Veterinary Immunology and Immunopathology 173 (2016) 27–33

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Evaluation of protective efficacy of a novel inactivated *Salmonella* Pullorum ghost vaccine against virulent challenge in chickens

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ARTICLE INFO

Article history:
Received 10 November 2015
Received in revised form 18 March 2016
Accepted 25 March 2016

Keywords:
Salmonella Gallinarum biovar Pullorum
Bacterial ghost
Vaccine
Chickens
Protective efficacy

ABSTRACT

Salmonella Gallinarum biovar Pullorum is the causative agent of pullorum disease in poultry, an acute systemic disease that results in a high mortality rate in young chickens. Vaccines have been considered in many developing countries where levels of infection are high and eradication is not a realistic option. An attenuated strain combined with protein E-mediated cell lysis was used to generate a safety enhanced *Salmonella* Pullorum ghost vaccine. Immune responses and protection induced by ghost vaccine in chickens were investigated following a prime-boost immunization administered via intramuscular and oral routes. Chickens from vaccinated groups showed significant increases in antigen-specific IgG, especially after booster immunization. Lymphocyte proliferation responses were also significantly increased in all immunized groups at 2-weeks post-final vaccination. The *Salmonella* Pullorum ghost vaccine provided satisfactory protection against virulent *Salmonella* Pullorum infection, as shown by the robust stimulation of both humoral and cell-mediated immune responses as well as the reduction in the number of bacterial recovered post-challenge. Moreover, the immune effects and survival rates indicated intramuscular injection is more efficient than oral vaccination. In conclusion, our results suggest that *Salmonella* Pullorum ghosts may be used as a safe and effective novel inactivated vaccine candidate to protect against virulent *Salmonella* Pullorum infection.

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1. Introduction

The bacterial genus *Salmonella* consists of more than 2500 documented serotypes, many of which are dangerous pathogens in humans or animals (Heithoff et al., 2015). *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Pullorum (*Salmonella* Pullorum) is highly adapted to fowl, in which it causes a widespread and devastating infection known as pullorum disease (PD). In the chickens, *Salmonella* Pullorum can affect young animals under 3 weeks of age, resulting in high mortality and horizontal transmission (Barrow and Freitas Neto, 2011). Additionally, it may persist for several months in the spleen and reproductive tract, leading to vertical transmission of the infection to eggs or progeny (Wigley et al., 2005). The disease has been eradicated from commercial poultry in some developed countries, but it continues to cause significant economic losses worldwide (Jones et al., 2001; Kang et al., 2012). The high level of infection, extended persistence in chickens, worsening problems of antibiotic resistance, and lack of an available eradication option call for a better approach to controlling *Salmonella* Pullorum infection (Pan et al., 2009).

Vaccination is an effective method for controlling and preventing salmonellosis (Babu et al., 2003; Liu et al., 2015). The efficacy of various live attenuated and killed *Salmonella* vaccines has been proven multiple times and these vaccines are used commercially worldwide. Many studies indicate that live vaccines are significantly more effective and protective than their inactivated counterparts. For example, the first effective live, rough mutant strain *Salmonella* Gallinarum 9R (Smith, 1956) has been used for decades to protect against fowl typhoid. However, there are several major disadvantages of live vaccines, including the potential for reversal to virulence through horizontal gene transfer, the public health problems associated with vaccinating food-producing animals, and environmental contamination via fecal shedding (Okamura et al., 2007). Conventional methods for producing inactivated vaccines,

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http://dx.doi.org/10.1016/j.vetimm.2016.03.015
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such as heat killing or formalin treatment, often result in a reduction in the ability of the vaccines to give full immunity by destroying the physical or chemical characteristics of components of bacterial surface structures (Jawale et al., 2002). Therefore an inexpensive, safe, protective and environment friendly vaccine is needed.

In recent years, inactivated bacterial ghost vaccines have been described as a novel approach to generating vaccine formulations against a wide variety of gram-negative pathogens (Muhammad et al., 2012). Controlled expression of the bacteriophage PhiX174 lysis gene E in gram-negative microorganisms induces expression of a 91-amino acid polypeptide that can assemble and penetrate the inner and outer membrane of the bacteria, forming a transmembrane tunnel. The high internal osmotic pressure of the cell expels the bacterial genome and cytoplasmic contents through the tunnel, leading to the generation of a non-living empty cell envelope, known as a bacterial ghost (Witte et al., 1990; Schön et al., 1995). Unlike the intense physical or chemical treatments involved in making traditional inactivated vaccines, the non-enzymatic activity of protein E means that bacterial ghosts retain all the functional and antigenic determinants of their living counterparts in a native state. Therefore, these ghost cells can induce significant humoral and cellular immune responses, resulting in effective immunoprotection, together with their adjuvant properties (Mader et al., 1997; Eko et al., 2000; Felnerova et al., 2004; Peng et al., 2011).

The focus of the present study was to develop a safety enhanced *Salmonella* Pullorum ghost vaccine. To accomplish this, a recombinant plasmid containing the bacteriophage PhiX174 lysis gene E, along with an enhancer, was constructed and introduced into an attenuated *Salmonella* Pullorum strain. *Salmonella* Pullorum ghosts were successfully prepared and shown to induce significant immune responses, confirming that this vaccine offers effective protection against challenge with a virulent *Salmonella* Pullorum strain in chickens.

2. Materials and methods

2.1. Bacterial strains, plasmids and animals

The *Salmonella* Pullorum strain (SP S06004) is virulent in chickens and is naturally resistant to nalidixic acid (Geng et al., 2009). A *spiC* deletion mutant strain, S06004Δ*spiC* (Geng et al., 2014), was used to construct the ghost vaccine. All strains were grown in Luria-Bertani (LB) broth supplemented with nalidixic acid (Nal, 100 μg/ml) or kanamycin (Km, 50 μg/ml) when needed. For construction of a lysis plasmid pBBR1MCS-E, the lysis gene E and thermo-sensitive λ*pR*-cI857 regulator cassette were cloned into a broad-host-rang cloning vector pBBR1MCS-2. The recombinant plasmid was propagated in *Escherichia coli* DH5α at 28 °C.

HY-line white chicken embryos, obtained from Jiangsu Institute of Poultry Sciences (China), were hatched in the laboratory. The broiler chickens were checked to confirm the absence of *Salmonella* infection by bacteriological examination as described below and for any clinical signs of enteric disease. All chickens were reared in wire cages with sufficient formulated feed and water at ambient temperature. All experimental and animal management procedures conformed to the guidelines of the Animal Welfare and Ethics Committees of Yangzhou University.

2.2. Preparation and characterization of Salmonella Pullorum ghosts

For the preparation of *Salmonella* Pullorum ghosts, transformation of the lysis plasmid pBBR1MCS-E into SP S06004Δ*spiC* cells was performed by electroporation. A single resulting colony of SP S06004Δ*spiC* strain harboring lysis plasmid pBBR1MCS-E was inoculated into LB broth containing 50 μg/ml Km and cultured overnight at 28 °C. Following incubation, 1 ml culture was added into 100 ml of LB broth containing 50 μg/ml Km and incubated at 28 °C until the culture reached OD_{600} = 0.4–0.6. To induce gene E-mediated bacteriolysis, the incubation temperature was then raised to 42 °C for about 6 h. After lysis, *Salmonella* Pullorum ghosts were harvested by centrifugation (4000*g* for 10 min), washed three times with sterile phosphate-buffered saline (PBS), resuspended in PBS (pH 7.2), and then stored at −80 °C. At various time points during induction (0–6 h post-induction), culture aliquots were serially diluted to the suitable concentration to monitor the viable cell counts as colony forming units (CFU). Lysis efficiency of SP S06004Δ*spiC* was then determined by the following formula: (1-CFU counts of ghosts post induction/CFU counts prior to induction) × 100%. A control strain was grown simultaneously using the same parameters for comparison (Jawale et al., 2014). The ghosts were fixed and then examined by scanning electron microscopy (SEM; S-4800 II, Hitachi) and transmission electron microscopy (TEM; Tecnai 12, Philips) as described previously (Yu et al., 2011), with minor modifications. Briefly, dehydration steps were 15 min each in 50%, 70%, 80%, 90%, 100% ethanol, a 1:1 mixture of 100% ethanol and isopentyl acetate, and pure isopentyl acetate. After lyophilization, samples were coated with a layer of gold and SEM examined.

2.3. Safety evaluation of the Salmonella Pullorum ghost vaccine

Prior to immunization assays, a safety evaluation was carried out to determine whether there were any adverse effects of the *Salmonella* Pullorum ghost vaccine on 1-day-old chickens. Briefly, the chickens were divided into four groups (n = 10 for each group) and intramuscularly inoculated with 10^7, 10^8, or 10^9 bacterial ghost cells or with PBS. The general condition of the chickens was observed daily, and the mortality, visible adverse reactions at injection site, and clinical symptoms of PD were recorded. Necropsies were performed at 3 weeks post-immunization to monitor the lesions or signs of pathology.

2.4. Immunization and challenge infection

Sixty 1-day-old chickens were divided into three groups (A, B, and C; n = 20 per group) to evaluate the protective efficacy of *Salmonella* Pullorum ghosts by employing a prime-boost vaccination strategy as described previously (Jawale and Lee, 2013). The chickens in group A were kept as a non-vaccinated control. Group B chickens were administered intramuscularly with *Salmonella* Pullorum ghosts (1×10^8 ghost cells/0.1 ml/chicken) at 1-day-old, and boosted at 14-days-old. Group C chickens were primed and boosted via oral route inoculation with *Salmonella* Pullorum ghosts (1×10^{10} ghost cells/0.1 ml/chicken) at the same time points. At 7 days post-final inoculation, all chickens received intramuscular challenge with 1×10^9 CFU/chicken wild-type *Salmonella* Pullorum strain S06004. Mortality and clinical symptoms were monitored daily for 14 days after the challenge. All surviving chickens were then sacrificed to identify any macroscopic organ lesions. Clinical scores were determined and recorded using a system similar to the one previously described (Mitra et al., 2013) with modifications. Each lesion was assigned a score of 0–5. A score of 0 indicated healthy organs with no lesions or signs of pathology whereas a score of 1 was assigned to indicate organs with the following signs of disease: hepatomegaly, splenomegaly, necrotic foci on the liver, and pale yellow nodules on the myocardium, for a maximum possible score of 4. The pathology score for dead birds in all groups was

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