Optimization of Immunization Procedure for *Eimeria tenella* DNA Vaccine pVAX1-pEtK2-IL-2 and Its Stability

Bucai Zhang^{1,2} · Cheng Yuan² · Xiaokai Song¹ · Lixin Xu¹ · Ruofeng Yan¹ · Muhammad Ali A. Shah³ · Changming Guo² · Shanyuan Zhu² · Xiangrui Li¹

Received: 10 January 2019 / Accepted: 23 May 2019 / Published online: 5 June 2019
© Witold Stefański Institute of Parasitology, Polish Academy of Sciences 2019

Abstract

Purpose To seek for the optimal immunization procedure of DNA vaccine pVAX1-pEtK2-IL-2 which was produced via cloning pEtK2 antigen gene of *Eimeria tenella* (*E. tenella*) and chicken IL-2 (chIL-2) gene into expression vector pVAX1.

Methods The doses, routes, times of inoculation and ages of the first inoculation of chickens were optimized. The stability of the vaccine, including store temperature and time, was also explored. The effects of the protective immunity against challenge infection were assessed according to average body weight gain, survival rate, oocyst output, lesion score and the anti-coccidial index (ACI).

Results The results suggested that intramuscular inoculation was the most efficient route to elicit immune response and 80 µg was the optimal immune dose. Two time injections induced more effective protection compared to single injection, the effect of the first injection at 14 days old was optimal. The immune efficacy of the vaccine stored at different time and temperature was very stable.

Conclusions The optimal immunization procedure for *Eimeria tenella* DNA vaccine pVAX1-pEtK2-IL-2 is 80 µg DNA, two time injections at 14 and 21 days old, respectively, by intramuscular inoculation.

Keywords *E. tenella* · pEtK2 antigen · DNA vaccine · Immunization procedure · Stability

Introduction

A single or several *Eimeria* species can cause avian coccidiosis. These intracellular protozoan parasites of genus *Eimeria* seriously weaken the feed utilization and growth of the commercial poultry. It causes huge economic losses worldwide [22]. Immediately after coccidial infection, both antibody and cell-mediated immune responses are activated, although cell-mediated immunity plays a major role in resistance to infection [15]. Immunization with DNA

plasmid encoding many *Eimeria* species antigens has been presented to provide effective protection against the challenge of *Eimeria* spp. because of its ability to elicit humoral and cellular immune responses in chickens, especially, induce significant changes in T cell subpopulations including CD4⁺, CD8⁺, γδ T⁺ and αβ T⁺ cells [15, 16, 23, 24].

Various factors determine the efficacy of DNA vaccines including plasmid delivered, immunization schedule, route or number of immunization, age of animals, and toxicity of the antigen to transfected host cell [13]. DNA vaccines are able to be delivered into the host via several routes including intravenous, intraperitoneal, intramuscular, intranasal, oral, intradermal, subcutaneous, gene gun and in ovo [19]. A given DNA-dose could effectively induce an immune response despite of body size [4, 5]. In about 70% of the reported cases of poultry DNA vaccination against viruses, *Eimeria* or bacteria, the plasmid DNA was immunized per injection with doses ranging from 50 µg to 200 µg, regardless of the bird species [19]. Generally, birds were inoculated with DNA vaccine at least two times by the same route to elicit protective immunity, the usual interval was 7 or

✉ Xiangrui Li
lixiangrui@njau.edu.cn

¹ MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, Jiangsu, People's Republic of China

² Jiangsu Agri-animal Husbandry Vocational College, Taizhou 225300, Jiangsu, People's Republic of China

³ Department of Pathobiology, PMAS Arid Agriculture University, Rawalpindi, Pakistan

14 days, since it is enough to permit the body's immune system to be effectively and sufficiently stimulated [19]. The storage temperature and time had a slight influence on the immune effects of DNA vaccine, which can be stably preserved as long as 6 months, even at room temperature [24].

pEtK2 antigen gene was selected from the cDNA library of the sporozoite of *Eimeria tenella*. The calmodulin-domain protein kinases (ECDPK) encoded by pEtK2 gene could stimulate the proliferation of T lymphocytes in chickens infected by coccidian, which could provide partial protection against the challenge with *Eimeria tenella* [7]. In previous study, we constructed two DNA vaccines pcDNA3.1b-TA4-IL-2 and pVAX1-pEtK2-IL-2. TA4 antigen of *Eimeria tenella* and chicken IL-2 gene were cloned into expression vector pcDNA3.1b, constructing DNA vaccine pcDNA3.1b-TA4-IL-2 [33]. *Eimeria tenella* pEtK2 antigen and chicken IL-2 were cloned into the eukaryotic expression vector pVAX1, producing DNA vaccine pVAX1-pEtK2-IL-2. The expression of aim genes in vivo was detected by reverse transcription-polymerase chain reaction (RT-PCR) and western blot. Results showed that both integrated genes pEtK2-IL-2 and TA4-IL-2 were successfully expressed in chicken leg muscle tissues [33]. Although the immunization procedure of pcDNA3.1b-TA4-IL-2 has been optimized [24], the two DNA vaccines were constructed by using different expression vectors (pcDNA3.1b and pVAX1) and different antigen genes of *Eimeria tenella* (TA4 and pEtK2) which might influence the immunization procedure of the DNA vaccine. In this research, we studied the optimal immunization procedure of the DNA vaccine pVAX1-pEtK2-IL-2, including dose, route, number of immunization and age of initial immunization of chicken, as well as its stability.

Materials and Methods

DNA Vaccines, Parasites and Animals

Recombinant DNA vaccine pVAX1-pEtK2-IL-2 was previously constructed in MOE Joint International Research Laboratory of Animal Health and Food Safety, Nanjing Agricultural University, China. Oocysts of *E. tenella* isolated from Jiangsu Province of China (JS) were proliferated and stored in 2.5% potassium dichromate solution at 4 °C. Oocysts of *E. tenella* were sporulated 7 days before the challenge infection using a previously described method [30]. Chinese Yellow chickens were purchased from a local hatchery farm, named Tangquan chicken farm located in suburb of Nanjing, and reared in a sterilized room in iron wire cage. Anti-coccidial drug-free water and food were available ad libitum. All animal experiments were ratified and evaluated by the Animal

Care and Use Committee of Nanjing Agricultural University (Approval No. 2010CB100750).

Animal Experimental Designs

At the beginning of each animal experiment, chickens were weighed individually, abandoning weaker or overweight ones, and randomly allocated into corresponding groups of 30 chickens each. Experimental groups and challenged control group were immunized with vaccine or sterile Tris-EDTA (TE), then challenged with *E. tenella*. Unchallenged control was injected with sterile TE and kept unchallenged.

Evaluation of Immune Protection

Protective efficacies of immunization were evaluated in terms of oocyst decrease rate, survival rate, average body weight gain, caecal lesion score and the anti-coccidial index (ACI). Caecal content from each bird was collected to determine oocyst output through McMaster's counting method. Oocyst decrease ratio was calculated as following: (the number of oocysts from challenged control groups – immunized groups)/challenged control groups $\times$ 100%. Survival rate was calculated as the number of surviving chickens divided by the number of initial chickens. Body weight gain of the chickens in each group was calculated by the body weight of the chickens at the slaughter subtracting the body weight at the time of challenge. Body weight gain rate was determined by following: (average body weight at the slaughter – average body weight at the challenge)/average body weight at the challenge $\times$ 100%. Relative weight gain rate strongly affected ACI for evaluating the efficacy of a vaccine or medicine and calculated as following: (body weight gain rate of each group/body weight gain rate of unchallenged control group) $\times$ 100%. Caecal lesion score of chickens in each group was estimated by the technique of Johnson and Reid [10]. The ACI was based on that of McManus et al. [18], which was a comprehensive index for evaluating the protective efficacy of a vaccine or medicine and determined by following: (survival rate + relative body weight gain rate) – (caecal lesion score + oocyst score). ACI is considered as “good” when greater than or equal to 180, “moderate” when range from 160 to 179, and “poor” when less than 160 [18]. Average body weight gain and caecal lesion scores were presented as the mean $\pm$ SD (standard deviation) and statistical analysis was treated with R statistic package [20]. For average body weight gain, differences of means between pairwise groups were performed by Turkey–Kramer technique, and results were regarded as significant when $P < 0.05$. Due to caecal lesion scores and oocyst output do not belong to Gaussian distribution, differences between groups were performed by pairwise comparisons

using Wilcoxon rank sum test, and results were regarded as significant when $P < 0.05$.

Optimization of Inoculation Doses

As shown in Table 1, 2-week-old chickens were distributed into 6 groups of 30 each randomly. Experimental chickens in groups 3, 4, 5 and 6 were intramuscularly immunized in legs with 25 µg, 50 µg, 80 µg and 100 µg of DNA vaccine, respectively. In group 1 (unchallenged control) and group 2 (challenged control), chickens were treated with sterile TE by intramuscular injection in legs. A booster immunization was given by the same method as primal immunization 1 week later. Seven days post the booster vaccination, all chickens were weighed individually and challenged orally with 5×10^4 sporulated oocysts of *E. tenella* except unchallenged control group. Chickens of unchallenged control group were given PBS orally. Seven days post-challenging infection, all experimental birds were weighed one-by-one again and killed by dislocation of cervical vertebrae joint and evaluated to assign the caecal lesion score. Meanwhile, caecal contents from each bird were collected and used to count the number of oocysts. Evaluating indexes including

oocyst decrease rate, survival rate, average body weight gain, lesion score and ACI of all groups were determined.

Optimization of Inoculation Routes

As shown in Table 2, 2-week-old chickens were distributed into 7 groups of 30 each randomly. Experimental chickens in groups 3, 4, 5, 6 and 7 were immunized with 80 µg of DNA vaccine by intramuscular injection, intravenous injection, subcutaneous injection, oral administration and intranasal administration, respectively. In group 1 (unchallenged control) and group 2 (challenged control), chickens were intramuscularly injected into legs with sterile TE. The subsequent booster immunization, challenging infection and evaluation of immune protection were implemented, just as described in the optimization of inoculation doses.

Optimization of Primal Inoculation Age and Inoculation Times

As shown in Table 3, 1-week-old chickens were allocated into 6 groups of 30 each randomly. Groups 1–6 were assigned as follows: group 1 unchallenged control, group 2 challenged control, group 3 inoculated once at 1 week old,

Table 1 Comparison of the effects of different immune doses of DNA vaccine pVAX1-pEtK2-chIL-2

Groups	Treatment	Average body weight gain (g)	Oocyst decrease rate (%)	Mean lesion score	Survival rate (%)	ACI
1	Unchallenged control	73.40 ± 17.97 ^a	–	0.00 ± 0.00 ^a	100	200.00
2	Challenged control	31.72 ± 11.30 ^b	0.00	3.40 ± 0.62 ^b	100	98.98
3	25 µg	51.30 ± 18.34 ^c	53.69	1.53 ± 0.73 ^{cd}	100	142.56
4	50 µg	57.62 ± 14.13 ^{cd}	74.05	1.43 ± 0.68 ^c	100	162.53
5	80 µg	70.08 ± 19.78 ^a	81.44	1.17 ± 0.38 ^c	100	184.87
6	100 µg	69.30 ± 17.96 ^a	80.04	1.27 ± 0.52 ^c	100	186.43

Significant difference ($P < 0.05$) between numbers with different letters (a, b, c, d). No significant difference ($P > 0.05$) between numbers with the same letter (a, b, c, d). “–” means no need for statistics. “ACI” means the anti-coccidial index

Table 2 Comparison of the effects of different immune routes of DNA vaccine pVAX1-pEtK2-chIL-2

Groups	Treatment	Average body weight gain (g)	Oocyst decrease rate (%)	Mean lesion score	Survival rate (%)	ACI
1	Unchallenged control	65.36 ± 17.05 ^a	–	0.00 ± 0.00 ^a	100	200.00
2	Challenged control	28.80 ± 9.57 ^b	0.00	2.93 ± 0.74 ^b	97	104.02
3	Intramuscular injection	64.20 ± 15.32 ^a	91.74	1.07 ± 0.25 ^c	100	190.22
4	Intravenous injection	61.25 ± 19.98 ^a	87.77	1.40 ± 0.72 ^c	100	181.53
5	Subcutaneous injection	54.96 ± 15.30 ^c	86.85	1.50 ± 0.73 ^d	100	166.63
6	Oral administration	47.82 ± 22.60 ^c	85.63	1.57 ± 0.82 ^d	100	158.51
7	Intranasal administration	51.70 ± 24.30 ^c	81.65	1.97 ± 0.85 ^d	100	160.62

Significant difference ($P < 0.05$) between numbers with different letters (a, b, c, d). No significant difference ($P > 0.05$) between numbers with the same letter (a, b, c, d). “–” means no need for statistics. “ACI” means the anti-coccidial index

Table 3 Comparison of the effects of different primal immunization age and immunization times of DNA vaccine pVAX1-pEtK2-chIL-2

Groups	Treatment	Average body weight gain (g)	Oocyst decrease rate (%)	Mean lesion score	Survival rate (%)	ACI
1	Unchallenged control	79.23 ± 17.70 ^a	–	0.00 ± 0.00 ^a	100	200.00
2	Challenged control	40.75 ± 18.08 ^b	0.00	3.67 ± 0.71 ^b	80	75.42
3	Inoculated once at 7 days old	41.83 ± 12.38 ^{bc}	49.84	3.03 ± 0.96 ^{bc}	97	107.35
4	Inoculated twice at 7 and 14 days old, respectively	62.53 ± 13.37 ^c	68.46	2.27 ± 1.05 ^c	100	153.21
5	Inoculated once at 14 days old	48.27 ± 17.35 ^{bc}	53.76	2.67 ± 1.06 ^{bc}	100	122.35
6	Inoculated twice at 14 and 21 days old, respectively	75.72 ± 21.30 ^a	80.72	1.37 ± 0.76 ^d	100	177.34

Significant difference ($P < 0.05$) between numbers with different letters (a, b, c, d). No significant difference ($P > 0.05$) between numbers with the same letter (a, b, c, d). “–” means no need for statistics. “ACI” means the anti-coccidial index

group 4 inoculated twice at 1 and 2 weeks old, respectively, group 5 inoculated once at 2 weeks old, group 6 inoculated twice at 2 and 3 weeks old, respectively. Chickens in groups 3, 4, 5 and 6 were immunized with 80 µg of DNA vaccine by intramuscular injection at corresponding immunization age. Chickens in groups 1 and 2 were treated with sterile TE by intramuscular injection in legs. The subsequent challenging infection and evaluation of immune protection were implemented just as described in the optimization of inoculation doses.

Optimization of Storage Temperature and Time

As shown in Table 4, 2-week-old chickens were distributed into 11 groups of 30 each randomly. Groups 1–11 were assigned as follows: group 1 unchallenged control, group 2 challenged control, group 3 immunized with DNA vaccine stored at –20 °C for 1 month, group 4 immunized

with DNA vaccine stored at –20 °C for 3 months, group 5 immunized with DNA vaccine stored at –20 °C for 6 months, group 6 immunized with DNA vaccine stored at 4 °C for 1 month, group 7 immunized with DNA vaccine stored at 4 °C for 3 months, group 8 immunized with DNA vaccine stored at 4 °C for 6 months, group 9 immunized with DNA vaccine stored at room temperature for 1 month, group 10 immunized with DNA vaccine stored at room temperature for 3 months, and group 11 immunized with DNA vaccine stored at room temperature for 6 months. Experimental chickens in groups 3–11 were intramuscularly immunized in legs with 80 µg of DNA vaccine. A booster immunization was given according to the previous method 1 week later. Unchallenged control and challenged control chickens were treated with sterile TE by intramuscular injection in legs. The subsequent challenging infection and evaluation of immune protection were implemented, just as described in the optimization of inoculation doses.

Table 4 Comparison of the effects of different storage temperature and time of DNA vaccine pVAX1-pEtK2-chIL-2

Groups	Treatment	Average body weight gain (g)	Oocyst decrease rate (%)	Mean lesion score	Survival rate (%)	ACI
1	Unchallenged control	45.55 ± 22.10 ^a	–	0.00 ± 0.00 ^a	100	200.00
2	Challenged control	13.77 ± 9.82 ^b	0.00	3.87 ± 0.35 ^c	100	82.11
3	Stored at –20 °C for 1 month	43.42 ± 21.05 ^a	73.36	1.10 ± 0.31 ^b	100	183.22
4	Stored at –20 °C for 3 months	43.87 ± 26.52 ^a	65.13	1.17 ± 0.38 ^b	100	182.04
5	Stored at –20 °C for 6 months	44.22 ± 17.53 ^a	73.68	1.20 ± 0.48 ^b	100	185.79
6	Stored at 4 °C for 1 month	42.42 ± 13.59 ^a	69.41	1.10 ± 0.31 ^b	100	183.32
7	Stored at 4 °C for 3 months	42.08 ± 27.85 ^a	65.46	1.10 ± 0.31 ^b	100	181.17
8	Stored at 4 °C for 6 months	43.22 ± 21.40 ^a	72.37	1.13 ± 0.35 ^b	100	185.12
9	Stored at room temperature for 1 month	42.40 ± 14.28 ^a	71.38	1.10 ± 0.31 ^b	100	183.22
10	Stored at room temperature for 3 months	42.37 ± 13.03 ^a	73.03	1.20 ± 0.41 ^b	100	180.32
11	Stored at room temperature for 6 months	44.17 ± 21.57 ^a	64.14	1.23 ± 0.43 ^b	100	185.02

Significant difference ($P < 0.05$) between numbers with different letters (a, b, c). No significant difference ($P > 0.05$) between numbers with the same letter (a, b, c). “–” means no need for statistics. “ACI” means the anti-coccidial index

Results

The Protective Effects of Different Immune Doses

Survival rates of chickens in groups from 1 to 6 were all 100%, and all experimental chickens were completely protected against the challenging infection. Average body weight gain was significantly reduced in challenged control group ($P < 0.05$) compared with unchallenged control group (Table 1). Meanwhile, chickens in experimental groups immunized with 25 μg , 50 μg , 80 μg and 100 μg of the DNA vaccine restored body weight gains compared with the challenged control group ($P < 0.05$). Furthermore, groups immunized with 80 μg and 100 μg showed significantly increased body weight gain relative to 25 μg and 50 μg ($P < 0.05$). In group inoculated with 80 μg of the DNA vaccine, the lowest and highest oocyst decrease rates were 20.43% and 98.47%, respectively, and the average oocyst decrease rate was 81.44%, which was higher than the other three doses. 27/30 (90%) of the immunizing chickens showed oocyst decrease rates beyond 50%, 3/30 (10%) of the immunizing chickens showed oocyst decrease rates below 50%, and 26/30 (86.7%) of the immunizing chickens showed oocyst decrease rates beyond 80%. Group 6 inoculated with 100 μg of the vaccine displayed a high average oocysts decrease ratio of 80.04%, approached to that of 80 μg of the DNA vaccine.

The caecal lesions were reduced significantly in experimental groups inoculated with DNA vaccine compared with challenged control group ($P < 0.05$). In group inoculated with 80 μg of DNA vaccine, cecal lesion score marked +1 was presented in 25/30 (83.3%) of the immunizing chickens, cecal lesion score marked +2 was presented in 5/30 (16.7%) of the immunizing chickens, and the average lesion score was +1.17, which was lower than those of the other three doses.

Groups of chickens immunized with 80 μg and 100 μg of the recombinant DNA vaccine resulted in ACIs more than 180, which displayed good protection against challenge infection with *E. tenella*.

According to the principle that the optimal dose of a vaccine or medicine was the lowest dose which could induce effective protective immunity, as well as considering three evaluating indexes including body weight gain, oocyst decrease rate and mean cecal lesion score as described above, it could draw a conclusion that 80 μg was the optimal dose of DNA vaccine pVAX1-pEtK2-IL-2.

The Protective Effects of Different Immune Routes

One chicken died of the challenge with *E. tenella* in challenged control group. Survival rates of chickens in group

1 and groups from 3 to 7 were all 100%. As Table 2 shows, average body weight gain had a significant decrease in challenged control group compared with the unchallenged control group ($P < 0.05$). By comparison, body weight gain of groups immunized by intramuscular injection, intravenous injection, subcutaneous injection, oral administration and intranasal administration restored compared with the challenged control group ($P < 0.05$). The body weight gain in groups immunized by intramuscular injection and intravenous injection increased significantly compared with the other three routes ($P < 0.05$). The highest and lowest oocyst decrease rates were 99.98% and 45.66%, respectively, in group inoculated by intramuscular injection. The average oocyst decrease rate was 91.74%, which was higher than the other four routes. We also found that oocyst decrease rates of 29/30 (96.7%) immunizing chickens was over 50%, oocyst decrease rates of 1/30 (3.3%) immunizing chickens was less than 50%, and oocyst decrease rates of 28/30 (93.3%) immunizing chickens was over 80%.

In regard to enteric lesion, significant alleviations in cecal lesions were noticed in experimental groups inoculated with the vaccine, but not in the challenged control group. In intramuscular injection group, cecal lesion score marked +1 was presented in 28/30 (93.3%) of the immunizing chickens, cecal lesion score marked +2 was presented in 2/30 (6.7%) of the immunizing chickens, and the average lesion score was +1.07, which was significantly lower than the other three routes, namely subcutaneous injection, oral administration and intranasal administration ($P < 0.05$), but the difference was not significant compared with the intravenous injection group ($P > 0.05$).

Alike results were discovered in regard to ACI, ACIs of groups inoculated with the vaccine by intramuscular injection, intravenous injection, subcutaneous injection, oral administration and intranasal administration were 190.22, 181.53, 166.63, 158.51, and 160.62, respectively, which revealed that intramuscular injection was the optimal route to induce protective immunity.

The Protective Effects of Different Primal Immunization Age and Times

One chicken and six chickens died from coccidial challenging infection in both group 3 inoculated once at 7 days old and group 2 challenged control, respectively. Survival rates of chickens in group 1 and groups from 4 to 6 were all 100%. As Table 3 shows, average body weight gain had a significant decrease in challenged control group compared with the unchallenged control group ($P < 0.05$). Booster immune groups, namely group 4 and group 6, significantly restored body weight gain and attenuated cecal lesions compared with the challenged control group ($P < 0.05$), also displayed

better alleviation in body weight gain–loss and cecal lesion than non-booster immune groups (group 3 and 5). Booster immune group 6 showed the best alleviation in body weight gain–loss and cecal lesion among four experimental groups (groups 3, 4, 5 and 6).

In chickens inoculated two times at 14 and 21 days old, respectively (group 6), the lowest and highest oocyst decrease rates were 30.41% and 92.76%, respectively, and the average oocyst decrease rate was 80.72%, which was higher than the other three groups (group 3, 4 and 5). Oocyst decrease rates of 28/30 (93.3%) immunizing chickens was over 50%, oocyst decrease rates of 2/30 (6.7%) of the immunizing chickens was less than 50%, and oocyst decrease rates of 26/30 (86.7%) immunizing chickens was over 80%.

ACIs of groups from 3 to 6 were 107.35, 153.21, 122.35 and 177.34, respectively. Alike findings were discovered in regard to ACI, booster immune groups, i.e., groups 4 and 6, displayed higher ACIs than those of non-booster immune groups, i.e., groups 3 and 5.

The Protective Effects of Different Storage Temperature and Time

Survival rates of chickens in groups 1–11 were all 100%, all experimental chickens were completely protected against the challenging infection. We can see from Table 4, average body weight gain in experimental groups 3–11 were significantly higher than that challenged control group ($P < 0.05$). There was no significant difference of average body weight gain among experimental groups 3–11 ($P > 0.05$).

Oocyst decrease rates of group 3, group 5, group 8 and group 10 were higher than the other immunization groups. There was no significant difference of mean cecal lesion score among experimental groups ($P > 0.05$).

ACIs of groups from 3 to 11 were 183.22, 182.04, 185.79, 183.32, 181.17, 185.12, 183.22, 180.32 and 185.02, respectively. ACIs of all experimental groups inoculated the vaccine were more than 180. Furthermore, ACIs of group 5, group 8 and group 11 were more than 185, which revealed completely immunization protection against *E. tenella* challenging infection.

Discussion

It is believed that unmethylated CpG motifs within *E. coli* DNA could cause immune stimulation [3]. Ampicillin-resistance gene existing in the backbone of pcDNA3.1 vector contains two short unmethylated CpG sequences, also named immunostimulatory DNA sequences (ISS), which plays an important role in the immune stimulatory effects of DNA vaccines [8, 11, 21]. The last products expressed by DNA vaccines with pcDNA3.1 vector including

ampicillin-resistance gene could elicit anaphylactic shock which was particularly dangerous for human health. pVAX1 is another plasmid vector designed for DNA vaccine development, which was constructed by modifying the vector, pcDNA3.1. However, pVAX1 vector contains kanamycin-resistance gene substituted for the ampicillin-resistance gene, which deleted the two CpG sequences (ISS). Although the immunization procedure of pcDNA3.1b-TA4-IL-2 has been optimized in our previous study [24], it is necessary to study immunization procedure of DNA vaccines constructed by different plasmid vectors. In this research, we studied immunization procedure of DNA vaccine pVAX1-pEtK2-IL-2 constructed using expression vector pVAX1.

The protective immunity elicited by DNA vaccines of poultry is usually not absolute or sufficient. Hence, to improve the protective immune responses induced by poultry DNA vaccines, some crucial strategies or methods need to be studied in depth, which concern optimization of inoculation doses, optimization of inoculation routes, optimization of primary inoculation age and inoculation times, optimization of the plasmid backbone and host codon usage, boosting vaccine uptake by host cells, combination of vaccine antigens and co-injection of immunomodulators [13, 19, 24]. Previous studies reported some DNA plasmids immunized, but not clearly told the basis of the dose choice in most cases. The dose usually ranged from 50 µg to 200 µg of plasmids. Few researchers who implemented the comparison of the effects of the protective immunity induced by different doses of the vaccines in chickens reported that the best doses for intramuscular immunization located range from 25 µg to 200 µg [24, 29, 32]. For example [31] found that when 3-day-old chickens were inoculated with 12.5 µg, 25 µg and 50 µg of pcDNA3.1-Et1A-TA4 by intramuscular injection at an interval of 7 days, the results presented that the 50 µg group achieved the highest ACI. In previous work, we constructed many monovalent or multivalent epitope DNA vaccines of the most relevant *Eimeria* species including *E. tenella*, *E. maxima*, *E. necatrix* or *E. acervulina*. Chickens were immunized intramuscularly with different doses of these vaccines ranging from 2 µg to 200 µg, the results showed that injection doses ranging from 25 µg to 100 µg of these vaccines could induce effective protection against corresponding *Eimeria* species, even partial cross-protection against other *Eimeria* spp. [24–28, 33]. Strangely, the best immune protection was not always achieved with the highest experimental doses [19]. Hence, more plasmids associated with stronger immune responses, but not necessarily linear correlation [1, 12]. Song et al. [23] reported that 1-day-old chickens inoculated intramuscularly with 5, 10, 50 and 100 µg of pMP13 plasmid showed significantly reduced oocysts after challenge infection with 5×10^3 *E. acervulina*. Both 5 µg and 50 µg plasmid groups were more effective in decreasing the oocyst output compared to both 10 µg and

100 µg, unexpectedly, effect of 100 µg immunization on oocyst reduction was the worst. However, in this study, ACIs of both 80 µg and 100 µg groups were 184.87 and 186.43, respectively, which were higher than that of 25 µg or 50 µg group, in keeping with what [31] reported.

Inoculation routes are very important for immunization procedure of poultry DNA vaccines. In the majority of cases, the DNA vaccine was inoculated by the intramuscular route, but there was no reference describing the fundamental immunological rational [19]. Nonetheless, the comparative study of the effect of different immunization routes was implemented by some researchers. For naked DNA plasmids, the intramuscular route was usually indicated as the most effective [14, 23, 24]. For example [23] found that intramuscular injection route presented more efficiency in reducing the oocyst output than subcutaneous route at dose 5 µg pMP13 when chickens were inoculated with the plasmid encoding a conserved antigen of *E. acervulina*. Likewise, when Song et al. [24] performed the comparison of the effects of five immune routes, the results showed that intramuscular route was the optimal one. Meanwhile, ACI of oral administration was over 180, which might be another efficient route for eliciting effective immunity against avian coccidiosis. In our study, the immune effects of five inoculation routes were also compared. Likewise, ACI of the intramuscular inoculation group was the highest, intravenous group was next and higher than 180, which afforded good protection against *E. tenella*.

The age of the first inoculation and inoculation times are also very important to DNA vaccine inoculation. A single injection of DNA vaccine was often insufficient to induce protective immunity. A booster with DNA vaccine was also able to induce some protective immunity [6]. Therefore, a booster immunization was often applied. This increased, for example, the level of antibodies induced by a DNA vaccine against avian influenza virus in chickens [9, 19].

Usually, broilers were slaughtered at the age of 4–5 weeks. The first DNA vaccination at the age of 2 weeks might not be practical for broilers. Therefore, immunization in ovo or at hatch might be an alternative. Ding et al. [6] found that in ovo inoculation with the EtMIC2 gene produced protective immunity against infection by *E. tenella*. However, Lim et al. [17] reported that the level of antibodies against AIV was higher in chickens inoculated for the first time at the age of 14 days than in those inoculated on the day of hatching. Actually, the immune system was less developed during embryonic development or hatching period or in younger chickens [2, 19]. Therefore, in ovo or at hatch inoculation of DNA vaccine could or could not achieve the desired effect need to be further studied.

In fact, age for the primary DNA vaccination had no absolute standard in the various published tests, which varied ranging from the day of hatching to 5–6 weeks, in general,

2–3 weeks, but the optimal age of primary immunization for every poultry DNA vaccine is unclear [19]. In our study, the first inoculation at 14 days old elicited more effective immunity against *E. tenella* challenge infection than that of the first inoculation at 7 days old, identical to Lim's [17] results.

In general, two inoculations achieved more effectively than single inoculation with higher dose of naked plasmids inducing better immune response. Song et al. [23] found that two inoculations by intramuscular injection with 10 µg or 100 µg DNA were more efficient in reducing the oocyst production of *E. acervulina* than that of single inoculation. Ding et al. [6] reported that two inoculations with pcDNA–EtMIC2 significantly increased body weight gain compared to single inoculation in *E. tenella* challenge infection. However, Song et al. [24] presented that there was no significant difference in ACIs of boosting and non-boosting groups. In our study, two inoculations elicited better protective immunity against *E. tenella* challenge infection compared to single inoculation, identical to Song's [23] and Ding's [6] results.

DNA vaccine can be preserved stably for a few weeks, up to 6 months, even at room temperature, on account of its structural, thermostable and chemical characters compared to traditional vaccines making up of inactivated, virulent organisms or attenuated vaccines [24]. In our study, there was no difference in ACIs of all experimental groups inoculated with preserved DNA vaccine, which were all more than 180, suggesting all the stored methods are feasible. Meanwhile, proving different storage temperature and time of DNA vaccine did not influence the effects of protective immunity against *E. tenella*, in accordance with what [24] reported.

Acknowledgements This work was supported by the Joint Research Project between National Natural Science Foundation of China and Pakistan Science Foundation (NSFC-PSF) (Grant no. 31661143017), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Colleges and Universities Nature Science Foundation of Jiangsu Province (17KJB230003) and the Qing Lan Project of Jiangsu Province (Grant no. 00000216049).

References

1. Barry MA, Johnston SA (1997) Biological features of genetic immunization. *Vaccine* 15(8):788–791. [https://doi.org/10.1016/S0264-410X\(96\)00265-4](https://doi.org/10.1016/S0264-410X(96)00265-4)
2. Brownlie R, Zhu J, Allan B, Mutwiri GK, Babiuk LA, Potter A et al (2009) Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Mol Immunol* 46:3163–3170. <https://doi.org/10.1016/j.molimm.2009.06.002>
3. Chen Y, Lenert P, Weeratna R, McCluskie M, Wu T, Davis HL et al (2001) Identification of methylated CpG motifs as inhibitors of the immune stimulatory CpG motifs. *Gene Ther* 8:1024–1032. <https://doi.org/10.1038/sj.gt.3301482>

4. Cox GJ, Zamb TJ, Babiuk LA (1993) Bovine herpesvirus 1: immune responses in mice and cattle injected with plasmid DNA. *J Virol* 67:5664–5667
5. Davis HL, McCluskie MJ, Gerin JL, Purcell RH (1996) DNA vaccine for hepatitis B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. *Proc Natl Acad Sci USA* 93:7213–7218. <https://doi.org/10.1073/pnas.93.14.7213>
6. Ding X, Lillehoj HS, Dalloul RA, Min W, Sato T, Yasuda A et al (2005) In ovo vaccination with the *Eimeria tenella* EtMIC2 gene induces protective immunity against coccidiosis. *Vaccine* 23:3733–3740. <https://doi.org/10.1016/j.vaccine.2005.01.144>
7. Dunn PP, Bumstead JM, Tomley FM (1996) Sequence, expression and localization of calmodulin-domain protein kinases in *Eimeria tenella* and *Eimeria maxima*. *Parasitology* 113(5):439–448. <https://doi.org/10.1017/S0031182000081506>
8. Elizabeth GR, Susana M, Javeed AS, Chang-you W, Joanna RK, Tara NT et al (2002) Vaccination with heat-killed leishmania antigen or recombinant leishmanial protein and CpG oligodeoxynucleotides induces long-term memory CD4⁺ and CD8⁺ T cell responses and protection against leishmania major infection. *J Exp Med* 195(12):1565–1573. <https://doi.org/10.1084/jem.20020147>
9. Jiang Y, Yu K, Zhang H, Zhang P, Li C, Tian G et al (2007) Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector. *Antiviral Res* 75:234–241. <https://doi.org/10.1016/j.antiviral.2007.03.009>
10. Johnson J, Reid WM (1970) Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. *Exp Parasitol* 28:30–36. [https://doi.org/10.1016/0014-4894\(70\)90063-9](https://doi.org/10.1016/0014-4894(70)90063-9)
11. Klinman DM, Yamshchikov G, Ishigatsubo Y (1997) Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J Immunol* 158:3635–3639
12. Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ et al (1997) Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from Plasmodium berghei malaria parasites. *J Immunol* 159(12):6112–6119
13. Leitner WW, Ying H, Restifo NP (2000) DNA and RNA-based vaccines: principles, progress and prospects. *Vaccine* 18(9):765–777. [https://doi.org/10.1016/S0264-410X\(99\)00271-6](https://doi.org/10.1016/S0264-410X(99)00271-6)
14. Li JR, Huang YW, Liang XY, Lu MJ, Li L, Yu L et al (2003) Plasmid DNA encoding antigens of infectious bursal disease viruses induce protective immune responses in chickens: factors influencing efficacy. *Virus Res* 98:63–74. <https://doi.org/10.1016/j.virusres.2003.08.017>
15. Lillehoj HS (1998) Role of T lymphocytes and cytokines in coccidiosis. *Int J Parasitol* 28:1071–1081. [https://doi.org/10.1016/S0020-7519\(98\)00075-7](https://doi.org/10.1016/S0020-7519(98)00075-7)
16. Lillehoj HS, Ding X, Quiroz MA, Bevenssee E, Lillehoj EP (2005) Resistance to intestinal coccidiosis following DNA immunization with the cloned 3-IE *Eimeria* gene plus IL-2, IL-15 and IFN-gamma. *Avian* 49(1):112–117
17. Lim KL, Jazayeri SD, Yeap SK, Mohamed Alitheen NB, Bejo MH, Ideris A et al (2013) Antibody and T cell responses induced in chickens immunized with avian influenza virus N1 and NP DNA vaccine with chicken IL-15 and IL-18. *Res Vet Sci* 95:1224–1234. <https://doi.org/10.1016/j.rvsc.2013.07.013>
18. McManus EC, Campbell WC, Cuckler AC (1968) Development of resistance to quinoline coccidiostats under field and laboratory conditions. *J Parasitol* 54(6):1190–1193
19. Meunier M, Chemaly M, Dory D (2016) DNA vaccination of poultry: the current status in 2015. *Vaccine* 34(2):202–211. <https://doi.org/10.1016/j.vaccine.2015.11.043>
20. R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>
21. Sato Y, Roman M, Tique H, Lee D, Corr M, Nguyen MD et al (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273:352–354. <https://doi.org/10.1126/science.273.5273.352>
22. Shirley MW, Smith AL, Tomley FM (2005) The biology of avian *Eimeria* with an emphasis on their control by vaccination. *Adv Parasitol* 60:285–330. [https://doi.org/10.1016/S0065-308X\(05\)60005-X](https://doi.org/10.1016/S0065-308X(05)60005-X)
23. Song KD, Lillehoj HS, Choi KD, Yun CH, Parcells MS, Huynh JT et al (2001) A DNA vaccine encoding a conserved *Eimeria* protein induces protective immunity against live *Eimeria acervulina* challenge. *Vaccine* 19:243–252. [https://doi.org/10.1016/S0264-410X\(00\)00169-9](https://doi.org/10.1016/S0264-410X(00)00169-9)
24. Song X, Xu L, Yan R, Huang X, Shah MA, Li X (2009) The optimal immunization procedure of DNA vaccine pcDNA-TA4-IL-2 of *Eimeria tenella* and its cross-immunity to *Eimeria necatrix* and *Eimeria acervulina*. *Vet Parasitol* 159:30–36. <https://doi.org/10.1016/j.vetpar.2008.10.015>
25. Song H, Yan R, Xu L, Song X, Shah MA, Zhu H, Li X (2010) Efficacy of DNA vaccines carrying *Eimeria acervulina* lactate dehydrogenase antigen gene against coccidiosis. *Exp Parasitol* 126:224–231. <https://doi.org/10.1016/j.exppara.2010.05.015>
26. Song H, Qiu B, Yan R, Xu L, Song X, Li X (2013) The protective efficacy of chimeric SO7/IL-2 DNA vaccine against coccidiosis in chickens. *Res Vet Sci* 94(3):562–567. <https://doi.org/10.1016/j.rvsc.2012.11.006>
27. Song X, Ren Z, Yan R, Xu L, Li X (2015) Induction of protective immunity against *Eimeria tenella*, *Eimeria necatrix*, *Eimeria maxima* and *Eimeria acervulina* infections using multivalent epitope DNA vaccines. *Vaccine* 33:2764–2770. <https://doi.org/10.1016/j.vaccine.2015.04.052>
28. Song X, Zhao X, Xu L, Yan R, Li X (2017) Immune protection duration and efficacy stability of DNA vaccine encoding *Eimeria tenella* TA4 and chicken IL-2 against coccidiosis. *Res Vet Sci* 111:31–35. <https://doi.org/10.1016/j.rvsc.2016.11.012>
29. Suarez DL, Schultz-Cherry S (2000) The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. *Avian Dis* 44:861–868. <https://doi.org/10.2307/1593058>
30. Tomley F (1997) Techniques for isolation and characterization of apical organelles from *Eimeria tenella* sporozoites. *Methods* 13:171–176. <https://doi.org/10.1006/meth.1997.0509>
31. Wu SQ, Wang M, Liu Q, Zhu YJ, Suo X, Jiang JS (2004) Construction of DNA vaccines and their induced protective immunity against experimental *Eimeria tenella* infection. *Parasitol Res* 94(5):332–336. <https://doi.org/10.1007/s00436-004-1185-6>
32. Xu J, Zhang Y, Tao J (2013) Efficacy of a DNA vaccine carrying *Eimeria maxima* Gam56 antigen gene against coccidiosis in chickens. *Korean J Parasitol* 51:147–154. <https://doi.org/10.3347/kjp.2013.51.2.147>
33. Xu Q, Song X, Xu L, Yan R, Shah MA, Li X (2008) Vaccination of chickens with a chimeric DNA vaccine encoding *Eimeria tenella* TA4 and chicken IL-2 induces protective immunity against coccidiosis. *Vet Parasitol* 156:319–323. <https://doi.org/10.1016/j.vetpar.2008.05.025>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.