organs of birds that survived until the end of the experiment (21 dpi). It is possible that these birds were able to overcome the infection because, in addition of presenting negative microbiological results, they were apparently healthy, consuming feed and water and producing eggs. Consistent findings were obtained with inoculated and sacrificed chicks: at the end of the experiments, it was not possible to recover SG from their liver, spleen, and ceca (Barrow *et al*., 1987; Jones *et al*., 2001), and the birds that survived were apparently healthy (Bumstead & Barrow, 1993).

The birds that died during the experiment presented classical fowl typhoid symptoms. Birds remained quiet in the corner of the cage, with their eyes closed, ruffled feathers, and presented greenish-yellow watery feces, which often contained blood. The presence of blood in the feces indicates sepsis, which causes gastrointestinal

hemorrhages and ulceration of the intestinal wall (Chappell *et al*., 2009).

In the present study, all analyzed egg samples were negative for SG, in agreement with previous studies using experimental infections (Berchieri Jr. *et al*, 2000; Berchieri Jr. *et al*, 2001; Oliveira *et al*, 2005). The vertical transmission of SG occurs easier in poultry strains that are genetically resistant to fowl typhoid.

Although *Salmonella* is able to penetrate the egg, there are other important extrinsic factors that may interfere in transmission, such as bacterial strain, temperature, humidity, number of microorganisms, and storage conditions (Messens *et al*., 2005). Some authors assert that eggs produced by SG-infected layers may not present any contamination (Oliveira *et al*., 2005), differently from layers that carry *Salmonella* Pullorum (SP) and may vertically transmit this agent through infected eggs due to the quantitative increase of SP in the reproductive tract during sexual maturation. It was found that gonadal hormones may aid the transport of infected macrophages to the reproductive system; however, the mode of SP transmission to the eggs has not been elucidated yet (Wigley *et al*., 2001).

CONCLUSIONS

Salmonella Gallinarum is virulent for Japanese quails and the mortality it causes is possibly influenced by the concentration of inoculated bacteria. SG may be recovered from the liver, spleen, cecum, lungs, and reproductive tract of experimentally-infected quails; however, inoculated birds may not show any clinical symptoms or shed the inoculated SG strain.

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