Research in Veterinary Science 107 (2016) 50–54

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Safety and protective efficacy of a *spiC* and *crp* deletion mutant of *Salmonella gallinarum* as a live attenuated vaccine for fowl typhoid

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ARTICLE INFO

Article history:
Received 1 June 2015
Received in revised form 7 April 2016
Accepted 22 May 2016

Keywords:
Salmonella gallinarum
Fowl typhoid
Live attenuated vaccine
Safety
Protective efficacy

ABSTRACT

With an aim to develop a safe, immunogenic fowl typhoid (FT) vaccine, the safety and efficacy of 1009Δ*spiC*Δ*crp*, a *spiC* and *crp* deletion mutant of *Salmonella gallinarum*, were evaluated in chickens. Three-day-old chickens were intramuscularly immunized with 1009Δ*spiC*Δ*crp* (1×10^7 CFU) and boosted 7 days later (at 10-days old) with the same dose and via the same route (vaccinated group). The vaccinated group showed no clinical symptoms and no differences in body weight compared to the unvaccinated control group. 1009Δ*spiC*Δ*crp* bacteria colonized and persisted in the liver and spleen of vaccinated chickens for >14 days, and significant specific humoral and cellular immune responses were induced. Vaccinated chickens were challenged with *S. gallinarum* strain SG9 at 21 days post-immunization (24-day-old chickens), and efficient protection was observed based on the mortality and clinical symptoms, as compared to those in the control group. These results demonstrate that 1009Δ*spiC*Δ*crp* can be used as a live attenuated vaccine.

1. Introduction

Fowl typhoid (FT), caused by *Salmonella gallinarum*, is a severe systemic disease characterized by severe anorexia, weight loss, lesions, depression, diarrhea, and high morbidity and mortality, that occurs in both young and adult chickens (Barrow and Freitas Neto, 2011). *S. gallinarum* can be transmitted to chicks through eggs, and FT outbreaks are of significant economic importance in many areas (Barrow and Freitas Neto, 2011; Bäumler et al., 2000).

Vaccination is an effective method for preventing *Salmonella* infections (Mastroeni et al., 2001). Killed vaccines consist of whole bacteria that are inactivated by different methods including heat, formalin, acetone or other treatments, and their major advantage is the absence of live organisms that may potentially pose a risk to human health. However, such vaccine strains can be easily eliminated by the host, and antigen expression is often so limited that adjuvants are usually required (Desin et al., 2013). The bacterial ghosts are one of the kinds of non-living vaccine, they are devoid of all cytoplasmic content but containing their natural outer membranes (Chaudhari et al., 2012). Live vaccines confers several advantages including the ability to administer vaccines orally to birds, and the induction of protective immunity through activation of both antibody and cell-mediated immune responses (Mastroeni et al., 2001; Desin et al., 2013). The currently available live *S. gallinarum* vaccine strain 9R has been used to prevent FT for nearly 60 years; however, this vaccine strain has several disadvantages, such as residual virulence, a low growth rate, and insufficient protection (Bouzoubaa et al., 1989; Kwon and Cho, 2011). More recently, a series of deletion mutants were generated and their protection efficiencies against FT were reported. For example, a *lon* and *cpxR* deletion strain of *S. gallinarum* offered efficient protection, although depression was temporarily observed after challenge (Matsuda et al., 2011). The *S. gallinarum* attenuated vaccine strains χ11571 (Δ*rfaH*489) and χ11570 (Δ*crp*-633) provided protection for 11/20 and 18/20 (Alive/Total) chickens, respectively (Mitra et al., 2013). In addition, the mortality rate of 5-day-old chickens orally vaccinated with a *cobS* and *cbiA* deletion mutant of *S. gallinarum* was only 15% (Penha Filho et al., 2010).

The *spiC* (also known as *ssaB*) gene encodes a *Salmonella* pathogenicity island-2 (SPI2) type III secretion system (T3SS) effector protein SpiC, which plays important roles in *Salmonella* infections. SpiC can inhibit fusion of *Salmonella*-containing vacuoles with lysosomes by interfering with intracellular trafficking (Uchiya et al., 1999). It is also necessary for the secretion of SseB, SseC, and SseD, which are components of the T3SS2 translocon (Freeman et al., 2002; Yu et al., 2002). SpiC is also a part of the SsaM/SpiC/SsaL regulatory complex, which mediates the switch from translocon protein secretion to effector translocation (Yu et al., 2010). The *crp* gene encodes cAMP receptor protein (CRP), which modulates genes involved in carbon source utilization, glycogen synthesis, flagellum synthesis, outer membrane proteins, and regulates the production of sugars (Poncet et al., 2009). The

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http://dx.doi.org/10.1016/j.rvsc.2016.05.007
0034-5288/

Table 1
The body weight of chickens after immunization.

Group	Mean body weight per chicken at dpi (g)		
	4	11	18
Vaccinated	66.929 ± 0.325	116.926 ± 0.577	188.239 ± 0.825
Control	67.334 ± 0.415	116.489 ± 0.493	189.059 ± 0.836

There were no significant differences between the groups at any time point ($P > 0.05$).

virulence of a *spiC* mutant of *Salmonella pullorum* is attenuated, and the lethal dose 50 of *spiC*-deleted *S. pullorum* S06004 was significantly higher than that of the parent strain (Geng et al., 2014). Strains of *S. gallinarum*, *Salmonella choleraesuis*, and *Salmonella typhimurium* with *crp* deletion mutations were also attenuated (Curtiss et al., 2009; Chen et al., 2010; Rosu et al., 2007).

Previous studies have shown that *spiC* and *crp* were related to virulence; however, no studies have examined the potential of using a *spiC* and *crp* deletion mutant of *S. gallinarum* to prevent FT. Recently, an *S. gallinarum* strain with deletions of *spiC* and *crp* was constructed, and the virulence of the mutant was markedly lower than that of a non-mutant strain (Cheng et al., 2015). In this study, we evaluated the safety and protective efficacy of a *spiC* and *crp* deletion mutant of *S. gallinarum* (1009Δ*spiC*Δ*crp*) as a live attenuated vaccine for FT, by monitoring the changes in body weight and clinical symptoms, as well as bacterial persistence, immune response, and protective effects in HY-line white chickens.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. gallinarum 1009 is a clinical isolate obtained from chickens with FT and is stored in our laboratory. Strain 1009Δ*spiC*Δ*crp*, a *spiC* and *crp* deletion mutant of *S. gallinarum* 1009, was constructed by allelic exchange using the suicide vector pGMB151 and λ Red recombineering. Analysis of the biological characteristics of the mutant strain showed that it was stably inherited and failed to utilize glucose or ferment mannose, maltose, and trehalose. In addition, growth analysis of the mutant strain showed lower colony forming units than the wild type strain. The virulence of the mutant was greatly attenuated, with an LD_{50} of 4.47×10^9 CFU, compared to 4.17×10^7 CFU for the WT strain (Cheng et al., 2015). The virulent *S. gallinarum* SG9 (nalidixic acid-resistant, Nal^r) strain was supplied by Dr. Barrow (Jones et al., 2001). Luria-Bertani (LB) broth, LB agar medium (containing 1.5% [w/v] agar), and XLT4 (Difco) agar were used for culturing bacteria at 37 °C. When needed, chloramphenicol (Cm) was added to the medium at a final concentration of 30 μg/mL.

2.2. Chickens

HY-line white chicken eggs were hatched, examined for any clinical signs of enteric disease, and were found to be negative for *Salmonella*. All chickens were maintained in wire cages and reared with commercial feed and drinking water throughout the experiment. All experimental and animal management procedures were performed with the permission of the Animal Care and Ethics Committee of Yangzhou University.

2.3. Changes in body weight and clinical symptoms

Eighty 3-day-old chickens were randomly assigned to two groups: the vaccinated group (n = 35) and the control group (n = 45). The vaccinated group was intramuscularly immunized with 1×10^7 CFU 1009Δ*spiC*Δ*crp* in 100 μL of phosphate buffered saline (PBS), and boosted 7 days later (10-day-old chickens) with the same dose and via the same route. The control group remained unimmunized and received equal volumes of PBS at the same times and via the same route. The body weights of these chickens were recorded at 4, 11, and 18 dpi (especially the days post first immunization), and their clinical symptoms were observed daily from 1 to 21 dpi.

2.4. Recovery of the vaccine strain from internal organs

To monitor the presence of the vaccine strain in the internal organs of the chickens after immunization, five chickens from each group were euthanized at 7, 10, 14, 18, and 21 dpi, and liver and spleen samples were aseptically removed. Samples were weighed and suspended in 1 mL of PBS and then individually homogenized. Various dilutions of the homogenates (100 μL each) were plated on XLT4 agar containing 30 μg/mL Cm and then incubated at 37 °C overnight. The number of 1009Δ*spiC*Δ*crp* colonies was counted and expressed as $\log_{10}$ CFU/g. Negative samples were reported as 0 CFU/g.

2.5. Enzyme linked immunosorbent assay (ELISA) for systemic IgG

Five blood samples were taken from each group, and *S. gallinarum* antigen-specific antibody levels were assessed by ELISA at 7, 14, and 21 dpi using heat-killed whole *S. gallinarum* bacteria as the coating antigen as previously described (Haneda et al., 2011). To determine *S. gallinarum* antigen-specific serum IgG levels, serum samples (1:50 dilution) were used as the primary antibody. The secondary antibody was horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Sigma, USA; 1:10,000 dilution). Bound HRP activity was determined using *o*-phenylenediamine dihydrochloride (Sigma). The OD_{492} was determined with an ELISA reader after the reaction was stopped with 2 M H_2SO_4. The titers are reported as the highest dilution at which the optical density was 2.1-fold higher than that of the negative control (P/N ≥ 2.1).

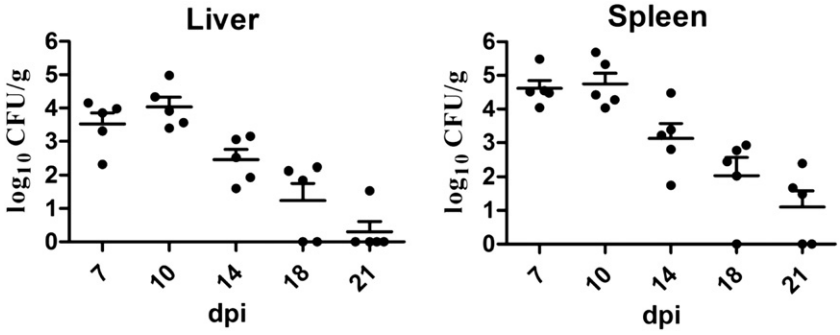

Fig. 1. Bacterial recovery from the liver and spleen of vaccinated chickens. The vaccinated group was intramuscularly immunized with 1×10^7 CFU of 1009Δ*spiC*Δ*crp* in 100 μL of PBS and boosted 7 days later (day 10) with the same dose via the same route. The control group (unimmunized) received equal volumes of PBS at the same times and via the same route. Values represent the mean ± SEM of $\log_{10}$ CFU/g. All the liver and spleen samples from chickens in the control group were negative.