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# Enhancement of humoral and cellular immunity in chickens against reticuloendotheliosis virus by DNA prime-protein boost vaccination

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ABSTRACT

Reticuloendotheliosis virus (REV) causes an oncogenic, immunosuppressive and runting syndrome in multiple avian hosts worldwide. In this study, an optimal vaccination strategy was developed to enhance the immune responses against REV infection. Chickens were vaccinated twice intramuscularly with plasmid pCAGgp90 encoding gp90 protein of REV, or with recombinant gp90 protein, or vaccinated with plasmid pCAGgp90 and then boosted with recombinant gp90 protein. The humoral immune responses were monitored by ELISA and virus neutralizing test. In addition, lymphocyte proliferation response, cytokine production and protection effectiveness against REV infection were also evaluated. Although all vaccinated groups developed immune responses, chickens primed with pCAGgp90 plasmid and boosted with rgp90 protein developed higher levels of antibodies compared with those immunized with pCAGgp90 plasmid or rgp90 protein alone. Furthermore, enhanced cellular immune responses were induced following priming with the pCAGgp90 plasmid and boosting with the rgp90 protein. In addition, the DNA prime-protein boost vaccine yielded 100% protection of chickens from REV viremia caused by challenge infection. These findings demonstrated that a DNA prime-protein boost vaccination strategy could enhance both humoral and cellular immune responses in chickens, highlighting the potential value of such an approach in the prevention of REV infection.



## 1. Introduction

Reticuloendotheliosis virus (REV) is a member of gammaretrovirus with a variety of strains [1], which causes an oncogenic, immunosuppressive and runting syndrome in multiple avian hosts [2]. The immunosuppression caused by REV infection increases the susceptibility to concurrent or secondary bacterial or viral infections and results in poor immune responses to other vaccines [3,4]. In addition, the co-infection of REV with other immunosuppressive viruses becomes more and more severe, which may be increased the tumor cases in chicken flocks in recent years [5]. REV can be transmitted vertically via eggs and horizontally by direct contact [6–8], mechanically by insects [9,10], or accidentally by injection of contaminated vaccines [11,12]. REV has a worldwide distribution, causing severe damages to the poultry industry [13–17]. Disease surveillance and vaccination are the best alternatives to culling for a better control and eradication of this disease. However, no commercial vaccine is currently available for REV prevention. The oncogenicity and the immunosuppressive ability of these viruses, their co-infection with other infectious viruses and their presence as contaminants in poultry biologics warrant development of a suitable vaccine [18].

The gp90 protein of REV is known to be the major candidate antigen for vaccines and disease serological diagnosis, which was associated with virus neutralization [19,20]. The genetic sequences of different REV strains show only minor variations [2], and the various strains of REV are antigenically similar [21], which suggested that a suitable vaccine expressing the gp90 protein of a single REV isolate may provide protective immunity against numerous REV-associated diseases.

Recently, several studies have suggested that heterologous prime-boost (i.e., the sequential use of two types of vaccines to deliver the same subunit antigen), can be more immunogenic than repeated administrations of either type of vaccine alone (homologous prime-boost) [22]. By using gp90 protein of REV as a model antigen, the aim of the present study is to develop a DNA prime-protein boost vaccine against REV infection in chickens. In the study, the immune responses and protection efficacy of three different vaccination strategies were evaluated: a DNA vaccine encoding gp90 protein of REV; a recombinant gp90 (rgp90) protein vaccine expressed from yeast *Pichia pastoris*; and a DNA prime-protein boost regimen. The results demonstrated that the priming of chickens with DNA and boosting with rgp90 enhanced both humoral and cellular immune responses against REV in chickens.

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## 2. Materials and methods

### 2.1. Viruses, cells and plasmids

REV HLJR0901 strain (GenBank ID: GQ415646) was isolated and stored at the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Science (CAAS) at −70 °C [23]. DF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). Primary chicken embryo fibroblast (CEF) cells were prepared from 10-day-old specific-pathogen-free (SPF) chicken embryos provided by HVRI, CAAS. The eukaryotic expression vector pCAGGS was kindly provided by Dr. J. Miyazaki (University of Tokyo, Tokyo, Japan).

### 2.2. Antibodies and primers

The mouse anti-gp90 monoclonal antibody (MAb) was prepared in our laboratory. All primers used were synthesized by Invitrogen (Beijing, China).

### 2.3. Animals

SPF White Leghorn Chickens, *Gallus domesticus*, were obtained from HVRI, CAAS and housed in the negative-pressure-filtered air isolators. Animal experiments were approved by the Animal Ethics Committee of the Institute and performed in accordance with animal ethics guidelines and approved protocols.

### 2.4. Construction of REV gp90 DNA vaccine

The full-length gp90 gene of REV HLJR0901 was amplified with the primers gp90F (5′-GACGAATTCGCCGCCACCATGGACTGTCTCACCAACCTC-3′) and gp90R (5′-TTTATCGATTCACTTATGACGCCCAGCGGTGTACTCG-3′) using the proviral cDNA extracted from REV-infected CEF cells as templates. The PCR product was cloned into pCAGGS vector under control of the Chicken β-actin promoter, and designated as pCAGgp90. The constructed plasmid was identified with right orientation by sequencing and purified by the Qiagen Plasmid Giga Kit (Qiagen, Santa Clarita, CA). Plasmid concentration was determined by spectrophotometry at 260 nm.

### 2.5. In vitro expression of the constructed plasmid

Monolayer of 80–90% confluent DF-1 cells in six-well plates were transfected with 4 μg of pCAGgp90 or pCAGGS along with the mock-treated negative controls using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA). At 48 h after transfection, the expression of gp90 protein from plasmid pCAgp90 was confirmed by indirect immunofluorescence assay (IFA) and western blotting using anti-gp90 MAb.

### 2.6. REV gp90 protein vaccine

The rgp90 protein was expressed in our laboratory using *P. pastoris* expression system (Invitrogen, Arlsbad, CA) [24]. Briefly, *Pichia* strain SMD1168/pPIC9k-gp90 was precultivated in 50 mL of BMGY medium and then resuspended in 20 mL of BMMY medium to induce rgp90 expression. Methanol was added to a final concentration of 0.5% every 12 h to maintain induction for 120 h at 30 °C. The culture supernatant was collected by centrifugation and detected by SDS-PAGE, followed by western blotting with anti-gp90 MAb. The gp90 protein concentration was determined by thin layer chromatogram scanning and Bradford total protein content assay (Bio-Rad).

### 2.7. Chicken immunization

A total of eighty 3-week-old SPF chickens were randomly divided into four groups (20 chickens each), each group was maintained in a negative-pressure isolator. Group 1 was immunized with 100 μg of pCAGgp90 plasmid. Group 2 was given 50 μg of rgp90 protein in Marcol 52 mineral oil adjuvant (ESSO, France). The group 3 chickens were first primed with 100 μg of pCAGgp90, followed by boosting with 50 μg of rgp90 protein in Marcol 52 mineral oil adjuvant. Chickens in group 4 were inoculated with 100 μg of pCAGGS as negative controls. All groups were inoculated twice at 3-week intervals by the intramuscular route. The optimal doses of DNA and protein were used in this study for immunization according to previous studies [25–28]. Serum samples were collected at weekly intervals for further analyses.

### 2.8. Protection efficacy against challenge with REV

At 6 weeks post first vaccination (w.p.f.v.), 15 chickens from each group were challenged intraperitoneally with $10^4$ $TCID_{50}$ of REV HLJR0901. These chickens were monitored daily for signs of illness after infection. For REV viremia detection, blood samples were aseptically collected in heparinized tubes at 7 days after infection. The blood genomic DNA was extracted using the Blood DNA Kit (Omega, USA) and detected by a real-time PCR assay as previous described [29]. Briefly, the Real-time PCR was carried out in a total volume of 25 μL containing 2.5 μL of 10× Ex Taq Buffer, 2 μL of dNTP (2.5 mM), 3 μL of $MgCl_2$ (25 mM), 1 μL of each primer (10 pM), 0.5 μL of probe (10 pM), 1 U of Ex Taq HS, 1 μL of DNA, and appropriate amount of $ddH_2O$. The reaction was undertaken with a pre-denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s and elongation at 72 °C for 20 s. In addition, the plasma samples were inoculated on CEF cells followed by further incubation for 7 days, and the cultures were checked for the evidence of virus by IFA with anti-gp90 MAb.

### 2.9. Serological assay

Serum samples were collected from all groups weekly until 6 w.p.f.v and analyzed using a commercial REV antibody test kit (IDEXX, Westbrook, Maine). The relative level of antibody titer was determined by calculating the sample to positive (S/P) ratio. Endpoint titers were calculated with the equation: $\log_{10}\text{titer} = 1.09\ (\log_{10}S/P) + 3.36$ (IDEXX, Westbrook, Maine). The sera with titers of higher than 1076 were considered REV antibody positive.

The neutralizing antibody titers in the sera were determined with virus neutralizing (VN) test at 6 w.p.f.v. Triplicates of heat-inactivated (56 °C for 30 min) serum samples were diluted serially by twofold in cell culture medium and mixed with an equal volume of 100 $TCID_{50}$ of HLJR0901. After 60 min incubation at 37 °C, the mixtures were added to CEF cells followed by further incubation for 7 days. At 7 days of incubation, the cultures were checked for evidence of virus by IFA with anti-gp90 MAb. The REV-specific neutralizing antibody titers were determined as the reciprocals of the highest dilutions of sera that caused complete neutralization.

### 2.10. Lymphocyte proliferation assay

Chicken splenic lymphocytes were isolated aseptically from five chickens of each group using chicken lymphocyte separation