Evaluation of the protective immune response induced by an rfbG-deficient Salmonella Enteritidis strain as a live attenuated DIVA vaccine in chickens

Xinwei Wang, Xilong Kang, Mingxing Pan, Ming Wang, Jiayue Zhang, and Hongqin Song

Corresponding Author(s): Hongqin Song, Yangzhou University

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Editor, Microbiology Spectrum
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Reviewer comments:

Reviewer #1 (Comments for the Author):

Overall this is an interesting study with well supported conclusions. Authors should add all statistical test methods used in the paper in the methods section and figure legends. Additionally, authors can elaborate on the importance of being able to differentiate between vaccinated and infected chickens using sera.

Reviewer #2 (Comments for the Author):

Evaluation of the protective immune response induced by an rfbG-deficient Salmonella Enteritidis strain as a live attenuated
DIVA vaccine in chickens by Wang et al.

The authors claim to have constructed a rfbG mutant of Salmonella enterica serovar Enteritidis (SE) that would be suitable as a live attenuated DIVA vaccine strain with protective properties against SE infections.

The topic of SE as a pathogen for poultry, but more importantly as a zoonotic pathogenic bacterium affecting humans, thus being a public health concern, is timely. This reviewer can imagine how much effort was put into the study. Nonetheless, the manuscript appears at this state somewhat preliminary as all sections of the manuscript need to be more elaborated. Many sections appear rather cryptic and are lacking details that are essential for the understanding of the executed experiments. Grammar and style should be carefully checked.

A very important aspect, although mentioned in the discussion section, is totally excluded from their own study: the colonization of the gastrointestinal tract of the chickens after oral uptake/inoculation of SE. This plays an important role in the natural environment as well as in the small scale and large scale industrial settings of poultry production. Many issues of food poisoning through SE occur when contaminated poultry carcasses are processed. Protection of poultry against systemic infection is important, indeed. But what about protection against gastrointestinal colonization and shedding of SE into the environment? The main aspect still is the problem that this zoonotic pathogen can easily be transferred to humans via poultry products and hens eggs.

Specific comments:

- It is rather safe to say that reversion of the rfbG mutation can be excluded. But what about the possibility of recombination with DNA fragments from other Salmonella strains that could potentially be present in the same bird at the time of vaccination? Please discuss.
- Abstract: first sentence: other serovars can cause salmonellosis as well. Please re-phrase
- Line 33-36: Re-phrase, please. A lethal challenge is per definition deadly. How can it be that birds do not show any signs of disease after a lethal challenge?
- Line 42: Find another word for "transformed".
- Line 59 and throughout the document: "Gram" starts always with a capital "G".
- Complete results section: Please provide sufficient details in all sub-sections.
- Line 119 and later in manuscript: what are "fatty changes"? Please explain.
- Line 213: Do the authors mean "constructed" rather than "contrasted"?
- Line 221 -223: How was the rfbG mutation confirmed? By sequencing?
- Lines 246 ff: Which volume of the samples was plated onto LB plates? What is the detection limit? Samples should have undergone an enrichment procedure in addition to "direct plating" How would LB medium discriminate against bacterial strains other than the challenge strain?
- Line 253: Please explain H&E stain.
- Table 2: Please elaborate what is the difference between the two PBS groups?
- Table 1: The respective experiment should be executed in duplicate and repeated with a larger number of birds. Were the birds commingled or housed in different rooms (potential "pen effect")?

Staff Comments:

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Evaluation of the protective immune response induced by an \textit{rfbG}-deficient \textit{Salmonella} Enteritidis strain as a live attenuated DIVA vaccine in chickens

Xinwei Wang, a,b,c,d Xilong Kang, a,b,c,d Mingxing Pan, a,b,c,d Ming Wang, a,b,c,d Hongqin Song a,b,c,d

a College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu 225009, China

b Jiangsu Key Laboratory of Zoonosis, Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, Jiangsu 225009, China
c Key Laboratory of Prevention and Control of Biological Hazard Factors (Animal Origin) for Agri-food Safety and Quality, Ministry of Agriculture of China, Yangzhou University, Yangzhou, Jiangsu 225009, China
d Joint International Research Laboratory of Agriculture and Agri-product Safety of the Ministry of Education, Yangzhou University, Yangzhou, Jiangsu 225009, China

Running Head: \textit{SALMONELLA ENTERITIDIS DIVA VACCINE}

#Address correspondence to Hongqin Song, hqsong@yzu.edu.cn.

*Present address: College of Veterinary Medicine, Yangzhou University, 48 East Wenhui Road, Yangzhou, Jiangsu 225009, China

Word count of the abstract: 222
Word count of the text: 2536
ABSTRACT: Salmonellosis is a widespread zoonotic disease caused by *Salmonella enterica* serovar Enteritidis (S. Enteritidis). *Salmonella* infections in humans are primarily caused by poultry and poultry products. Vaccination is an effective method to prevent *Salmonella* infections. In this study, we constructed a live attenuated DIVA (differentiation of infected and vaccinated animals) vaccine candidate, Z11ΔrfbG, and evaluated its protective effectiveness and DIVA potential in chickens. Compared to the virulent wild-type strain, the 50% lethal dose (LD50) of the *rfbG* mutant strain increased 61-fold, confirming its attenuation. High serum levels of S. Enteritidis-specific IgG titers indicated that a significant humoral immune response was induced in the vaccinated group. The 5×10⁶ and 5×10⁷ colony-forming unit (CFU)-vaccinated chicken groups showed no clinical symptoms, pathological changes, or death after the lethal challenge. In contrast, the control group showed severe clinical symptoms and pathological alterations. Z11ΔrfbG vaccination significantly reduced *S. Enteritidis* colonization in the spleen and liver. In addition, the Z11ΔrfbG vaccinated group exhibited a negative response to the serological test, whereas the virulent wild-type Z11 infection group was strongly positive for the serological test, showing a DIVA capability of Z11ΔrfbG vaccination. Overall, our findings show that the *rfbG* mutant strain may be transformed into a live attenuated vaccine that can be used in chickens without compromising the ability to differentiate between the sera of infected and vaccinated animals.

IMPORTANCE: *S. Enteritidis* is a highly adapted pathogen that causes significant economic losses in the poultry industry around the world. Vaccination is an effective method of controlling *S. Enteritidis* infections. Here, we demonstrated that *S. Enteritidis* can
Z11 Δ rfbG has the potential to be a safe, immunogenic, and DIVA vaccine candidate for the control of *Salmonella* infections in chickens. Z11ΔrfbG not only provided effective protection in chickens, but also had ability to distinguish between infected and vaccinated chickens by serological tests.

**Keywords:** *Salmonella* Enteritidis, *rfbG*, live attenuated vaccine, DIVA, Chicken
INTRODUCTION

Salmonellosis is a broad term that refers to acute and chronic infections caused by *Salmonella* in animals and humans and is a serious public health concern (1). *Salmonella*, a gram-negative rod, is a major foodborne zoonotic pathogen with a wide host range (2). *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) is a global concern, causing recessive infections in adult chickens and bacterial excretion into the external environment through feces, resulting in challenging pathogen purification, systemic infections, and high mortality (3). In recent years, it has been reported that *S. Enteritidis* is gradually replacing *S. Typhimurium* as the most common *Salmonella* serotype (4). Additionally, the widespread use of antibiotics has led to the emergence of multiple antibiotic-resistant bacteria. Therefore, there is an urgent need for an efficient vaccine to manage this major zoonotic infection (4). Owing to their ability to stimulate the cellular and humoral adaptive immune responses, live *Salmonella* vaccines are considered more effective against intestinal and systemic infections than inactivated vaccines (5, 6).

Lipopolysaccharide (LPS), an important component of the outer membrane of gram-negative rods, primarily influences the phenotype, virulence, and immune cross-reaction of *Salmonella* strains (7, 8). LPS is primarily composed of O antigen, core polysaccharides, and lipid A (9). The *rfa*, *rfb*, and *rfc* gene clusters are the main genes involved in LPS biosynthesis (10). The *rfb* gene cluster regulates biosynthesis of the O antigen chain, with *rfbG* encoding CDP-glucose 4,6-dehydratase dehydratase (11). Jiao et al. (12) showed that *rfbG* downregulation leads to LPS deficiency in *S. Enteritidis* which reduced *S. Enteritidis* virulence in a mouse model, and that its gene locus can be used as a
marker. However, the effects of the S. Enteritidis rfbG-deficient strain in chicken remain unknown.

This study aimed to determine whether attenuated levels of S. Enteritidis rfbG-deletion mutants in a chicken model would protect poultry against virulent S. Enteritidis challenge, while also allowing us to distinguish between the sera of vaccinated and infected chickens.

RESULTS

Virulence of the vaccine candidates. The virulence of the Z11ΔrfbG and Z11 strains (rfbG-deletion mutant strain and wild-type S. Enteritidis strain, respectively) was evaluated in 7-day-old SPF chickens after intramuscular immunization. As shown in Table 1, the LD$_{50}$ (50% lethal dose) of Z11ΔrfbG was $1.9 \times 10^9$ CFU (colony-forming unit), which was 61-fold higher than that of the virulent wild-type Z11 ($3.1 \times 10^7$ CFU). These results indicated that the virulence of Z11ΔrfbG was attenuated compared to that of the wild-type strain.

Immune protective efficacy of Z11ΔrfbG after bacterial challenge. To evaluate the protective efficacy of Z11ΔrfbG, chickens were vaccinated intramuscularly with Z11ΔrfbG and challenged with the virulent wild-type strain Z11. The survival percentages of these chickens are shown in Table 2. Following the challenge with Z11, two chickens died in the $5 \times 10^5$ CFU-vaccinated group. Meanwhile, 4 out of 15 non-vaccinated chickens died in the control group. However, none of the chickens in the $5 \times 10^6$ and $5 \times 10^7$ CFU groups died. As shown in Figure 1, there was a significant weight loss in the non-vaccinated group compared to the vaccinated and control groups at 11
days post-challenge (DPC). However, there was no significant difference between the 5×10^6 and 5×10^7 CFU-inoculated groups and the control group. Slight and temporary diarrhea was observed following challenge in the vaccinated group compared to the control group, whereas persistent weight loss, diarrhea, ruffled feathers, and depression were observed following challenge in the non-vaccinated group.

**Clearance of bacteria in the spleen and liver.** Liver and spleen samples from three chickens in each group were aseptically collected 14 DPC to evaluate the bacterial clearance in the internal organs of the chickens. The bacterial isolates were identified in the liver and spleen of the non-vaccinated group but not in the liver or the spleen of the vaccinated groups at 14 DPC. These results indicated that the vaccine promoted bacterial clearance from the liver or spleen.

**Histological analysis after challenge with Z11.** Liver lesions were observed at 14 DPC and histological analysis of the liver was performed using Hematoxylin and eosin (H&E) staining. As shown in Figure 2, no obvious lesions were detected in the livers of the 5×10^6 and 5×10^7 CFU-vaccinated groups compared to those of the control group. However, white nodules were observed in the liver of the non-vaccinated group (Figure 2A), and the liver cells of the 5×10^5 CFU-vaccinated group and the non-vaccinated group had fatty changes which were more severe in the non-vaccinated group (Figure 2B).

**Humoral immune responses after immunization.** Serum S. Enteritidis-specific IgG antibodies were evaluated in chickens following Z11ΔrfbG immunization to determine the efficacy of Z11ΔrfbG in inducing humoral immune responses. As shown in Figure 3,
against *S. Enteritidis* in the Z11ΔrfbG vaccinated group was detected and increased dramatically 10 days after the second immunization. The 5×10⁶ and 5×10⁷ CFU-
vaccinated groups had significantly higher *S. Enteritidis*-specific IgG antibody titers than the control group.

**The DIVA capability of the Z11ΔrfbG vaccine.** The DIVA capability of the Z11ΔrfbG vaccine was evaluated using the slide agglutination test or the Biocheck *Salmonella* group D antibody ELISA test to detect LPS-specific serum antibodies. The slide agglutination test showed that the serum samples from chickens immunized with Z11ΔrfbG failed to agglutinate with the commercialized agglutination antigens on day 14 post-immunization (Figure 4). However, serum samples from chickens infected with the virulent wild-type strain Z11 agglutinated with *S. Enteritidis* antigens (Figure 4).

**DISCUSSION**

*Salmonella enterica* causes high morbidity and death in humans, both in terms of the number of infections and severity of the disease. In contrast to the *Salmonella enterica* serovars with a limited host range, such as *S. Typhi*, *S. Dublin*, and *S. Gallinarum*, *S. Enteritidis* has a large host range (13). Additionally, poultry and poultry-associated products are among the most important vehicles for human *Salmonella* infections (14). Vaccination in chickens is an important strategy currently used to reduce the levels of *Salmonella* in poultry flocks (15). A live attenuated *Salmonella* vaccine must be sufficiently attenuated, immunogenic, and protective. A variety of *Salmonella* mutant strains have been suggested as candidate vaccines. In a previous study, we constructed a mutant of the *S. Enteritidis* Z11 strain that lacks *rfbG* (16). The objective of the present
study was to evaluate the safety, protective efficacy, and DIVA capability of the rfbG-deficient mutant S. Enteritidis Z11 strain as a live attenuated DIVA vaccine candidate.

Live attenuated *Salmonella* vaccines are avirulent. Some LPS-deficient mutants result in attenuation of *Salmonella* (17). Jiao et al. (12) showed that the virulence of rfbG mutant strains was attenuated in a mouse model, and that rfbG mutant strains are safe for mammals. Similarly, this study showed that the LD$_{50}$ of Z11ΔrfbG was 61-fold higher than that of wild-type Z11 in a chicken model, indicating that the virulence of Z11ΔrfbG was significantly attenuated compared to that of virulent wild-type strain Z11 in chickens. In contrast, rfaH, an essential gene for LPS biosynthesis, does not affect the virulence of *S. Gallinarum* (18). Although the virulence of *S. Pullorum* S06004ΔspiCΔrfaH was significantly attenuated compared to that of wild-type S06004, it was mainly the spiC deletion that exerted the attenuating effect (19).

The early immune response against *Salmonella* relies on the innate immunity within the gut mucosa. In general, antibodies are necessary for resistance to systemic *Salmonella* infections, and as the infection progresses, an effective immune response to *Salmonella* relies on humoral immunity, which can provide effective protection to the host (20-21). A previous study reported that a novel live *S. Enteritidis* vaccine strain, JOL919, induced significant systemic immunoglobulin responses in chickens after inoculation (22). Another study reported that IgG levels were significantly higher in chickens vaccinated with the cobS and cbiA mutant *S. Gallinarum* strains than in the control group (23). Similarly, in this study, all chickens immunized with 5×10$^6$ and 5×10$^7$ CFU Z11ΔrfbG had significantly higher levels of *S. Enteritidis*-specific serum IgG than the non-
vaccinated group. These results demonstrated that the \textit{rfbG} mutant can induce strong humoral responses.

A standard vaccine not only induces a strong immune response but also has high immune protection efficacy. In this study, the survival rate of the $5 \times 10^7$ and $5 \times 10^6$ CFU-vaccinated groups was 100% after two intramuscular injections. Furthermore, chickens immunized with $5 \times 10^6$ and $5 \times 10^7$ CFU of Z11\textit{ΔrfbG} showed no clinical symptoms. Slight pathological changes were observed in the organs of $5 \times 10^5$ CFU-vaccinated chickens, whereas more severe fatty changes were detected in unvaccinated chickens after the challenge. \textit{S. Enteritidis} colonize the gastrointestinal tract in avians, which is frequently followed by an invasion of systemic areas, including the spleen and liver (24). Braukmann et al. (25) showed that a live \textit{Salmonella enterica} vaccine effectively inhibited the invasion and colonization of the challenge strain. Furthermore, a study reported that bacterial clearance occurred one week after \textit{Salmonella} infections (26). Sven et al. (27) demonstrated that a live \textit{Salmonella Enteritidis} vaccine inhibits systemic invasion after early infection with \textit{S. Typhimurium} and \textit{S. Infantis}. Our results showed that intramuscular immunization in 7-day-old chickens with a mutant strain of \textit{S. Enteritidis} \textit{rfbG} resulted in lower levels of the challenge strain and complete clearance of bacteria in the liver and spleen, which is consistent with previous studies. Therefore, the \textit{rfbG} mutant showed a strong protective efficacy.

The DIVA vaccine, an identifiable vaccine, can be used to distinguish immunized animals from infected animals. Most of the attenuated mutant strains used in DIVA vaccines lack specific antigens (such as LPS and neuraminidase); therefore, LPS is a suitable target for the construction of the DIVA vaccine (28). We have previously shown
that rfbG plays a role in LPS synthesis (12). In the present study, the Z11ΔrfbG
vaccinated group exhibited a negative serological test result, while the virulent wild-type
Z11 infection group showed a strong positive result for the serological test. These results
indicated that the rfbG mutant allows for the differentiation between infected animals and
vaccinated animals. Therefore, it may be used in combination with herd-level Salmonella
surveillance.

In conclusion, this study demonstrated that S. Enteritidis Z11ΔrfbG has the potential to
be a safe, immunogenic, and DIVA vaccine candidate for the control of Salmonella
infections. Attenuated S. Enteritidis Z11ΔrfbG elicited a strong humoral immune
response and provided effective protection in chickens. In addition, vaccination with
Z11ΔrfbG did not affect our ability to distinguish between infected and vaccinated
chickens by serological tests.

MATERIALS AND METHODS

Experimental animals. Healthy SPF chickens (7-day-old) were obtained from
Zhejiang Lihua Agricultural Technology Co., Ltd, China. All animal experiments were
approved by the Animal Welfare and Ethics Committees of Yangzhou University and
complied with the guidelines of the Institutional Administrative Committee and Ethics
Committee of Laboratory Animals (IACUC license number: SYXK [Su] 2016–0020).

Bacterial strains. Virulent wild-type S. Enteritidis strain Z11 was a clinical isolate
obtained from S. Enteritidis-infected chickens and stored in our laboratory. The rfbG-
deletion mutant strain, Z11ΔrfbG, was contrasted using a homologous recombination
technique mediated by a suicide plasmid, as previously described (16). Briefly, upstream
and downstream fragments of the \textit{rfbG} gene were amplified using PCR. The pDM4 plasmid was digested using a restriction endonuclease \textit{Xba I} (TaKaRa). The purified plasmid and the upstream and downstream fragments were fused using the ClonExpress MultiS One Step Cloning Kit (Vazyme Biotechnology Co., Ltd., Nanjing, Jiangsu, China). Recombinant plasmids were transferred into X7213 cells and sequenced. Single crossover mutants were obtained by conjugal transfer of recombinant suicide plasmids into the Z11 strain. The \textit{rfbG}-deletion mutant was screened on 15\% sucrose Luria-Bertani (LB) plates. The \textit{S. Enteritidis} Z11\textDelta \textit{rfbG} strain was used as a DIVA vaccine candidate in this study.

\textbf{Assessment of bacterial virulence.} The virulence of \textit{S. Enteritidis} Z11 and Z11\textDelta \textit{rfbG} vaccines was evaluated in chickens by determining the 50\% lethal dose (LD\textsubscript{50}). Sixty-six 7-day-old SPF chickens were used in this study. Thirty chickens in the wild-type group were randomly assigned to five groups (n=6). Each group was injected intramuscularly with a 10-fold dilution (1\times 10^{5} to 1\times 10^{9} CFU) of Z11. Thirty chickens in the deletion mutant group were randomly assigned to one of five groups (n=6). Each group was injected intramuscularly with a 10-fold dilution (2\times 10^{6}–2\times 10^{10} CFU) of Z11\textDelta \textit{rfbG}. Six chickens were inoculated with 100 \mu L of phosphate-buffered saline (PBS) via the same route as the control group. Chicken death was monitored daily for 14 days post-infection. LD\textsubscript{50} was calculated using the Reed–Muench method (29).

\textbf{Immune protection assessment.} Seventy-five 7-day-old SPF chickens were randomly assigned to three groups, namely the control (no vaccine, no challenge) (n=15), bacterial challenge without immunization (n=15), and live vaccine groups with the bacterial challenge (n=45). SPF chickens in the vaccine groups were randomly assigned
to three groups (n=15). Each group was administered 100 μL of diluted suspensions of
Z11ΔrfbG containing 5×10⁸, 5×10⁷, or 5×10⁶ CFU/mL in PBS by intramuscular
injection. Control chickens received 100 μL PBS via the same route. After the first
immunization, the vaccine groups were administered a booster dose at 17 d of age. These
chickens, as well as those in the unvaccinated group (28-day-old), were challenged
intramuscularly with 7×10⁸ CFU of the Z11 strain 11 days after the second
immunization. Deaths and clinical symptoms were recorded daily for 14 days after the
challenge.

**Bacterial clearance assay.** Bacterial clearance in the internal organs of the chickens
was evaluated. Liver and spleen samples from three chickens in each group were
aseptically collected at 14 DPC. The samples were then weighed and homogenized in 1
mL of PBS. Homogenates were 10-fold serially diluted and subsequently inoculated onto
LB agar plates at 37 °C for 12–16 h. Bacterial colonies were calculated as log10 CFU/g.

**Histological analysis.** Sections of the spleen and liver were collected from chickens at
14 DPC, and tissue samples were fixed in 10% neutral-buffered formalin. Paraffin-
embedded sections were stained with H&E stain (30) and were observed at 100 × and
400 × magnifications using an optical microscope.

**Serum IgG test.** Humoral immune responses were evaluated by determining the S.
Enteritidis-specific IgG antibody levels using enzyme-linked immunosorbent assay
(ELISA), as previously described (31), using Z11ΔrfbG as the coating antigen. Serum
samples were collected from chickens in each group on day 10 after the second
immunization and then serially diluted to be used as the primary antibody. The secondary
antibody used was horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG
(1:10,000 dilution; Sigma-Aldrich, St. Louis, MO, USA). HRP activity was determined using a 3,3,5,5-tetramethylbenzidine substrate solution (Solarbio, Beijing, China), and the optical density (OD<sub>450</sub>) value was determined using an ELISA reader (BioTek, Winooski, VT, USA).

**DIVA capability assessment for the Z11ΔrfbG vaccine.** The DIVA capability of the Z11ΔrfbG strain was evaluated using the serological method to detect LPS-specific serum antibodies by a slide agglutination test or a commercial ELISA kit. Fifteen chickens were randomly divided into 3 groups (n=5). Cells were infected with Z11ΔrfbG, Z11, or PBS. Serum was collected 14 days later and used to detect LPS antibodies. The slide agglutination test was performed using commercialized agglutination antigens obtained from Zhonghai Biotech Co., Ltd. (Beijing, China) according to the manufacturer’s instructions. ELISA was performed using the *Salmonella* group D antibody test kit (BioCheck, Inc., San Francisco, CA, USA) according to the manufacturer’s instructions (19).

**Statistical analysis.** GraphPad Prism 5 software (San Diego, CA, USA) was used for data analysis. Data are presented as mean ± standard error (SEM). Statistical analysis was set as *P* < 0.05 (*), < 0.01 (**), or 0.001 (***)

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**Table 1.** The 50% lethal dose ($LD_{50}$) of the *S. Enteritidis* Z11 and Z11ΔrfbG strains in chickens.

| Strains         | Challenge dose (CFU) | Number of deaths/Total number of chickens | LD$_{50}$ (CFU) |
|-----------------|----------------------|-------------------------------------------|-----------------|
| Z11             | 1×10$^9$             | 6/6                                       |                 |
| Z11ΔrfbG        | 1×10$^8$             | 5/6                                       |                 |
| Z11ΔrfbG        | 1×10$^7$             | 1/6                                       | 3.1×10$^7$      |
| Z11ΔrfbG        | 1×10$^6$             | 0/6                                       |                 |
| Z11ΔrfbG        | 1×10$^5$             | 0/6                                       |                 |
| Blank           | PBS                  | 0/6                                       | /               |
Table 2. The protective efficacy of intramuscular Z11ΔrfbG vaccination.

| Vaccination | Challenge |
|-------------|-----------|
| **Strain** | **Dose (CFU)** | **Number** | **Strain** | **Route** | **Dose (CFU)** | **Survivors/Total** | **Survival rate (%)** |
| Z11ΔrfbG | $5 \times 10^5$ | 15 | Intramuscularly | $7 \times 10^8$ | 15/15 | 100 |
| PBS | - | 11/15 | - | 15/15 | 100 |

395
Figure 1. The bodyweight of chickens after the challenge. Chickens of vaccinated and non-vaccinated groups were intramuscularly challenged with $7 \times 10^8$ colony-forming units (CFU) of the virulent wild-type strain (Z11), and the control group received 100 μL of PBS. The bodyweights of these chickens were recorded 11 days post-challenge (DPC). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with the bodyweight of control group chickens. Data are presented as mean ± SEM.

Figure 2. A diagram showing the pathological anatomy (A) and histological analysis (B) of the liver after the bacterial challenge. (A) The liver pathological anatomy diagram was observed 14 days post-challenge (DPC). The arrows indicate the lesions. (B) The histopathological changes in the livers of chickens were examined by H&E staining 14 DPC. The results were observed at 100 × and 400 × magnification using an optical microscope. Arrows in the liver sections represent the fatty changes. The circle in the liver section indicates the necrotic foci.

Figure 3. Determination of serum IgG levels. The enzyme-linked immunosorbent assay was used to identify S. Enteritidis-specific IgG antibody titers in the serum of chickens from each group 10 days following the second inoculation. *, $P < 0.05$, and ***, $P < 0.001$ compared with the control group. Data are presented as mean ± SEM.

Figure 4. The DIVA capability of Z11ΔrfbG. The serum was collected from chickens infected with Z11ΔrfbG, Z11, or PBS for 14 days and used for the detection of LPS antibodies. Agglutination assay was performed using commercialized agglutination antigens.
Figure 1.
Figure 2.
Figure 3.

S. Enteritidis-specific IgG titers

Day 10 after the second immunization
Figure 4.

\[\text{Z11} \quad \text{Z11}\Delta\text{rfbG} \quad \text{PBS}\]
Table 1. The LD$_{50}$ of the *S. Enteritidis* Z11 and Z11ΔrfbG in chickens.

| Strains          | Challenge dose (CFU) | Number of deaths/Total number of chickens | LD$_{50}$ (CFU) |
|------------------|----------------------|------------------------------------------|----------------|
|                  | 1×10$^9$             | 6/6                                      |                |
|                  | 1×10$^8$             | 5/6                                      |                |
| Z11              | 1×10$^7$             | 1/6                                      | 3.1×10$^7$     |
|                  | 1×10$^6$             | 0/6                                      |                |
|                  | 1×10$^5$             | 0/6                                      |                |
|                  | 2×10$^{10}$          | 5/6                                      |                |
|                  | 2×10$^9$             | 4/6                                      |                |
| Z11ΔrfbG         | 2×10$^8$             | 0/6                                      | 1.9×10$^9$     |
|                  | 2×10$^7$             | 0/6                                      |                |
|                  | 2×10$^6$             | 0/6                                      |                |
| Blank            | PBS                  | 0/6                                      | /              |
Table 2. The protective efficacy of the Z11ΔrfbG after intramuscular vaccination.

| Vaccination | | Challenge | | Survivors/ | Survival |
|-------------|-------------|-----------|-------------|-----------|
| | | | | Total rate (%) | |
| Strain | Route | Dose (CFU) | Number | Strain | Route | Dose (CFU) | |
| Z11ΔrfbG | Intramuscularly | 5×10^5 | 13/15 | 86.7 |
| PBS | | - | 11/15 | 73.3 |
| PBS | | - | 15/15 | 100 |
Figure 1. The bodyweight of chickens after the challenge. Chickens of vaccinated and non-vaccinated groups were intramuscularly challenged with $7 \times 10^8$ colony-forming units (CFU) of the virulent wild-type strain (Z11), and the control group received 100 μL of PBS. The bodyweights of these chickens were recorded 11 days post-challenge (DPC). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with the bodyweight of control group chickens. Data are presented as mean ± SEM.
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Figure 4. The DIVA capability of Z11ΔrfbG. The serum was collected from chickens infected with Z11ΔrfbG, Z11, or PBS for 14 days and used for the detection of LPS antibodies. Agglutination assay was performed using commercialized agglutination antigens.
September 5, 2022

Dear Editor:

We have revised the manuscript of Spectrum01574-22 (Evaluation of the protective immune response induced by an $rfg$-deficient *Salmonella* Enteritidis strain as a live attenuated DIVA vaccine in chickens) and made changes as suggested by the reviewers and the editor. All the reviewers’ and editorial comments have been accepted.

Enclosure:

1. The revised manuscript and all changes of the manuscript marked in yellow color;
2. The letter detailed our responses to all the comments passed by the reviewers and the editor.

Thank you for your attention!

Yours sincerely

Hongqin Song

Yangzhou University
The letter accepting changes to the reviewer 1

Dear reviewer:

Thank you very much for your valuable comments on our manuscript. We have revised the manuscript of Spectrum01574-22 (Evaluation of the protective immune response induced by an *rfbG*-deficient *Salmonella* Enteritidis strain as a live attenuated DIVA vaccine in chickens) and made changes as you suggested.

Reviewer 1: Overall this is an interesting study with well supported conclusions. Authors should add all statistical test methods used in the paper in the methods section and figure legends. Additionally, authors can elaborate on the importance of being able to differentiate between vaccinated and infected chickens using sera.

1. Authors should add all statistical test methods used in the paper in the methods section and figure legends.

   **Answer:** Thanks for your kind advice. In the methods section (line 310) and in figure legends, we added the statistical test methods used in the results (one-way ANOVA and Bonferroni's multiple comparison test).

2. Additionally, authors can elaborate on the importance of being able to differentiate between vaccinated and infected chickens using sera.

   **Answer:** Thank you for your kind suggestion. Because *Salmonella* vaccination may interfere with existing serologic monitoring, the development of a vaccine that distinguishes between immunized and infected animals is necessary. In line 204, we describe the importance of distinguishing between immunized and infected animals.
The letter accepting changes to the reviewer 2

Dear reviewer:

Thank you very much for your valuable comments on our manuscript. We have revised the manuscript of Spectrum01574-22 (Evaluation of the protective immune response induced by an *rfbG*-deficient *Salmonella* Enteritidis strain as a live attenuated DIVA vaccine in chickens) and made changes as you suggested.

Reviewer 2:

Evaluation of the protective immune response induced by an *rfbG*-deficient *Salmonella* Enteritidis strain as a live attenuated DIVA vaccine in chickens by Wang et al.

The authors claim to have constructed a *rfbG* mutant of *Salmonella enterica* serovar Enteritidis (SE) that would be suitable as a live attenuated DIVA vaccine strain with protective properties against SE infections.

The topic of SE as a pathogen for poultry, but more importantly as a zoonotic pathogenic bacterium affecting humans, thus being a public health concern, is timely. This reviewer can imagine how much effort was put into the study. Nonetheless, the manuscript appears at this state somewhat preliminary as all sections of the manuscript need to be more elaborated. Many sections appear rather cryptic and are lacking details that are essential for the understanding of the executed experiments. Grammar and style should be carefully checked.

Answer: Thank you for your kind advice. We described the experimental methods and results in detail in the manuscript, such as a detailed description of the experimental
groups (line 254). In view of the grammar problem, we also had a professional embellishment company (Editage, https://www.editage.cn) to polish the manuscript and revise it.

A very important aspect, although mentioned in the discussion section, is totally excluded from their own study: the colonization of the gastrointestinal tract of the chickens after oral uptake/inoculation of SE. This plays an important role in the natural environment as well as in the small scale and largescale industrial settings of poultry production. Many issues of food poisoning through SE occur when contaminated poultry carcasses are processed. Protection of poultry against systemic infection is important, indeed. But what about protection against gastrointestinal colonization and shedding of SE into the environment? The main aspect still is the problem that this zoonotic pathogen can easily be transferred to humans via poultry products and hens eggs.

Answer: Thank you for your valuable suggestions for us. Therefore, based on your suggestion, we examined the colonization of bacteria in the liver, spleen, and cecum after challenge by a repeated bacterial colonization assay, and the results showed that the bacterial load in the vaccinated group was significantly reduced in the liver, spleen, and cecum compared with the non-vaccinated group, indicating that the \( \text{rfbG} \)-deficient strain accelerated the clearance of bacteria in the liver, spleen, and cecum, thus reducing the colonization.

Your question about how to prevent the transmission of \textit{Salmonella} Enteritidis to humans through poultry products and eggs is very important to us, but since our
experimental design was developed based on published studies (1, 2), our study is still in the preliminary stage of vaccine protection. We will further investigate at a later stage, based on your suggestions, whether \textit{rfbG}-deficient strains can reduce the transmission of \textit{Salmonella} enterica pathogens in poultry products and eggs.

Specific comments:

1. It is rather safe to say that reversion of the \textit{rfbG} mutation can be excluded. But what about the possibility of recombination with DNA fragments from other \textit{Salmonella} strains that could potentially be present in the same bird at the time of vaccination? Please discuss.  

   Answer: Thank you for your suggestion. All the chickens we used in the experiment were SPF chickens, and before we started the experiment, we would strictly sterilize the isolator to ensure that it was completely sterile, and we also set up a control group to eliminate the contamination of other bacteria.

2. Abstract; first sentence: other serovars can cause salmonellosis as well. Please re-phrase.  

   Answer: Thank you very much for your comments. We are very sorry for this loose remark. We have rewritten the original sentence in line 26 as "\textit{Salmonella enterica} serovar Enteritidis (\textit{S. Enteritidis}), one of the zoonotic pathogens, not only results in significant financial losses for the global poultry industry, but it also has the potential to spread to humans through poultry and poultry products".

3. Line 34-39: Re-phrase, please. A lethal challenge is per definition deadly. How can it be that birds do not show any signs of disease after a lethal challenge?
Answer: Thank you for your kind advice. We have rewritten the original sentence in line 35 as "After challenge, the non-vaccinated group showed serious clinical symptoms (diarrhea, decreased appetite, depression, weight loss), pathological changes (white nodules in the liver and fatty lesions in liver cells) and death. In contrast, there were no clinical symptoms, pathological changes, or death in the $5 \times 10^6$ and $5 \times 10^7$ colony-forming unit (CFU)-vaccinated groups".

4. Line 42: Find another word for "transformed".

Answer: Thank you for your suggestion. We have rewritten the original sentence in line 44 as "Overall, our findings demonstrate the viability of the rfbG mutant as a live attenuated chicken vaccine that can discriminate between animals that have been immunized and those that have been infected."

5. Line 59 and throughout the document: "Gram" starts always with a capital "G".

Answer: Considering to your suggestion, we changed it to "Gram-negative".

6. Complete results section: Please provide sufficient details in all sub-sections.

Answer: Thank you for your kind advice. We have described the results in detail, such as the results of bacterial clearance (line 105) and changes in body weight (line 124) after challenge.

7. Line 119 and later in manuscript: what are "fatty changes"? Please explain.

Answer: Under normal circumstances, in addition to fat cells, other cells generally do not see or only a small number of lipid droplets, such as the emergence of lipid droplets in these cells or a significant increase in lipid droplets, called steatosis.

8. Line 213: Do the authors mean "constructed" rather than "contrasted"?
Answer: We are very sorry for our incorrect writing. We have changed "contrasted" to "constructed" in line 231.

9. Line 221 -223: How was the $rfbG$ mutation confirmed? By sequencing?

Answer: We verified whether the $rfbG$ was deleted successfully by PCR. The detailed process is as follows: the outer primers and inner primers of $rfbG$ were designed respectively, and the deletion of $rfbG$ was determined by amplifying the sequences of outer primers and inner primers. Using the wild-type strain Z11 as the negative control, if the sequence amplified by the outer primers was smaller than that of the negative control and the size of the different band met the size of the $rfbG$ band but the inner primers could not amplify the sequence, the $rfbG$ deletion was successful. We have added a method to verify the deletion strain in line 240.

10. Lines 246 ff: Which volume of the samples was plated onto LB plates? What is the detection limit? Samples should have undergone an enrichment procedure in addition to "direct plating" How would LB medium discriminate against bacterial strains other than the challenge strain?

Answer: 1. 100 µL of tissue slurry was added dropwise to each LB plate. 2. We carried out bacteriological analysis according to the description of Barrow et al. (3). The steps of the bacterial colonization test are as follows: aseptically collect the organ tissues, place them in homogenized tubes containing sterile PBS, grind them to obtain tissue slurry, dilute the tissue slurry gradient 10 times, select the appropriate dilution gradient for plate coating according to the specific situation, incubate them at 37°C for 12-16 hours, and calculate the bacteria load of the organ the next day based on the
bacterial count results and the mass of the collected organ tissues. 3. PCR was used to distinguish between wild strains and deletion strains. In line 105 of the manuscript, we mention that before challenge (7 and 10 days after the second immunization), no bacteria were isolated from the liver, spleen, and cecum of the vaccinated group and the non-vaccinated group, indicating that there were no \( rfbG \) deletion strains in the vaccinated group and the non-vaccinated group. After challenge, we use PCR to verify the bacteria on the LB plate. The results show that the bacteria on the LB plate are wild strains. (For specific methods of PCR, see question 9)

11. Line 253: Please explain H&E stain.

Answer: Hematoxylin and eosin (H&E) stain is one of the most basic and widely used techniques in histology and pathology teaching and research. Hematoxylin staining solution is alkaline and can stain the basophilic structures of tissues (such as ribosomes, nuclei and ribonucleic acid in cytoplasm) into blue-purple; eosin is an acidic dye and can stain the eosinophilic structures of tissues (such as intracellular and intercellular proteins) into pink, so that the morphology of the whole cellular tissue is clearly visible. The tissue sectioning method is a very common test method in teaching, research, and pathology testing, and H&E stain is the most common staining method used in the process of making sections.

12. Table 2: Please elaborate what is the difference between the two PBS groups?

Answer: One group of PBS was not inoculated with the \( rfbG \) deletion strain but was challenged. We named it the non-vaccinated group in line 255. The other group of PBS was not inoculated with the \( rfbG \) deletion strain and was not challenged. We
named it the control group in line 254.

13. Table 1: The respective experiment should be executed in duplicate and repeated with a larger number of birds. Were the birds commingled or housed in different rooms (potential "pen effect")?

Answer: According to your suggestion, we expanded the number of samples and re-carried out the experiment. The experimental method and results have been modified in this manuscript. The results showed that there was no cross-contamination between different groups of chickens raised in different isolators and a control group.

Reference

1. Penha Filho RA, de Paiva JB, Arguello YM, et al. 2009. Efficacy of several vaccination programmes in commercial layer and broiler breeder hens against experimental challenge with *Salmonella enterica* serovar Enteritidis. Avian Pathol 383:67-375. DOI: 10.1080/03079450903183645.

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3. Barrow PA, Lovell MA. 1991. Experimental infection of egg-laying hens with *Salmonella enteritidis* phage type 4. Avian Pathol 20:335-348. DOI: 10.1080/03079459108418769.
October 22, 2022

Dr. Hongqin Song
Yangzhou University
Yangzhou
China

Re: Spectrum01574-22R1 (Evaluation of the protective immune response induced by an rfbG-deficient Salmonella Enteritidis strain as a live attenuated DIVA vaccine in chickens)

Dear Dr. Hongqin Song:

Your manuscript has been accepted, and I am forwarding it to the ASM Journals Department for publication. You will be notified when your proofs are ready to be viewed.

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Editor, Microbiology Spectrum

Journals Department
American Society for Microbiology
1752 N St., NW
Washington, DC 20036
E-mail: spectrum@asmusa.org