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Eimeria necatrix Infection in Chickens: Protective Immunity Induced by Immunization with the Recombinant Gametocyte Antigen EnGAM22

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ABSTRACT

***Background*:** Coccidiosis is a significant parasitic disease in poultry, leading to considerable economic losses. The macrogametocyte stage of *Eimeria* species has been identified as a potential target for protective immunity. Previous studies have indicated that antigens derived from this stage can provide immunity against coccidiosis. The objective of the study was to evaluate the effects of recombinant EnGAM22 vaccination on resistance to experimental *E. necatrix* infection and correlated disease resistance with humoral and cell-mediated immunity in Chickens.

***Materials, Methods & Results*:** To evaluate the immunogenicity of the recombinant protein rEnGAM22, chickens were subcutaneously injected with 25, 50, or 75 µg of the protein along with Freund's adjuvant. Post-immunization, the chickens were challenged orally with sporulated *Eimeria necatrix* oocysts. The effectiveness of the vaccine was measured through various parameters, including fecal oocyst output, lesion scores, body weight gain, serum antibody levels, and cytokine responses. Chickens vaccinated with 50 µg of rEnGAM22 and subsequently challenged with sporulated *E. necatrix* oocysts showed reduced fecal oocyst shedding and lesion scores compared to other immunized groups and the infected control group, with the exception of the live oocyst group. There was no significant difference in body weight between the immunized groups and the infected control group. Additionally, rEnGAM22 induced higher levels of anti-rEnGAM22 serum antibodies, particularly 7 days after secondary immunization, especially in the 50 µg group. Serum levels of IL-2, IL-4, IL-10, and IFN-γ were highest in the group receiving 50 µg of rEnGAM22, with IL-2 and IL-4 showing a stronger response compared to IL-10 and IFN-γ. These findings suggest that rEnGAM22 provides some level of protection against *E. necatrix* infection and could be considered for developing a recombinant subunit vaccine against coccidiosis.

***Discussion*:** The findings of this study demonstrate that the recombinant protein rEnGAM22 from *Eimeria necatrix* gametocytes has the potential to confer protective immunity against coccidiosis in poultry, particularly at a dosage of 50 µg. The reduction in fecal oocyst shedding and lesion scores in chickens vaccinated with 50 µg of rEnGAM22 suggests that this dosage is optimal for eliciting an effective immune response. These results are consistent with the reported protective role of macrogametocyte stage antigens in previous studies, reinforcing the potential of targeting this stage in the lifecycle of *Eimeria* species for vaccine development. The study also highlights the ability of rEnGAM22 to induce a strong humoral immune response, as evidenced by the increased production of anti-rEnGAM22 serum antibodies. The significant rise in antibody levels 7 days after secondary immunization, particularly with the 50 µg dose, indicates high immunogenicity and memory response induction. This is further supported by elevated cytokine levels, especially IL-2 and IL-4, markers of Th1 and Th2 immune responses. The dominance of IL-2 and IL-4 over IL-10 and IFN-γ suggests a balanced Th1/Th2 response, which is crucial for effective immunity against intracellular parasites like *Eimeria*. Interestingly, there was no significant difference in body weight between the immunized groups and the infected control group, indicating that while rEnGAM22 vaccination reduces parasite load and tissue damage, it does not completely prevent the impact of infection on growth performance. This suggests that while rEnGAM22 offers some protection, it may need to be combined with other antigens or adjuvants to improve efficacy and reduce the impact of infection on poultry production. The study highlights the potential of rEnGAM22 as a recombinant subunit vaccine candidate for coccidiosis, but further research is required to optimize formulation, dosage, and assess long-term protection, including combining it with other immunogenic proteins for broader protection against multiple *Eimeria* species.

Keywords: coccidiosis, *Eimeria necatrix*, gametocyte antigen, immunization, cytokines.

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INTRODUCTION

Coccidiosis has severe economic impacts on the commercial poultry industry worldwide, with global costs in excess of £10.36 billion in 2016 [3]. While prophylactic medications have been effective, drug-resistant parasites are emerging, necessitating alternative control strategies [4]. Vaccination with live vaccines, using oocysts from wild-type or attenuated parasites, is the leading alternative, but it has limitations, including pathogen spread, antigenic variation, and high production costs [6,27,14,11]. Thus, new immunological approaches for the control of this disease are urgently needed. Subunit vaccines derived from intrinsic parasitic antigens or recombinant proteins from cloned DNA may overcome these difficulties [22]. To date, only one subunit vaccine against coccidiosis in chickens, CoxAbic®, has reached the commercial market.

Eimeria necatrix is the 2nd most pathogenic species after *E. tenella* [12], mainly infects chickens 8 weeks and older, causes high mortality on free ranges, and increases coccidiosis. In this research, the gametocyte protein EnGAM22 from *E. necatrix* was analyzed. EnGAM22 is a gametocyte protein involved in oocyst wall formation in *E. necatrix*[15,25]. In our previous study, we found that recombinant EnGAM22 protein expressed in a bacterial expression vector was recognized not only by convalescent serum from *E. necatrix*-infected chickens but also by that from *E. tenella*- and *E. maxima*-infected chickens[15]. In the current study, we assessed the effects of recombinant EnGAM22 vaccination on resistance to experimental *E. necatrix* infection and correlated disease resistance with humoral and cell-mediated immunity.

MATERIALS AND METHODS

Animals and parasites

One-day-old Suqin yellow chickens were purchased from the Poultry Institute, Chinese Academy of Agricultural Sciences[1], bred in a coccidian-free environment, and provided with water and food ad libitum. The *E. necatrix* strain originally isolated and maintained in our laboratory was propagated by oral passage in 3-4-week-old chickens. Feces were collected on 7-12 days post-infection (PI), and sporulated oocysts were purified and stored at 4°C in 2.5% potassium dichromate until required[15].

Production of recombinant EnGAM22

Recombinant EnGAM22 (rEnGAM22) with no signal peptide was produced in *Escherichia coli* BL21 (DE3)[2] as a fusion product with an N-terminal tag of 6 histidines. Expression of the recombinant protein was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)[3], and the protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column[4] according to our previous study [15]. Endotoxin was depleted by ToxinEraser endotoxin removal kit[4].

Immunization and challenge

The experimental design is illustrated in Table 1. Chickens were assigned to 1 of 7 groups (n=15 per group): rEnGAM22 protein immunized (25 μg, 50 μg, 75 μg), adjuvant control, live oocyst, infected and phosphate buffered saline (PBS) control. At 9 days of age, rEnGAM22 protein immunized groups were subcutaneously immunized with 0.1 mL recombinant protein (250, 500, or 750 μg/mL) in an equal volume of Freund's complete adjuvant (FCA)[5]. The adjuvant control group was given 0.1 mL PBS in an equal volume of FCA. The live oocyst group was orally immunized with 1,000 sporulated oocysts. At 16 days of age, animals in rEnGAM22 protein immunized groups were given a booster injection with 0.1 mL recombinant protein (250, 500, or 750 μg/mL), and the adjuvant control group was given 0.1 mL PBS in an equal volume of Freund's incomplete adjuvant (FIA)[5].

Seven days after the 2nd immunization (23 days of age), each group was divided into 2 subgroups. One subgroup (A, n=10) was orally given 25,000 sporulated oocysts, whereas the 2nd subgroup (B, n=5) was given 5,000 sporulated oocysts. For subgroup A, body weight gain was measured between the day of the 1st immunization and the day before infection (9-23 days of age). Body weight gain was measured a 2nd time between 0-8 days PI (23-31 days of age). Lesion scores were assessed on day 8 PI (31 days of age) on a scale [from 0 to 4] by 2 independent observers according to the Johnson and Reid method [12]. For subgroup B, fecal oocyst shedding was measured between days 7 and 17 PI (30-40 days of age) using the McMaster counting method [24].

Detection of serum antibody levels

Blood was collected from 5 randomly-selected chickens from each group on the day before the

1st immunization (9 days of age), 7 days following primary immunization (16 days of age), and 7 days following secondary immunization (23 days of age). Blood was stored at 4°C for 4 h and serum was obtained by centrifugation at 2,500 *g* for 15 min. Indirect enzyme-linked immunosorbent assays (ELISAs) were employed to detect IgY antibodies against EnGAM22. Briefly, 96 well plates were coated with 1 μg/well of purified rEnGAM22 overnight at 4°C. Plates were washed with 0.05% Tween-20/PBS (PBST) 3 times for 15 min prior to being blocked with 1.0% bovine serum albumin (BSA)5 in PBS for 1.5 h at 37°C. After blocking, diluted serum (1:200) was added and incubated at 37°C for 1.5 h. After washing 3 times with PBST, the combined antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-chicken IgY (H + L) antibody (dilution: 1:20,000)[4] at an optical density at 450 nm (OD450) using an ELIASA[6]. All samples were run in triplicate.

Determination of serum cytokine levels

The cytokines interleukin (IL)-2, IL-4, IL-10 and interferon (IFN)-γ in the serum of 5 chickens from each group were quantified on the day before immunization (9 days of age) as well as 7 days after both primary and secondary immunization using commercial IL detection kits[7] according to the manufacturer's instructions. All serum samples were run in triplicate.

Statistical analysis

Data were analyzed using a one-way analysis of variance with SPSS 22.0 software for Windows[8]. Treatment mean values were compared using Duncan's multiple range tests. Differences were considered to be significant at $P < 0.05$.

RESULTS

Expression and western blot analysis of rEnGAM22

The expressed rEnGAM22 fusion protein was purified using Ni-NTA affinity purification column as previously described [15], and was found to be approximately 29 kDa, which differed from the calculated value (23.3 kDa). This phenomenon has been explained in our previous research [15]. The fusion protein was recognized by mouse anti-6×His monoclonal antibody (Figure 1A), convalescent chicken sera (Figure 1B), and mouse anti-rEnGAM22 polyclonal antibodies (Figure 1C). After being concentrated, the final concentration of the purified protein was 10.0 mg/L. The encoding sequence of EnGAM22 was submitted to GenBank in our previous research [15], and the accession number is KF649255.

Protective efficacy of rEnGAM22

We evaluated the immune protective effects of rEnGAM22 using parasitological criteria (oocyst excretion and intestinal lesion scores) and growth performance (Table 2, Figure 2). Chickens immunized with 50 μg rEnGAM22 had reduced fecal oocyst shedding between days 7 and 17 PI compared with other immunized groups and the infected control group, although the greatest reduction was recorded in the live vaccine group. Neither 75 μg nor 25 μg of rEnGAM22 were effective in decreasing the number of fecal oocysts (Figure 2A). Similarly, chickens vaccinated with 50 μg rEnGAM22 demonstrated reduced lesion scores compared with other immunized and non-immunized groups, and only slightly higher scores than the live vaccine group (Figure 2B). These results are similar to previous research on GAM82, which showed that a lower dose (30 μg) reduced intestinal lesion scores [14]. For 1st weight gain (9-23 days of age), there were no differences among groups, which illustrated that the addition of adjuvant had no negative effect on the chickens. For second weight gains (23-31 days of age), there was no difference in the body weight of any group compared to that of the infected control group. In contrast, increased weight gain was observed in group immunized with live oocysts (Figure 2C).

Antibody (IgY) response against rEnGAM22

Serum antibody levels were measured using indirect ELISA. No antibody was detected in any group the day before immunization (data not shown). Similarly, there was no obvious difference between groups 7 days after primary immunization. However, 7 days after secondary immunization, chickens immunized with rEnGAM22 showed elevated serum antibody levels compared with non-immunized groups ($P < 0.05$), and the group immunized with 50 μg rEnGAM22 had the highest levels (Figure 3).

Serum cytokine response to rEnGAM22

IL-2 responses to immunization are shown in Figure 4. At 7 days following primary immunization (16 days of age), IL-2 levels were significantly increased in each group, the group immunized with 75

μg rEnGAM22 had the highest levels, and there was no significant difference between the group immunized with 50 μg rEnGAM22 and the group given the live vaccine. At 7 days following secondary immunization (23 days of age), the group immunized with 50 μg rEnGAM22 had the highest levels of IL-2, which were significantly higher ($P < 0.5$) than those of the other groups immunized with protein but were not significantly different from those of the group given the live vaccine. No difference was detected between the 2 control groups (infected and PBS control).

IL-4 responses to immunization are shown in Figure 4. At 7 days following primary immunization (16 days of age), IL-4 levels were significantly elevated in every group. Levels were highest in the group immunized with 50 μg rEnGAM22, but there was no significant difference between the groups immunized with 50 or 75 μg rEnGAM22. At 7 days following secondary immunization (23 days of age), the group immunized with 50 μg rEnGAM22 had the highest levels of IL-4, although they were similar to the groups immunized with 75 μg rEnGAM22 and the live vaccine.

IL-10 responses to immunization are shown in Figure 4. The most significant response was observed in the group immunized with 50 μg rEnGAM22 ($P < 0.5$) 7 days following primary immunization. Although the group immunized with live vaccine showed the highest response 7 days following secondary immunization, there was no significant difference between this group and the group immunized with 50 μg rEnGAM22.

IFN-γ responses to immunization are shown in Figure 4. IFN-γ levels were significantly elevated in every group 7 days following primary and secondary immunization, although the group immunized with 50 μg rEnGAM22 showed the most significant response.

Overall, there was no difference in the 4 serum cytokines the day before immunization (9 days of age). The group immunized with 25 μg rEnGAM22 and the adjuvant control group had the lowest cytokine levels overall. The group immunized with 50 μg rEnGAM22 showed the greatest immune response with the highest concentration of the 4 cytokines ($P < 0.5$) but had similar levels of IL-2, IL-4, IL-10, and IFN-γ to the live vaccine group and similar levels of IL-2 and IL-4 to the group immunized with 75 μg rEnGAM22. Of these 4 cytokines, the IL-2 and IL-4 responses were greater than those of IL-10 and IFN-γ.

Table 1. Experimental design and immunization schedule.

Groups	No. of chicken		primary immunization (d)	Secondary immunization (d)	Immunization route	Infection days (d)	1st weight gain (d)	2nd weight gain (d)	Infection dose	
	Group A	Group B							Group A	Group B
75 μg	10	5	9	16	Subcutaneous injection	23	9~23	23~31	2.5×10^4	0.5×10^4
50 μg	10	5	9	16	Subcutaneous injection	23	9~23	23~31	2.5×10^4	0.5×10^4
25 μg	10	5	9	16	Subcutaneous injection	23	9~23	23~31	2.5×10^4	0.5×10^4
Adjuvant control	10	5	9	16	Subcutaneous injection	23	9~23	23~31	2.5×10^4	0.5×10^4
Live vaccine	10	5	9	/	Oral gavage	23	9~23	23~31	2.5×10^4	0.5×10^4
Infected control	10	5	/	/	/	23	9~23	23~31	2.5×10^4	0.5×10^4
PBS control	10	5	/	/	/	/	9~23	23~31	/	/

Note: "/"means no items.

Table 2. Effects of immunoprotection of rEnGAM22 on 3 parameters of disease resistance.

Groups	1st average weight gain (g)	1st relative weight gain (%)	2nd average weight gain (g)	2nd relative weight gain (%)	Servival rate (%)	Average lesion score (mean ± SD)	Oocyst output per bird	Oocyst reduction (%)
75 μg	183.71 ± 2.86^{a}	114.2	34.87 ± 9.22^{a}	25.9	100.0	2.45 ± 0.45^{bc}	7.94×10^{7}	-9.4§
50 μg	170.89 ± 3.11^{a}	99.9	42.85 ± 7.37^{a}	28.7	100.0	2.40 ± 0.44^{bc}	5.75×10^{7}	20.8
25 μg	177.03 ± 3.80^{a}	110.0	42.44 ± 5.56^{ab}	31.6	90.0	3.00 ± 0.36^{bc}	7.99×10^{7}	-10.1§
Adjuvant control	175.72 ± 2.29^{a}	109.2	45.01 ± 4.75^{ab}	33.5	90.0	3.40 ± 0.28^{c}	7.78×10^{7}	-10.7§
Live oocyst	171.71 ± 3.98^{a}	106.7	75.37 ± 15.95^{b}	56.0	100.0	2.30 ± 0.29^{b}	0.50×10^{7}	93.1
Infected control	182.07 ± 3.47^{a}	113.1	55.72 ± 5.77^{ab}	41.4	90.0	2.95 ± 0.36^{bc}	7.26×10^{7}	0.0
PBS control	171.07 ± 4.92^{a}	100.0	149.24 ± 5.92^{c}	100.0	100.0	0.00 ± 0.00^{a}	0.00	0.0

Note: Values with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test. '§' means no reduction, but elevation.

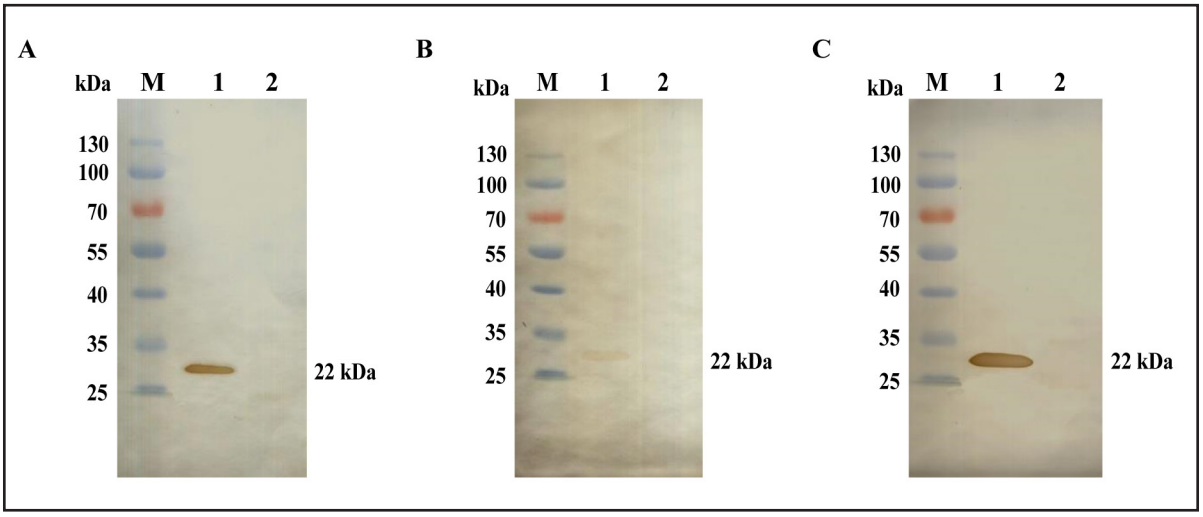

Figure 1. Expression and western blot analysis of rEnGAM22 protein. A- Purified rEnGAM22 protein was analyzed by western-blotting with anti-6×His monoclonal antibody. Lane 1: prestained protein marker. Lane 2: expressed recombinant protein *E. coli* BL21. Lane 3: wildtype vector transferred bacterial *E. coli* BL21. B- Purified rEnGAM22 protein was analyzed by western-blotting with the convalescent chicken sera. Lane 1: prestained protein marker. Lane 2: expressed recombinant protein in *E. coli* BL21. Lane 3: wildtype vector transferred bacterial *E. coli* BL21. C- Purified rEnGAM22 protein was analyzed by western-blotting with mouse anti-rEnGAM22 polyclonal antibodies. Lane 1: prestained protein marker. Lane 2: expressed recombinant protein in *E. coli* BL21. Lane 3: wildtype vector transferred bacterial *E. coli* BL21.

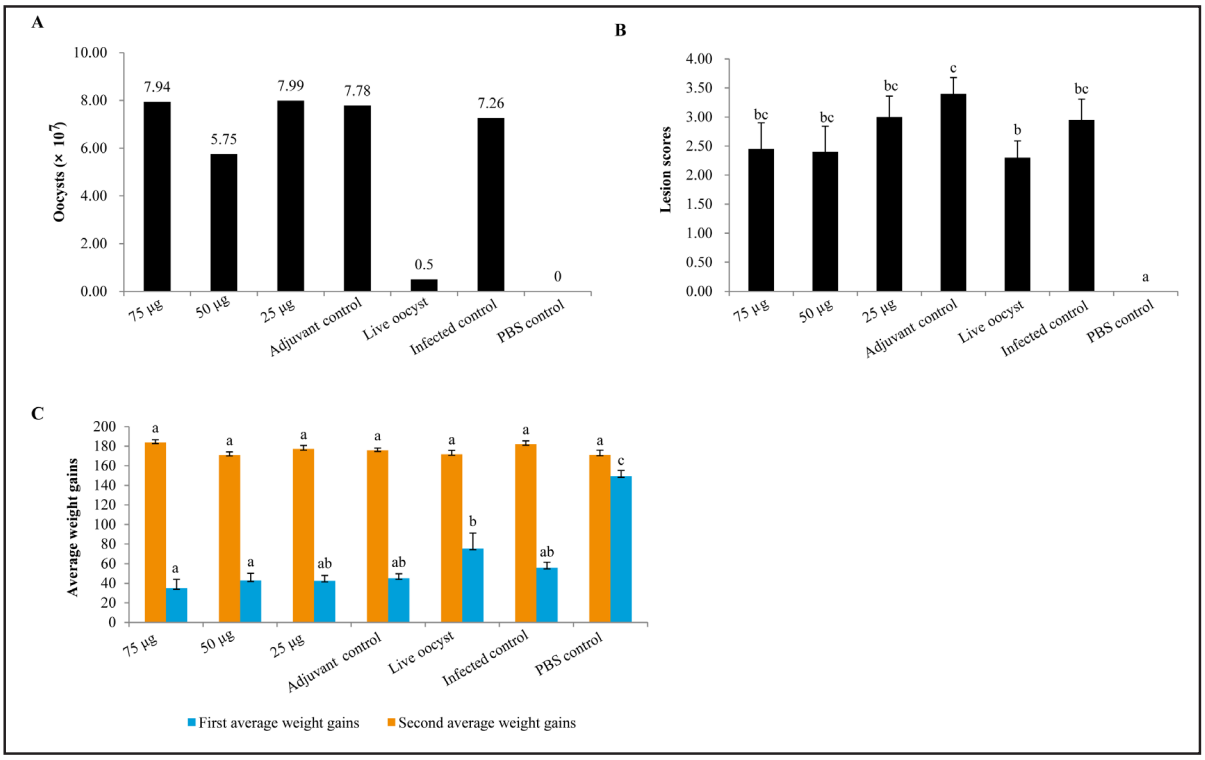

Figure 2. Effects of immunoprotection of rEnGAM22 on 3 parameters of disease resistance. Chickens were immunized with 75, 50, or 25 μg of rEnGAM22, adjuvant control, live vaccine, infected or PBS control and fecal oocyst shedding (A), lesion scores (B) and average weight gains (C) were determined. [The bars of (B) and (C) represent the mean ± S.D. value (n=10), and bars with different letters are significantly different ($P < 0.05$) according to the Duncan's multiple range test].

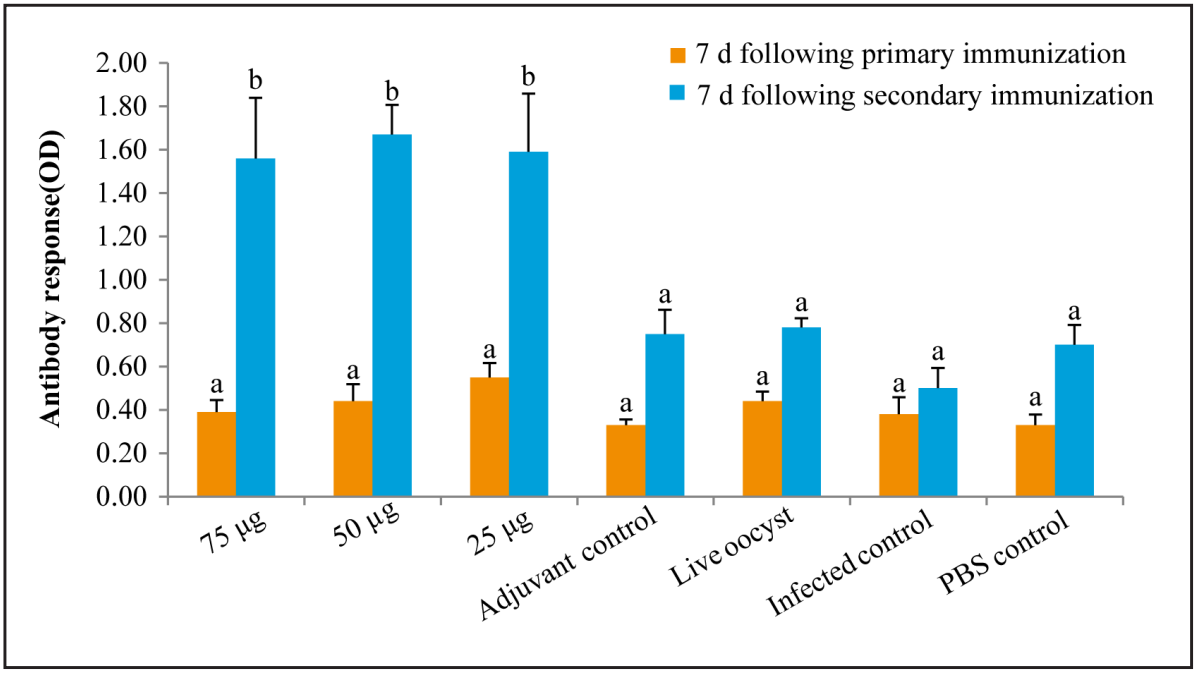

Figure 3. Antibody (IgY) response against rEnGAM22. Chickens were immunized with 75, 50, or 25 μg of rEnGAM22, adjuvant control, live vaccine, infected or PBS control and antibodies were detected by ELISA at 7 days following primary and secondary immunization. [Each bar represents the mean ± S.D. value (n = 5), and bars with different letters are significantly different ($P < 0.05$) according to the Duncan's multiple range test].

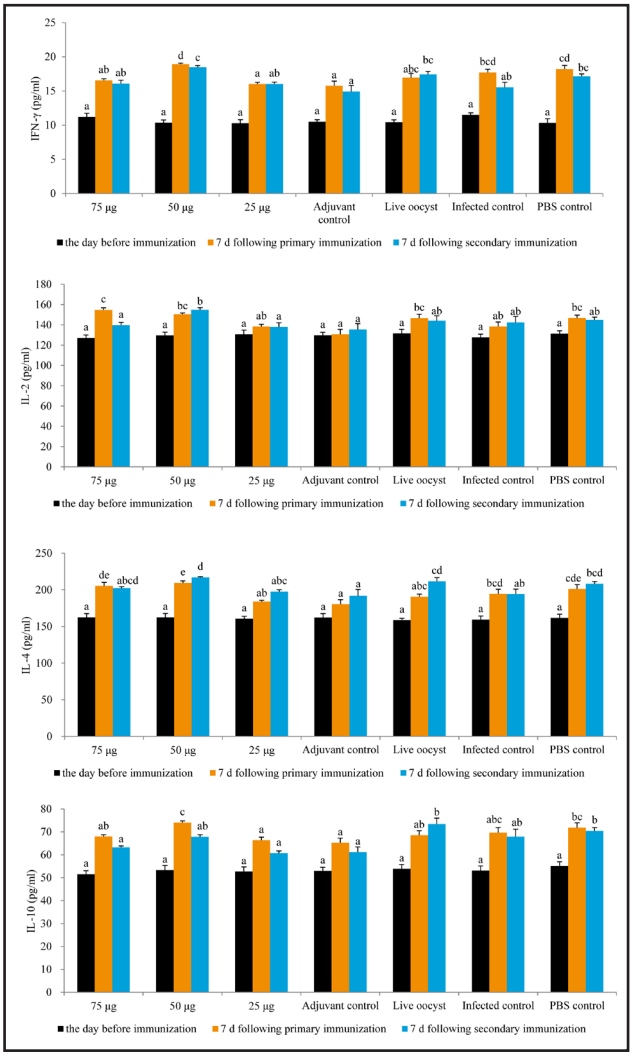

Figure 4. Serum cytokines response against rEnGAM22 immunization. Chickens were immunized with 75, 50, or 25 μg of rEnGAM22, adjuvant control, live vaccine and infected or PBS control and IFN-γ, IL-2, IL-4 and IL-10 were detected by ELISA at 7 days following primary and secondary immunization. [Each bar represents the mean ± S.D. value (n = 5), and bars with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test].

DISCUSSION

This study was designed to evaluate the effects of the prokaryotic-expressed product of *gam22* of *Eimeria necatrix* on host immunity following experimental infection with *E. necatrix*. The findings of this study were as follows: 1) immunization with the rEnGAM22 protein before *E. necatrix* infection was associated with reduced fecal oocyst shedding and intestinal lesions compared with infected controls; 2) immunization with rEnGAM22 induced high levels of anti-rEnGAM22 antibodies and increased levels of IL-2, IL-4, IL-10, and IFN-γ, cytokines that are associated with the immuno-protective mechanism against parasite infection, and 3) immunization with 50 μg rEnGAM22 provided the greatest protection.

There have been many gametocyte antigens studied for use in immunizations against many species of live parasites, including *E. maxima* [2], *E. tenella* [20], *Plasmodium falciparum* [23], and *Cryptosporidium parvum* [17]. However, there have been few reports of gametocyte antigens from *E. necatrix*. Previously, we successfully cloned, sequenced, and expressed the EnGAM22 protein. This purified rEnGAM22 protein was labeled by mouse anti-rEnGAM22 polyclonal antibodies and convalescent chicken sera, which demonstrated the improved immunogenicity provided by this gametocyte protein.

There have been reports that *gam82* of *E. maxima* is effective in reducing oocyst shedding and lesion scores and inducing humoral and cell mediated immunity when compared to non-vaccinated controls[10,16]. Our research showed that the purified rEnGAM22 protein greatly reduced oocyst shedding compared to non-vaccinated and infected controls, presumably due to GAM22-reactive antibodies. Additionally, lesion scores were also reduced. However, this study failed to detect a significant effect on body weight gain. These results are similar to previous research [10,16,1,7].

Eimeria infection can induce specific immunity involving both cellular and humoral immune mechanisms mediated by antibodies, lymphocytes, and cytokines [18]. T lymphocytes represent the prevailing component of protective immunity in avian coccidiosis[21]. Although a concrete role for humoral immunity in the response to poultry coccidiosis has yet to be defined, responses have been shown to be mediated by T helper (Th) cytokines, both Th1 and Th2 [17]. IL-4, and IL-10 are Th2-related cytokines, whereas IFN-γ and IL-2 are Th1-related cytokines. IL-2 and IL-10 reportedly stimulate the proliferation of T lymphocytes, B cells, and natural killer (NK) cells when infected with *Eimeria* [5,26]. IL-4 is crucial in B cell differentiation and proliferation to produce antigen-specific serum antibodies and improve immune responses [9,8]. IFN-γ can activate the nitric oxide pathway, thereby inhibiting the intracellular development of parasites and reducing oocyst shedding [13,19]. In this study, we detected the serum cytokines IL-2, IL-4, IL-10, and IFN-γ 7 days after both primary and secondary immunization and found that IL-2 and

IL-4 levels increased significantly in the group immunized with 50 and 75 μg rEnGAM22 as well as the live vaccine group 7 days after primary immunization. This was likely due to the fact that these cytokines played essential roles in B cell proliferation and the production of antibodies against *Eimeria* infection. In addition, IL-10 and IFN-γ levels increased slowly during the same period but were slightly lower 7 days after secondary immunization compared with primary immunization. These results are similar to those of previous research on the immuno-protective effect of the *E. maxima* gametocyte antigen GAM82 [10]. It has been speculated that the slow increase in cytokines at the time of booster immunization is associated with normal immune homeostatic mechanisms [10].

Small doses of live oocysts can successfully protect against coccidiosis. In this study, compared with the cellular and humoral immune responses to live oocysts, immunization with 50 μg rEnGAM22 protein provided better immuno-protection than 75 μg and 25 μg immunized groups. This was likely due to the higher specificity of EnGAM22 to activate immune responses than whole oocyst proteins, or to the short period of exposure to the vaccine strain of live oocysts in the live vaccine group. Whether immune responses in the live vaccine group would be higher if exposure time was increased requires further researched. In addition, some antibodies were detected by ELISA in the adjuvant control, infected control, and PBS control groups as well as in the live oocyst vaccine group. We speculated that this may be due to bacterial proteins contamination in the purified rEnGAM22, as it was difficult to ensure a completely sterile environment for preparing and purifying recombinant proteins. Additionally, chickens may also ingest bacteria and produce antibodies. As shown by ELISA results (the 96-well plates were coated with rEnGAM22 to detect chicken antibodies coming from chickens), the adjuvant control, infected control, PBS control and live oocyst vaccine groups also showed some reactions, even though they were not immunized with recombinant protein.

CONCLUSION

In summary, purified rEnGAM22 protein stimulated anti-rEnGAM22 antibodies, induced multiple cytokines, and protected chickens against experimental *E. necatrix* infection to some degree. This study established a theoretical basis for immuno-prophylaxis and will contribute to the development of a recombinant subunit vaccine against *E. necatrix* coccidiosis.

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Ethical approval. The study protocol was approved by the Animal Care and Use Committee of the College of Veterinary Medicine of Yangzhou University (Approval ID: SYXK [Su] 2016-0020). Animal care and all animal procedures were conducted according to the guidelines of the Animal Research Ethics Committee of Yangzhou University (Protocol No. NSFC2020-SYXY-27).

Declaration of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors alone are responsible for the content and writing of this paper.

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