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Research paper

An immunogenic *Salmonella* ghost confers protection against internal organ colonization and egg contamination

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ABSTRACT

The tightly regulated expression of the PhiX174 lysis gene *E* from a multi-copy plasmid led to the stable production of an *Salmonella* Enteritidis bacterial ghost. The present study was conducted to evaluate induction of the humoral and cell-mediated immune responses induced after single or double intramuscular immunization with the *S.* Enteritidis ghost and to assess its protective effect on colonization of the intestinal tract, visceral and reproductive organs, internal egg contamination, and egg production of laying chickens. A total of 60 chickens were equally divided into three groups ($n = 20$); group A (non-immunized control), group B (immunized at 8 and 16 weeks of age) and group C (immunized at 16th week of age). Chickens from both immunized groups B and C demonstrated significant increases in plasma IgG, intestinal secretory IgA levels, and antigen-specific lymphocyte proliferative responses. The population of CD3+CD4+ positive T cells in the immunized chickens was also significantly increased after immunization and virulent challenge. In addition, the immunized groups B and C showed significantly higher egg production and a lower percentage of *S.* Enteritidis contaminated eggs after challenge compared to those of group A. A comparison of challenge strain isolation from the immunized-challenged and non-immunized-challenged layer hens showed that the double immunization group induced excellent protection against intestinal, liver, splenic, and ovarian *Salmonella* colonization; however, the single immunized chickens showed lower counts only in the splenic and ovarian organs. Overall, the data give compelling evidence that vaccination with the *S.* Enteritidis ghost induced robust protective immunity against experimental avian salmonellosis and may contribute to the reduce incidence of egg contamination.

1. Introduction

Salmonella enterica serovar Enteritidis (*S.* Enteritidis), a Gram-negative facultative intracellular organism, is recognized worldwide as an important enteric zoonotic pathogen associated with food borne illness in humans (Howard et al., 2012). Layer chickens infected with *S.* Enteritidis appear to be asymptomatic, but a systemic phase of infection is responsible for colonization of the internal organs including the gastrointestinal tract, hematolymphoid tissue, and reproductive organs (Shivaprasad et al., 1990). In addition, contaminated eggs produced by infected laying hens are thought to be the main source of human infection (Keller et al., 1995). *S.* Enteritidis either enters eggs by penetration through the eggshell from contaminated feces after or during oviposition, or directly contaminates the internal egg contents from *S.* Enteritidis infection of the reproductive tract (Gantois et al., 2009).

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Table 1
Plasmids, bacterial strains and primers used in this study.

Plasmids/Bacteria	Description	Reference
Plasmids		
pMMP172	*asd*+ vector containing *PhiX174* Lysis gene E with double regulatory ghost cassette	Jawale et al. (2014)
Bacterial strains		
JOL860	Wildtype *S.* Enteritidis	Lab stock
JOL1254	*asd* gene knockout *S.* Enteritidis	Lab stock
JOL1373	JOL1254 containing pMMP172	This study
JOL 1182	*S.* Enteritidis virulent challenge strain, isolated from chicken	Lab Stock
Primers		
OMPC-F	5′ ATCGCTGACTTATG-CAATCG 3′	Jawale et al. (2012)
OMPC-R	5′ CGGGTTGCGT-TATAGGTCTG 3′	
SEsp-F	5′ TGTGTTTTATCTGAT-GCAAGAGG 3′	
SEsp-R	5′ TGAAC-TACGTTCGTTCTTCTGG 3′	

The use of antimicrobials can be effective for controlling *S.* Enteritidis infection in layer flocks (Seo et al., 2000); however, this practice may aggravate the risk of antibiotic residues in eggs and tissues (Donoghue, 2003) or increase the emergence of multiple antibiotic-resistant bacteria (Hall, 2010). Therefore, vaccination of laying chickens might be the most effective way to reduce internal organ colonization and egg contamination with *S.* Enteritidis (Barrow, 2007). The use of killed, subunit, and live, attenuated vaccines are reported in the literature (Cooper et al., 1994; Davison et al., 1999; De Buck et al., 2005; Nandre et al., 2013). Live, attenuated *S.* Enteritidis vaccine candidates are able to replicate, colonize, and invade the intestinal and visceral organs of chickens leading to the induction of strong immunity in the vaccinated chickens; however, a number of safety concerns must be addressed such as shedding through feces (Tan et al., 1997) and egg contamination (Cooper et al., 1994). Killed and subunit vaccines possess excellent safety profiles and are capable of inducing strong antibody responses and weak cell mediated immune responses; however, they confer only partial protection against intestinal colonization, fecal shedding, systemic spread, and egg contamination (Gast et al., 1993; Davison et al., 1999; De Buck et al., 2005; Nakamura et al., 2004). Experimental evidence regarding the immunogenic potential of bacterial ghosts is well documented (Jawale et al., 2012). Genetically inactivated bacterial ghosts are produced by controlled expression of the PhiX174 lysis gene *E*, leading to the formation of a transmembrane tunnel structure in the cell wall of gram-negative bacteria through which the cytoplasmic contents are expelled (Szostak et al., 1996). As such, bacterial ghosts are capable of inducing strong humoral and cell-mediated immune responses (Haslberger et al., 2000; Kudela et al., 2005). The antibody-mediated immune responses are effective against *S.* Enteritidis when it is present in the extracellular state; however, as *S.* Enteritidis is capable of multiplying at intracellular locations in immune cells, particularly macrophages, the induction of the cell-mediated immune response is pivotal for protecting against the systemic phase of salmonellosis (Chappell et al., 2009). The presence of vaccination-induced protective immunity in laying chickens may reduce the dissemination of *S.* Enteritidis infection in systemic tissues including the reproductive tract, thereby limiting or preventing egg contamination (Barrow, 2007).

In the present study, *S.* Enteritidis ghosts were generated using a modified ghost cassette, where expression of the PhiX174 lysis gene *E* was controlled and stabilized by cloning it between a sense λpR and an antisense P_{araBAD} promoter (Jawale et al., 2014). We evaluated this new *S.* Enteritidis ghost vaccine with regard to induction of the humoral and cell-mediated immune responses and its effect on colonization of the intestinal tract, visceral and reproductive organs, and eggs in vaccinated and non-vaccinated layer hens challenged with wild-type *S.* Enteritidis. The effect of virulent challenge infection on the egg production status of laying chickens was also determined.

2. Materials and methods

2.1. Bacterial strains, plasmids, and primers

The bacterial strains and plasmid vectors used in this study are listed in Table 1. The *asd* gene deleted *S.* Enteritidis strains were grown at 37 °C in LB broth containing 50 μg/ml diaminopimelic acid (DAP). All bacterial strains were stored at −80 °C in growth medium containing 20% glycerol.

2.2. Generation of S. Enteritidis ghosts

The ghost cassette with stringent regulation of the PhiX174 lysis gene *E* was cloned in the *asd*+ multi-copy plasmid, pMMP172 (Jawale et al., 2014). The chromosomal *asd* gene knockout *S.* Enteritidis strain JOL1254 was transformed with ghost vector pMMP172, generating the resulting strain JOL1373. The JOL1373 strain was grown until mid-logarithmic phase at 28 °C in nutrient broth containing 0.2% L-arabinose. The cells were collected by means of centrifugation, washed twice, re-suspended in 100 mL nutrient broth without L-arabinose, and shifted to 42 °C for induction of gene *E*-mediated lysis. After lysis for 48 h, the ghost cells were harvested, washed twice with sterile PBS (pH 7.4), and stored at −70 °C. The lysis efficiency was evaluated by plating serially diluted lysed culture on Brilliant Green Agar (BGA, Becton, Dickinson and Company, USA). *S.* Enteritidis was completely lysed when the ghost cells were harvested 48 h after the induction of lysis.