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Immune protection duration and efficacy stability of DNA vaccine encoding *Eimeria tenella* TA4 and chicken IL-2 against coccidiosis

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ABSTRACT

In our previous study, an effective DNA vaccine encoding *Eimeria tenella* TA4 and chicken IL-2 was constructed. In the present study, the immunization dose of the DNA vaccine pVAX1.0-TA4-IL-2 was further optimized. With the optimized dose, the dynamics of antibodies induced by the DNA vaccine was determined using indirect ELISA. To evaluate the immune protection duration of the DNA vaccine, two-week-old chickens were intramuscularly immunized twice and the induced efficacy was evaluated by challenging with *E. tenella* at 5, 9, 13, 17 and 21 weeks post the last immunization (PLI) separately. To evaluate the efficacy stability of the DNA vaccine, two-week-old chickens were immunized with 3 batches of the DNA vaccine, and the induced efficacy was evaluated by challenging with *E. tenella*. The results showed that the optimal dose was 25 μg. The induced antibody level persisted until 10 weeks PPI. For the challenge time of 5 and 9 weeks PLI, the immunization resulted in ACIs of 182.28 and 162.23 beyond 160, showing effective protection. However, for the challenge time of 13, 17 and 21 weeks PLI, the immunization resulted in ACIs below 160 which means poor protection. Therefore, the immune protection duration of the DNA vaccination was at least 9 weeks PLI. DNA immunization with three batches DNA vaccine resulted in ACIs of 187.52, 191.57 and 185.22, which demonstrated that efficacies of the three batches DNA vaccine were effective and stable. Overall, our results indicate that DNA vaccine pVAX1.0-TA4-IL-2 has the potential to be developed as effective vaccine against coccidiosis.

1. Introduction

Avian coccidiosis caused by *Eimeria* infection is one of the most common and economically important diseases of poultry worldwide (Kaboudi et al., 2016; Shirley et al., 2005). *Eimeria tenella* is one of the seven *Eimeria* species that infects domestic chickens and results in severe lesion of caeca, body weight loss, hemorrhagic diarrhea, haemorrhaging and death (Witcombe and Smith, 2014). The annual cost of losses to the global poultry industry caused by coccidiosis has been predicted to exceed US $3 billion (Blake and Tomley, 2014).

Current control strategy relies primarily on routine chemoprophylaxis using anticoccidial drugs. However, alternative methods are needed due to the rapid emergence of drug-resistant parasites and drug residues in chicken products (Chapman and Jeffers, 2014; Clarke et al., 2014). Immunization is a practical alternative to chemotherapy for the control of coccidiosis. However, most current commercial vaccines consisting of live oocysts of attenuated or non-attenuated coccidia are expensive and difficult for large-scale manufacturing. Furthermore, live vaccines have the risk of vaccinal pathogenicity, the potential reversion to virulence and coccidiosis breakout (Del Cacho et al., 2016; Williams, 2002). Therefore, it is urgent to develop safe and effective vaccine against avian coccidiosis.

DNA vaccines have been suggested as a promising alternative strategy against coccidiosis without the disadvantages associated with chemoprophylaxis and live vaccines (Blake and Tomley, 2014; Pereira et al., 2014; Song et al., 2015). In our previous work, a DNA vaccine encoding *Eimeria tenella* TA4 and chicken IL-2 was constructed and induced effective protection against challenge of *Eimeria* (Xu et al., 2008). The immunization procedure of the DNA vaccine including immunization doses, routes, times and age was optimized and the storage period was evaluated (Song et al., 2009). A practical vaccine against coccidiosis should confer stable protection between different bathes as well as long-lasting protection covering the production cycle of chickens. In

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order to address these issues, the present study was performed to evaluate the efficacy stability and immune protection duration of DNA vaccine pVAX1.0-TA4-IL-2 against *E. tenella* to provide reference for clinical application of the DNA vaccine.

2. Materials and methods

2.1. Plasmid, parasite and experimental animals

Plasmid pET28a-TA4 and pVAX1.0-TA4-IL-2 encoding *E. tenella* TA4 and chicken IL-2 were prepared in our lab (Xu et al., 2008; Song et al., 2009). Three batches of plasmid were prepared using HighPure Maxi Plasmid Kit (TIANGEN, China) according to manufacturer's protocol. Oocysts of *E. tenella* were collected and sporulated 7 days before the challenge infection using a previously described protocol (Tomley, 1997). The purity of *E. tenella* was determined with ITS1-PCR (Jenkins et al., 2006; Haug et al., 2007). New-hatched Hy-Line layer chickens (commercial breed W-36) were reared under coccidian-free conditions in wire cages until the end of experiment. Food and water without anticoccidia drugs were available ad libitum. All animal experiments were evaluated and approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (approval number: 2010CB100750).

2.2. Optimization of immunization doses of DNA vaccine pVAX1.0-TA4-IL-2

In our previous study, we have demonstrated that 25 μg was the optimal dose among 25, 50, 100 and 200 μg (Song et al., 2009). Nevertheless, whether a lower dose could provide better efficacy than 25 μg remained unknown. To clarify this issue, two lower doses were designed (2, 10, 25, 50, 100 and 200 μg) to optimize the immunization doses of DNA vaccine pVAX1.0-TA4-IL-2 following the animal experimental design and evaluation of immune protection described in Section 2.6 and shown in Table 1.

2.3. Evaluation of antibody dynamics induced by the DNA vaccine using indirect ELISA (IE)

2.3.1. Antigen preparation of indirect ELISA

Recombinant plasmid pET28a-TA4 was expressed through *E. coli* BL21 (DE3) and purified with His-Bind Purification Kit (Novagen). The purity of recombinant protein was determined by SDS-PAGE. The concentration of the recombinant protein was estimated using Bicinchoninic Acid kit (Sigma-Aldrich, USA). Stocks protein were prepared and stored at −80 °C for the further agar diffusion test and indirect ELISA.

2.3.2. Establishment of optimal TA4 protein and serum dilutions

To collect the positive and negative reference chicken sera, ten three-week-old chickens were orally inoculated with 5×10^3 sporulated oocysts of *E. tenella* for 2 times with 10-days intervals. Blood was collected from wing vein of the chickens and the serum was collected for titer determination by agar diffusion assay 10 days post the last dose (Baker et al., 1991; Constantinoiu et al., 2007). A third even fourth dose would be given unless the titers of sera were beyond 1:16. The serum was collected and stored at −20 °C as positive reference serum for indirect ELISA. Serum was collected from the unchallenged chickens and stored at −20 °C as negative positive reference for indirect ELISA.

A checkerboard titration employing the positive and negative reference sera was performed to determine the best antigen protein concentration for coating the microplate and the best dilutions of sera and conjugate as previously described (Crowther, 2009).

2.3.3. Immunization and serum samples collection

Two-week-old chickens were randomly divided into immunized group and non-immunized group of 35 each. Immunized group of chickens were immunized with 25 μg of plasmid pVAX1.0-TA4-IL-2 by leg intramuscular injection. Chickens of phosphate buffered saline (PBS) control group were injected with sterile PBS at the same injection site. A booster immunization was given 1 week later with the same amount of components as the first immunization. From 1 week to 10 weeks post primary immunization (PPI), blood samples were collected from the wing vein of immunized group and control group at 1-week intervals respectively. The sera were collected and stored at −20 °C for the further determination by ELISA.

2.3.4. Determination of TA4-specific antibody levels by indirect ELISA

After checkerboard titration, TA4-specific antibody levels in the serum collected from one to ten weeks post primary immunization were determined by indirect ELISA as previously described (Constantinoiu et al., 2007). Briefly, Maxisorp TM microplates (Nunc, Denmark) were employed and all immunoreagents were assayed in 100 μl volumes. Between all steps of the reaction, microplates were washed 3 × 5 min with PBS buffer (pH 7.2) containing 0.05% Tween 20 (PBS-T). Test wells of microplate were coated with 1 μg of TA4 recombinant antigen (optimized in 2.9.2) diluted in 0.05 M carbonate buffer pH 9.6 and incubated overnight at 4 °C. Non-specific binding sites were blocked by incubation with 200 μl of 2% skim milk powder (SMP) in PBS-T for 1 h at 37 °C. Subsequently, serum diluted 1:80 in PBS-T containing 2% SMP (PBS-TS) were added and the plates were incubated for 2 h at 37 °C. After incubation with sera the wells were filled with rabbit anti-chicken IgG antibody peroxidase conjugate (Sigma) diluted 1:2000 in PBS-TS and incubated for 1 h at 37 °C. After 10-minute incubation with 3,3′,5,5′-Tetramethylbenzidine(TMB) (Sigma) in the dark, 50 μl 2 M·H_2SO_4 was filled in each well to stop the enzymatic reaction. Absorbance was read at 450 nm (OD450) with a microplate spectrophotometer. The antibody titer curve was made using Microsoft Excel 2010.

2.4. Evaluation of immune protection duration of the DNA vaccine

As shown in Table 2, five challenge times (5, 9, 13, 17 and 21 weeks PLI) were designed to evaluate the immune protection duration of DNA vaccine pVAX1.0-TA4-IL-2 following the animal experimental design and evaluation of immune protection described in Section 2.6 and shown in Table 2.

Table 1
Optimization of immunization doses of DNA vaccine pVAX1.0-TA4-IL-2.

Groups	Average body weight gain (g)	Relative body weight gain (%)	Mean lesion scores	Oocyst output ($\times 10^6$)	Oocyst decrease ratio(%)	ACI
Unchallenged control	35.85 ± 17.97^c	100	0.00 ± 0^a	0.00 ± 0^a	100.00	200
Challenged control	19.30 ± 11.30^a	53.80	3.2 ± 0.86^d	1.3467 ± 1.25^a	0.00	80.37
2 μg	33.39 ± 15.74^c	93.61	1.454 ± 0.58^b	0.4068 ± 0.36^b	69.80	166.64
15 μg	36.47 ± 17.05^d	105.68	1.448 ± 0.72^b	0.3509 ± 0.56^b	73.94	190.2
25 μg	38.32 ± 12.07^d	108.66	1.324 ± 0.50^b	0.3494 ± 0.56^b	74.06	194.42
50 μg	33.98 ± 14.70^b	91.61	1.786 ± 0.93^c	0.4193 ± 0.31^b	68.86	172.75
100 μg	16.06 ± 21.94^c	46.26	1.857 ± 0.57^c	0.7538 ± 0.30^c	44.03	126.69
200 μg	19.60 ± 18.03^b	54.43	2.029 ± 0.99^c	0.8890 ± 3.23^c	33.99	124.14

Note: Significant difference ($P < 0.05$) between numbers with different letters. No significant difference ($P > 0.05$) between numbers with the same letter.