RESEARCH ARTICLE

Development of Subunit Vaccine against Poultry Coccidiosis

Vijayashanthi Ramalingam, Raman Muthusamy*, Kasthuri Bohra, Madan Nithyanantham, Azhahianambi Palavesam, Dhinakarraj Gopal

Abstract

Coccidiosis has a major impact on the economy of the poultry industry worldwide, caused by the protozoan parasite of the genus *Eimeria* inhabiting the intestinal tract and caecum. The current method of prophylactic use of anti-coccidial drugs led to the emergence of drug-resistant isolates with the presence of drug residues in meat and egg. Of the alternate control strategies available, use of recombinant protein-based vaccines was reported to achieve 30 to 90% reduction in parasite replication with a reduction in intestinal lesion score and improved feed conversion ratio along with bodyweight gain. In the present study, an immunization trial was conducted on 75 chickens using the bivalent recombinant candidates comprising of gametocyte antigen (Gam82) from *Eimeria maxima* and microneme protein 1 (EtMIC1) from *Eimeria tenella* had been found to offer higher protection against the experimental challenge using field isolates of mixed *Eimeria* oocysts (as Group III) for upto 69.23% than those two recombinant protein namely Gam82 (Group I- 34.49%) and EtMIC1 (Group II- 46.15%) given separately, for up to 6 weeks of post-immunization.

Keywords: ELISA, Gametocyte antigen (Gam82), Immunization trial, Intestinal lesion score, Microneme protein 1, Poultry coccidiosis.

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Introduction

Chicken coccidiosis is one of the devastating diseases of commercial poultry caused by the protozoan parasite of the genus *Eimeria*, estimated to incur a global economic loss in excess of Great Britain Pound (GBP) 10.4 billion and considered one of the top ten diseases of livestock (Blake *et al.,* 2020).

The clinical infestation of coccidiosis results in severe morbidity due to reduced feed conversion ratio (FCR), poor weight gain, and malabsorption, leading to a negative impact on the poultry production system worldwide. Long term usage of chemoprophylactic and therapeutic anti-coccidial drugs led to the emergence of drug-resistant field strains (Shirley *et al.,* 2007 and Shivaramaiah *et al.,* 2013) and drug residues in the food chain (Chapman *et al.*, 2002; Peek and Landman, 2011 and Soutter *et al.,* 2020).

Of the several alternate chicken coccidiosis control strategies available, the attenuated live anti-coccidial oocyst vaccines are the most viable strategy providing higher immune response and are widely used in several countries (Smith *et al.*, 2002). Currently, the production of such live, attenuated *Eimeria* oocyst vaccines is laborious. It is passaged in target host kept in the battery cages and is considered the major setback to the industries (Barbour *et al.,* 2015). This has necessitated the concept of recombinant anti-coccidial vaccine development, which provides long-term immunity in vaccinated birds (Blake *et al.,* 2017). More research has to be followed on developing and evaluating multivalent recombinant anti-coccidial vaccines using two or more species of Eimeria (Song *et al.*, 2015 and Liu *et al.,* 2018). Hence, the present study was carried out to find appropriate candidate antigens in developing recombinant anti-coccidial vaccine from indigenous strains of poultry coccidia to induce robust long-term protective immunity.

Translational Research Platform for Veterinary Biologicals, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram, Chennai-600051, Tamil Nadu, India.

Corresponding Author: Raman Muthusamy, Translational Research Platform for Veterinary Biologicals, Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram, Chennai-600051, Tamil Nadu, India, e-mail: raman.tanuvas@gmail.com

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Materials and Methods

Propagation of Monospecific *Eimeria* Culture

Fifty-day-old broiler chicks were raised in a secluded experimental animal house. Of which, 25 chicks were challenged with pure monospecific oocyst culture of *E. tenella* on Day-17 to Day-19 and kept in a separate cage. Similarly, 25 chicks were challenged with pure monospecific oocyst culture of *E. maxima* on Day-26 using oral gavage. The chicks were observed for oocyst shedding since 4 day

post-challenge (dpc), and the oocysts were harvested and stored in 2.5% potassium dichromate solution in well-aerated condition until its usage in the experiment.

Recombinant Protein Production

The oocyst purification, sporulation, and excystation of *E. tenella* and *E. maxima* were done as followed by Chapman and Shirley (2003) for extraction of EtMIC1 and Gam82 gene, respectively. Briefly, the coccidial oocysts were recovered by flotation technique using saturated salt solution followed by decontaminated using 4% (v/v) hypochlorite solution. The oocysts were sporulated in 2.5% potassium dichromate solution (w/v) by incubating for 48 hours at 25°C in a shaking incubator. The sporulated oocysts were vortexed with 1 mm diameter glass beads (Sigma Aldrich, USA) and then treated with Hank's balanced salt solution (Sigma-Aldrich, USA) containing 0.25% trypsin (w/v) and 1% sodium taurodeoxycholate (w/v) (Sigma-Aldrich, USA) at 41°C with constant agitation for 4 hours. The purity of *E. tenella* and *E. maxima* were assessed for ribosomal Internal Transcribed Spacer 1 (ITS 1) by species-specific nested- PCR as described by Lew *et al.* (2003).

Intestinal scraping of *E. maxima* experimentally infected birds was used for isolation of total RNA using Trizol reagent (Invitrogen, USA). cDNA was prepared, and Gam82 coding sequence was amplified using the primer sequence as described by Jang *et al.* (2010). Whereas the EtMIC1 cDNA was amplified using a specific primer pair as described by Mohana Subramanian *et al.* (2008) (Table 1). An amplicon of both EtMIC1 and Gam82, cDNA was cloned into pET28a (+) plasmid (Novagen, NJ) downstream from an NH_2-terminal His_6 epitope tag, the clone sequences were verified and transformed chemically into competent *Escherichia coli* BL21 (DE3) Star (Invitrogen, USA).

Recombinant protein expressed in *E. coli* clone was induced for 4-liter culture with 1 Mm Isopropyl β-D-thiogalactoside (IPTG, Bangalore Genei, India) using mass fermentation technique (New Brunswick Scientific) at 150 rpm for 16 hours at 25°C (pH of 7.3). The bacterial culture was incubated at 37°C for 4 hours and centrifuged at 6100 X g for 30 minutes. The cell pellet was lysed using a lysis buffer (10 mM PBS-Phosphate Buffered Saline containing 1 mg/mL of lysozyme, Sigma Aldrich, USA) followed by sonication. The recombinant His6 tagged proteins were purified using Ni-NTA chromatography under native conditions (Spriesterbach *et al.*, 2015). The purified protein was dialyzed overnight against 1X PBS at 4°C, and the protein concentrations of purified proteins were estimated using Bicinchoninic Acid Kit (Sigma-Aldrich, USA). Purified recombinant proteins were stored in 2 mL cryovials at -20°C until further analysis.

Experimental Design

The experimental trial was conducted from January 2020 to March 2020 at TRPVB animal house. A total number of 75 coccidia-free commercial broilers (IAEC approval letter no. 1803/DFBS/IAEC/2019, dated: 31.12.2019) were purchased from commercial suppliers as 0-day old broiler chicks. The chicks were divided into 5 groups, with 15 birds in each group. Group-I (Gam82), Group-II (EtMIC1), Group-III (Gam82+ EtMIC1), Group-IV (Infected control), Group-V (Uninfected control), and were housed under an animal containment facility before primary immunization. The birds were on a coccidiostat-free diet during the experiment. The birds were immunized using Montanide ISA 71 VG adjuvant (Seppic, France) with recombinant proteins, and experimental infection was induced as described in Table 2.

Evaluation of Efficacy of Immunization

The biological samples were collected and analyzed to evaluate the vaccine efficacy by measuring performance parameters (body weight gain), parasitological (OPG, Lesion score) and serological parameter (Antibody titer) as mentioned in Table 3. The vaccine efficacy was evaluated using the intestinal lesion score of immunized and control groups (3 birds/day/group) from 7 dpc to 12 dpc as described by Raman *et al.* (2011). The gross intestinal lesion score scale was recorded depending on the severity of lesions from 0 (no lesions) to 4 (most severe lesions).

The purified proteins were characterized using 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described by Laemmli (1970) using vertical slab gel electrophoresis system (Bio-Rad Ltd, USA) and broad range molecular weight marker (Bangalore Genei, India). The gel was stained with Coomassie Brilliant Blue stain overnight or electrotransfer to poly vinylidene fluoride (PVDF) membrane as described by Towbin *et al.* (1979). The purified proteins were trans-blotted on a PVDF membrane using Bio-Rad Trans-Blot. A 2% skim milk in 1X PBS was used for blocking the non-specific site of the protein and then washed with 1X PBS for three times at room temperature. The PVDF membrane was incubated in 1:25 (v/v) of immune chicken sera mixed in 2% skim milk powder

Table 1: Primers used for Target cDNA Amplification

Protein	*Primer*	*Primer sequence*	*Product Size (Kbp)*	*GenBank accession no.*
EtMIC1	Forward	5'- AGCTGGATCCACCAGCTCTGGCCAGGATCAGGTG-3'	2.025	EU093966
	Reverse	5'-TCTAGAGCGGCCGCTGCCCACATCTCTGATTGTTC-3'		
Gam 82 (Jang *et al.*, 2010)	Forward	'5 -AGCTGGATCCACCAGCTCTGGCCAGGATCAGGTG-3'	1.7	AAO47083
	Reverse	5'-TCTAGAGCGGCCGCTGCCCACATCTCTGATTGTTC-3'		

Table 2: Immunization and oocyst challenge schedule

Group	*No. of Birds*	*Recombinant protein used*	*Primary immunization (Day-7)*	*Secondary immunization (Day-14)*	*Dose of sporulated oocyst challenged (Day-21)*
I	15	Gam82	50 µg Gam-82 emulsified in Montanide ISA 71 VG adjuvant, I/M	50 µg Gam-82 emulsified in Montanide ISA 71 VG adjuvant, S/C	20,000 virulent mixed oocyst culture of *E. maxima*, *E. tenella*, *E. acervulina* (Oral)
II	15	EtMIC1	50 µg EtMIC1 emulsified in Montanide ISA 71 VG adjuvant, I/M	50 µg EtMIC1 emulsified in Montanide ISA 71 VG adjuvant, S/C	20,000 virulent mixed oocyst culture of *E. maxima*, *E. tenella*, *E. acervulina* (Oral)
III	15	Gam82+EtMIC1	50 µg Gam82+EtMIC1 emulsified in Montanide ISA 71 VG adjuvant, I/M	50 µg Gam82+EtMIC1 emulsified in Montanide ISA 71 VG adjuvant, S/C	20,000 virulent mixed oocyst culture of *E. maxima*, *E. tenella*, *E. acervulina* (Oral)
IV	15	Uninfected control	-	-	-
V	15	Infected control	-	-	20,000 virulent mixed oocyst culture of *E. maxima*, *E. tenella*, *E. acervulina* (Oral)

I/M –Intramuscular
S/C- Subcutaneous

Table 3: Work Plan

S.No	*Parameters*	*Time Line*	*Methodology*
1	Body weight gain	7, 14, 28, 42, and 56 dpi	Standard weighing balance (Mohana Subramanian *et al.,* 2008)
2	Oocyst output	5 dpc to 12 dpc	Modified Mc Master egg counting technique using flotation method (Zajac and Conboy, 2012)
3	Intestinal lesion score	7 dpc to 12 dpc	Post-mortem intestinal lesions after 7 dpc (Raman *et al.,* 2011)
4	Antibody response	0, 7, 14, and 21 dpi	Indirect ELISA (Mohana Subramanian *et al.*, 2008)

(SMP) in 1 X PBS as primary antibody for an hour at room temperature, while rabbit anti-chicken IgG HRP conjugated antibody in 2% SMP at the concentration of 1:400 was used as a secondary antibody.

Antibody responses in immunized birds were assessed by the protocol as described by Mohana Subramanian *et al.* (2008) with few modifications. In brief, two separate high-affinity MaxiSorp™ ELISA plates (Nunc-Immuno™, Denmark) were each well coated with 150 ng of recombinant EtMIC1 and Gam82 protein in 0.05 M carbonate buffer pH 9.6, respectively. The plates were incubated overnight at 4°C followed by blocking the wells with 1.5% (w/v) bovine serum albumin (Sigma-Aldrich, USA) in 1X PBS (pH 7.4). Each well was added with two fold serial dilution beginning with 1:100 of immunized bird sera. Horse Radish Peroxidase Conjugated Rabbit anti-chicken IgG, whole molecule (1:20,000 dilution; Sigma-Aldrich, USA) was used to detect the antigen-specific chicken antibody. The enzymatic reaction was developed with 3,3′,5,5′- tetramethylbenzidine (TMB, Sigma-Aldrich, USA) and 0.03% hydrogen peroxide as substrate. Optical Density (OD) values were measured at 450 nm using ELISA microplate reader (TECAN Infinite®200 PRO, Switzerland).

Results and Discussion

Total protein concentrations of affinity-purified EtMIC1 and Gam82 were 6.17 mg/L and 3.461 mg/L, respectively. Similarly, total protein concentration of 4.0 mg/L and 4.2 mg/mL to

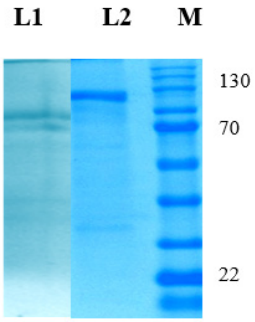

Figure 1: 12% SDS-PAGE analysis of recombinant Gam 82 (L1) and EtMIC1 (L2) resolved under reducing condition stained with Coomassie brilliant blue stain. Protein marker (M)

7 mg/L of purified Gam82 and EtMIC1 were obtained by Jang *et al.* (2010), Qi *et al.* (2013), and Mohana Subramanian *et al.* (2008), respectively. 10% SDS-PAGE analysis of affinity-purified EtMIC1 and Gam82 revealed prominent protein bands at 100 kDa and 82 kDa, respectively, under reducing conditions after being stained with Coomassie Blue (Figure 1). While, a prominent reactive band was detected at the range of 82 and 100 kDa for recombinant Gam82 and EtMIC1, respectively, using their specific chicken antisera in immunoblot analysis (Figure 2). A prominent protein band was noticed at the range of 100 kDa for the purified recombinant EtMIC1 protein on 10% SDS-PAGE analysis, and a seroreactive band using anti-EtMIC1 chicken sera was detected at 100 kDa in immunoblot (Mohana Subramanian *et al*., 2008).

Ni-NTA affinity-purified recombinant *E. coli*-expressed Gam82 protein revealed a protein band at 70 kDa on SDS-PAGE analysis and confirmed the antibody response using the specific monoclonal antibody raised against His-epitope in immunoblot analysis (Jang *et al.,* 2010). 12% SDS-PAGE analysis, affinity-purified Gam82 antigen, and Gam82 cell lysate revealed 3 predominant bands at the range of 60 to 80 kDa and at 75 kDa, respectively. While, immunoblot analysis detected a reactive band at 82 kDa and 75 kDa for native and purified recombinant Gam82 protein using chicken Affinity Purified Gametocyte Antigen (APGA) (Belli *et al.,* 2004).

A prominent protein band was noticed at the range of 115.8 kDa, while characterizing amylose resin purified recombinant EtMIC1 protein in 10% SDS-PAGE analysis. While, a sharp reactive band was noticed at 115.8 kDa and 69.3 kDa for recombinant *E. coli*-expressed EtMIC1 and EtMIC1-VD (Von Willebrand factor type A domain), respectively, using anti- MBP monoclonal antibody raised against MBP fusion protein (Qi *et al.,* 2013). Chen *et al.* (2015) characterized various fragments of EtMIC1 expressed yeast vector (EBY 100/ pCT EtMIC1-I to EtMIC1-III) at the range of 49.33, 75.57, and 41.66 kDa, respectively, in the immunoblot analysis using anti-EtMIC1 antibody. Li *et al.* (2020) noticed a prominent protein band at the molecular weight of 130 kDa for purified recombinant EtMIC1 protein on 12% SDS-PAGE analysis, and the reactivity band was observed at 130 kDa using its specific antisera in the immunoblot analysis. The immunoblot analysis of soluble protein of *E.maxima* sporozoite revealed 4 seroreactive bands at 70, 90, 95, and 130 kDa as observed earlier by Haung *et al.* (2018) in similar studies.

An increase in body weight gain was observed in uninfected control birds (Group-V), followed by birds that received recombinant proteins such as EtMIC1 (Group-II), Gam82 along with EtMIC1 (Group-III), and Gam82 alone (Group-I) at 35 dpi (Figure 3). The increased raise was observed from 21-day post-immunization (dpi), with the highest body weight gain was observed on 49 dpi.

The higher body weight gain in immunized birds was noticed with recombinant EtMIC1 on 7 dpc (Mohana Subramanian *et al.,* 2008). In previous studies conducted by Jang *et al.* (2010) and Qi *et al.* (2013) observed that the birds immunized with a higher dose of recombinant Gam82 (60 µg) and EtMIC1 (100 µg), gained more body weight gain than birds received a lower dose (Gam82 at the dose of 30 µg and EtMIC1 at the dose of 50 µg) of recombinant proteins.

In previous studies conducted by Jang *et al.* (2010) and Qi *et al.* (2013) used a higher dose of recombinant Gam82 and EtMIC1, respectively. The birds immunized with a higher dose of recombinant Gam82 and EtMIC1, respectively, observed more weight gain (81.2 and 88.5%) than birds immunized with a lower dose of recombinant Gam82 and EtMIC1 (Jang *et al.* 2010; Qi *et al.* 2013). While in the present study, a moderately higher body weight gain (75%) was observed in birds that received both EtMIC1 and a combination of EtMIC1 and Gam82, indicating the higher efficacy of the bivalent recombinant vaccine in controlling the multiplication of coccidial parasite.

The oocyst output in birds immunized with either Gam82 (Group I) or EtMIC1 (Group II) had prolonged shedding of oocyst than birds that received the combined recombinant EtMIC1 and Gam82 in the observation made from 5 dpc continuously for 7 days using modified Mc Master technique (Figure 4). Mohana Subramanian *et al.* (2008) compared the vaccination efficacy of EtMIC1 immunized bird group and showed 61% reduction in oocyst output and marginal reduction in lesion score compared with mock immunized bird groups with FMDV 3AB. Chen *et al.* (2015) observed reduced oocyst shedding by 74 to 79% in birds immunized with three various fragments of EtMIC1 protein. Whereas the infected control group showed reduced OPG to 30% only.

Birds immunized with higher dose (60 µg/mL) of recombinant Gam82 protein exhibit reduced oocyst output (an average of 1.1 X 10^7 oocysts/birds) from 5 to 10 dpc compared with other immunized groups with a lower dose of Gam82, 3-1E protein (30 and 60 µg/mL) and positive control (Jang *et al.,* 2010). Qi *et al.* (2013) observed that the

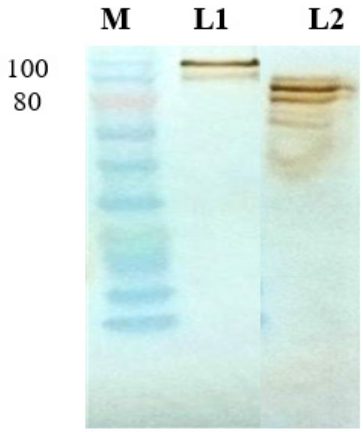

Figure 2: Western blot analysis of purified recombinant anti-coccidial proteins such as EtMIC1 (L1), Gam82 (L2) reacted with specific immune chicken sera (1:25) as primary antibodies followed by rabbit anti-chicken IgG HRP conjugated antibody (1:400). M-Prestained protein marker

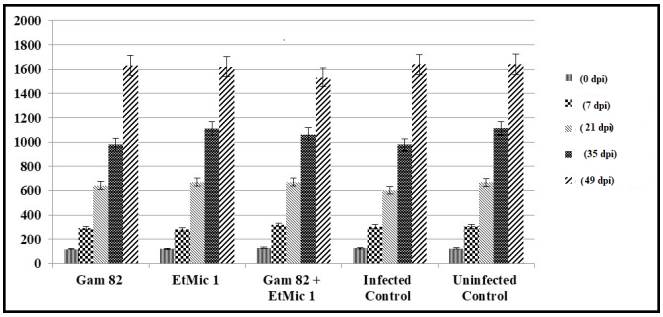

Figure 3: Average body weight gain (in grams) of birds immunized with recombinant anti-coccidial vaccine compared with infected and uninfected bird groups on 0 dpi, 7 dpi, 21, 35, and 49 dpi

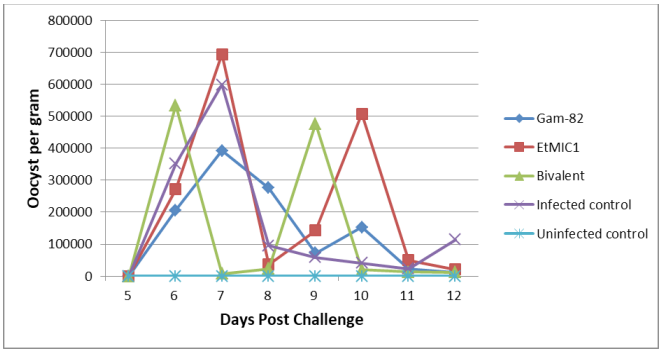

Figure 4: Oocyst output (Oocyst Per Gram) showed birds immunized with recombinant Gam 82 protein imparted less oocyst count on 7 dpc compared with other immunized bird groups.

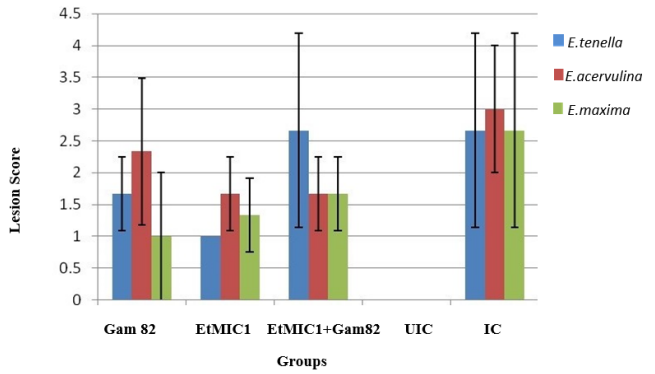

Figure 5: Average lesion score on 7 dpc showed birds immunized with recombinant EtMIC1 protein have less intestinal pathology than other immunized and infected control (IC). Uninfected control (UIC)

higher dose of EtMIC1 (100 µg/mL) showed lesser OPG count compared with a lower dose of EtMIC1, EtMIC1-VD (50 µg/mL), and positive control.

In the present study, the intestinal lesions were less pronounced in all the immunized groups than infected control group (Figure 5). Qi *et al.* (2013) reported that similar intestinal pathology in birds that received higher doses of EtMIC1 (100 µg/birds) with Anti-Coccidial Index (ACI) of 167.2 in his experiment. On the contrary, Jang *et al.* (2010) observed a positive correlation of reduced intestinal lesion when birds received a lower dose (30 µg) of purified Gam82 protein than those birds who received a higher dose of purified protein (60 µg) and non-immunized infected control.

In the present study, profound antibody response was observed in birds that were immunized with a combination of recombinant Gam82 and EtMIC1 (Group-III) than birds that received either of the protein separately (Group-I and Group-II) and control groups of birds (Group-IV and V). Similarly, higher antibody response was observed in the birds that immunized with EtMIC1 (expressed in Yeast vector-EBY-100 pCTCON-2) compared with control groups (Chen *et al.* 2015) and placebo group (FMDV 3AB) of birds (Mohana Subramanian *et al.,* 2008).

On the contrary, Belli *et al.* (2004) observed that the birds immunized with a lower dose of Gam82 (5 µg) showed higher antibody response compared with the higher dose (10 µg) group even after 12 weeks post-immunization.

Conclusion

In the present study, the average body weight gain and protection rate of immunized bird groups with EtMIC1 was higher (11.85 and 46.15%) than Gam82 Group (0.34 and 34.49%) at 6 weeks after primary immunization, while the immunized birds with the combination of these two candidate proteins observed higher protection rate (69.23%), with average body weight gain of 7.66%. Hence, both monovalent and bivalent recombinant proteins can be used for vaccine development as they have induced the host immune system to produce robust protective immunity even in case of mixed infection with pathogenic poultry *Eimeria* spp. However, the vaccine efficacy study can be further enhanced by developing a recombinant, anti-coccidial vaccine with various vectors and incorporating candidate antigens.

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