



Reversion to Virulence Evaluation of a 9R Vaccine Strain of *Salmonella enterica* Serovar Gallinarum in Commercial Brown Layers

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ABSTRACT

The live vaccine Cevac S. Gallinarum, made from a rough strain of *Salmonella enterica* subspecies *enterica* serotype Gallinarum is used for preventing fowl typhoid, a disease that still causes considerable economic losses in countries with a developing poultry industry. The objective of this paper was to evaluate a possible reversion to virulence of the strain used in a vaccine in commercial brown layers. Only *Salmonella*-free chicks were utilized. One hundred twenty (120) 12-day-old Dekalb brown layers divided in two trials were used. The first trial had six groups of 15 birds each. Birds of group 1 were vaccinated with 10 doses of Cevac S. Gallinarum subcutaneously and 10 doses orally, in a total of 20 doses of vaccine. Then the birds of groups 2, 3, 4, and 5 received inocula that contained feces and a pool of organs with fragments of liver, heart, spleen, and cecal tonsils obtained from the immediately previous group. The second trial had three groups with 10 birds each. Birds in group 7 received inocula containing a pool of organs from birds of group 5 from trial 1, whilst the birds in group 8 were vaccinated subcutaneously with one dose of vaccine. Both trials included negative control groups (6 and 9). Throughout the experimental period, birds were monitored for reactions to the vaccination on the site of administration, clinical signs, and *post-mortem* lesions. In each passage, in addition to the birds euthanized to provide the inocula material, two birds from each group were euthanized for assessment of possible lesions, and their organs (liver, heart, spleen and cecal tonsils) were cultured in an attempt to isolate the vaccine strain. Except for one bird from group 1, that had a local reaction on the site of vaccination - a small vesicle with less than 0.5 mm that persisted until the third day post vaccination -, no other bird had any local reaction to the vaccine or any visible clinical alteration. Birds in group 8 did not present any reaction or clinical alteration because of the vaccine. We only managed to re-isolate the vaccine strain in the inocula made from organs of birds in group 1. We confirmed the isolation by means of biochemical tests, serology, and acriflavine agglutination test. All other cultures made from organs or feces, from all the other experimental groups did not show any growth of the vaccine strain or any other *Salmonella* serovar, suggesting that the vaccinated birds did not shed the SG9R vaccine strain. No bird presented any clinical symptoms or died during the trials, and no gross lesions were observed in the *post-mortem* examinations. Under the controlled conditions and time-frame of the present experiment, it was possible to conclude that the rough 9R strain of *Salmonella* Gallinarum present in the vaccine Cevac S. Gallinarum (Ceva Campinas Ltda. - Campinas, SP - Brazil) did not revert to virulence.



INTRODUCTION

With the growth of the poultry industry, avian salmonellosis became a limiting factor in poultry farms, in which can cause significant economic losses in all stages of production. Avian salmonellosis is the term that designates a large group of acute or chronic bird diseases caused by one or more bacteria of the genus *Salmonella* (Gast, 1997). These bacteria are rods from the *Enterobacteriaceae* family, and most of them are mobile. They are aerobic or facultative anaerobic organisms that ferment sugars, producing gas and H₂S, and that have a complex antigenic structure (somatic "O", flagellar "H", and capsular "K" antigens) (Gast, 1997; Barrow, 2000).

Fowl typhoid is a disease caused by *Salmonella enterica* subspecies *enterica* serotype Gallinarum, characterized by septicemia and toxemia. It is more commonly observed in adult commercial layers, but it can also affect breeders (Berchieri Júnior & Oliveira, 2007). The infection causes high morbidity and up to 60% mortality in experimentally-infected birds (Jones *et al.*, 2001). It has a worldwide distribution and, although it is strictly controlled in European countries and in North America, it is still present in countries with a developing poultry industry and in those where poultry are reared in back yards. In Brazil, attention was drawn to the disease due to the 80's and 90's outbreaks, representing up to 65% of the total isolates of *Salmonella* spp. (Berchieri Júnior & Oliveira, 2007).

Live vaccines against fowl typhoid are available in the market. They are made from rough 9R strains originated from attenuated 9S strains of *Salmonella* Gallinarum (Gordon *et al.*, 1959). This kind of vaccine produces better immunological results and offers better protection to birds as compared to inactivated vaccines (Gast *et al.*, 1993). It also induces immune responses without causing severe damage to the birds, although there are reports that they can cause some of the clinical alterations observed in field fowl typhoid (Berchieri Júnior, 2000). In addition, it has been recently reported that the virulence (mortality rate after experimental challenge) of a laboratory freeze-dried SG strain doubled after *in vivo* passage in chickens (Audisio & Terzolo, 2001). Similar to other injectable vaccines, SG9R stimulates the production of IgG and IgM, making it difficult to differentiate vaccinated from challenged birds. And, as it is a live vaccine, it may also remain in the reproductive system and be transmitted through the eggs (Bersot, 2006). Inactivated vaccines may induce effective protection against the invasion of organs by

Salmonella, although they are not effective in controlling the colonization of the intestines by these bacteria (Nakamura *et al.*, 1994; Lee *et al.*, 2005).

The objective of this paper was to evaluate the possibility of reversion to virulence of the 9R *Salmonella* Gallinarum strain present in the commercial vaccine Cevac S. Gallinarum designed for the control of fowl typhoid and other salmonellosis cases in poultry.

MATERIAL AND METHODS

Vaccine and vaccination

A live lyophilized vaccine against *Salmonella* Gallinarum (Cevac S. Gallinarum, batch 003/07 and diluents 001/07, both within the shelf life recommended by the manufacturer) was used in this trial. The vaccine and diluents were produced by Ceva Campinas Ltda., located in Campinas, SP, Brazil. The vaccine contained a minimum concentration of 2x10⁷ colony forming units (CFU)/dose. Birds of group 1 in trial 1 and group 8 in trial 2, respectively, were vaccinated at 12 and 13 days of age, respectively, after reconstitution and homogenization of vials containing 1000 doses of vaccine with 50 ml of diluent. Dilution was calculated to supply 10 regular doses of the vaccines in a 0.5 ml final volume. All birds in group 1 from trial 1 were double vaccinated: 0.5 ml subcutaneously (lower dorsal area of the neck) and 0.5 ml orally, and therefore received the equivalent to 20 doses/bird. All birds of group 8 in trial 2 received one dose of the vaccine subcutaneously.

Birds and facilities

The experiment was conducted at the poultry house of the Department of Avian Pathology of the School of Veterinary Medicine (FMVZ), UNESP, Botucatu campus, SP, Brazil, where 200 Dekalb day-old brown layers were housed in heated wire cages and received water and non-medicated feed *ad libitum*. Brown layers were used because they are highly susceptible to infections by *Salmonella* Gallinarum (Berchieri Jr. *et al.*, 2000; Freitas, *et al.*, 2007). The birds were vaccinated against Marek's Disease (HVT and Rispens strains), fowlpox, and infectious bursal disease at the hatchery. In order to ascertain that the birds were free from *Salmonella*, immediately before housing, 40 birds were euthanized by cervical dislocation, and had their liver, heart, cecal tonsils, and yolk sac content collected and cultured specifically for *Salmonella* isolation as previously described (Andreatti Filho, 2007). Cloacal swabs were collected from the remaining birds and cultured for



Salmonella presence using the same procedure described above. At 12 days of age another group of 40 birds was euthanized and their organs were again collected for specific *Salmonella* isolation culture. The remaining birds (120), which had been distributed into the experimental groups, were once again submitted to cloacal swabs for *Salmonella* isolation procedures. On the same day (12 days of age), blood samples were collected from all birds for serological analysis (FlockChek *Salmonella* Enteritidis Antibody Test Kit; Idexx Laboratories, Westbrook, Maine, USA) for the detection of *Salmonella* Enteritidis circulating antibodies. All procedures used for microbiological investigations followed the guidelines of the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA - Brasil, 2003). All birds were identified with plastic numbered tags that were attached to their wings.

Inocula

Feces - During the period of 24 hours after vaccination (group 1) or administration of inocula (groups 2, 3, 4, and 5) and during the 5 following days, 7.5g of feces from each group were suspended in buffered peptone water (BPW) and homogenized. The whole suspension was divided into 15 aliquots and each was orally administered to all birds of the subsequent group. Each bird received 5ml of the feces suspension using a graded pipette.

Pool of organs - Every day, for three consecutive days, two birds in each group were euthanized, starting 48 hours after vaccination or administration of the inocula. Fragments of liver, heart, spleen, and cecal tonsils were collected, macerated in 30ml BPW, and filtered. All birds of the subsequent groups received 1ml of the final suspension using a graded pipette.

Experimental design

Trial 1 - Twenty four hours after vaccination of the birds of group 1, feces collection started and lasted for five consecutive days. Organs collection started 48 hours after vaccination and lasted for three consecutive days. The inocula made from feces and organs of the birds of group 1 were administered to birds of group 2 during the same periods in which they were collected. This means that all birds of group 2 were inoculated 5 times with feces and three times with the pool of organs, on days 1 to 5 and 2 to 4, respectively, after the vaccination of birds of group 1. The same procedure, collection periods, and administration of feces and pool of organs were sequentially performed

with groups 2, 3, 4, and 5. The birds that remained after all collections in each of the groups were monitored for 14 days after vaccination or inoculation, and eventually euthanized to check if there were any clinical gross lesions and to collect fragments of liver, heart, spleen, and cecal tonsils, which were submitted to a specific culture for the isolation of *Salmonella* (Andreatti Filho, 2007).

Trial 2 - On the seventh day following the first administration of feces, 10 birds of group 5 were euthanized to check for clinical gross lesions and to collect fragments of liver, heart, spleen, and cecal tonsils. After the pool of organs was prepared (as previously described), 0.5 ml were subcutaneously inoculated in all 10 birds of group 7 in trial 2. All birds of group 8 were vaccinated with one subcutaneous dose (0.5 ml on the neck) of Cevac S. Gallinarum vaccine. All birds of groups 7, 8, and 9 were clinically observed for 21 days following the vaccination or inoculation. Then they were euthanized and inspected for clinical gross lesions. Fragments of liver, heart, spleen, and cecal tonsils were collected and submitted to a specific culture for isolating *Salmonella* (Andreatti Filho, 2007).

Groups 6 and 9 were the negative controls in trials 1 and 2, respectively. Birds of those groups did not receive any inoculum or treatment, but they were also euthanized, sampled, and analyzed as the birds in the other experimental groups.

Figure 1 shows the experimental design scheme for both trials.

Culture for the isolation of the vaccine antigen

Aliquots of all the inocula made from feces or from the pool of organs in all passages between the experimental groups were obtained and submitted to a specific culture for the isolation of the vaccine strain or any other serovar of *Salmonella*. The aliquots were seeded in tubes containing 10ml tetrathionate broth and selenite cystine broth, and were incubated for 24 hours at 37°C. Then they were seeded on Petri dishes containing brilliant green agar and MacConkey agar, and incubated as described for the isolation of the bacteria. The enriching selective broths and the seeding on the selective agars were kept for a period of 40 hours. The colonies that were isolated from each one of the media and that had typical *Salmonella* characteristics were seeded on triple sugar iron agar, urea broth, and lysine iron agar. The colonies with typical characteristics on these media were

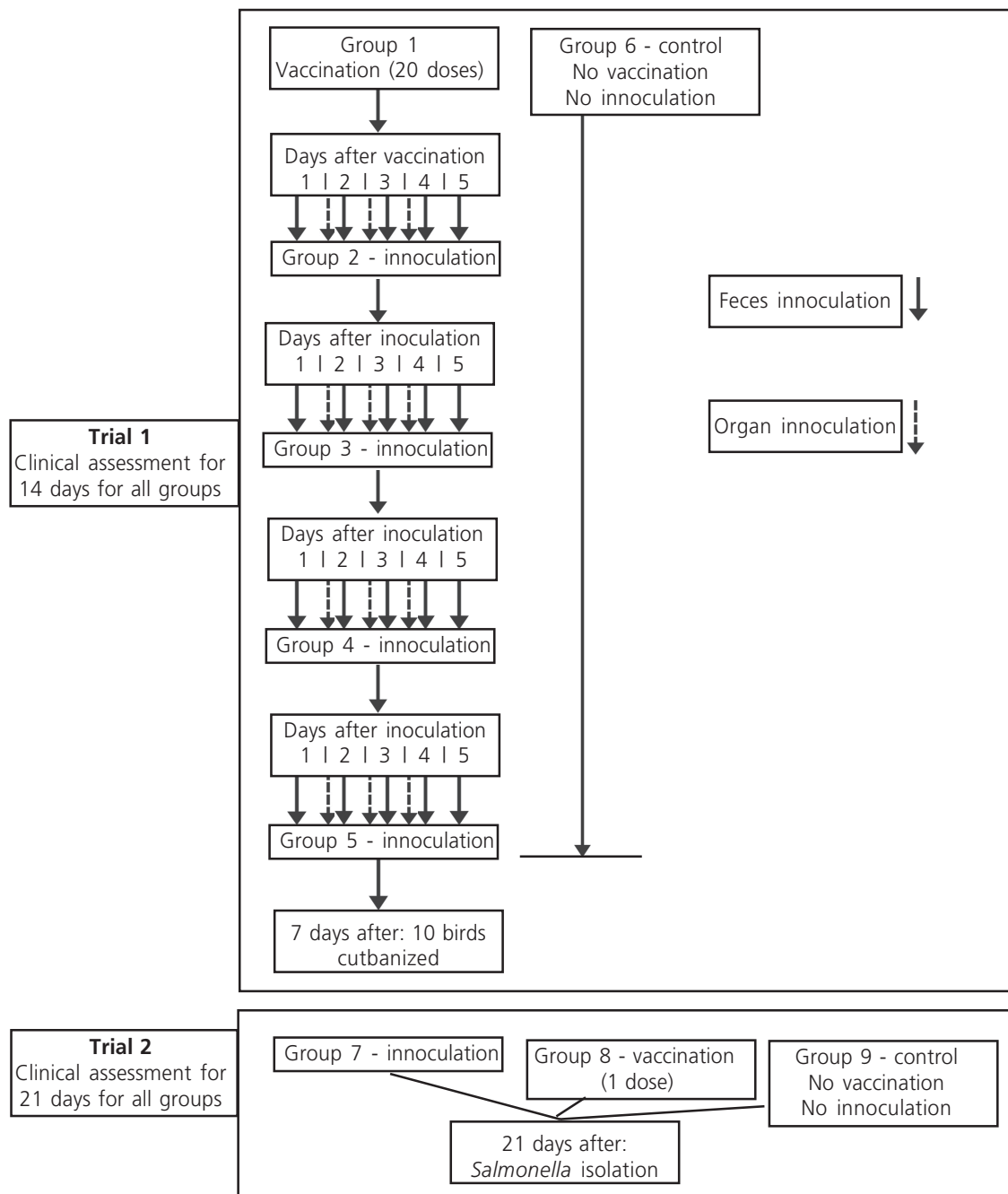


Figure 1 - Experimental design for evaluation of possible reversion to virulence of the *Salmonella enterica* serovar Gallinarum 9R vaccine strain present in the commercial vaccine Cevac S. Gallinarum (Ceva Campinas - Campinas, SP, Brazil).

biochemically confirmed by culture in SIM medium (sulfide, indole, and motility), Simmons citrate, methyl red, Voges-Proskauer, arginine, ornithine, malonate, glucose (gas), lactose, sucrose, mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, arabinose, and raffinose. Simultaneously to the biochemical analyses, we submitted the colonies with typical *Salmonella* characteristics to serological tests using polyvalent somatic antiserum (Poli O), polyvalent flagellar

antiserum (Poli H), and group-D somatic antiserum (factor 9) (Andreatti Filho, 2007). The 1% acriflavine agglutination test was used to confirm the rough strain (Carter & Subronto, 1973; MERCOSUL, 1997).

RESULTS AND DISCUSSION

The birds were monitored for local reactions to the vaccine, clinical signs of disease, and *post-mortem* gross



lesions throughout the experimental period. In each of the passages between the different experimental groups, two birds from each group were euthanized to check if there were any lesions, and their organs (liver, heart, spleen, and cecal tonsils) were submitted to culture in an attempt to isolate the vaccine strain. The organs were also used to make the inocula (pool of organs and feces). We did not find any *Salmonella* or lesions caused by it in any of the microbiological or macroscopic analyses, respectively.

Except for one bird of group 1, which had a local reaction on the vaccination site characterized by a <0.5mm vesicle that remained visible for 3 days after vaccination, no other bird had any visible clinical alteration or local reaction, even when receiving the equivalent to 10 doses of the vaccine, subcutaneously on the neck in addition to 10 oral doses. Birds of group 8 received a single subcutaneous dose of the vaccine and did not present any local reaction or clinical alteration. These results are similar to those observed by Lee *et al.* (2005) when brown Hy-line layers were vaccinated subcutaneously with one or ten doses of a vaccine against fowl typhoid, at the ages of two, four, and six weeks of age. None of the birds in the present experiment had any symptoms, lesions, or mortality during the three weeks following the first vaccination. Barrow *et al.* (1987) reported that the use of the 9R vaccine strain is effective when it is administered correctly. However, the same authors stated that the possibility of reversion to virulence is not totally clear, as the vaccine strain has the same plasmid considered essential to virulence, which is found in field virulent samples of *Salmonella* Gallinarum. The 9R strain of *S. Gallinarum* produced hepatitis and splenic lesions without mortality in meat-type and brown-egg-producing strains of chickens, but not in Leghorns (Silva *et al.*, 1981) and for this reason we used brown layers in the experiment. Zhang-Barber *et al.* (1998), using a single oral immunization with *S. Gallinarum* nuoG mutant, reduced mortality in 2-week-old chickens following challenge with virulent *S. Gallinarum* from 75% to less than 8%.

The SG9R strain of the vaccine was isolated only in organs from birds of group 1. This was confirmed by means of biochemical tests, serology, and acriflavine agglutination test. In all other cultures made from feces or organs of birds in trial 1 and 2, there was no growth of the vaccine strain or of any other *Salmonella* serovar, indicating that the birds vaccinated with the equivalent to 20 doses of the vaccine did not shed the vaccine SG9R strain. No bird died during the trials, and

no clinical signs or gross lesions suggesting an infection by *Salmonella* spp. were observed in the post-mortem examinations of the birds. Wigley *et al.* (2005), orally inoculating three-week-old SPF white Leghorn birds with a vaccine sample of *Salmonella* Gallinarum at a concentration of 10⁸CFU/ml, observed gross lesions characterized by moderate to severe increase in liver and spleen size. The microbiological cultures made from the liver and spleen of those birds were positive for the same bacteria two weeks after inoculation.

The 9R strain of *Salmonella* Gallinarum has been used in poultry industry in several countries for many decades, and field observations and field trials results indicate that it is safe and presents reasonable effectiveness to be used as one of the tools of a comprehensive biosecurity program directed to *Salmonella* control in poultry farms (Barrow, 2007).

CONCLUSION

Under the controlled conditions and time-frame of the present experiment, it is possible to conclude that the rough 9R strain of *Salmonella* Gallinarum present in the vaccine Cevac S. Gallinarum (Ceva Campinas Ltda. Campinas, SP, Brazil) did not revert to virulence. Notwithstanding, it would be appropriate that further controlled and long-term field studies be conducted to ensure neither transmission nor acquisition of virulence plasmids between the 9R strain and field strains of *Salmonella* Gallinarum. This subject was not specifically covered in the present study.

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