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Effect of doses and administration routes of 9R vaccine on protection of Japanese quails against experimental infection with *Salmonella* Gallinarum

[*Efeito das doses e vias de administração da vacina 9R na proteção de codornas japonesas contra infeccção experimental com* Salmonella *Gallinarum*]

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ABSTRACT

Coturniculture has increased significantly in the last decades. There are several pathogens that can affect these birds. Among the diseases, fowl typhoid stands out as a disease with a potentially great impact to the poultry industry. The objective of this the study was to evaluate the effect of doses and administration routes of live 9R vaccine on protection of Japanese quails against experimental infection with *Salmonella* Gallinarum (SG). Two hundred and fifty birds were used, divided into five groups: G1, oral vaccination with one dose; G2, oral vaccination with 2 doses; G3, subcutaneous vaccination with one dose; G4, subcutaneous vaccination with two doses and G5 not vaccinated. All birds from all five groups were challenged with SG at an age of 45 days. SG was quantified in the periods of one, four, seven and twelve days after the challenge. The presence of clinical signs and macroscopic lesions of the disease were observed. The groups vaccinated by subcutaneous route had a higher egg production and lower mortality rate. Birds receiving a dose of the vaccine by subcutaneous route also showed lower amount of SG in the liver and spleen seven days after the challenge.

Keywords: quail, *Salmonella* Gallinarum, 9R vaccine, administration route

RESUMO

A coturnicultura tem aumentado significativamente nas últimas décadas. Existem vários patógenos que podem afetar essas aves. Entre as doenças, o tifo aviário se destaca como uma doença de grande impacto para a indústria avícola. O objetivo deste estudo foi avaliar o efeito de doses e vias de administração da vacina viva 9R na proteção de codornas japonesas contra infecção experimental por Salmonella *Gallinarum (SG). Foram utilizadas duzentos e cinquenta aves, divididas em cinco grupos: G1, vacinação oral com uma dose; G2, vacinação oral com 2 doses; G3, vacinação subcutânea com uma dose; G4, vacinação subcutânea com duas doses e G5 não vacinado. Todas as aves dos cinco grupos foram desafiadas com SG aos 45 dias de idade. A SG foi quantificada nos períodos de um, quatro, sete e doze dias após o desafio. Foi observada a presença de sinais clínicos e lesões macroscópicas da doença. Os grupos vacinados por via subcutânea apresentaram maior produção de ovos e menor taxa de mortalidade. Aves recebendo uma dose da vacina por via subcutânea também apresentaram menor quantidade de SG no fígado e baço sete dias após o desafio.*

Palavras-chave: codorna japonesa, Salmonella *Gallinarum, vacina 9R, via de administração*

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INTRODUCTION

Salmonella infections, mostly caused by *S.* Gallinarum, *S.* Pullorum, *S.* Typhimurium and *S.* Enteritidis, are the most worrying and difficult poultry problem to control (Andreatti Filho, 2007; Berchieri Júnior and Oliveira, 2007). Salmonellosis can determine restrictions to trade of products of animal origin for causing foodborne infections in humans through ingestion of contamined food, in addition to damages due to high mortality of birds, medication costs, reduction in egg production and quality of chicks and increased costs of prevention and control measures (Shivaprasad, 2000; Penha *et al.*, 2008; Omwandho and Kubota, 2010). *Salmonella* Gallinarum (SG), a cause of fowl typhoid and responsible for high mortality (Silva *et al*., 2013) and systemic infection, can be isolated in the feces of infected birds and transmitted by sick and carrier birds (Berchieri Júnior and Oliveira, 2007; Shivaprasad and Barrow, 2008).

Vaccine-induced immunity reduces the amount of *Salmonella* in the intestine of the birds. Properly vaccinated birds better resist infection and react against invasive bacteria more quickly and effectively than unimmunized birds (Siqueira, 2009). The best known and widely used salmonellosis vaccine is 9R, made with a rugose strain of SG (Berchieri Júnior and Oliveira, 2007). Due to the scarcity of literature regarding fowl typhoid affecting quails as well as the preventive methods of this disease in these birds, studies that demonstrate such results are necessary, exploring microrganisms of interest to poultry farming. The objective of the current trial is to study the effect of doses and administration routes of live 9R vaccine on protection of Japanese quails against experimental infection with SG.

MATERIAL AND METHODS

This study was approved by the Ethics Committee for the Use of Animals (CEUA) - Protocol 0059/2017. Two hundred and sixty (260) one-day old lay Japanese quail chicks (*Coturnix coturnix japonica*) were housed in heated wire cages, receiving water and feed *ad libitum*. Immediately before being housed, 10 birds were euthanized in order to confirm the absence of *Salmonella* spp., through an established methodology (Andreatti Filho, 2007).

The birds remained in a single group until the 10 days of age, when they were randomly divided into five groups of 50 animals each, with the following design:

Group 1 (G1): birds vaccinated by oral route at 10 days of age;
Group 2 (G2): birds vaccinated by oral route at 10 and 25 days of age;
Group 3 (G3): birds vaccinated by subcutaneous route at 10 days of age;
Group 4 (G4): birds vaccinated by subcutaneous route at 10 and 25 days of age;
Group 5 (G5): non-vaccinated control birds.

Immunization was performed orally and subcutaneously (SC) with the live 9R strain (Bio-gallinarum 9R®, Biovet, Brazil) with a dose of 0.2mL/bird. All birds from all five groups were challenged with SG at an age of 45 days. The challenge was carried out by oral route using a gavage needle, inoculating a wild type of SG strain (0.2mL/bird) containing $8.1x10^8$ CFU/mL (Colony-forming units) resistant to nalidixic acid (Nal) and rifampicin (Rif) was developed to allow quantification. On days 1, 4, 7 and 12 after the challenge three intact eggs from each treatment were collected and five birds of each treatment were euthanized by cervical displacement and the liver, spleen, cecum and ovary were individually collected for SG quantification.

For SG quantification in the organs, they were macerated and homogenized with PBS in the 1:10 ratio, getting a 10^{-1} dilution followed by other dilutions until 10^{-8}, with a subsequent plating in duplicate of 0.1mL on AVB Nal/Rif plates, cultivated for 24 hours at 40°C, obtaining the amount of CFU. In the eggs, SG was quantified in the shell by immersing the three intact eggs in 22.5mL of PBS in plastic bags and gently mixed, followed by a quantification procedure similar to that performed in the organs. Then, the same eggs were immersed in a 70% ethanol solution for five minutes. After drying, all contents of the three eggs were removed and homogenized in 45mL of PBS followed by the SG quantification already described. The PBS volume used was readjusted according to Katayama *et al.* (2012).

At the same time, organ and egg macerates (shell and contents) were enriched separately in test tubes containing the Tetrathionate and Rappaport Vassiliadis broths, incubated at 40ºC for 24 hours with subsequent plating in AVB Nal/Rif, to confirm the presence of SG in the negative cases of bacterial counts.

The evaluation of egg production was submitted to Kruskal-Wallis non-parametric test, complemented with Dunn's multiple comparison test (Zar, 2009). The mortality evaluation was submitted to Goodman's association test, complemented by multiple comparisons between and within multinomial populations (Goodman, 1964, 1965), considering the 5% level of significance. The evaluation of the SG count in the organs, egg shell and egg contents was performed by the non-parametric variance technique for the two-factor model (treatment and sacrifice moment), complemented by Dunn's multiple comparison test (Zar, 2009), considering the 5% level of significance.

RESULTS AND DISCUSSION

The daily monitoring of the birds enabled the verification of clinical signs such as apathy, bristled feathers, dehydration, hemorrhagic diarrhea and prostration, similar to that observed by Silva *et al*. (2016), Zancan *et al.* (2016) and Batista (2017). The mortality of SG-infected birds can occur from four to five days after exposure and may persist up to almost two weeks (Shivaprasad, 2000; Silva *et al.*, 2013). In addition to the clinical signs mentioned above, a reduction in egg production was observed, with mortality beginning two days after SG administration, persisting for up to 12 days, similar to that observed in chickens by Batista (2017). Also in relation to mortality (Table 1), it was found that in all vaccinated groups, the number of living birds was higher than that of dead birds, whereas in the control group there was no difference when the experimental period was analyzed from the second vaccinal dose performed in the treatments of groups 2 and 4, corroborating with the one observed by Zancan (2013), in which the vaccination decreased bird mortality.

It is also worth noting the protection of 58% of the unvaccinated control group after challenge, suggesting a natural genetic resistance of the species that may also occur with chickens or by the characteristic of strains or inoculated dose that causes low mortality.

Table 1. Protection of Japanese quails vaccinated with live 9R strain of *Salmonella* Gallinarum (SG) orally and subcutaneously route and subsequently challenged with SG

Treatment groups*	No dead birds / total challenged	Protection (%)
G1 - Oral at 10 days	8 / 50bA**	84 %
G2 - Oral at 10 and 25 days	11 / 50bA	78 %
G3 - SC at 10 days	3 / 50aA	94 %
G4 - SC at 10 and 25 days	3 / 50aA	94 %
G5 - Control not vaccinated	21 / 50cA	58 %

*Oral and subcutaneous (SC) vaccinated groups in different ages. All birds from all groups were challenged with SG at 45 days of age. ** Lowercase letters: comparison between groups with mortality response set; uppercase letters: mortality comparison inside the group.

The control group had a higher mortality, which was significantly different from the groups that received the oral vaccine (G1 and G2), which in turn had higher mortality than the groups receiving the vaccine by subcutaneous route (G3 and G4), regardless of the amount of doses. The irrelevancy of the amount of doses corresponds with the findings of Penha Filho (2009) in which the administration of one or two doses of vaccine in chicken and Zancan (2013) with the administration of two or three doses confirmed partial protection to the birds against the challenge by wild strain of SG. Specifically, in quails, Tirabassi *et al.*, 2016, reported that the 9R vaccine provides protection against fowl typhoid.

A degeneration and/or necrosis of skeletal musculature of the chickens was noted by Zancan *et al.*, 2016, as well as in this experiment. The productivity analysis during the week before the challenge with SG shows that there was no significant difference between the

experimental groups. However, during the 2-week period after the challenge with SG, it was observed that groups 3 and 4 (one and two subcutaneous vaccine doses, respectively) showed a better egg production, and the control group did not differ from the other experimental groups.

The macroscopic changes observed in euthanized birds in different analysis periods, such as hepatomegaly, splenomegaly, liver and spleen necrosis and hemorrhage, hemorrhage and atrophy in the ovary (ovarian follicles), and hemorrhage of cecal tonsils are commonly described by other authors in birds challenged with SG (Deshmukh *et al*., 2007; Casagrande *et al.*, 2014; Silva *et al*., 2016; Batista, 2017). It is also common to find changes in air sacs, lungs, heart, intestine and distended gallbladder (Zancan *et al.*, 2016).

Silva *et al.* (2013) reisolated SG in the liver and lung, four days after the challenge in 16% of the inoculated quails. In quails challenged with a larger quantity of SG there was re-isolation of the bacterium in liver, spleen and lung in 33% of birds four days after the challenge and in the ovary/oviduct of 50% of the birds one week after the challenge. Batista (2017) also reisolated SG in the liver and spleen of the birds three days after the challenge. Om the 7th day after the challenge with SG a significantly higher amount of the bacterium (Table 2) was quantified in the liver and spleen of the control group (G5) in comparison to the birds vaccinated by subcutaneous route at 10 days of age (G3). In the same period, the control group presented higher amount of SG when compared to the 1st day after the challenge.

In relation to SG quantification in cecum (Table 3), there was no significant difference between treatments. The highest amount of SG in birds vaccinated by oral route at 10 days of age (G1), birds vaccinated by oral route at 10 and 25 days of age (G2) and birds vaccinated by subcutaneous route at 10 and 25 days of age (G4) occurred on the 4th day after the challenge, when compared to the 12th day after the challenge with SG in each of the different treatments.

Table 2. Mean amount of *Salmonella* Gallinarum (SG) in the liver and spleen of Japanese quails vaccinated with live 9R strain of SG orally and subcutaneously and subsequently challenged with SG. Values expressed in Log_{10}

Treatment*	Days after the challenge				p value
	1	4	7	12	
G1 - Oral at 10 days	0	2.9	2.5 ab	0	P> 0.05
G2 - Oral at 10 and 25 days	0	2.3	2.5 ab	2.0	P> 0.05
G3 - SC at 10 days	0	1.6	0 a	2.0	P> 0.05
G4 - SC at 10 and 25 days	0	2.3	2.9 ab	0	P> 0.05
G5 - Control not vaccinated	0 A**	2.3 AB	3.5 ab	2 AB	P< 0.05
P value	P> 0.05	P> 0.05	P< 0.05	P> 0.05	

*Oral and subcutaneous (SC) vaccinated groups in different ages. All birds from all groups were challenged with SG at 45 days of age. ** Lowercase letters: amount comparison between groups (column); uppercase letters: comparison of body weight inside the group (line).

Table 3. Mean amount of *Salmonella* Gallinarum (SG) in the cecum of Japanese quails vaccinated with live 9R strain of SG orally and subcutaneously route and subsequently challenged with SG. Values expressed in Log_{10}

Treatment*	Days after the challenge				p value
	1	4	7	12	
G1 - Oral at 10 days	3.5 AB**	4.1 B	2.1 A	0 A	P< 0.05
G2 - Oral at 10 and 25 days	3.9 AB	4.5 B	3.8 AB	0 A	P< 0.05
G3 - SC at 10 days	3.7	4.1	1.9	1.6	P> 0.05
G4 - SC at 10 and 25 days	3 AB	4.5 B	3 AB	0 A	P< 0.05
G5 - Control not vaccinated	4.7	4	3.4	2.6	P> 0.05
p value	P> 0.05	P> 0.05	P> 0.05	P> 0.05	

*Oral and subcutaneous (SC) vaccinated groups in different ages. All birds from all groups were challenged with SG at 45 days of age. ** Uppercase letters: amount comparison inside the group (line).

There was no significant difference between the experimental groups in the SG quantifications in the yolk sac, egg shell and in the egg contents, similar to that observed by Berchieri Júnior *et al*. (2000). In an experiment using *Salmonella* Pullorum (SP), Okamura *et al.* (2007) showed that bacteria containing *Salmonella* Enteritidis strain reduced the vertical transmission of SP in laying birds.

CONCLUSION

The present study concludes that the immunization of quails with 9R brings significant benefits against infection by *Salmonella* Gallinarum. The birds vaccinated via subcutaneous route with one dose at 10 days of age presented the best results, especially regarding the isolation of SG in the liver and spleen, higher egg production and lower mortality. Studies regarding the efficacy of Salmonella vaccines in quails are of great importance due to lack of research on the species. *Salmonella* Gallinarum is virulent for Japanese quail, causing economic losses. Experimentally infected birds present clinical signs and anatomopathological changes similar to those presented by commercial laying hens affected by the pathogen.

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