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Safety and Efficacy of a *Salmonella* Gallinarum *ΔcobSΔcbiA* Strain with Potential to Prevent Chicken Infections by *Salmonella* Gallinarum and *Salmonella* Enteritidis

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ABSTRACT

New vaccine design techniques have allowed the development of effective vaccine strains against *Salmonella* infections in which the risks of reversion to the wild type and virulence is null. The mutant strain *Salmonella* Gallinarum Δ*cob*SΔ*cbi*A was previously shown to be avirulent in chickens. In this study, this strain was tested as a vaccine against *Salmonella* Gallinarum (SG) and *S.* Enteritidis (SE) infections, and its protection levels, safety and possible risks of reversion to virulence after vaccination of layers were evaluated. Birds were vaccinated at five days of age or at five and 25 days of age. At 45 days of age, brown and white layers were challenged with SG and SE wild strains, respectively. Two assays to test the possibility of reversion to virulence were performed. Five successive bacterial passages in brown layers were carried out in the first assay. In the second assay, brown layers received a ten-fold concentrated inoculum of the SGΔ*cob*SΔ*cbi*A strain and were evaluated for clinical signs and mortality. In both experiments, no birds that received the inoculation of the attenuated strain died. Additionally, the use of the mutant strain as a vaccine provided good protection levels against both challenge strains.

INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Gallinarum (SG) is the causal agent of fowl typhoid. This disease is characterized by the systemic infection of the host and high mortality rates, reaching up to 80% of the affected flocks (Shivaprasad, 2000; Jones *et al.*, 2001). Over the past years, this disease has been prevented by the development of new vaccination approaches, together with multiple health strategies on the rearing farms. Live attenuated SG vaccine strains have demonstrated to be effective against the infection by the heterologous serovar *Salmonella* Enteritidis (SE) (Penha Filho et al., 2009; Penha Filho *et al.*, 2010). However, non-typhoidal serotypes are difficult to control in the field, because the epidemiology of these *Salmonella* serotypes involves several components present in the environment. These pathogens can infect and persist in different hosts, such as wild birds, rodents, and insects, which may cause the massive contamination of commercial flocks and are particularly associated with horizontal transmission (Guard-Petter, 2001; Berchieri Jr. *et al.*, 2000, Shivaprasad, 2000). In order to reach effective *Salmonella* control, an adequate immune response using non-pathogenic-attenuated strains should be the main objective in a biosafety program (Mastroeni *et al.*, 2000).

In previous studies, mutants of SG were constructed with the deletion in eight genes involved in the bacterial anaerobic respiratory pathway. Among the mutants, a SG strain with double deletion in genes *cob*S and

*cbi*A, belonging to the *cob* operon, lost its virulence for chickens (Paiva *et al.*, 2009ab). Additionally, studies were conducted to assess the use of this strain as a potential vaccine for the protection of chickens against SG and for cross-protection against SE, as this vaccine strain is considered much less harmful to human beings than live vaccines containing viable SE cells (Lee et al., 2007). The mutant strain SGΔ*cob*SΔ*cbi*A reduced mortality rates caused by the wild type SG and the excretion of SE by infected chickens (Penha Filho et al., 2010). These findings are very significant, because chickens are considered the most important source of the human foodborne salmonellosis agent (Galanis *et al.*, 2006, Belgian Laboratory for *Salmonella*, 2010). Since birds can be infected at any time of their lives, improving their immunity could be a valuable approach to protect chickens that produce eggs either for breeding (parent flocks) or for human consumption, and the use of vaccination as part of a general biosecurity program could enhance the control of chicken infection by these two *Salmonella* serotypes. Although it has been shown that live vaccines against *Salmonella* can stimulate a complete immune response, these strains can only be used in the field only after studies on their possible residual virulence and stability of attenuation are completed.

Aiming at ensuring that the mutant SG Δ*cob*SΔ*cbi*A is a safe vaccine strain, this study evaluated its virulence attenuation and its potential to reverse to virulence.

MATERIAL AND METHODS

Birds

Commercial lines of brown and white lines table-egg female layers, highly susceptible to fowl typhoid and *S.* Enteritidis infection, respectively (Berchieri Jr. *et al.*, 2000; Penha Filho *et al.*, 2010), were used in the experiments. All birds were obtained at day of hatch and were reared and fed as recommended by the manual of each genetic line.

At arrival, the *Salmonella*-free status of the birds was checked by the inspection of the transport boxes using drag swabs to detect *Salmonella* spp. (Zancan *et al.*, 2000). All birds were negative.

The experiments followed the approaches adopted by Berchieri *et al.* (2001a, b). Birds were housed in rooms with controlled environment in battery cages, receiving water and feed *ad libitum* throughout the experiments.

Bacterial strains

The description of the construction of the attenuated nalidixic acid-resistant strain of *Salmonella* Gallinarum, carrying deletion on *cob*S and *cbi*A genes (SGD*cob*SD*cbi*A) was described by Paiva *et al.* (2009b). A pathogenic strain of *Salmonella* Gallinarum resistant to nalidixic acid was used to challenge the brown layers and a pathogenic strain of *Salmonella* Enteritidis resistant to nalidixic acid and spectinomycin was used to challenge the white-egg layers. Both bacterial strains were donated by Dr. P.A. Barrow (University of Nottingham, Leicestershire, UK) and previously shown to be invasive in brown and white varieties of layers (Berchieri Jr. *et al.*, 2001ab).

Preparation of the inocula

The inocula consisted of cultures of each strain (SGΔ*cob*SΔ*cbi*A, SG and SE) in Luria-Bertani (LB) broth (Invitrogen 12780-052) prepared in a shaking incubator (100rpm) at 37ºC overnight. Ten dilutions of each culture were plated on Brilliant Green Agar (BGA) (Oxoid CM0263) containing novobiocin (1µg/mL) and nalidixic acid (20 µg/mL) (BGA Nal/Nov), incubated at 37ºC for 24h, to determine their titers. The cultures used to prepare the inocula contained approximately 10^8 colony forming units (CFU) per mL.

Assessment of the attenuation of the *SGΔcobSΔcbiA* strain

Virulence reversion assessment

Experiment 1: Successive bacterial passages *in vivo*

Two groups of 20 brown layers each were used. Birds in one group were orally inoculated with 1.0 mL of an overnight broth culture of SGΔ*cob*SΔ*cbi*A, while those in the second group were subcutaneously inoculated in the nape of the neck. In this case, the broth culture was centrifuged at 4000rpm and the pellet was resuspended in equal volume of PBS pH7.4. Two, five and seven days post-inoculation (dpi) three birds from each group were sacrificed by cervical dislocation and the presence of the SGΔ*cob*SΔ*cbi*A strain was investigated in the liver and spleen. Swabs taken from these organs were directly plated onto Brilliant Green Agar (CM0263, Oxoid) containing nalidixic acid (25µg/mL) (BGANal). Additionally, swab tubes were cultured in selenite broth (CM0395 Oxoid)

containing novobiocin (40µg/mL) (SN) for enrichment. Swabs and plates were incubated at 37ºC for 24h. . In the absence of colony growth in the plates, new plating was performed with the enriched swabs *Salmonella* colonies were purified in LB agar containing discs with the antimicrobial substances nalidixic acid, spectinomycin, and kanamycin. The remaining birds were reared to 21 dpi, when they were sacrificed by cervical dislocation and inspected for the presence of SGΔ*cob*SΔ*cbi*A in the liver and spleen, by the same methods previously described.

The assay was carried out five times. In the first assay, 5-d-old birds were inoculated either with 0.5 mL orally or 0.2 mL subcutaneously. In the second assay, 12-d-old birds were inoculated with the same doses and the same routes. In the third assay, birds were 25 days old and received 1.0 mL of the inocula orally and 0.2 subcutaneously. In the fourth assay, 6-d-old birds were inoculated as described in the first assay. In the fifth assay, birds were 23 days old and were inoculated as described in the third assay. The SGΔ*cob*SΔ*cbi*A recovered from liver or spleen was inoculated in the birds in the following test and so on, up to the fifth.

Experiment 2: High dose of SGΔ*cob*SΔ*cbi*A inoculum

Brown layers at 14 days of age were divided into four groups of 20 birds each. Two groups were inoculated with 0.5 mL orally and in the other two groups with 0.2 mL subcutaneously, as described above. In both cases, one group received the inoculum of SGΔ*cob*SΔ*cbi*A (containing 4.1 x 10^8 CFU/mL) and the other group received the same volume of the inoculum, but which contained ten times more bacteria (5.8 x 10^9 CFU/mL). Birds were inspected during 28 days.

Vaccination with *SGΔcobSΔcbiA*

Prevention against SGNal^r

One hundred and twenty three chicks of a commercial brown layer strain were separated in three groups of 41 birds each. Birds in group A and B were vaccinated at five and 25 days of age and birds in group C were not vaccinated. Group A birds were inoculated by gavage with 0.5 mL of SGΔ*cob*SΔ*cbi*A broth culture containing 10^8 CFU/mL. Group B birds received 0.2 mL of SGΔ*cob*SΔ*cbi*A broth culture resuspended in PBS pH 7.4, subcutaneously in the nape of the neck. Group C birds were not vaccinated. At 45 days of age, all birds were orally challenged with 1.0 mL of the wild SG culture, containing 10^8 CFU/mL. Mortality was recorded for four weeks. At the end of this period, all surviving birds were sacrificed and their livers were swabbed. The swabs were enriched in SN broth at 37ºC for 24h, and plated onto BGA Nal/Nov for the isolation of SGNal^r.

Prevention against SENal^{r}Spec^r

Three groups of 35 chicks of a commercial white layer strain were vaccinated as described above. At the age of 45 days, all birds were orally challenged with 2.0 mL of SE culture with 10^8 CFU/mL.

In this assay, systemic infection and fecal shedding of the challenge strain were evaluated. At two, five, seven, 14, 21 and 28 dpi, five birds from each group were sacrificed by cervical dislocation and the viable count of the challenge strain was estimated in the liver, spleen and cecal contents. Twice a week, at three, seven, 10, 13, 17, 21, and 24 dpi, cloacal swabs were taken from all remaining birds and inspected for fecal shedding of SE, as described above.

RESULTS

Assessment of the attenuation of the *SGΔcobSΔcbiA* strain

Virulence reversion assessment

SGΔ*cob*SΔ*cbi*A was isolated up to seven dpi from birds from both groups after each inoculation. The remaining birds did not die and did not present any signal of fowl typhoid and also, SGΔ*cob*SΔ*cbi*A was not recovered from their liver or spleen at 28 dpi. This situation persisted until the last inoculation.

In experiment 2, birds that received the higher dose of the inoculum (10-fold of the original concentration) by both routes were examined for 28 days and did not show any signs of fowl typhoid.

Vaccination with *SGΔcobSΔcbiA*

Prevention against SG

The results are presented in Table 1. Birds were vaccinated at five and 25 days of age and challenged at 45 days of age. Mortality was lower in the vaccinated groups. The group of birds that received the vaccine by subcutaneous route presented significantly ($p < 0.05$) higher protection.

Table 1 - Cumulative mortality of brown layers orally vaccinated with SGΔcobSΔcbiA and challenged with wild Salmonella Gallinarum strain.

Vaccination route	Cumulative mortality (dpi) in groups with 41 birds.													
	5	6	7	8	9	10	11	12	13	19	20	21	28	Total (%)
Oral	1	3	11	21	24	26	27	-	28	-	-	-	-	68,29
SC	-	2	3	4	5	7	-	-	-	-	-	9	-	21,95
Unvacc.	4	16	26	38	39	-	41	-	-	-	-	-	-	100

SC: subcutaneous; Unvacc.: unvaccinated birds.

Prevention against SE

Three groups with 35 chicks of a commercial white layer strain were vaccinated as in the previous experiment. At 45 days, all birds were orally challenged with 2.0 mL of SE culture containing 10^8 CFU/mL. In this assay, systemic infection and fecal shedding of the challenge strain were evaluated.

Data (log_{10}) relative to the presence of the challenge strain in cecal contents, and in the liver and spleen are shown in Figure 1.

Table 2 shows the data on the fecal excretion of SE.

DISCUSSION

Infections by *Salmonella* have become frequent in commercial chicken flocks mainly due to failures in the biosecurity program. In order to prevent the indiscriminate use of antibiotics in the field, the best way to control these infections is to administer safe vaccines, capable of generating effective immune response against these bacteria (Baumler *et al.*, 2000). The SGΔ*cob*SΔ*cbi*A strain showed good efficacy in the protection of chickens against SG and SE (Penha Filho *et al.*, 2010), and therefore, in the present study, this double knocked-out mutant was tested for safety, protection and stability *in vivo*.

Two different experiments were performed to verify possible risks of reversion of the tested strain to virulence. In experiment 1, after five successive passages *in vivo* using the SGΔ*cob*SΔ*cbi*A strain that was re-isolated from each previous passage, no clinical signs or changes characterizing fowl typhoid were observed in any of the birds in any of the groups, either orally or subcutaneously inoculated. These results indicate that this strain has very stable deletions in its genome. The known mutation regions in the *cob*S and *cbi*A genes are the attenuating factors in this strain. The large deletion contributes to the stability of the gap established in the gene, preventing it to reverse to its virulent pattern. A trial to evaluate the risk of reversion to virulence of the SG9R vaccine strain (Okamoto *et al.*, 2010) reported that this strain is not attenuated by metabolic mutations, yet it maintains its attenuation after five successive passages *in vivo*. It should be considered that deleted mutants present much lower risks of reversion to virulence.

Table 2 - Number of positive birds for the SE challenge strain after swabbing the cloaca of birds orally or subcutaneously vaccinated with the *Salmonella* Gallinarum strain *ΔcobSΔcbiA* ($\chi 2$ $p < 0.05$).

	GROUPS								
	Orally vaccinated			Subcutaneously vaccinated			Unvaccinated		
DPI	D	E	T	D	E	T	D	E	T
3	17	4	21/35	17	13	30/35	28	3	31/35
7	5	4	9/30	12	9	21/30	21	6	27/30
10	2	2	4/25	6	4	10/25	7	6	13/25
13	1	1	2/25	0	1	1/25	4	2	6/25
17	2	0	2/20	1	0	1/20	1	0	1/20
21	2	0	2/20	4	0	4/20	2	0	2/20
24	0	0	0/15	0	1	0/15	0	0	0/15
Total	28	12	40/170 a	40	28	67/170ab	63	17	80/170 bc

DPI: days post-infection; D: Positive by direct platting; E: Positive after enrichment. T: total result, or number of positive birds/number of swabbed birds.

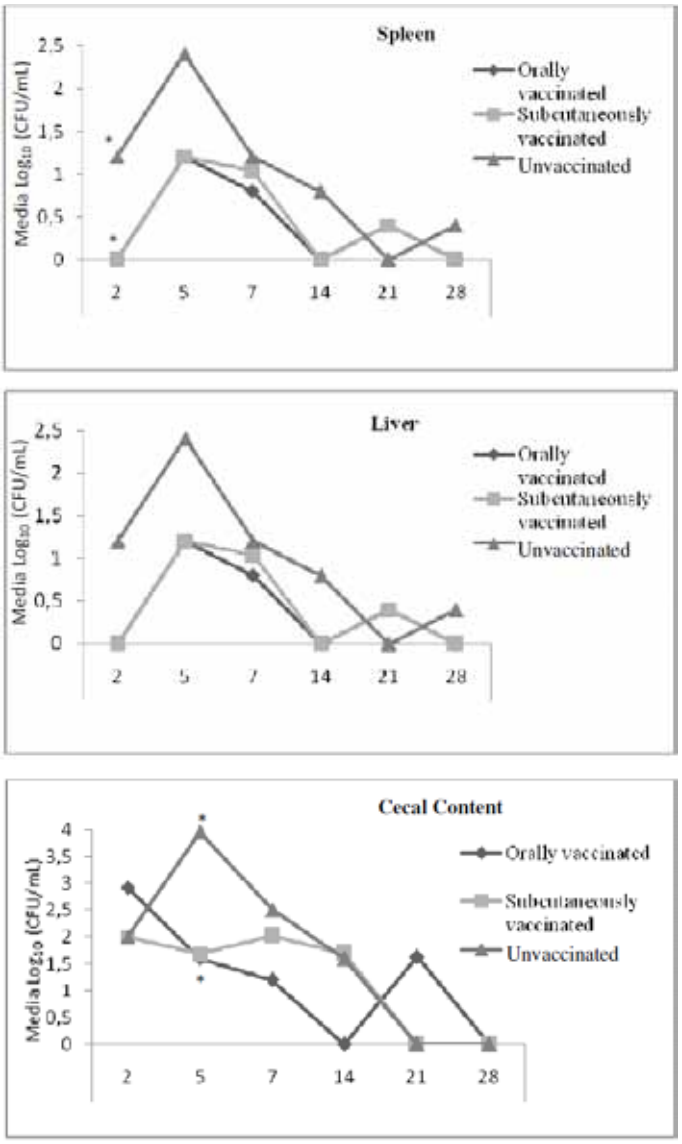

Figure 1 - Viable number (Log10 CFU/mL) of the challenge *Salmonella* Enteritidis strain Nalr Specr in the spleen, liver and cecal contents of birds vaccinated by oral or subcutaneous routes with the *Salmonella* Gallinarum *ΔcobSΔcbiA* strain; (*Tukey's test, $p < 0.05$). Mean of a group of five birds.

In experiment 2, even after the inoculation of a 10-fold concentration of the inoculum, which dose was higher than the typically applied, no apparent clinical signs of fowl typhoid were observed, and birds remained healthy until the last day of the experiment. Based on the results from both experiments, it is possible to suggest that the deletions in the genome of the attenuated SGΔ*cob*SΔ*cbi*A strain are stable and this strain has no residual virulence for chickens. Also, this strain presents low risk of reversion to virulence under typical field conditions. Considering that *cob*S and *cbi*A are metabolic genes and have no direct connection to the virulence mechanism, such as those performed by the *Salmonella* pathogenicity islands (Jones *et al.*, 2001), it is interesting that full virulence does not depend only on the synthesis of virulence factors. In this bacterium, it also depends on the expression of other regulatory factors, which may vary according to the environment (Miller & Mekalanos, 1990).

The use of this attenuated strain as vaccine was tested against SG and SE, using the oral and subcutaneous routes. As shown by the results in Table 1, vaccination reduced the mortality caused by the wild SG strain, but the protection was significant only in the group subcutaneously vaccinated. The immunogenic properties of the attenuated strains seem to be different according to the inoculation route. The levels of protection in chickens that were vaccinated with an *aro*A mutant of SG also differed depending on the administration route, confirming our results. The intramuscular vaccination was more effective than the oral application (Griffin & Barrow, 1993).

The SGΔ*cob*SΔ*cbi*A to protect chickens against the SE challenge was also applied using two routes: oral and subcutaneous. Differently from the vaccination against SG, the results in Figure 1 and Table 2 show that the oral application was more effective to control SE infection and fecal excretion than the subcutaneous vaccination. These data demonstrate that the immunological mechanisms involved in the response to each bacterium can be different, and therefore, the application route may also help to modulate the immune response of different challenge strains.

Our results demonstrated that this live SG vaccine has stable deletions and low risk of reversion to virulence. Furthermore, the levels of protection provided by this strain make it a good candidate for a vaccine to be used in the field. It was again shown that the immune response is a biological phenomenon that may be affected to variations, such as the route of administration, which, in the present study, stimulated different immune responses against the two serotypes tested.

Further studies are in progress to determine the biological factors involved in the immune response generated by this attenuated strain.

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