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Improved immune responses against avian influenza virus following oral vaccination of chickens with HA DNA vaccine using attenuated *Salmonella typhimurium* as carrier

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ABSTRACT

This study evaluates the immune responses of single avian influenza virus (AIV) HA DNA vaccine immunization using attenuated *Salmonella enterica* sv. Typhimurium as an oral vaccine carrier and intramuscular (IM) DNA injection. One-day-old specific-pathogen-free (SPF) chicks immunized once by oral gavage with 10^9 *Salmonella* colony-forming units containing plasmid expression vector encoding the HA gene of A/Ck/Malaysia/5858/04 (H5N1) (pcDNA3.1.H5) did not show any clinical manifestations. Serum hemagglutination inhibition (HI) titer samples collected from the IM immunized chickens were low compared to those immunized with *S. typhimurium*.pcDNA3.1.H5. The highest average antibody titers were detected on day 35 post immunization for both IM and *S. typhimurium*.pcDNA3.1.H5 immunized groups, at 4.0 ± 2.8 and 51.2 ± 7.5, respectively. *S. typhimurium*.pcDNA3.1.H5 also elicited both $CD4^+$ and $CD8^+$ T cells from peripheral blood mononuclear cells (PBMCs) of immunized chickens as early as day 14 after immunization, at 20.5 ± 2.0 and $22.9 \pm 1.9\%$, respectively. Meanwhile, the $CD4^+$ and $CD8^+$ T cells in chickens vaccinated intramuscularly were low at 5.9 ± 0.9 and $8.5 \pm 1.3\%$, respectively. Immunization of chickens with *S. typhimurium*.pcDNA3.1.H5 enhanced IL-1β, IL-12β, IL-15 and IL-18 expressions in spleen although no significant differences were recorded in chickens vaccinated *via* IM and orally with *S. typhimurium* and *S. typhimurium*.pcDNA3.1. Hence, single oral administrations of the attenuated *S. typhimurium* containing pcDNA3.1.H5 showed antibody, T cell and Th1-like cytokine responses against AIV in chickens. Whether the T cell response induced by vaccination is virus-specific and whether vaccination protects against AIV infection requires further study.

1. Introduction

The basic principle of DNA vaccination is the induction of an immune response by injection of naked DNA encoding the targeted gene into the host cells [1]. A major disadvantage of DNA vaccination is its low efficiency, which is inherent in the way that vaccines are currently delivered, and its requirement of relatively large amounts of purified plasmids [2]. Plasmid purification under good manufacturing practice conditions is costly. In addition, DNA vaccination by direct intramuscular injection is time consuming, laborious, expensive and stressful to the chickens, and thus is not suitable for use in the poultry industry where mass vaccination is often practiced. It is

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essential to develop carrier systems that improve DNA vaccine efficacy.

To induce a strong and long-lasting immune response, effective DNA delivery technologies are required that can induce high and continued levels of antigen production and stimulation at appropriate target sites. Recently, a variety of Gram-positive and Gram negative bacteria have been used as carriers for efficient delivery of either DNA vaccine constructs or vaccine antigens [3]. This strategy allows administration of DNA vaccines *via* mucosal surfaces as well as delivery of the plasmid DNA directly to professional antigen presenting cells (APCs), which can elicit humoral and cellular responses against the pathogens from which the target genes are derived [4–7].

Oral administration of the attenuated *Salmonella typhimurium* strain SV4089 containing pcDNA3 expressing *Eimeria tenella* 5401 antigen could induce strong humoral and cellular immunity, and offered partial protection of chickens against *E. tenella* challenge [7]. In another study by Li et al. [4] oral administration of attenuated *S. typhimurium* containing plasmids encoding infectious bursal disease virus (IBDV) polyprotein offered good protection to chickens against virulent IBDV challenge. Recently, Pan et al. [6] have shown that DNA vaccine delivered by attenuated *S. typhimurium* aroA$^-$ strain SL7207 and boosting with a conventional killed vaccine confers protection on chickens against infection with H9 subtype of avian influenza virus.

The pathogenicity of the bacteria such as *S. typhimurium* can be reduced significantly by various attenuation methods while still retaining their invasion capacity and thus deliver the heterologous genes into eukaryotic cells. *S. typhimurium* strain SV4089 is a double mutant (Dam$^-$ and PhoP$^-$), derived from the wild-type *S. typhimurium* strain SL1344. This mutant strain of *Salmonella* is not pathogenic to chickens at a dose level as high as 10^{10} colony-forming units (CFU)/ml delivered orally [4,7], whilst the oral LD_{50} of wild-type SL1344 in chickens is $\sim 10^4$ CFU/ml [8]. Although the host immune response to *Salmonella* infection in chickens is not well characterized, recent work examining the production of proinflammatory cytokines and chemokines following serovar *typhimurium* infection in young chickens revealed that many elements of the avian host response are similar to those in mammalian models [9].

Protective immunity against avian influenza depends largely on the development of immune response against HA glycoprotein [10]. A wide variety of vaccines against AIV has been developed and tested in experimental conditions but only inactivated whole AIV virus vaccines and live recombinant fowlpox virus vectored expressing HA vaccines have been licensed and widely used in various countries [11]. Recently, reverse genetics approach using AIV and other avian viruses such as the Newcastle disease virus have been used to develop AIV vaccines against highly pathogenic AIV in chickens [12–15]. Various experimental DNA vaccines and vectored vaccines based on influenza hemagglutinin antigens have shown efficacy in chickens against challenge with the AIV [10]. Although DNA vaccination is an attractive vaccination approach in chickens, it may not be suitable for use where mass vaccination is often practiced. This study was carried out to assess the immune responses following single immunizations of HA DNA vaccines using oral administration of attenuated *Salmonella enterica* sv. Typhimurium and intramuscular injection, respectively.

2. Materials and methods

2.1. Attenuated Salmonella strain and AIV HA

Attenuated *S. enterica* sv. Typhimurium (*S. typhimurium*) strain SV4089, a double mutant [*dam*$^-$*228*::Mu*d*J (Dam$^-$) *phoP7953*::Tn*10* (PhoP$^-$)], derived from the wild-type *S. typhimurium* strain SL1344 was kindly provided by Dr. Josep Casadesus, Department of Molecular Genetics, University of Sevilla, Spain. The eukaryotic expression plasmid, pcDNA3.1 (Invitrogen, USA) expressing the AIV A/Ck/Malaysia/5858/04 (H5N1) HA gene (pcDNA3.1.H5) used in this study was as previously constructed by Jalilian et al. [16].

2.2. Transformation and stability of pcDNA3.1.H5 into S. typhimurium

A single colony of attenuated *S. typhimurium* strain SV4089 was grown in LB broth to an optimal density (OD600) 0.6–0.8 and resuspended in ice-cold ultrapure H_2O. The plasmids pcDNA3.1.H5 and control plasmid, pcDNA3.1 were purified (Qiagen, Germany) and transformed into *S. typhimurium* competent cells by electroporation set at 2.5 kV, 25 μF and 200–400 Ω (Gene Pulser, Bio-Rad, USA). The transformed culture was then plated onto LB plates with 50 μg/ml ampicillin. To determine the stability of the transfected plasmids, the bacterial cultures were passaged for approximately 100 generations (25 days) without antibiotic selection. The percentage stability of the plasmid was estimated by calculating the number of cells containing the plasmid at each passage divided to the number of *Salmonella*. Resistant colonies harboring the DNA vaccine minigene were cultured and stored after confirmation by PCR and fluorescence *in situ* hybridization (FISH) on the targeted AIV HA gene.

2.3. Preparation of Salmonella as vaccine inoculum

Bacterial strains were cultured at 37 °C in Luria Bertani (LB) broth or agar containing 100 μg/ml ampicillin, 25 μg/ml novobiocin (NO) and 20 μg/ml nalidixic acid (NA). The culture was streaked onto NO-NA-XLT4 plates, incubated for an additional 24 h at 37 °C, and examined for the presence of *S. typhimurium* colonies by PCR. The bacterial colonies were cultured in LB medium, collected by centrifugation and diluted to a concentration of approximately 10^9 CFU/ml.

2.4. Vaccination of chickens

One-day-old SPF chickens were purchased from Malaysian Vaccines and Pharmaceuticals, Bangi, Selangor. Cloacal swabs were collected from these chicks to determine their freedom from *Salmonella*. A total of three trials