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Protection of chickens against fowl typhoid using field vaccine programs formulated with the live attenuated strain *Salmonella Gallinarum* ΔcobSΔcbiA

Proteção de aves contra o tifo aviário utilizando a cepa atenuada **Salmonella Gallinarum ΔcobSΔcbiA** *em diferentes programas vacinais de campo*

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ABSTRACT: *Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) is a host-specific bacteria that causes the fowl typhoid (FT). This disease is highly pathogenic to commercial chickens, specially brown layers and breeders, causing acute septicemia followed by high morbidity and mortality. Vaccination is extensively adopted in the fields as a biosafety tool for prevention of isolated infections and outbreaks in commercial poultry flocks. The present study evaluated the use of an attenuated SG with deletions on genes *cob*S and *cbi*A (SGΔ*cob*SΔcbiA) as a live vaccine, using vaccination schemes adjusted for field conditions. To this end, brown layers were used in two different experiments, to evaluate the long-term protection, necessary in the fields. The vaccination scheme on the first experiment consisted of two doses, the first at 4^{th} week-of-age and the booster dose at 8^{th} week-of-age with challenge at 16^{th} week-of-age with wild SG strain. On the second experiment, the vaccination was carried out by different routes using three doses of the live vaccine, at 4^{th}, 8^{th} and 12^{th} weeks-of-age, and the challenge was done at 20^{th} weeks-of-age. After the challenge, the mortality was recorded during 28 days, and the egg production (experiment 2) was evaluated and compared with the group of unvaccinated layers. In both experiments, the mortality was significantly reduced, and the egg production was not affected in vaccinated layer-hens. In summary, this study shows the efficacy and the protection of different vaccination schemes against FT that can be applied under field conditions in commercial poultry farms.

KEYWORDS: immunity; attenuated; eggs; layer-hens; infection.

RESUMO: *Salmonella enterica* sorovar Gallinarum biovar Gallinarum (SG) é uma bactéria hospedeira específica que causa o tifo aviário (TA). Essa doença é altamente patogênica em aves comerciais, especialmente galinhas poedeiras de linhagem vermelha e aves reprodutoras pesadas, causando septicemia aguda, e consequentemente, alta morbidade e mortalidade. A vacinação é amplamente utilizada no campo como uma ferramenta de biossegurança para a prevenção de infecções isoladas e surtos nas granjas avícolas comerciais. O atual estudo avaliou o potencial vacinal de uma cepa viva atenuada de SG com deleções nos genes *cob*S e *cbi*A (SGΔ*cob*SΔcbiA), utilizando esquemas de vacinação formulados para utilização em campo. Para isso, as galinhas poedeiras de linhagem vermelha foram utilizadas em dois experimentos diferentes, para avaliar a proteção a longo prazo, necessária no campo. O esquema de vacinação no primeiro experimento consistiu em duas doses, a primeira na quarta semana de vida e a dose de reforço na oitava, e o desafio na 16ª semana com a estirpe selvagem SG. No segundo experimento, a vacinação foi realizada por diferentes rotas usando três doses da vacina viva, na quarta, na oitava e na décima segunda semana de vida, e o desafio foi feito na 20ª semana de vida. Após o desafio, a mortalidade foi acompanhada por 28 dias, e no experimento 2 a produção de ovos também foi avaliada e comparada com o grupo de galinhas não vacinadas. Em ambos os experimentos, a mortalidade foi significativamente reduzida, e a produção de ovos não foi afetada nos grupos de galinhas poedeiras vacinadas. Este estudo mostra a eficácia da proteção dos diferentes programas de vacinação contra o TA, que podem ser aplicados em granjas comerciais em condições de campo.

PALAVRAS-CHAVE: imunidade; vacina viva atenuada; ovos; galinha; infecção.

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INTRODUCTION

Salmonella enterica subsp. *enterica* sorovar Gallinarum biovar Gallinarum (SG) is a non-motile, host-specific bacteria that causes fowl typhoid, a disease of elevated economic impact for the poultry industry (BARROW; FREITAS NETO, 2011; ROSU et al., 2007). This biovar causes septicemic infection in young and adult chickens, resulting in high mortality rates within infected chickens (SHIVAPRASAD, 2000). Infection of poultry can lead to the development of clinical or subclinical symptoms, depending on the chicken variety, genetic resistance, immune response of the host and general nutritional and health status of the flock (FREITAS NETO et al., 2007). The infections by SG are usually acute, and the spread of the bacteria is more intense if carcasses of infected dead chickens are exposed to other chickens (BARROW et al., 1994; BERCHIERI JR. et al., 2000). SG can survive in the animal environment, and, due to the constant and slow horizontal transmission, the mortality is persistent in affected flocks, making it difficult to eradicate the infection (FEBERWEE et al., 2001b).

Legislations and biosafety programs in Brazil allow the vaccination of commercial chickens for the prevention of this disease, except in breeder flocks (BRASIL, 2003). The utilization of vaccines to protect chickens against fowl typhoid, using attenuated strains of *Salmonella* Gallinarum, is a desirable feature once this serotype is not pathogenic for humans. Currently, live vaccines (LV) against SG are frequently used in the fields. The attenuated strains are able to colonize internal organs, without clinical symptoms, however they induce an efficient immune response (DE PAIVA et al., 2009; LEE et al., 2005; SHEHATA et al., 2013). The use of LV prepared with attenuated strains of SG has showed good immunogenicity and capacity to induce a strong cellular and humoral immune response in experimental models (MATSUDA et al., 2011a; 2011b; PENHA FILHO et al., 2012). Previous work from our group showed that the mutant strain SGΔ*cob*SΔcbiA has had the potential to protect chickens against SG infection in experimental models (PENHA FILHO et al., 2010; 2012).

Therefore, the present study was designed to evaluate the efficacy of the attenuated strain SGΔ*cob*SΔcbiA to stimulate long-term protection against fowl typhoid using vaccination schemes applicable in field conditions.

MATERIALS AND METHODS

Chickens

Two-hundred and ten commercial brown layer-hens, susceptible to fowl typhoid, were used. Chickens were tested at arrival for *Salmonella* spp., and all chickens used in these experiments were negative for this bacterium. Animal experiments were approved by the Committee of Ethics in Animal Welfare (Proc. N. 008971/11).

Inoculums and vaccines

Mutant strain of SG with deleted genes *cob*S and *cbi*A (SGΔ*cob*SΔ*cbi*A), attenuated for commercial chickens, was constructed according to the previously described (DE PAIVA et al., 2009) and used LV. The construction and use of the mutant strain was approved by the National Technical Committee of Biosecurity (Protocol no. 2982/2011).The pathogenic wild strain of SG 287/91 was used to challenge the chickens. A commercial bacterin against *Salmonella enterica* subsp. *enterica* sorovar Enteritidis (SE) was used as the killed vaccine (KV). The SG inoculums were prepared in LB broth (Oxoid, United Kingdom) culture containing nalidixic acid (30 μg/mL) (Sigma-Aldrich, United States), at 37ºC for 24 hours. The culture was centrifuged for 20 minutes at 5,000 g at 20ºC. The pellet was suspended in phosphate-buffered saline (PBS), pH 7.4, at the concentration of 10^8 colony forming units (CFU)/mL. Oral vaccination was performed by the inoculation of 1 mL of PBS containing 10^8 CFU/mL of SGΔ*cob*SΔ*cbi*A into the crop of the chickens. The subcutaneous (SC) vaccination was done in the neck using 0.5 mL of PBS containing 10^8 UFC/mL of SGΔ*cob*SΔ*cbi*A for each chicken. The KV was administered intramuscularly (IM), according to the manufacturer instructions. The challenge was done by the oral route, with1 mL of PBS containing 10^8 UFC/mL of the wild strain SG 287/91.

Experimental design

Vaccination schemes used in both experiments 1 and 2 are described in Table 1. After the challenge, the mortality was recorded until 28 days post-infection (experiments 1 and 2), and the egg production by adult chickens was recorded and compared with unvaccinated chickens (experiment 2) to evaluate the effect of vaccination and challenge in the productive period of layer-hens. Clinical symptoms and general health status were evaluated throughout the experiments, after vaccinations and challenge.

Bacteriology

At 28 dpi, all surviving chickens from all groups (experiments 1 and 2) were euthanized, and swabs were harvested from liver and spleen to assess the presence of challenge strain SG 287/91 in internal organs. Immediately after sampling, swabs were inoculated in LB broth (Oxoid, United Kingdom) and incubated at 37ºC for 24 hours and subsequently plated in Brilliant Green Agar (Oxoid, United Kingdom) containing nalidixic acid (30 μg/mL) (Sigma-Aldrich, United States).

Statistical analysis

The mortality rate, the egg production and SG isolation results were analyzed by the nonparametric chi-square method considering the significance levels of 5% ($p<0.05$).

RESULTS

Experiment 1: efficacy of two vaccine doses against fowl typhoid

The use of two doses of LV administered by different routes in chickens from groups A and B showed the same protection efficacy against SG 287/91 challenge as in group C, which received a mixed vaccination with one dose of LV at 4 weeks-of-age and one dose of KV at 8 weeks-of-age. The mortality rates in groups A, B and C reached 3.3% (1/30) and were significantly lower ($p<0.05$) than in unvaccinated control (group D), which had 33% (10/30) of mortality after challenge.

At 28 dpi, all surviving chickens were euthanized for bacteriological examination, and results are shown in Table 2. The bacteriology showed that 48.2% (14/29) of chickens in group A were still positive for SG 287/91. In group B, 68,9% (20/29); in group C, 34.5% (10/29); and in group D (unvaccinated chickens), 40% (8/20) of surviving chickens carried the challenge strain (SG 287/91) in liver or spleen samples at 28 dpi.

Experiment 2: efficacy of three vaccine doses and egg production after SG challenge

The use of three doses of LV, administered orally, conferred protection of 80% in group E, once the mortality rate reached 20% (6/30). Similar protection was noticed in group F, which was vaccinated with two oral doses, and the third booster dose was given via SC, showing a mortality rate of 16.7% (5/30). The protection was significantly higher in the vaccinated groups (E and F) in comparison with the unvaccinated chickens in group G, in which the mortality rate reached 50% (15/30) ($p<0.05$).

At 28 dpi, all surviving chickens were euthanized for bacteriological examination, and results are shown in Table 3. The bacteriology showed that 54.1% (13/24) of chickens in group E were still positive for SG 287/91. In group F, 64% (16/25) and, in group G, (unvaccinated chickens) 40% (6/15) of surviving chickens carried the challenge strain (SG 287/91) in liver or spleen samples at 28 dpi.

Table 1. Vaccination schemes using two or three doses.

Experiment	Groups	n	Age of chickens (weeks)			Challenge SG 287/81 (w)
			4^{th} w	8^{th} w	12^{th} w	
1	A	30	LV/oral	LV/oral	-	16^{th}
1	B	30	LV/SC	LV/oral	-	16^{th}
1	C	30	LV/oral	KV/IM	-	16^{th}
1	D*	30	-	-	-	16^{th}
2	E	30	LV/oral	LV/oral	LV/oral	20^{th}
2	F	30	LV/SC	LV/oral	LV/oral	20^{th}
2	G*	30	-	-	-	20^{th}

*Negative control group; w: weeks of age; LV: live vaccines; SC: subcutaneous; IM: intramuscularly.

Table 2. Isolation of wild SG 287/91 at 28 dpi in liver and spleen samples of surviving chickens from groups A, B and C (vaccinated) and group D (unvaccinated).

	Groups			
	A	B	C	D
Positive/surviving (%)	14/29 (48.2)	20/29 (68.9)	10/29 (34.5)	8/20 (40.0)

Different letters indicate statistically significant difference ($p<0.05$).

Table 3. Isolation of wild SG 287/91 at 28 dpi in liver and spleen samples of surviving chickens from groups E and F (vaccinated) and group G (unvaccinated).

	Groups		
	E	F	G
Positive/surviving (%)	13/24 (54.1)[a]	16/25 (64.0)[a]	6/15 (40.0)[b]

Different letters indicate statistically significant difference ($p<0.05$).

The onset of lay was at 17 weeks-of-age for hens in experiment 2, and the egg production was recorded until 24 weeks-of-age (28 dpi). The results in Table 4 show that the production of eggs was significantly higher in vaccinated groups E and F ($p<0.05$) in comparison with control group G. The challenge affected laying from 6 to 12 dpi, when feed consumption was reduced and egg production was interrupted in all groups. Although the vaccinated hens showed fast recovery after challenge and returned the laying, whilst unvaccinated layers (group G) showed severe clinical symptoms and onset of mortality.

DISCUSSION

The poultry production worldwide is constantly affected by fowl typhoid (OIE, 2015), resulting in morbidity and mortality of chickens and causing high production losses. Brown layer-hens and meat varieties (broilers) are highly susceptible to infection and develop clinical signs ranging from low feed consumption, green-yellowish diarrhea, fever, prostration, which frequently result in mortality and culling of flocks (FREITAS NETO et al., 2007; GARCIA et al., 2009; SHIVAPRASAD, 2000).

The reduced mortality rates recorded in experiment 1 demonstrate that the use of two doses of LV or the combination of LV with a booster dose of KV have similar efficacies to protect chickens against the most advanced development of fowl typhoid. The mortality was 10 fold reduced in vaccinated groups (3.3%) in comparison with unvaccinated group (33%). Considering that the layer-hens in experiment 1 were challenged on the 16^{th} weeks-of-age, which is the onset of sexual maturity, and a period of stress and vulnerability of the host organism to respond to pathogens, the vaccination allowed these chickens to control the development of fowl typhoid and survive the challenge. The strain SGΔ*cob*SΔ*cbi*A showed good efficacy to protect chickens from mortality caused by SG infection in the early ages, before reaching sexual maturity (PENHA FILHO et al., 2010). However, under field conditions, different factors interfere in poultry immune responses and vaccination efficacy, such as nutrition, stressful conditions, infections, sexual maturation and age, thus booster doses are often used in vaccination programs to confer a long lasting immunity (FEBERWEE et al., 2001a).

Table 4. Cumulative number of eggs laid by adult hens vaccinated with three doses in experiment 2, from 17 to 24 weeks-of-age (28 dpi).

	Groups		
	E	F	G
Total egg production	127^{a}	117^{a}	39^{b}

Different letters indicate statistically significant difference ($p<0.05$).

Therefore, in the present study the use of one dose of LV in combination with KV showed the same efficacy as the use of two doses of LV to prevent mortality caused by wild SG strain. The use of three doses of SGΔ*cob*SΔ*cbi*A showed to be beneficial to the recovery of layer after challenge, once all chickens interrupted the egg production at 6 dpi; however, the immunized returned to lay eggs at 12 dpi, whilst the control group showed mortality and ceased production. Considering the benefits, the use of vaccines has been indicated by different studies, especially in areas with outbreaks or imminent challenge (FEBERWEE et al., 2001a; FRASER et al., 2007).

The use of vaccination also showed to be advantageous to layer flocks considering the recovery time, allowing the birds to clear the bacteria during the first week of infection and return to lay eggs after a short period. As shown in Table 4, the production of eggs is significantly higher in vaccinated adult layers. The decreased egg production in unvaccinated chickens was not only due to the mortality in this group, but also related to the pathology caused by the bacterial proliferation in the host organism, causing gross lesions in important organs for the systemic homeostasis. Throughout the experimental period after challenge the clinical symptoms daily evaluated were decreased in all vaccinated birds, using two or three doses of LV, whilst in unvaccinated animals severe clinical symptoms preceded death, including anorexia, weakness, greenish diarrhea, loss of appetite, fluffed feather and fever. The use of a live attenuated strain of SG, with deletions on *cpx*R and *lon* genes, could also protect chickens against fowl typhoid, and only slight anorexia and depression were temporarily observed by the authors, while in the unvaccinated group, birds showed severe infection followed by mortality of 100% (MATSUDA et al., 2011b).

The observations reported by many different authors affirm that excretion of SG in feces and eggs is not detectable with current methods, thus vaccination against this disease should control the bacterial spread to the host organism decreasing the bacterial numbers in organs in a short period of time, reducing the risk of death and horizontal transmission of this bacteria specially through the contact with carcasses or other infected tissues (BERCHIERIJR. et al., 2001; SHIVAPRASAD, 2000). The efficacy of the attenuated strain SGΔ*cob*SΔ*cbi*A was shown in experimental models; however, in the current study, we show the capacity of this strain to protect against development of clinical fowl typhoid in adult birds vaccinated with applicable schemes. The long-term protection is necessary and desirable for the use of a vaccine in the fields (COLLINS, 1974; DOUGAN et al., 2011). The results shown in the present study suggest the good capacity of the attenuated strain to protect adult layer-hens in the fields, controlling the development of the disease in a shorter period and reducing losses related to animal deaths or lower eggs production.

CONCLUSIONS

The attenuated strain of SG showed a protection rate that ranged from 80 to 96.7% in chickens vaccinated with two and three doses.

The booster immunization using one (groups A, B and C) or two booster doses (groups E and F) were capable to stimulate the long-lasting immune response, protecting chickens against wild SG challenge at 16^{th} and 20^{th} weeks-of-age.

The vaccination with attenuated SG strain showed to be safe and supported a fast recovery of chickens after challenge, allowing good egg laying rates in comparison with unvaccinated chickens.

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