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Efficacy Of Several *Salmonella* Vaccination Programs Against Experimental Challenge With *Salmonella* Gallinarum In Commercial Brown Layer and Broiler Breeder Hens

■ Author(s)

Paiva JB de[1]
Penha Filho RAC[1]
Argüello YMS[1]
Silva MD da[1]
Gardin Y[2]
Resende F[3]
Berchieri Junior A[1]
Sesti L[3]*

[1] Avian Pathology Group. FCAV/Unesp, Jaboticabal, SP, Brazil.
[2] CEVA SANTÉ ANIMALE - Libourne Cedex, France, 35501.
[3] CEVA SAÚDE ANIMAL - Paulínia, SP, Brazil.

■ Mail Address

Luiz Sesti
Ceva Saúde Animal
Paulinia, SP, Brasil
Phone +55 19 8136 0569
Fax +55 19 3833 7722

E-mail: luiz.sesti@ceva.com

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ABSTRACT

The protective effect of various *Salmonella* vaccines regimens against an experimental *Salmonella* Gallinarum challenge (SGNalr strain at 12 wk of age) was evaluated in two experiments. In Experiment 1 commercial brown layers were vaccinated according to one of the following programs: (i) two doses of a SE bacterin (Layermune SE; group 1); (ii) a first dose of a live SG9R vaccine (Cevac SG9R) followed by a SE bacterin (Layermune SE; group 2); (iii) one dose of each of two different multivalent inactivated vaccines containing SE cells (Corymune 4 & Corymune 7; group 3) or (iv) not vaccinated (group 4). In Experiment 2, broiler breeders were given the same vaccination treatments except for the group vaccinated with the multivalent vaccines. Overall, in both experiments, all vaccination schemes were effective in reducing mortality after challenge with a SG field strain. Primary vaccination with an initial dose of a live SG9R vaccine followed some weeks later by a dose of an inactivated SE bacterin was the most effective ($p<0.05$) vaccination program against mortality induced by field SG experimental challenge in both experiments.

In conclusion, *Salmonella* vaccination programs containing SE bacterins alone or in combination with a live SG9R vaccine are effective in preventing mortality induced by infection of field SG. Nevertheless, it is important to emphasize that any vaccination program against any *Salmonella* serotype will only be effective if it is part of a sound and comprehensive biosecurity program designed for *Salmonella* control in poultry farms.

INTRODUCTION

Salmonella enterica serovar Gallinarum (SG) is the etiologic agent of Fowl Typhoid, a severe systemic disease of chickens and other galliform birds (Shivaprasad, 2000). *Salmonella* Gallinarum is a non-motile host-specific bacterium in domestic poultry. Infection in chickens occurs at all ages and is characterized by severe hepatomegaly and splenomegaly accompanied by liver with bronzing aspect, anemia, and septicemia (Shivaprasad, 2000). The disease is dose-dependent and differences in pathogenicity may be found depending upon the susceptibility of the infected genetic line of chickens (Oliveira *et al*., 2005).

S. Gallinarum is primarily associated with the mononuclear phagocyte system and resides prematurely within macrophages in the liver and spleen. SG can be found in the gastrointestinal tract early in the infection after oral contamination or at the final stage when the birds are dying (Barrow *et al*., 1994; Wigley *et al*., 2002). Regarding the epidemiology of fowl typhoid, the most important transmission route is horizontal, and very little information is available on direct evidences of transmission of the pathogen through the eggs to the progeny (Hall, 1949). *S.*

Gallinarum infection generally results either in mortality of susceptible birds or bacterial clearance in resistant birds within three to four weeks of the initial infection, although occasionally persistent infection may occur (Wigley *et al*., 2002; Wigley, 2004).

Mortality and morbidity rates due to Fowl Typhoid may reach up to 80%. Fowl typhoid has been eradicated from Australia, North America, and most European countries, where rigorous biosecurity and specific control programs including vaccination and good management practices have been largely applied. However, it is still of considerable economic importance in many countries of Africa, Asia, and Central and South America (Pomeroy & Nagajara, 1991; Lee *et al*., 2003). The most effective means of control is a combination of stringent biosecurity and management procedures and eradication (Calnek *et al*., 1997). Removal of birds that had died from disease from the environment, reduced the resultant mortality/morbidity and is regarded as a very useful measure for control of the Fowl Typhoid (Oliveira *et al*., 2005).

Vaccination to prevent or reduce *Salmonella* infection in poultry has been accepted worldwide. Presently in Brazil there are commercially available *Salmonella* Enteritidis (SE) bacterins and live attenuated *Salmonella* Gallinarum vaccines. Commercial layer and broiler industries in Brazil have gradually accepted routine vaccination as a preventive intervention method to reduce the *Salmonella* in industrial farms.

SE bacterins contain bacteria organisms that were inactivated and suspended in water-in-oil or aluminum hydroxide adjuvant. Bacterins stimulate high levels of circulating antibodies in commercial layers, which persist into the laying period (Timms *et al*., 1990; Barbour *et al*., 1993; Timms *et al*., 1994). Those SE bacterins provide cross-protection against *S.* Gallinarum and other serotypes. Some disadvantages of bacterins are the labor cost for administration and the post-vaccination stress due to tissue reaction at the site of injection, which is caused by the release of bacterial cell wall endotoxins subsequent to vaccine antigen metabolization in the birds (Nakamura *et al*., 1994).

For fowl typhoid prevention, inactivated *Salmonella* vaccines and live SG vaccine using the 9R strain have been introduced (Lee *et al*., 2005). Killed vaccines can be efficacious in reducing *Salmonella* in poultry. They are safe because there is no reversion to virulence, no spreading in the environment and are considered good enough to protect chickens when applied in large-scale poultry production (Barrow *et al*., 1991; De Buck *et al*., 2004). Nevertheless, live vaccines are considered to have advantages over killed vaccines as far as induced immunity is concerned (Van Immerseel *et al*., 2005). Live vaccines, by causing the expression of all appropriate antigens in vivo, induce better protection against *Salmonella* because they stimulate both cell-mediated and humoral immunity and expression of all appropriate antigens *in vivo*, while inactivated ones mainly stimulate the production of antibodies only against the antigens present at the time of *in vitro* harvesting (Collins, 1974; Gast *et al*., 1993; Barrow & Wallis, 2000). Killed vaccines may also be rapidly destroyed and eliminated from the host, and they are generally considered as unable to induce activation of cytotoxic T cells (Barrow & Lovell, 1991; Nagajara & Rajashekara, 1999). It is widely accepted that cell-mediated immunity is more important than humoral responses in the protection against *Salmonella*, especially in infections caused by host-specific serotypes (Collins, 1974; Mastroeni *et al*., 1993; Van Immerseel *et al*., 2005; Barrow, 2007).

The strain 9R of SG is routinely administered to chickens in countries with endemic fowl typhoid (Smith, 1956; Shivaprasad, 2000). The 9R strain developed in the 1950s has a semi-rough lipopolysaccharide structure that reduces the virulence of that microorganism (Smith, 1956 a,b; Silva *et al*., 1981; Silva, 1984; Feberwee *et al*., 2001a,b). This vaccine may also provide some protection against *Salmonella* Enteritidis and *Salmonella* Typhimurium (Barrow *et al*., 1991; Audisio & Terzolo, 2002; Tan *et al*., 2008ab). SG9R vaccine presented acceptable safety and efficacy in young layer hens even when administered at 4 weeks of age (Lee *et al*., 2005). In addition, no evidence of fecal shedding of the vaccine strain was found (Feberwee *et al*., 2000). However, there have been reports of SG9R vaccine strain fecal shedding for a maximum time of 24 hours after vaccination (Silva *et al*., 1981).

Live vaccine 9R strain induces cellular and humoral responses in chickens, and both immune responses reach their peaks at similar times. Bacterial clearance three weeks post-vaccination coincides with an increase in circulating anti-*Salmonella* antibodies, increased cytotoxic T cell proliferation directed to *Salmonella* cells, and increased expression of interferon gamma (Wigley *et al*., 2005). A slight increase in the expression of the pro-inflammatory cytokine interleukin-Iβ was detected early in the infection (Wigley *et al*., 2005).

Some primary breeder and commercial layer producers administer the live SG or ST vaccine early in the pullets' life, followed by a SE bacterin at the end of rearing (Nassar *et al*., 1994; Schaller, 1996; Cookson & Maiers, 2004). The combined use of live and killed vaccines had not been studied at that time, but a broader range of protection against other serotypes with this live/killed vaccine approach would certainly be expected.

This study assessed the efficacy of commercial killed SE vaccines alone or in combination with a commercial live SG9R vaccine in controlling an experimental SG challenge.

MATERIAL AND METHODS

Birds

Female birds from a commercial strain of brown table-egg layers (Dekalb white; Granja Planalto, Uberlândia, MG, Brazil) were used in the Experiment 1. Brown layers are highly susceptible to *Salmonella* Gallinarum infections (Berchieri Jr. *et al*., 2000; Freitas, *et al*., 2007). In Experiment 2, female birds from a commercial strain of broiler breeders (Cobb 500; Cobb-Vantress Brasil, Guapiaçú-SP-Brazil) were used. They were obtained at one day of age and were reared and fed according to the recommendations of the production manuals of each strain.

At arrival, all birds were inspected according to Zancan *et al*. (2000) to confirm if they were free from *Salmonella* sp infection and antibodies against SE.

All birds were housed in the same house in separate sets of five cages containing 6 birds each (Experiment 1) or sets of four cages containing 5 birds each (Experiment 2). Experiment 2 was carried out first, and there was a downtime period of few weeks for cleaning and disinfection before Experiment 1 started.

Vaccines

The vaccines used were commercial vaccines produced by CEVA-Phylaxia (Cevac Corymune 4K & 7K; Budapest, Hungary), CEVA Biomune (Layermune SE; Lenexa, USA), and CEVA Campinas (Cevac SG9R; Campinas, Brazil).

Cevac Corymune 4K contains an inactivated combination of Avibacterium paragallinarum serotypes A, B and C, and *Salmonella* Enteritidis strain, homogenized with aluminum hydroxide adjuvant and thiomersal as a preservative. Cevac Corymune 7K contains an inactivated combination of *Avibacterium paragallinarum* serotypes A, B and C, *Salmonella* Enteritidis strain, La Sota strain of Newcastle Disease virus, Massachussetts strain of the Infectious Bronchitis virus, and B8/78 strain of the EDS virus, homogenized with oil adjuvant and thiomersal as a preservative. Layermune SE is an inactivated bacterial vaccine (bacterin) that contains multiple selected strains of *Salmonella* Enteritidis (SE) in oil adjuvant. Cevac SG9R contains live *Salmonella* Gallinarum strain (strain 9R; at least 10^7 CFU per dose) naturally attenuated and non-pathogenic for chicken, in freeze-dried form.

At 5 and 9 weeks of age, birds in each group were either vaccinated intramuscularly in the breast muscle (Cevac Corymune 4K & 7K and Layermune SE) or subcutaneously in the dorsal lower part of the neck (Cevac SG9R) as recommended by the manufacturers.

Challenge

A virulent *Salmonella* Gallinarum 9S strain, isolated from diseased chickens was used. A spontaneous mutant isolate resistant to nalidixic acid (SGNalr) was used to allow recovery.

The inocula consisted of overnight cultures in LB broth (DIFCO-244620) prepared in shaking incubator (100rev/min) at 37°C for 24h. These cultures contained approximately $8x10^8$ colony forming units (CFU)/mL.

At 12 weeks of age all chickens were orally inoculated directly into the crop with 2mL of a broth culture of the bacterial strain.

Experimental design

Table 1 - Experiment 1 - Vaccination program and SE challenge in commercial table-egg layers.

Group (# of birds)	Vaccination program		SG Challenge at 12 wk of age (2 mL containing ~$8x10^8$ CFU per mL)
	1st dose (5 wk)	2nd dose (9 wk)	
1 (30)	Layermune SE	Layermune SE	SGNalr
2 (30)	Cevac SG9R	Layermune SE	SGNalr
3 (30)	Corymune 4K	Corymune 7K	SGNalr
4 (30)	Not vaccinated	Not vaccinated	SGNalr

Table 2 - Experiment 2 - Vaccination program and SE challenge in broiler breeders.

Group (# of birds)	Vaccination program		SG Challenge at 12 wk of age (2 mL containing ~$8x10^8$ CFU per mL)
	1st dose (5 wk)	2nd dose (9 wk)	
1 (20)	Layermune SE	Layermune SE	SGNalr
2 (20)	Cevac SG9R	Layermune SE	SGNalr
3 (20)	Not vaccinated	Not vaccinated	SGNalr

For the two experiments, mortality was recorded

over a period of 28 days post-infection. Mortality rates were analyzed using the Chi-Square test ($p<0.05$).

RESULTS

All birds in both experiments were completely negative for *Salmonella* spp at arrival.

Experiment 1. Cumulative mortality data in commercial brown layer hens are shown in Table 1 and Figure 1. A considerable reduction in mortality was observed in the groups of birds vaccinated with SE inactivated vaccines (Groups 1 and 3; $p<0.05$). No mortality occurred in brown layer hens vaccinated with a live SG9R vaccine (Cevac SG9R) plus an inactivated bacterin (Layermune SE).

Experiment 2. Cumulative mortality data in broiler breeder hens are shown in Table 2 and Figure 2. Both vaccination schemes reduced mortality during the four-week period after challenge. However, protection was significant (lower mortality; $p<0.05$) only in birds vaccinated initially with the live SG9R vaccine (Cevac SG9R) followed by a dose of the inactivated SE vaccine (Layermune SE).

DISCUSSION

The primary source of *S.* Gallinarum infection in poultry flocks is other infected poultry and vertical transmission; thus, introduction of these organisms in poultry flocks can be reasonably well controlled by

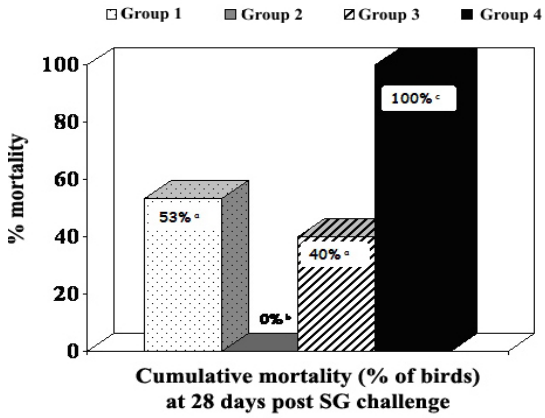

Figure 1 - Cumulative % mortality of commercial brown layer hens four weeks post-challenge with *Salmonella* Gallinarum (bars with % followed by different superscript letters are statistically different [$p<0.05$]).

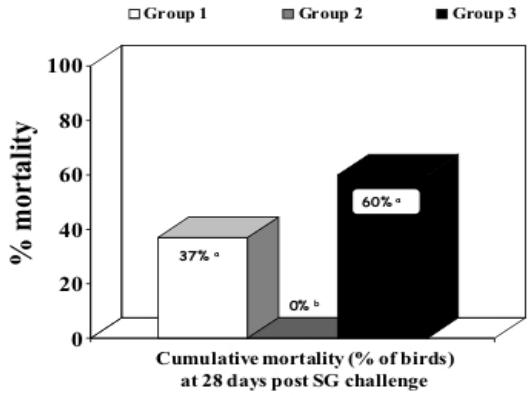

Figure 2- Cumulative % mortality of broiler breeder hens four weeks post-challenge with Salmonella Gallinarum (bars with % followed by different superscript letters are statistically different [$p<0.05$]).

Table 1 - Cumulative mortality of commercial brown layers hens vaccinated at 5 and 9 weeks old and challenged at 12 weeks old with *Salmonella* Gallinarum (total mortality figures [# of dead birds / total] followed by different superscript letters are statistically different [2; $p<0.05$]).

Treatments	Cumulative mortality during 28 day post challenge																								Total	
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	# dead birds / total	(%)
Group 1			1	4	10	14	15	16																	16/30 a	53.3 a
Group 2																									0/30 b	0 b
Group 3		2		5	7	8	11	12																	12/30 a	40 a
Group 4	9	18	26	30			31																		31/31 c	100 c

Table 2 - Cumulative mortality of broiler breeder hens vaccinated at 5 and 9 weeks old and challenged at 12 weeks old with *Salmonella* Gallinarum (total mortality figures [# of dead birds / total] followed by different superscript letters are statistically different [2; $p<0.05$]).

Treatments	Cumulative mortality during 28 day post challenge																								Total	
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	# dead birds / total	(%)
Group 1				2	4				5								7								7/20 a	37 a
Group 2																									0/20 b	0 b
Group 3		1	3	9	10	12																			12/20 b	60 c

standard biosecurity measures to minimize the risk of contact with infected flocks or people, equipment, and other fomites that may have originated from an infected site (Shivaprasad, 2003). However, in high field infection pressure farms, particularly multiage ones, vaccination presents an additional and effective control tool (Barrow, 2007).

Killed vaccines have been used to control *Salmonella* infections in poultry with variable success. Killed vaccines protect chickens against massive *Salmonella* challenge (particularly the non-typhoid serotypes) at any age (Timms *et al*., 1994). Nonetheless, they may reduce but not totally eliminate the microorganism from internal organs (Gast *et al*., 1993), perhaps because humoral immunity alone is unlikely to fully protect against SE, as complete protection against *Salmonella* requires the induction of both humoral and cellular immunity (Miyamoto *et al*., 1999). Notwithstanding, the recent development of novel adjuvant technology is very promising for the development of totally safe, inactivated *Salmonella* vaccines capable of inducing potent immune stimulation targeting different weapons of the chickens' immune system (Barrow, 2007).

It has been proposed that cell-mediated immunity is more important than humoral immunity for tissue clearance of *Salmonella*, whereas humoral responses seem to be the key in reducing intestinal colonization (Hassan & Curtiss 1994; Barrow & Wallis, 2000; Babu *et al*., 2004). An ideal vaccine should promote protection of birds against mucosal and systemic infection by effectively stimulating both immune responses (Van Immerseel et al., 2005). Although some authors (Barrow & Wallis, 2000; Meyer *et al*., 1992, Zhang-Barber, 1999) demonstrated that killed *Salmonella* vaccines induce only partial immune response, in the present work this response was good enough to significantly prevent mortality in the groups of birds challenged with a virulent SGNalr strain ($p < 0.05$). These findings are very interesting since the same vaccines were also assessed against a SE challenge and demonstrated significant efficacy (Penha Filho *et al*., unpublished data). *Salmonella* Gallinarum and *Salmonella* Enteritidis belong to the same serogroup (D1) and share the same "O' somatic antigenic formula (1,9,12; Ewing, 1986) which explains the cross protection provided by the SE bacterin against SG (Kingley & Baumler, 2000). It has been demonstrated that *Salmonella* vaccines can elicit cross-immunity against members of the same serogroup. The ability of a SE bacterin to cross protect against other *Salmonella* has been demonstrated by field use in Latin America (Norton & Lozano, 1997). In an endemically SG-contaminated layer farm, cumulative mortality by Fowl Typhoid during an 11 wk period was significantly lower in a house where birds were vaccinated with a SE bacterin (1.8 % mortality) as compared to the average mortality in non-vaccinated houses (8.1% mortality; Norton and Lozano, 1997).

According to Liu *et al*. (2001), inactivated vaccines can decrease SE fecal excretion and that effect may depend on their composition. A study conducted by Barbour *et al*. (1993) comparing six inactivated SE vaccines showed variable reduction in SE fecal excretion. The same was observed by Freitas *et al*. (2008) comparing three commercial SE bacterins. These authors suggested that several factors could be responsible for this effect, such as adjuvant type and composition, *Salmonella* Enteritidis strain, inactivation method, etc. These factors could explain the different protection results observed in birds vaccinated with Layermune SE or Corymune bacterins in Experiment 1, although in the present study a SG and not a SE challenge was used. There is very scarce information on the effectiveness of *Salmonella* Gallinarum inactivated bacterins. In the recent article of Haider *et al*. (2007), it was demonstrated that a SG bacterin made from field isolates was able to induce significant seroconversion, although no information was provided in regard to protection against an experimental challenge.

In the present experiments, the best protection was observed in groups of birds vaccinated with the live SG 9R vaccine. Vaccination against host-specific *Salmonella* serotypes that cause severe systemic disease induces strong serotype-specific protective immunity (Smith, 1956; Barrow & Wallis, 2000).

A number of studies (Smith, 1956; Harbourne, 1957; Gordon & Luke, 1959; Gordon *et al*., 1959; Lee *et al*., 2005) have shown that the SG9R vaccine strain as an effective means for fowl typhoid prevention. Mortality in highly susceptible chicks exposed to virulent strains of *S.* Gallinarum was limited by SG9R vaccine (Silva *et al*., 1981). Lee *et al*. (2005) showed that a 9R vaccine provided excellent protection and is safe for vaccination of 4 week-old chickens. Indeed, in the present studies, birds that received the SG9R strain by subcutaneous route showed no evidence of disease, while SGNalr challenge strain was highly virulent to both genetic lines of birds assessed.

In this study we did not find any differences in the protection induced by the SG9R vaccine among the

genetic lines used in both experiments. However, the vaccination program with two doses of an inactivated SE bacterin (Layermune SE + Layermune SE) apparently had lower effectiveness, which may indicate lower susceptibility to fowl typhoid by meat-type chickens. Regardless possible differences in susceptibility to a SG challenge and despite of the genetic evolution of commercial bird lines throughout the past decades, the present situation is similar to that previously described by Gordon *et al*. (1959). Notwithstanding Bumstead *et al*. (1993) state that the modern genetic lines of commercial birds exhibit different patterns of immunity to *Salmonella*.

There is quite little scientific information available in literature on the control of Fowl Typhoid through vaccination programs adopting live and inactivated *Salmonella* vaccines. However, several published experiments indicate that such combination programs using live (including the SG9R strain) plus inactivated *Salmonella* vaccines can be very effective against SE infections in layer hens (Nassar *et al*., 1994; Schaller, 1996; Cookson & Maiers, 2004). It is not possible to directly compare our study with the previously mentioned SE experiments as the vaccination schemes and vaccines used are quite different. However, it stands very clear that the combination of an initial live *Salmonella* vaccine followed some weeks later by an inactivated product may be a very useful and effective program in preventing fowl typhoid.

In conclusion, vaccination programs containing SE bacterins alone or in combination with a live SG9R vaccine induced variable protection against mortality caused by infection of field SG, depending on the specific vaccination program used. Bacterins only, although having significantly decreased mortality in challenged brown layers, did not have a significant effect on broiler breeder hens. The combination of an initial dose of a live SG9R vaccine followed some weeks later by a dose of an inactivated SE bacterin was the most effective vaccination program.

Nevertheless, it is crucial to emphasize that any vaccination program against any *Salmonella* serotype will only be effective if it is part of a sound and comprehensive biosecurity program designed for *Salmonella* control.

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