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Development and evaluation of protective immunity of a ROP27 DNA vaccine against *Eimeria tenella* in chickens

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ABSTRACT

This study aimed to develop and evaluate the protective immunity of a ROP27 DNA vaccine against *Eimeria tenella* (*E. tenella*) in chickens. ***E. tenella*** is a parasitic protozoan that poses a significant threat to the poultry industry. The rhoptry protein 27 **(ROP27)** of *E. tenella* has been shown to have immunoprotective properties. However, traditional protein expression methods are time-consuming and labor-intensive, limiting large-scale production. In this study, we developed a pVAX-ROP27 DNA vaccine and confirmed its expression in chickens using RT-PCR and Western blot analysis. The protective immunity of the DNA vaccine was evaluated through an animal experiment with different immunization doses. The results confirmed the successful construction of the pVAX-ROP27 DNA vaccine and its in vivo expression. Chickens immunized with the vaccine at different doses showed significant improvements in average weight gain, relative weight gain rate **(RWG)**, cecal lesion score reduction **(RLS)**, and oocyst reduction rate, as well as a decrease in oocysts per gram **(OPG)**. The group immunized with 100 μg/feather of pVAX-ROP27 exhibited the most significant effect, achieving an anticoccidial index **(ACI)** of 179.80. Additionally, levels of IL-2, IFN-γ, IL-6, IgG, and IgY significantly increased with the number of immunizations, whereas IL-4 and IL-10 showed no significant differences. Histopathological analysis of the ceca revealed that lesions were least severe in the 100 μg/feather pVAX-ROP27 immunized group. These findings suggest that the pVAX-ROP27 DNA vaccine offers immune protection against *E. tenella* infection and could serve as a promising candidate for preventing and controlling chicken coccidiosis.

Introduction

Chicken coccidiosis is a widespread disease caused by intracellular parasitic protozoa (Mesa-Pineda et al., 2021; Shirley et al., 2005). The incidence rate of chicken coccidiosis on intensive farms ranges from 20% to 80%, with a mortality rate of 60% to 80% (Gao et al., 2024). The global economic loss attributed to this disease is estimated at approximately 10.4 billion pounds annually (Blake et al., 2020). There are seven common types of chicken coccidia, with *E. tenella* being one of the most harmful, characterized by a widespread epidemic range and significant economic impact (Attia et al., 2023; Badri et al., 2024).

Currently, the prevention and control measures for chicken coccidiosis primarily depend on drug therapy and vaccine immunization. However, the emergence of drug residues and drug-resistant strains has shifted the focus towards vaccine immunization (Fatoba and Adeleke, 2018; Vermeulen et al., 2001). The main types of coccidiosis vaccines include virulent vaccines, attenuated vaccines, and subunit vaccines (Chen et al., 2023). Although virulent vaccines can generate strong protective immunity, their high virulence often leads to outbreaks of chicken coccidiosis. Attenuated live vaccines demonstrate effective prevention, yet they retain some pathogenicity, which can adversely affect chicken production performance and pose a risk of toxicity reversion (Lin et al., 2017). In recent years, novel subunit vaccines have attracted significant attention from researchers as a means to prevent and control chicken coccidiosis through advancements in DNA recombination technology (Venkatas and Adeleke, 2019).

Subunit vaccines are classified into recombinant subunit vaccines and DNA vaccines, among others. DNA vaccines involve the cloning of foreign DNA that encodes antigen proteins into eukaryotic expression vectors. The recombinant plasmid DNA is directly injected into the animal, allowing the host cells to utilize their transcription machinery to synthesize antigen proteins. This process activates the immune system,

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triggering immune responses (Venkatas and Adeleke, 2019). DNA vaccines offer several advantages over traditional vaccines. For instance, the proteins expressed by the vaccine are generated within the host's cells, eliminating the risk of virulence reversion. Research conducted by Geriletu et al. shows that both pcDNA4.0-MZ and pcDNA4.0-MZ-IL17 DNA vaccines significantly alleviate cecal lesions (Geriletu et al., 2011). Furthermore, Xu et al. developed the pcDNA3.0-TA4-IL-2 DNA vaccine, which demonstrated a substantial reduction in cecal lesions, achieving an ACI of 190 (Xu et al., 2008). Similarly, Shi et al. created the pVAX1-MIC2-IL-18 DNA vaccine, resulting in a notable reduction in OPG and an ACI of 171.17 post-immunization compared to the control group (Shi et al., 2014). Zhang et al. developed the pVAX-*Tg*ROP21 recombinant DNA vaccine, which elicited a robust immune response, protecting experimental animals from the highly virulent RH strain of *Toxoplasma gondii* (Zhang et al., 2018). In conclusion, DNA vaccines have demonstrated a positive immune protective effect.

The rhoptries are essential for the invasion and survival of apicomplexan parasites within host cells. They house two distinct subpopulations of proteins, including rhoptry proteins **(ROP)** from the bulb compartment. ROP plays a pivotal role in interacting with host cell signaling pathways, serving as a key determinant of virulence and triggering various downstream responses. Furthermore, ROP may represent a promising vaccine candidate against *E. tenella* (Oakes et al., 2013). Liu et al. expressed the recombinant protein *Et*ROP17 **(r*Et*-ROP17)**, which significantly decreased oocyst output and cecal lesions, suggesting that *Et*ROP17 could serve as an effective candidate vaccine for *E. tenella* (Liu et al., 2022). Studies have demonstrated that recombinant protein vaccines derived from *Et*ROP30 and *Et*ROP35 can enhance relative body weight in chickens, reduce cecal lesions, decrease oocyst output, and elevate antibody levels (Bingxiang et al., 2022; Wang et al., 2022). These findings indicate that rhoptry genes hold considerable promise as candidate vaccines.

E. tenella inflicts the most severe damage to the host during the sporozoite and schizont stages (Burrell et al., 2020). Prior research has established that rhoptry protein 27 (ROP27) exhibits high expression during the schizont stage and that the recombinant protein r*Et*ROP27 demonstrates strong immunogenicity (Li et al., 2023). However, the process of protein expression is time-consuming and labor-intensive, making it impractical for large-scale applications. Furthermore, it remains uncertain whether the *Et*ROP27 DNA vaccine can effectively prevent and control *E. tenella* infection. Accordingly, this study aims to select *Et*ROP27 as the target gene for constructing the pVAX-ROP27 DNA vaccine and to evaluate its immunogenicity. The findings of this research may not only provide a theoretical framework for the clinical application of DNA vaccines but also inspire novel approaches for preventing *E. tenella* infections and developing vaccines, thereby contributing significant scientific and practical insights.

Materials and methods

Ethics statement

All experiments involving animals were conducted in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC). This study received approval from the Animal Protection and Utilization Committee at Shanxi Agricultural University, China (SXAU-EAW-2023C.2023C.AZ.010007270).

Funding

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Experimental animals, parasite and antiserum

1-Day-old SPF chickens were hatched from SPF breeder embryos provided by Beijing Boehringer Ingelheim Weitong Laboratory Animal Technology Co., Ltd (Beijing, China). The chickens were raised under strict pathogen-free conditions. Appropriate temperatures for their age were maintained, and feed and water were provided ad libitum; no antibiotics or anticoccidial drugs were used. Rabbit antiserum against recombinant ROP27 protein was provided by the Veterinary Pathology Laboratory at the College of Veterinary Medicine, Shanxi Agricultural University. The strain used in this experiment is the Shanxi virulent strain of *E. tenella* (*Et*SX01), which was incubated in a 2.5% potassium dichromate ($K_2Cr_2O_7$) solution, provided by the Veterinary Pathology Laboratory at the College of Veterinary Medicine, Shanxi Agricultural University.

Reagents

The following reagents were used: the pMD™ 18-T vector Cloning Kit, QuickCut™ KpnI, QuickCut™ XhoI, E. coli DH5α Competent Cells, DL2000 DNA Marker, T4 DNA Ligase, BCA Protein Assay Kit, and PrimeScript™ RT Reagent Kit with gDNA Eraser, all purchased from Takara (Osaka, Japan). LB broth powder, LB agar powder, Ampicillin, Kanamycin, BSA, Triton X-100, Tris, SDS, HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L), and the ECL detection kit were obtained from Solarbio (Shanghai, China). GoTaq® Green Master Mix was sourced from Promega (Wuhan, China), whereas the SanPrep Column DNA Gel Extraction Kit was purchased from Sangon Biotech. E.Z.N.A® Endo-free Plasmid Mini Kit II and E.Z.N.A® Endo-free Plasmid DNA Maxi Kit were acquired from Omega (Wuhan, China). The Chicken ELISA Kit was obtained from J&L Biological (Shanghai, China).

Construction of DNA vaccine pVAX-ROP27

The *Et*ROP27 sequence (Cluster-10347.5943) was utilized to design specific primers with Primer Premier 5. The primer sequences are as follows: forward primer for *Et*ROP27, 5'-GGTACCGATGGGTAACGAGTCTCTGC-3', which incorporates the KpnI restriction site; and reverse primer, 5'-CTCGAGTCAGGGGCCACCCCCTTTGA-3', incorporating the XhoI restriction site. Using *E. tenella* sporozoite cDNA as a template, the target gene was amplified by PCR employing the GoTaq® Green Master Mix kit (Promega, Wuhan, China). The PCR reaction was performed under the following conditions: 95°C for 2 min, followed by 35 cycles consisting of 95°C for 30 s, 59°C for 30 s, and 72°C for 70 s, and a final extension at 72°C for 8 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel at 120 V for 30 min. The target DNA band was excised from the gel using a gel imaging analysis instrument. The target fragment was subsequently purified and recovered employing the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China), following the manufacturer's instructions. The concentration and quality of the purified fragment were determined using a full-wavelength microplate reader. The purified target fragment was then cloned into the pMD18-T vector. A single bacterial colony was selected for amplification and culture, and the sample was sent to Sangon Biotech (Shanghai, China) for sequencing analysis. The bacterial solution with the correct sequence was selected for plasmid extraction using the E.Z.N.A® Endo-free Plasmid Mini Kit II (Omega, Wuhan, China). Both the plasmid and pVAX DNA were digested with KpnI and XhoI endonucleases, and the digested products were purified using the aforementioned method. The target fragment was subsequently cloned into the eukaryotic expression vector pVAX. Following sequencing confirmation, the correctly constructed plasmid was designated pVAX-ROP27 (Shi et al., 2023).

The expression of ROP27 mRNA in vivo was detected by PCR

The recombinant plasmid pVAX-ROP27 was injected into the leg muscles of 14-day-old chickens at a dosage of 100 μg/feather. The control group received an injection of PBS. Seven days post-injection, RNA was extracted from the muscle at the injection site for both the control and experimental groups. The quality and concentration of the extracted RNA were assessed using a full-wavelength microplate reader. Genomic DNA was removed, and the RNA was reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Osaka, Japan). PCR was performed on the target genes using the GoTaq® Green Master Mix kit (Promega, Wuhan, China). The PCR amplification procedure was as follows: 95°C for 2 min; followed by 35 cycles of 95°C for 10 s, 59°C for 30 s, and 72°C for 70 s; and a final extension of 72°C for 5 min. After PCR amplification, the product was loaded onto a 1% agarose gel, and results were visualized using a gel imaging analyzer following electrophoresis at 120 V for 35 min.

Detection of the expression of ROP27 protein in vivo by Western blot

The injection method and sampling times were aligned with those utilized for RT-PCR. After homogenization, the muscle tissue was added to a RIPA lysate (containing protease and phosphatase inhibitors, with 0.1M EDTA in a 1:50 ratio) and incubated at 4°C for 10 min. The homogenate was then centrifuged at 12,000 r/min for 10 min to isolate total protein. The protein concentration was determined according to the instructions provided with the BCA Protein Assay Kit (TaKaRa, Osaka, Japan). The supernatant was combined with a 5× protein loading buffer (4:1) and heated for 10 min. The samples were subsequently separated using a 10% polyacrylamide gel at 80 V for 30 min and 120 V for 2 h. Transfer of proteins was performed onto a 0.45 μm nitrocellulose membrane, which was then blocked with 5% BSA at room temperature for 2 h. The membrane was incubated overnight at 4°C with a rabbit anti-ROP27 antibody (1:400) and was washed five times with 1× TBST (Solarbio, Beijing, China), with each wash lasting 5 min. Following this, the membrane was incubated at 37°C for 2 h with HRP-conjugated goat anti-rabbit IgG (1:5000), followed by five washes with TBST (10 min each). Finally, Superstar ECL Plus Ready-to-use was added to visualize the signal using a highly sensitive chemical fluorescence imager.

Animal experiment

A total of 240 one-day-old chickens were randomly divided into eight groups: one unchallenged group, one challenged group, one pVAX control group, and one pVAX-ROP27 group. In the pVAX-ROP27 group, each chicken leg was injected with pVAX-ROP27 at doses of 12.5 μg, 25 μg, 50 μg, 100 μg, and 200 μg. Immunization occurred at 14 days of age, followed by a booster dose at 21 days of age. The remaining two groups received PBS injections concurrently. All groups, with the exception of the unchallenged control group, were challenged with 5×10^4 fresh sporulated oocysts of *E. tenella* at 28 days of age and were dissected 7 days post-challenge (Fig. 1).

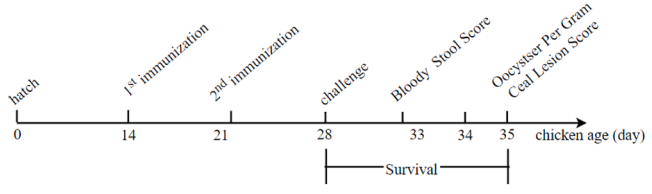

Figure 1. Schematic outline of the animal experiments.

Evaluation of immunogenicity of pVAX-ROP27

The clinical symptoms of chickens in each group were monitored for 7 consecutive days post-challenge, and mortality was recorded. Bloody stool scores were recorded on day 5 post-challenge. On day 7 post-challenge, OPG was measured, and cecal lesions were scored following fasting and weighing. Survival rate, relative weight gain rate **(RWG)**, lesion score reduction rate **(RLS)**, oocyst reduction rate, and anti-coccidial index **(ACI)** were calculated.

On day 7 post-infection, fresh feces from each group were collected, weighed, and thoroughly mixed. A 2 g sample from each group was taken for OPG determination using the McMaster method. Specifically, 10 mL of saturated saline was added to the 2 g fecal sample, mixed thoroughly, followed by the addition of 50 mL of saturated saline. The mixture was filtered through a 60-mesh fecal sieve. After filtration, the liquid was gently shaken, and a small amount of the filtrate was drawn with a disposable pipette and placed in the McMaster counting chamber. The sample was allowed to settle for 1–2 min, and the number of oocysts in both chambers was counted under a microscope. The average count was multiplied by 200 to determine the number of OPG of feces. (Long, et al., 1976).

The formula for calculating average weight gain was: Average weight gain = (final total weight of chickens in each group - initial total weight of chickens in each group) / number of chickens per group. RWG = (average weight gain of immunized or infected chickens / average weight gain of blank control chickens) × 100%.The survival rate was calculated as: **Survival rate** = number of surviving chickens / initial number of chickens in each group. The cecal lesion score ranged from 0 (none) to 4 (severe) and was evaluated by three independent observers, according to Johnson and Reid (1970). **RLS** was calculated using the formula: RLS = (average lesion score in the challenged group - average lesion score in the immunized challenged group) / average lesion score in the challenged group (Long, Millard, Joyner, and Norton, 1976). The **oocyst reduction rate** was calculated as: Oocyst reduction rate = (oocyst output in the challenged group - oocyst output in the immune group) / oocyst output in the challenged group × 100% (Lillehoj and Ruff, 1987). The **ACI** was calculated using the formula: ACI = (RWG + survival rate) × 100 − (mean lesion score × 10) − (OPG value). ACI ≥ 180 indicates good protection, 160 ≤ ACI < 180 indicates moderate protection, 120 ≤ ACI < 160 indicates limited protection, and ACI < 120 indicates no protection (Chapman, 1998).

Except for the challenged group and the pVAX control group, which had their ceca collected from chickens that died on day 5, the ceca of the other groups were collected on day 7 for histopathological examination and hematoxylin-eosin staining.

Detection of serum antibodies and cytokines by ELISA

Serum antibody and cytokine concentrations were assessed using ELISA. Blood samples were collected from three chickens in each group at the following time points: before the first immunization, after the booster immunization, before the challenge, and post-challenge (14 days, 21 days, 28 days, and 35 days). Whole blood was allowed to clot at room temperature for 2 h, followed by centrifugation at 2000×g for 20 min. The supernatant was carefully collected and stored at -80°C for later analysis. IgG, IgY, IL-2, IFN-γ, IL-10, IL-6, and IL-4 concentrations were measured using commercially available ELISA kits.

Image and statistical analyses

Data were expressed as mean ± SD. Statistical comparisons were made using one-way analysis of variance with SPSS 17.0 statistical software (Chicago, IL, USA). Histograms and other graphical representations were generated using GraphPad Prism 5.0 software (San Diego, CA, USA). All experiments were conducted in triplicate. A p-value of less than 0.05 was considered statistically significant.

Results

Gene clone and plasmid construction

The *Et*ROP27 gene was specifically amplified, resulting in a target band of approximately 1068 bp following agarose gel electrophoresis (Fig. 2A). After gel recovery and purification, the concentration was determined to be 342 μg/μL, with an OD_{260}/OD_{280} ratio of 1.91, indicating good purity. The recombinant vector pMD18T-ROP27 was successfully constructed, and positive bacterial clones were selected for sequencing (Fig. 2B). Once sequencing confirmed the accuracy of the construct, the pMD18T-ROP27 plasmid was extracted, yielding a concentration of 680 μg/μL and an OD_{260}/OD_{280} ratio of 1.95. Double digestion of pMD18T-ROP27 and pVAX1 was then performed, followed by recovery and purification (Fig. 2C). The concentration of ROP27 after recovery was 202 μg/μL, with an OD_{260}/OD_{280} ratio of 1.90, indicating good purity. The concentration of pVAX1 was 250 μg/μL, with an OD_{260}/OD_{280} ratio of 1.98. The target fragment was subsequently cloned into the pVAX vector. Positive colonies were identified using bacterial liquid PCR, followed by double digestion and sequencing analysis (Fig. 2D). Ultimately, the eukaryotic expression vector pVAX-ROP27 was successfully constructed.

The expression of EtROP27 in vivo

The expression of ROP27 was analyzed in both the experimental group (injected with pVAX-ROP27) and the control group (injected with PBS) using RT-PCR and Western blot techniques. The RNA concentration in the experimental group was found to be 950 μg/μL, with an OD_{260}/OD_{280} ratio of 1.95. In contrast, the control group exhibited a concentration of 820 μg/μL and an OD_{260}/OD_{280} ratio of 1.89, indicating good purity in both groups. The total protein concentration measured 7.5 μg/μL in the experimental group, compared to 6.2 μg/μL in the control group. Notably, the experimental group displayed a specific about 1068 bp band, whereas no band was detected in the control group (Fig. 3A), confirming successful transcription of ROP27 in chickens. Furthermore, Western blot analysis identified specific bands at approximately 38.07 kDa in the experimental group, and no bands were observed in the control group (Fig. 3B), thereby confirming the expression of ROP27 in chickens.

The immunoprotective effect of pVAX-ROP27

The immunoprotective effect of pVAX-ROP27 was evaluated through various parameters associated with *E. tenella* infection, including average weight gain, RWG, survival rate, average cecal lesion score, lesion score reduction rate, OPG, oocyst reduction rate, bloody stool score, and ACI. When compared to the unchallenged control group, the challenge control group, along with the 12.5 μg/feather, 25 μg/feather, 50 μg/feather, and 200 μg/feather pVAX-ROP27 groups, showed a significant decrease in average weight gain and RWG. However, there was no significant difference in the average weight gain for the 100 μg/feather pVAX-ROP27 group. Except 100 μg/feather pVAX-ROP27 group RWG was no significant differences, all pVAX-ROP27 dosage groups exhibited significant increases in average weight gain, RWG, lesion score reduction rate, and oocyst reduction rate when compared to the challenge control group. Additionally, significant reductions in average cecal lesion scores and OPG values were observed across all pVAX-ROP27 dosage groups. Within a certain range, increasing the immune dose corresponded with improvements in all measured parameters.

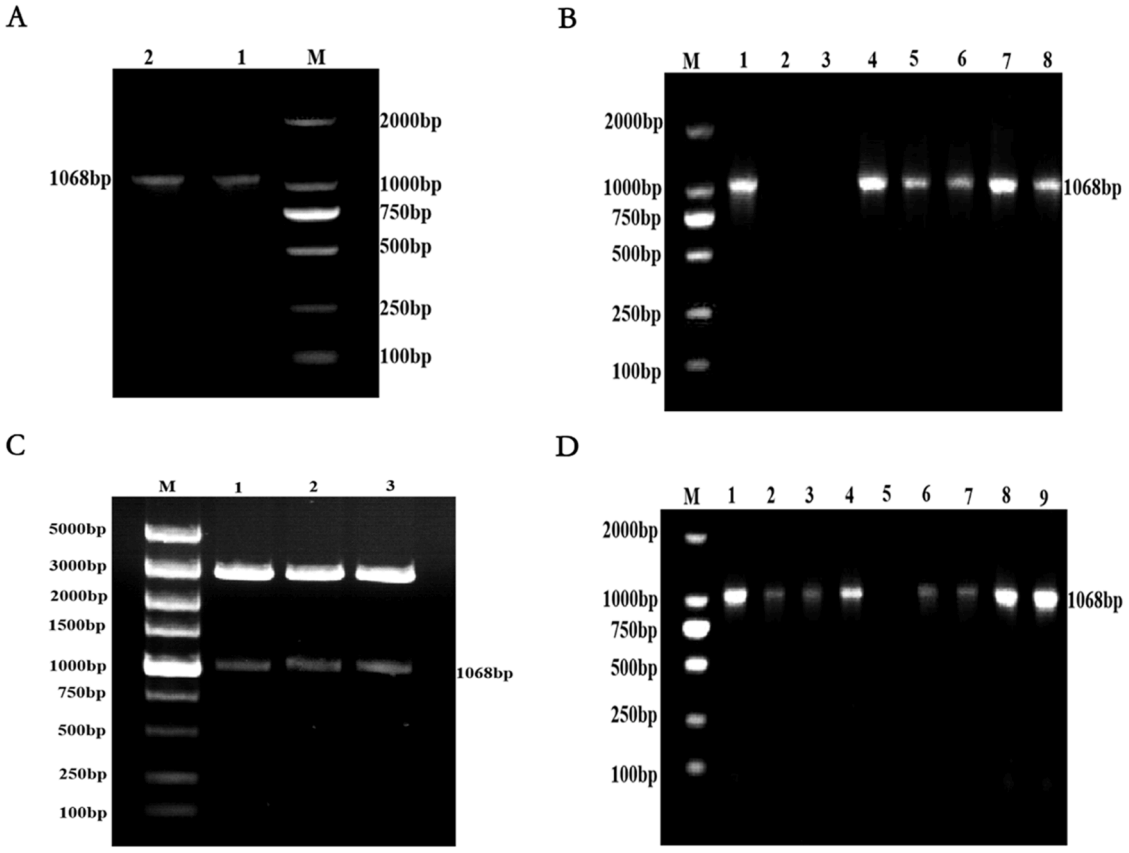

Figure 2. The construction of pVAX-ROP27 expression vector.
(A) *Et*ROP27 gene amplification. (B) The bacterial solution PCR results of pMD18T-ROP27. (C) The results of double enzyme digestion of pMD18T-ROP27. (D) The bacterial solution PCR results of pVAX-ROP27.

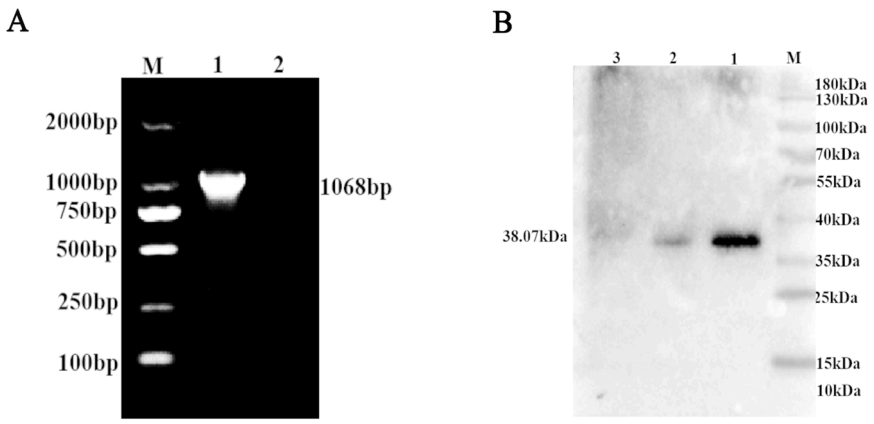

Figure 3. The expression of EtROP27 in vivo
(A): RT-PCR detection of ROP27 gene transcription in the pVAX-ROP27 injected muscle. M, DNA molecular weight marker DL2000; lane 1, pVAX-ROP27 injected muscle; lane 2, non-injected muscle. (B): Western blot detection of the ROP27 gene expression in the pVAX-ROP27 injected. M, protein molecular weight marker; lane 1, purified ROP27 protein; 2, pVAX-ROP27 injected muscle; lane 3, non-injected muscle.

Notably, the group receiving100 μg/feather of pVAX-ROP27 demonstrated the most pronounced effects, achieving an ACI of 179.80 (Table 1 and Fig. 4).

PVAX-ROP27 reduces histological damage in the cecum

The unchallenged group exhibited intact cecal glands, with no histopathological changes or necrosis observed (Fig. 5A). Conversely, the challenged group and the pVAX control group displayed significant damage to the cecal structure. Most intestinal villi were broken and disintegrated, whereas the intestinal glands showed necrosis and rupture. Numerous schizonts, appearing round or elliptical, were identified within the mucosal epithelial cells and intestinal adenocytes. Capillaries in the mucosal lamina propria and submucosa were markedly dilated and congested with an abundance of erythrocytes, leading to severe hemorrhaging and blood-filled contents that also contained numerous schizonts (Fig. 5B-C). In the 12.5μg and 25μg pVAX-ROP27 groups, the cecal structure appeared incomplete, characterized by widespread shedding of the chicken cecal mucosal epithelium. The intestinal glandular structure was extensively disrupted, with the intestinal villous epithelium and glandular cells filled with a large number of gametophytes and oocysts of various developmental stages. The intestinal contents contained oocysts of *E. tenella* at different developmental stages, along with blood, indicating a heavy infection (Fig. 5D-E). In the 50μg and 200μg pVAX-ROP27 groups, the intestinal mucosal epithelium exhibited local detachment and mild degeneration. Although the intestinal glandular structure remained intact, the lamina propria and submucosal capillaries exhibited dilation and bleeding. Additionally, a small number of oocysts at various developmental stages and gametocytes were observed in both the intestinal villous epithelial cells and glandular cells (Fig. 5F-H). In the 100μg pVAX-ROP27 group, the four layers of the cecum were clearly discernible, with intact intestinal villi and glands. A small amount of bleeding was noted in the submucosa. A very limited number of coccidian oocysts at various developmental stages and gametocytes were present in the epithelial cells of the intestinal villi and glandular cells, indicating a mild infection (Fig. 5G).

PVAX-ROP27 induces humoral and cellular immunity

As the number of immunizations increased, concentrations of IgG (Fig. 6 A), IgY (Fig. 6 B), IL-2 (Fig. 6 C), IFN-γ (Fig. 6 D), and IL-6 (Fig. 6 F) across all pVAX-ROP27 dose groups also exhibited a significant increase ($P < 0.05$), with the 100 μg dose group demonstrating the highest levels. In comparison to concentrations prior to the first immunization, the level of IL-10 (Fig. 6 E) significantly increased following the initial immunization in the 50 μg, 100 μg, and 200 μg pVAX-ROP27 groups .

Table 1
Evaluation of the protective efficacy of ROP 27

Group		Average weight gain	RWG %	survival rate %	average cecal lesion score	RLS %	OPG×10^4	Ooocyst reduction rate %	Bloody stools	ACI
Unchallenged group		300.44± 20.87^{Aa}	100^{Aa}	100	0^{Aa}	100.00^{Aa}	0^{Aa}	100.00^{Aa}	0.00	200
challenged group		175.80 ±9.18^{Bb}	58.51 ±3.06^{Bb}	60	3.50±0.53^{Bb}	0.00^{Bb}	247.50 ±13.42^{Bb}	0.00^{Bb}	3.00	73.51
Pvax control group		171.89 ±44.99^{Cb}	57.21 ±14.97^{Cb}	60	3.40±0.52^{Cb}	2.86 ±14.75^{Cb}	248.75 ±15.31^{Cb}	0.00^{Cb}	3.00	73.21
pVAX-ROP27 group	12.5μg	189.94 ±34.20^{Dc}	63.22 ±11.38^{Db}	90	2.70±0.48^{Dc}	22.86 ±13.80^{Dc}	117.50 ±21.04^{Dc}	52.53±8.50^{Dc}	2.00	125.22
	25μg	215.50 ±30.91^{Ed}	71.73 ±10.29^{Ec}	100	2.60±0.52^{Ed}	25.71 ±14.75^{Ed}	92.50 ±20.49^{Ed}	62.63±8.28^{Ed}	2.00	144.73
	50μg	248.18 ±53.13^{Fe}	82.61 ±17.68^{Fd}	100	1.40±0.52^{Fe}	60.00 ±14.75^{Fe}	67.50 ±15.73^{Fe}	72.73±6.36^{Ed}	1.00	167.60
	100μg	269.81 ±38.81^{Af}	89.80 ±12.92^{Ae}	100	0.90±0.32^{Gf}	74.29 ±9.04^{Gf}	50.00 ±22.08^{Gf}	79.80±10.22^{Gf}	1.00	179.80
	200μg	258.44 ±43.35^{Gg}	86.04 ±14.43^{Gf}	100	1.40±0.70^{Hg}	60.00 ±19.98^{Hg}	68.75 ±25.29^{Hg}	72.22±10.22^{Hg}	1.00	171.04

Note: The same capital letters represent non-significant differences compared with the unchallenged group ($P > 0.05$), and different capital letters represent significant differences compared with the unchallenged group ($P < 0.05$); the same lowercase letters represent non-significant differences compared with the challenged group ($P > 0.05$), and different lowercase letters represent significant differences compared with the challenge group ($P < 0.05$).

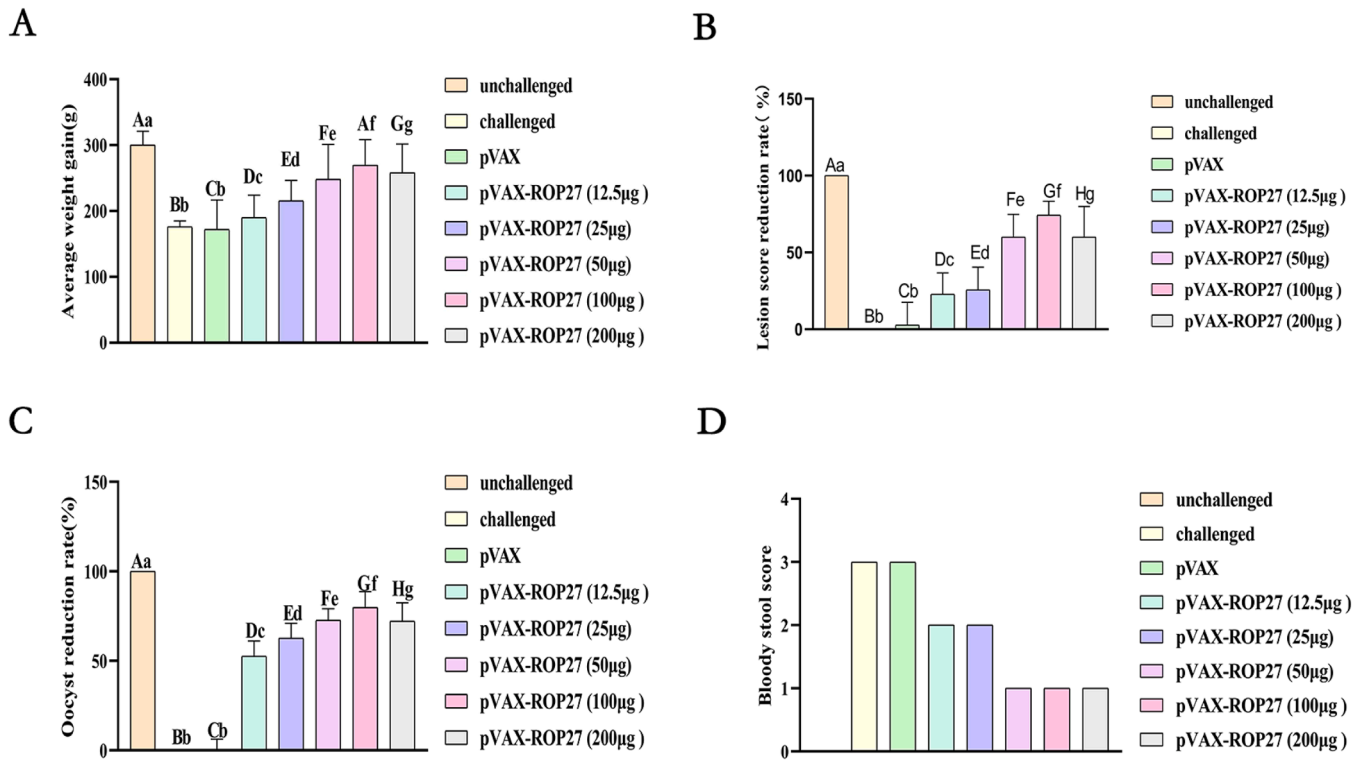

Figure 4. Effects of pVAX-ROP27 protein against the *E.tenella* challeng.
(A) Average weight gain; (B) Lesion score reduction rate; (C) Oocyst reduction rate; (D) Bloody stool score. The same capital letters represent non-significant differences compared with the unchallenged group ($P > 0.05$), and different capital letters represent significant differences compared with the unchallenged group ($P < 0.05$); the same lowercase letters represent non-significant differences compared with the challenged group ($P > 0.05$), and different lowercase letters represent significant differences compared with the challenge group ($P < 0.05$).

Similarly, the concentration of IL-4 significantly rose after the first immunization in the 25 μg, 50 μg, and 100 μg pVAX-ROP27 groups . Following the second immunization, no significant differences in IL-10 concentrations were observed in the 100 μg and 200 μg pVAX-ROP27 groups. Additionally, no significant differences were noted in IL-4 concentrations (Fig. 6 G) among the 25 μg, 50 μg, and 100 μg pVAX-ROP27 groups. In conclusion, pVAX-ROP27 mediates both cellular and humoral immunity, with a stronger emphasis on cellular immunity; the 100 μg dosage was identified as the optimal dose.

Discussion

The complex life cycle of *E. tenella* presents significant challenges in the prevention and control of chicken coccidiosis, resulting in considerable economic losses within the poultry industry (Mesa-Pineda, Navarro-Ruiz, Lopez-Osorio, Chaparro-Gutierrez, and Gomez-Osorio, 2021). The effectiveness of anticoccidial drugs is increasingly compromised by the emergence of drug-resistant strains and a rise in chemical residues (Witcombe and Smith, 2014). Within the field of parasitology, there is a growing consensus that immunization should take precedence over drug treatment for the prevention of coccidiosis. Therefore, the urgent challenge remains to identify a suitable vaccine for the prevention and control of chicken coccidiosis.

E. tenella is classified within the Apicomplexa protozoa group, which is characterized by an apical complex comprising an apical polar ring and specialized secretory organelles, including microfilaments, rhoptries, and dense granules (Olajide et al., 2022). Rhoptries, as the largest organelles of the apical complex, serve as crucial virulence factors in Apicomplexa protozoa. They play a vital role in the invasion of host cells, the formation of parasitophorous vacuoles **(PV)**, and the modulation of host cell functions (Chapman et al., 2013). Shi et al. constructed the r*Et*ROP21 recombinant protein and assessed its efficacy through animal experiments. The results indicated that chickens immunized with r*Et*ROP21 demonstrated a significant increase in average weight gain, a reduction in average lesion scores and oocyst output, and achieved an ACI of 163.2, highlighting that *Et*ROP21 elicits a robust immune response (Shi et al., 2023).

Li immunized chickens with varying doses of the r*Et*ROP27 recombinant protein and found that a medium dose elicited the optimal immune response, significantly reducing oocyst output and lesion scores whereas increasing average weight gain, achieving an ACI of 171.47 (Li, Lv, Zheng, and Wei, 2023). Therefore, *Et*ROP27 emerged as a promising candidate antigen for the development of a coccidiosis vaccine. In this study, a pVAX-ROP27 DNA vaccine was constructed. The results from RT-PCR and Western Blot analyses demonstrated that ROP27 could be effectively expressed in chickens. Different doses of pVAX-ROP27 were administered via intramuscular injection into the leg muscle of 14-day--old chickens, followed by a booster immunization at 21 days of age. Evaluation of its immunogenicity revealed that pVAX-ROP27 significantly increased average weight gain, reduced oocyst output, and improved conditions related to bloody stool and cecal lesions. Among the doses tested, 100 μg/feather proved to be the most effective, achieving an ACI of 179.80. Notably, there was a discernible difference in immune response between this and the r*Et*ROP27 recombinant protein. Zhao et al. constructed pET28a-*Et*SAG4 and pEGFP-N1-*Et*SAG4 vaccines and immunized animals using doses of 50 μg and 100 μg, respectively. The ACIs recorded were 155.89 and 163.77 for pET28a-*Et*SAG4, and 156.66 and 168.24 for pEGFP-N1-*Et*SAG4 (Zhao et al., 2020). Geng et al. developed subunit and DNA vaccines utilizing *Et*SAG6 and *Et*SAG15. Their results indicated that the DNA vaccines pEGFP-N1-*Et*SAG6 and pEGFP-N1-*Et*SAG15 provided more effective immune protection against *E.tenella* compared to the subunit vaccines r*Et*SAG6 and r*Et*SAG15 (Geng et al., 2022). These findings are in agreement with the results of the current study; however, further

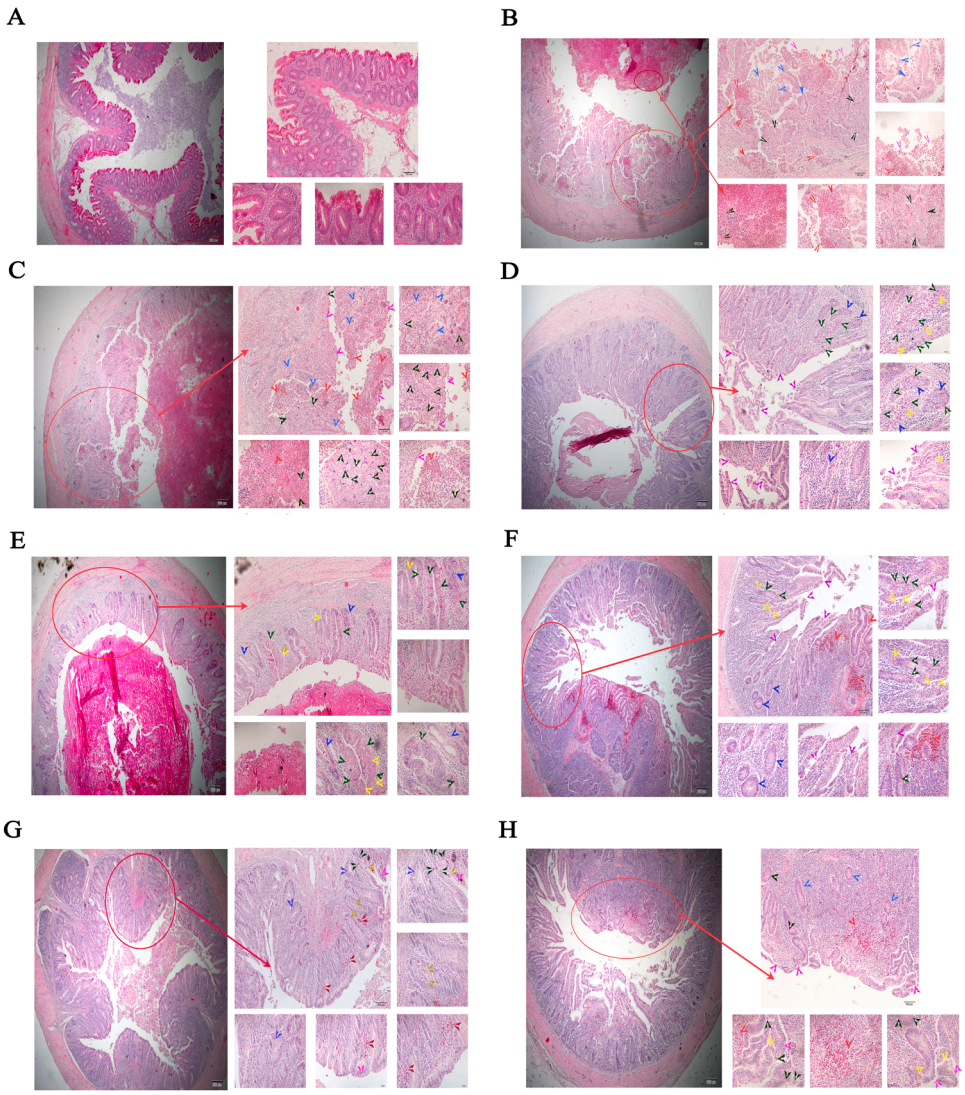

Figure 5. Histopathological sections of chicken cecum in each group.
(A) represent the unchallenge group; (B) represent the challenge group; (C) represent the pVAX control group; (D) represent the 12.5 μg pVAX-ROP27 group; (E) represent the 25 μg pVAX-ROP27 group; (F) represent the 50 μg pVAX-ROP27 group; (G) represent the 100 μg pVAX-ROP27 group; (H) represent the 200 μg pVAX-ROP27 group. Purple arrows represent damaged intestinal villi; yellow arrows represent coccidia at various stages of development; green arrows represent schizont; red arrows represent hemorrhagic erythrocytes; blue arrows represent ruptured intestinal glands.

exploration is necessary to understand the underlying mechanisms.

The histopathological results were consistent with those reported by Suprihati and Yunus, who observed numerous oocysts within the lamina propria of the cecum, severe hemorrhage, complete desquamation of the epithelium, and edema of the lamina muscularis following *E. tenella* infection (Suprihati and Yunus, 2018). In comparison to the control group, the structure of the cecum in the 100 μg pVAX-ROP27 group was intact, with only minimal lesions. These findings align with those reported by Desouky A. Y. and Chen H. L. (Chen et al., 2020; Desouky et al., 2021).

After the plasmid DNA of the DNA vaccine was ingested, the majority of the plasmid DNA associated with MHC I molecules, facilitating recognition by $CD8^+$ T cells. Under the stimulation of IL-2 and IFN-γ, $CD8^+$ T cells were activated, initiating cellular immunity. A smaller proportion of the plasmid DNA bound to MHC II molecules and was recognized by T helper **(Th)** cells. Under the influence of IL-4, these Th cells differentiated into activated Th2 cells, which were subsequently recognized by $CD4^+$ Th cell receptors, thereby activating humoral immunity (Bessay, et al., 1996; Gaghan, et al., 2022; Kundu, et al., 2017; Wallach, 2010). In this study, the results indicated that levels of IgG, IgY, IL-2, IFN-γ, and IL-6 in different pVAX-ROP27 dosage groups significantly increased with the number of immunizations ($P < 0.05$), with 100 μg proving to be the most effective dose. Notably, the concentrations of IL-2 and IFN-γ showed the most prominent changes. Furthermore, the levels of IL-10 and IL-4 increased significantly after immunization compared to pre-immunization levels, although they remained stable with subsequent immunizations.Zhao et al. inoculated chickens with the pEGFP-N1-EtSAG4 eukaryotic vector and observed that, compared to the control group, the concentrations of IgY, IFN-γ, and IL-17 were significantly increased, whereas levels of IL-4 and IL-10 showed no significant changes (Zhao et al., 2020). The study by Geriletu et al. demonstrated that expression levels of IL-2 and IFN-γ in the DNA vaccine immunization group were significantly higher than those in the control group (Geriletu, Xu, Xurihua, and Li, 2011). Zhang immunized animals with pVAX-ROP21 and found that levels of IgG and IFN-γ were significantly increased, whereas IL-4 and IL-10 levels showed no significant changes (Zhang et al., 2018). These findings are consistent with previously reported studies. Collectively, these results indicate that the pVAX-ROP27 DNA vaccine induces both cellular and humoral immunity, with cellular immunity being the dominant response.

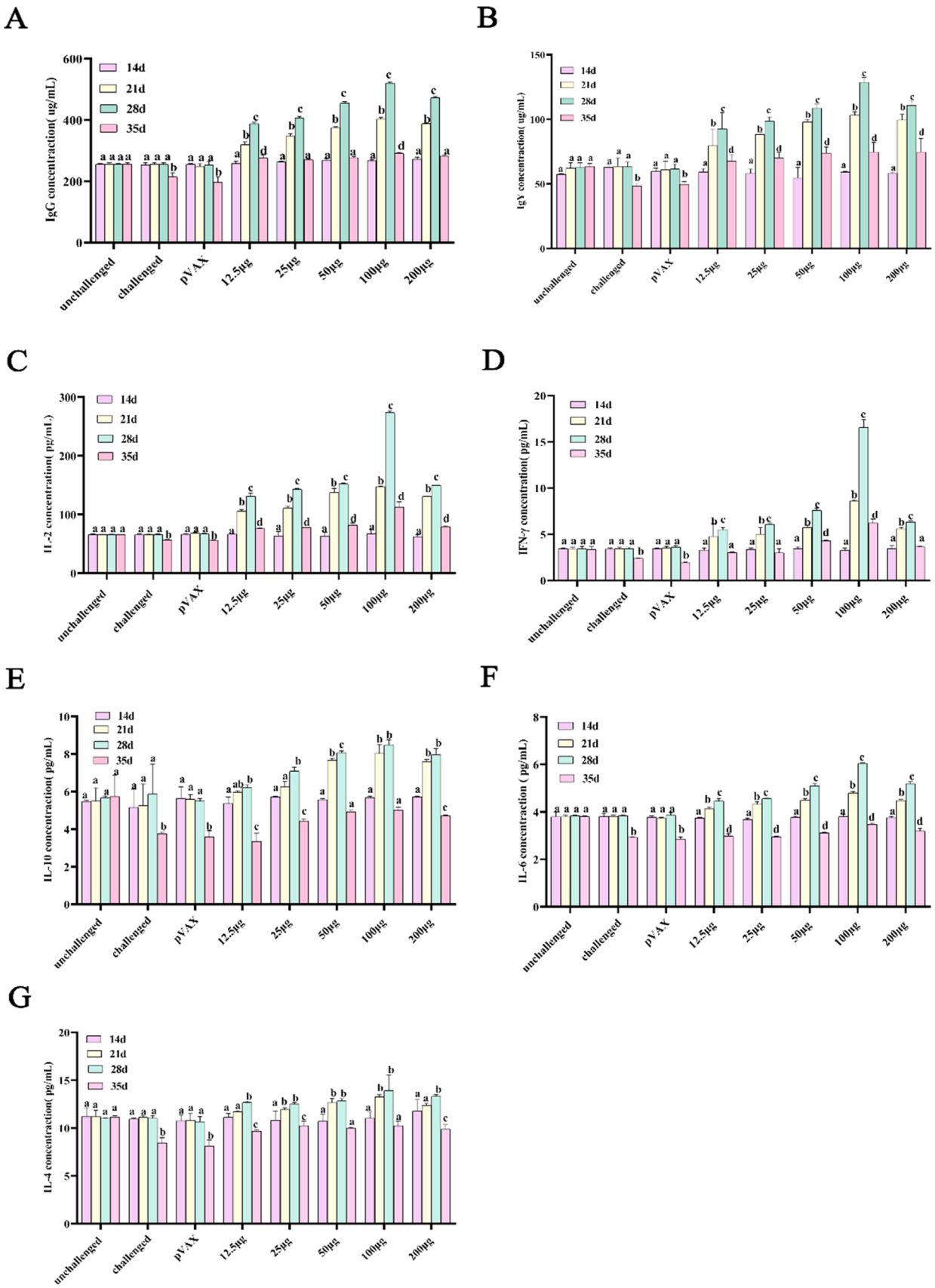

Figure 6. Concentration of serum antibodies and cytokines antibodies.
(A) IgG; (B) IgY; (C) IL-2; (D) IFN-γ; (E) IL-10; (F) IL-6; (G) IL-4. Different letters in the same group indicate significant differences ($P< 0.05$). Same letters in the same group indicate non-significant differences ($P> 0.05$).

The immune response elicited by a DNA vaccine can be significantly influenced by various factors, including the inoculation method, route, dosage, age at first immunization, and frequency of immunization. Notably, the route of DNA inoculation plays a critical role in determining the type of immune response generated; specifically, intramuscular and intradermal inoculations tend to induce Th1 responses, whereas epidermal injections are more likely to elicit Th2 responses. Fynan et al. conducted a comparative analysis of the immune effects associated with different DNA vaccination routes, such as intramuscular, intravenous, intranasal, intradermal, subcutaneous, and intraperitoneal injections. Their findings demonstrated that, with the exception of intraperitoneal injection, all other routes conferred some degree of protective effect, with intramuscular and intravenous injections yielding the most favorable results (Fynan et al., 1993). Muscle cells exhibit a DNA uptake and expression capacity that is 100 to 1,000 times greater than that of other tissues. Song et al. optimized the immunization regimen for the pcDNA-TA4-IL-2 DNA vaccine by testing doses of 25, 50, 100, and 200 μg/feather. Their results indicated that the most effective immune response was achieved with a dose of 25 μg/feather. The study further compared the effects of subcutaneous injection, oral administration, intravenous injection, intramuscular injection, and intranasal administration, revealing that the ACI for intramuscular injection reached 193.97, which significantly outperformed other immunization methods (Song et al., 2009). Therefore, this study opted to administer the vaccine via intramuscular injection into the thigh. The optimal inoculation doses can vary among different DNA vaccines. To determine the ideal dose of pVAX-ROP27, this study evaluated doses of 12.5, 25, 50, 100, and 200 μg, identifying 100 μg as the most effective dose. The study also found that as the immune dose increased, the immune effect correspondingly improved. However, it was noted that excessively high doses could lead to a diminished immune response, consistent with findings from previous research. Although the precise mechanism behind this phenomenon remains unclear, it is hypothesized that an increase in immunization dose, with a constant injection volume, leads to increased protein expression up to a certain saturation threshold, beyond which immune efficacy declines. Similar findings have been documented in other studies. For instance, Zhang et al. investigated the immune effects of the pVAX1-pEtK2-IL-2 DNA vaccine with doses of 25 μg, 50 μg, 80 μg, and 100 μg, concluding that 80 μg was the most effective dosage (Zhang et al., 2019). In another study, Zhao et al. examined the immune protective effects of recombinant protein or DNA vaccines at doses of 50 μg or 100 μg *in vivo*, determining that the pEGFP-N1-EtSAG4-16-22 plasmid at 50 μg exhibited the highest ACI at 173.11 (Zhao et al., 2021).

Conclusion

The construction of the DNA vaccine pVAX-ROP27 was successfully achieved in this study. *In vivo* expression and animal experiments demonstrated that pVAX-ROP27 induced both humoral and cellular immunity, exhibiting strong immunogenic properties. These findings establish a foundation for selecting DNA vaccine antigens and present a novel strategy for preventing chicken coccidiosis and developing related vaccines.

However, this study has certain limitations. It focused solely on the optimal immunization dose of the DNA vaccine. Further investigation is necessary to understand the immune mechanisms and determine the optimal immunization regimen. Additionally, the safety and stability of DNA vaccines for clinical use remain uncertain. Furthermore, the presence of numerous species of chicken coccidia with complex life cycles, each possessing specific antigens, poses a challenge. A single-antigen DNA vaccine may not fulfill market demands; therefore, it is essential to select protective antigens from different life stages or species of coccidia to construct a multivalent vaccine that elicits more effective immune protection. Therefore, the screening of additional candidate vaccine antigens remains a critical challenge for the advancement of DNA vaccines. Further research in this field is required.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104955.

References

Attia, M.M., Mohamed, R.I., Salem, H.M., 2023. Impact of Eimeria tenella experimental infection on intestinal and splenic reaction of broiler chickens. J. Parasit. Dis. 47, 829–836. https://doi.org/10.1007/s12639-023-01629-z.

Badri, M., Olfatifar, M., Hayati, A., Bijani, B., Samimi, R., Abdoli, A., Nowak, O., Diaz, D., Eslahi, A.V., 2024. The global prevalence and associated risk factors of Eimeria infection in domestic chickens: a systematic review and meta-analysis. Vet. Med. Sci. 10, e1469. https://doi.org/10.1002/vms3.1469.

Bessay, M., Le Vern, Y., Kerboeuf, D., Yvoré, P., Quéré, P., 1996. Changes in intestinal intra-epithelial and systemic T-cell subpopulations after an Eimeria infection in chickens: comparative study between E acervulina and E tenella. Vet. Res. 27, 503–514.

Bingxiang, W., Ningning, Z., Yakun, W., Lingyu, S., Hongmei, L., Zhang, X., Zhao, X., 2022. Characterization of the Eimeria tenella rhoptry protein with a nuclear localization sequence (EtROP30). Parasitol. Res. 121, 1507–1516. https://doi.org/10.1007/s00436-022-07499-3.

Blake, D.P., Knox, J., Dehaeck, B., Huntington, B., Rathinam, T., Ravipati, V., Ayoade, S., Gilbert, W., Adebambo, A.O., Jatau, I.D., Raman, M., Parker, D., Rushton, J., Tomley, F.M., 2020. Re-calculating the cost of coccidiosis in chickens. Vet. Res. 51, 115. https://doi.org/10.1186/s13567-020-00837-2.

Burrell, A., Tomley, F.M., Vaughan, S., Marugan-Hernandez, V., 2020. Life cycle stages, specific organelles and invasion mechanisms of Eimeria species. Parasitology. 147, 263–278. https://doi.org/10.1017/S0031182019001562.

Chapman, H.D., 1998. Evaluation of the efficacy of anticoccidial drugs against Eimeria species in the fowl. Int. J. Parasitol. 28, 1141–1144. https://doi.org/10.1016/s0020-7519(98)00024-1.

Chapman, H.D., Barta, J.R., Blake, D., Gruber, A., Jenkins, M., Smith, N.C., Suo, X., Tomley, F.M., 2013. A selective review of advances in coccidiosis research. Adv. Parasitol. 83, 93–171. https://doi.org/10.1016/B978-0-12-407705-8.00002-1.

Chen, C., Su, J., Lu, M., Xu, L., Yan, R., Li, X., Song, X., 2023. Protective efficacy of multiepitope vaccines constructed from common antigens of Eimeria species in chickens. Vet. Res. 54, 119. https://doi.org/10.1186/s13567-023-01253-y.

Chen, H.L., Zhao, X.Y., Zhao, G.X., Huang, H.B., Li, H.R., Shi, C.W., Yang, W.T., Jiang, Y. L., Wang, J.Z., Ye, L.P., Zhao, Q., Wang, C.F., Yang, G.L., 2020. Dissection of the cecal microbial community in chickens after Eimeria tenella infection. Parasit. Vectors. 13, 56. https://doi.org/10.1186/s13071-020-3897-6.

Desouky, A.Y., Ammar, N.I., Mohamed, A.M., Elhawary, N.M., Sultan, K., 2021. Immunological and histopathological evaluation of Eimeria tenella oocysts Egyptian local isolate vaccine and its comparative efficacy with a commercial live vaccine. Trop. Biomed. 38, 8–13. https://doi.org/10.47665/tb.38.1.002.

Fatoba, A.J., Adeleke, M.A., 2018. Diagnosis and control of chicken coccidiosis: a recent update. J. Parasit. Dis. 42, 483–493. https://doi.org/10.1007/s12639-018-1048-1.

Fynan, E., Robinson, H., Webster, R., 1993. Use of DNA encoding influenza hemagglutinin as an avian influenza vaccine. DNA Cell Biol. 12, 785–789.

Gaghan, C., Adams, D., Mohammed, J., Crespo, R., Livingston, K., Kulkarni, R.R., 2022. Characterization of vaccine-induced immune responses against coccidiosis in broiler chickens. Vaccine 40, 3893–3902. https://doi.org/10.1016/j.vaccine.2022.05.043.

Gao, Y., Sun, P., Hu, D., Tang, X., Zhang, S., Shi, F., Yan, X., Yan, W., Shi, T., Wang, S., Zou, J., Yin, G., Liu, X., Dong, H., Suo, X., 2024. Advancements in understanding chicken coccidiosis: from Eimeria biology to innovative control strategies. One Health Adv. 2, 6. https://doi.org/10.1186/s44280-024-00039-x.

Geng, T., Luo, L., Wang, Y., Shen, B., Fang, R., Hu, M., Zhao, J., Zhou, Y., 2022. Evaluation of immunoprotective effects of recombinant proteins and DNA vaccines derived from Eimeria tenella surface antigen 6 and 15 in vivo. Parasitol. Res. 121, 235–243.

Geriletu, L., Xurihua, X., Li, X., 2011. Vaccination of chickens with DNA vaccine expressing Eimeria tenella MZ5-7 against coccidiosis. Vet. Parasitol. 177, 6–12. https://doi.org/10.1016/j.vetpar.2010.11.041.

Johnson, J., Reid, W.M., 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.

Kundu, K., Garg, R., Kumar, S., Mandal, M., Tomley, F.M., Blake, D.P., Banerjee, P.S., 2017. Humoral and cytokine response elicited during immunisation with recombinant Immune Mapped protein-1 (EtIMP-1) and oocysts of Eimeria tenella. Vet. Parasitol. 244, 44–53. https://doi.org/10.1016/j.vetpar.2017.07.025.

Li, M., Lv, X., Zheng, M., Wei, Y., 2023. The immunoprotective effect of ROP27 protein of Eimeria tenella. Animals. (Basel) 13. https://doi.org/10.3390/ani13223500.

Lillehoj, H.S., Ruff, M.D., 1987. Comparison of disease susceptibility and subclass-specific antibody response in SC and FP chickens experimentally inoculated with Eimeria tenella, E. acervulina, or E. maxima. Avian Dis. 31, 112–119.

Lin, R.Q., Lillehoj, H.S., Lee, S.K., Oh, S., Panebra, A., Lillehoj, E.P., 2017. Vaccination with Eimeria tenella elongation factor-1alpha recombinant protein induces protective immunity against E. tenella and E. maxima infections. Vet. Parasitol. 243, 79–84. https://doi.org/10.1016/j.vetpar.2017.06.003.

Liu, X., Mu, B., Zheng, W., Meng, Y., Yu, L., Gao, W., Zhu, X., Liu, Q., 2022. Identification and Protective efficacy of Eimeria tenella Rhoptry Kinase Family protein 17. Animals. (Basel) 12. https://doi.org/10.3390/ani12050556.

Long, P.L., Millard, B.J., Joyner, L.P., Norton, C.C., 1976. A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. Folia Vet. Lat. 6, 201–217.

Mesa-Pineda, C., Navarro-Ruiz, J.L., Lopez-Osorio, S., Chaparro-Gutierrez, J.J., Gomez-Osorio, L.M., 2021. Chicken coccidiosis: from the parasite lifecycle to control of the disease. Front. Vet. Sci. 8, 787653. https://doi.org/10.3389/fvets.2021.787653.

Oakes, R.D., Kurian, D., Bromley, E., Ward, C., Lal, K., Blake, D.P., Reid, A.J., Pain, A., Sinden, R.E., Wastling, J.M., Tomley, F.M., 2013. The rhoptry proteome of Eimeria tenella sporozoites. Int. J. Parasitol. 43, 181–188. https://doi.org/10.1016/j.ijpara.2012.10.024.

Olajide, J.S., Qu, Z., Yang, S., Oyelade, O.J., Cai, J., 2022. Eimeria proteins: order amidst disorder. Parasit. Vectors. 15, 38. https://doi.org/10.1186/s13071-022-05159-0.

Shi, T.Y., Zhou, S.H., Kong, Y.R., Fu, Y., Liu, Y., Yan, W.C., Zhou, Y.X., Zhang, L., Hao, L. L., Sun, H.C., 2023. A rhoptry protein, localizing in the bulb region of rhoptries, could induce protective immunity against Eimeria tenella infection. Front. Immunol. 14, 1277955. https://doi.org/10.3389/fimmu.2023.1277955.

Shi, W., Liu, Q., Zhang, J., Sun, J., Jiang, X., Geng, J., Wang, F., Xiao, Y., Li, H., Zhao, X., 2014. Co-expression of EtMic2 protein and chicken interleukin-18 for DNA vaccine against chicken coccidiosis. Res. Vet. Sci. 97, 64–70. https://doi.org/10.1016/j.rvsc.2014.05.001.

Shirley, M.W., Smith, A.L., Tomley, F.M., 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.

Song, X., Xu, L., Yan, R., Huang, X., Shah, M.A., 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.

Suprihati, E., Yunus, M., 2018. Evaluation of the antigenicity and immunogenicity of Eimeria tenella by reproductive index and histopathological changes of cecal coccidiosis virulent live vaccine in broiler chickens. Afr. J. Infect. Dis. 12, 104–110. https://doi.org/10.2101/Ajid.12v1S.15.

Venkatas, J., Adeleke, M.A., 2019. A review of Eimeria antigen identification for the development of novel anticoccidial vaccines. Parasitol. Res. 118, 1701–1710. https://doi.org/10.1007/s00436-019-06338-2.

Vermeulen, A.N., Schaap, D.C., Schetters, T.P., 2001. Control of coccidiosis in chickens by vaccination. Vet. Parasitol. 100, 13–20. https://doi.org/10.1016/s0304-4017(01)00479-4.

Wallach, M., 2010. Role of antibody in immunity and control of chicken coccidiosis. Trends. Parasitol. 26, 382–387. https://doi.org/10.1016/j.pt.2010.04.004.

Wang, B., Zhao, N., Sun, J., Sun, L., Li, H., Wu, Z., Li, H., Zhang, X., Zhao, X., 2022. Identification and characterization of Eimeria tenella rhoptry protein 35 (EtROP35). Vet. Sci. 9. https://doi.org/10.3390/vetsci9090465.

Witcombe, D.M., Smith, N.C., 2014. Strategies for anti-coccidial prophylaxis. Parasitology. 141, 1379–1389. https://doi.org/10.1017/S0031182014000195.

Xu, Q., Song, X., Xu, L., Yan, R., Shah, M.A., 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.

Zhang, B., Yuan, C., Song, X., Xu, L., Yan, R., Shah, M.A.A., Guo, C., Zhu, S., Li, X., 2019. Optimization of immunization procedure for Eimeria tenella DNA vaccine pVAX1-pEtK2-IL-2 and its stability. Acta Parasitol. 64, 745–752. https://doi.org/10.2478/s11686-019-00090-4.

Zhang, Z., Li, Y., Wang, M., Xie, Q., Li, P., Zuo, S., Kong, L., Wang, C., Wang, S., 2018. Immune protection of Rhoptry protein 21 (ROP21) of toxoplasma gondii as a DNA vaccine against toxoplasmosis. Front. Microbiol. 9, 909. https://doi.org/10.3389/fmicb.2018.00909.

Zhao, P., Li, Y., Zhou, Y., Zhao, J., Fang, R., 2020. In vivo immunoprotective comparison between recombinant protein and DNA vaccine of Eimeria tenella surface antigen 4. Vet. Parasitol. 278, 109032.

Zhao, P., Wang, C., Ding, J., Zhao, C., Xia, Y., Hu, Y., Zhang, L., Zhou, Y., Zhao, J., Fang, R., 2021. Evaluation of immunoprotective effects of recombinant protein and DNA vaccine based on Eimeria tenella surface antigen 16 and 22 in vivo. Parasitol. Res. 120, 1861–1871. https://doi.org/10.1007/s00436-021-07105-y.