Rhomboid Protein 2 of Eimeria Maxima Provided Partial Protection Against Infection by Homologous Species

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Abstract

Background: Rhomboid-like proteases (ROMs) are considered as a new candidate antigen for developing new-generation vaccine due to their important role involved in the invasion of apicomplexan protozoa. In prior works, we obtained a ROM2 sequence of *Eimeria maxima* (EmROM2) which is the homologous gene with ROM2 of *Toxoplasma gondii*. This study was conducted to evaluate the immunogenicity and protective efficacy of EmROM2 recombinant protein (rEmROM2) and EmROM2 DNA (pVAX1-EmROM2) against infection by *Eimeria maxima* (*E. maxima*).

Methods: Western blot assay was conducted to analyze the immunogenicity of rEmROM2. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot assay were performed to determine the transcription and expression of pVAX1-EmROM2 recombinant plasmid. EmROM2-induced changes in transcriptional level of cytokines, T lymphocytes subsets and specific serum IgG antibody were detected through qPCR (quantitative real-time PCR), flow cytometry and indirect ELISA, respectively. Ultimately, a vaccination-challenge trial was performed to evaluate the protective efficacy of rEmROM2 and pVAX1-EmROM2 against infection with *E. maxima*.

Results: The purified rEmROM2 was recognized with chicken anti-*E. maxima* serum. After vaccination with pVAX1-EmROM2, apparent transcription and translation of EmROM2 were observed in the vaccinated chickens. Vaccination with rEmROM2 and EmROM2 DNA significantly upregulated the proportion of CD8+ and CD4+ T lymphocytes, the transcription level of cytokines (IFN-γ, IL-2, IL-4, IL-10, IL-17, TGF-β and TNF SF15) and serum IgG antibody response. Meanwhile, the vaccination significantly alleviated enteric lesions, weight loss, and reduced oocyst output caused by challenge infection of *E. maxima*, and provided anticoccidial index (ACI) of more than 160, indicating partial protection against *E. maxima*.

Conclusions: Vaccination with rEmROM2 and pVAX1-EmROM2 activated notable humoral and cell-mediated immunity and provided partial protection against infection by *E. maxima*. These results demonstrated that EmROM2 protein and DNA are promising vaccine candidates against *E. maxima* infection.

1. Background

Avian coccidiosis, a kind of intestinal parasitic protozoa disease, seriously impairs the poultry industry worldwide with a loss of beyond $3 billion USD per year [1]. Currently, controlling of this disease mainly depends on the usage of anticoccidial drugs worldwide [2, 3]. However, extensive drug use has caused drug resistance and drug residues in food extensively concerned by people [4, 5]. Live vaccine is the main alternative control strategy to chemical prophylaxis [2]. Nevertheless, some conventional live vaccines are costly and not fully efficient [6]. Furthermore, attenuated live vaccines may return to pathogenic form [7]. Vaccination with new generation vaccines including DNA vaccine and subunit vaccine is a promising strategy alternative to conventional treatments [6, 8]. For developing new generation vaccines, it is
important to identify protective antigens. Accordingly, research efforts have been put in to find novel vaccine targets over the past several decades [9].

A series of specialized parasite molecules are required when apicomplexan protozoa entry into the host cell [10]. Rhomboid-like proteases (ROMs) involve in the process of invasion of apicomplexan parasites [11]. Invasion by majority of apicomplexan parasites are carried out by adhesions, which mediate binding to the receptor molecules of host cell. It is thought that ROMs can cleave adhesins result in parasites can break away from receptors and finally entry into the host cell completely [12, 13]. Therefore, ROMs can be regarded as a new candidate antigen for developing new-generation vaccine [13, 14].

There are many reports about the ROMs family in apicomplexan protozoa, such as *Plasmodium* spp., *Toxoplasma gondii, Theileria annulata* and *T. parva* [13, 15, 16]. Meanwhile, several researches concerning the protective immunity induced by ROMs of *Eimeria* have been published. For example, the protective efficacy of some vaccines (such as rFPV-rhomboid, rBCG pMV261-Rho and rBCG pMV361-Rho) about ETRH01 rhomboid-like protein gene from *Eimeria tenella (E. tenella)* have been evaluated [9, 17, 18]. Up to now, however, there are still few reports on the immunogenicity and protective efficacy of *E. maxima* ROMs.

In prior works, we obtained a ROM2 sequence of *E. maxima*. And in this study, the recombinant protein and eukaryotic expression plasmid of EmROM2 were used as subunit vaccine and DNA vaccine. Meanwhile, the immunogenicity and protective efficacy induced by EmROM2 were evaluated. Our results provide a promising vaccine candidate antigen against *E. maxima*.

## 2. Methods

### 2.1. Plasmids, parasites, and animals

The prokaryotic expression vector pET-32a and eukaryotic expression vector pVAX1 were bought from Novagen (Darmstadt, Germany) and Invitrogen (Carlsbad, California, U.S.A), respectively. The oocysts of *E. maxima* were derived from our laboratory, propagating, harvesting and sporulating by the method previously described [19]. New-hatched Hy-Line chickens were raised in strictly sterilized chicken cages and fumigated henhouse. The food and water provided are free of anticoccidial drugs. SD rats (180 g-200 g) were purchased from Qinglongshan Breeding Farm in Nanjing. Animal experiments were approved by the Committee on Experimental Animal Welfare and Ethics of Nanjing Agricultural University (approval number: PAT2020001).

### 2.2. Cloning of EmROM2 and construction of recombinant plasmids of pET-32a-EmROM2 and pVAX1-EmROM2

Using micro glass balls to break the sporulated oocysts of *E. maxima* by whirl mix [19]. The total RNA of *E. maxima* sporozoites was extracted by a E.Z.N.A.™ Total RNA Kit I (OMEGA, Norcross, Georgia, U.S.A) follows the instruction. Then, HiScript II Q RT SuperMix (Vazyme, Nanjing, China) was utilized to generate
the cDNAs. RT-PCR was conducted to amplify EmROM2 gene using the specific primers (Table 1). Finally, the PCR products were cloned into prokaryotic expression vector pET-32a and eukaryotic expression vector pVAX1 to create recombinant plasmids pET-32a-EmROM2 and pVAX1-EmROM2 respectively. Concisely, EmROM2 gene and pET-32a vector were cleaved by BamHI and Hind III, while EmROM2 gene and pVAX1 vector were cleaved by BamHI and EcoRI, and finally ligated at the same enzyme sites. Double-enzyme digestion and sequencing were used to verify the recombinant plasmids.

| Table 1 | Primers used for the construction of pET-32a-EmROM2 and pVAX1-EmROM2 |
|---------|------------------------------------------------------------------------|
| **Plasmids** | **Primers** |
| pET-32a-EmROM2 | Forward: 5′-CGGATCCATGGCGCGGGTTCATACTT-3′ |
| | Reverse: 5′-CCAAGCTTTCAGGCGCAACTACGGGGGAG-3′ |
| pVAX1-EmROM2 | Forward: 5′-CGCGGATCCATGGCGCGGGTTCATACTT-3′ |
| | Reverse: 5′-CCGGAATTCTCAGGCGCAACTACGGGGGAG-3′ |

2.3. Preparation of rEmROM2 and anti-rEmROM2 serum

The expression plasmid of pET-32a-EmROM2 was transformed into the *E. coli* BL21 (DE3) to express rEmROM2, which was purified using a protein purification kit of His Trap™ FF (GE Healthcare, U.S.A). Then a ToxinEraser™ Endotoxin Removal Kit (Genscript, Nanjing, China) was used to remove the endotoxin to eliminate possible interference. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect the purified rEmROM2. The rat anti-rEmROM2 serum was prepared by the protocol previously described [8] for the detection of Western blot. Meanwhile, the serum from non-injected rat was used as a negative control.

2.4. Western blot recognition of rEmROM2 by chicken anti-*E. maxima* serum

Chicken anti-*E. maxima* serum was obtained by the method previously described [8, 20]. A Western blot assay was carried out with the above chicken antiserum as primary antibody (serum from uninfected chicken was set as negative control), and horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG (Sigma-Aldrich, Darmstadt, Germany) as secondary antibody. Briefly, rEmROM2 was separated through SDS-PAGE, next, transferred to a nitrocellulose membrane (Merck millipore, Darmstadt, Germany). Subsequently, the membrane was blocked with 5% bovine serum albumin (BSA) (Takara Biomedical Technology, Dalian, China) in PBST (phosphate buffered saline-Tween) overnight at 4 °C, afterwards incubated with chicken anti-*E. maxima* serum (1: 100) and goat anti-chicken IgG (1:4500), respectively. Finally, 3, 30-diaminobenzidine (DAB) was used to detect the bound antibody [8].
2.5. Detection of transcription and expression of the pVAX1-EmROM2 in vivo through RT-PCR and Western blot

Fourteen-day-old healthy chickens were divided into two groups at random and vaccinated with 100 µg of pVAX1-EmROM2 and 100 µg of pVAX1 by intramuscular injection of leg, respectively. The pVAX1-injected muscle and non-injected muscle were set as empty and negative controls. One week later, muscle samples were collected from the injected and non-injected sites. After grinding in a mortar, total RNA of muscle tissue was extracted using RNAiso Plus (Takara Biomedical Technology, Dalian, China) follows the product instruction. Then the residual recombinant plasmid was removed by digestion with DNase I (Takara Biomedical Technology, Dalian, China) to eliminate possible interference. With the RNA as template, the transcription of EmROM2 gene was determined through RT-PCR with its specific primers (Table 1). RIPA solution (Thermo Scientific, Waltham, MA, U.S.A) was used to treat the pVAX1-EmROM2-injected muscles for 2 h. Then Western blot assay was performed with the supernatant collected. The rat anti-rEmROM2 serum was set as primary antibody (the non-injected rat serum was set as negative control), and HRP-conjugated anti-rat IgG (Sigma-Aldrich, Darmstadt, Germany) as secondary antibody to detect the translation of pVAX1-EmROM2 in vivo [8].

2.6. Immunogenicity analysis of EmROM2

2.6.1. Experimental design

Fourteen-day-old healthy chickens were divided into 6 groups (30 chickens per group) at random. In experimental groups, 200 µg of rEmROM2 and 100 µg of pVAX1-EmROM2 were injected into the leg muscles separately. Simultaneously, 200 µg of pET-32a tag protein and 100 µg of pVAX1 were injected in vector control groups. Sterile PBS (phosphate buffered saline) was injected in PBS control groups. When the chickens were 21 days old, a booster vaccination was conducted as described above.

2.6.2. Flow cytometry analysis of splenic T lymphocytes subsets

One week after the primary and booster vaccination, the proportion of CD8+ and CD4+ T lymphocytes was detected with the spleens of 5 chickens from each group. Splenic lymphocytes were collected according to the previously protocol [21]. Under dark conditions, lymphocytes (1 × 10^7 cells/ml) were incubated with mouse anti-chicken CD3+ and mouse anti-chicken CD8+, mouse anti-chicken CD3+ and mouse anti-chicken CD4+ (SouthernBiotech, Birmingham, AL, U.S.A) for 45 min at 4°C, respectively. Then PBS was used to wash the cells twice by centrifugation (2500 rpm, 3 min, 4°C). T lymphocytes subsets analysis was conducted using BD FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, U.S.A).

2.6.3. Detection of cytokines transcription through quantitative real-time PCR
The total RNA of splenic lymphocytes was extracted by a E.Z.N.A.™ Total RNA Kit I (OMEGA, Norcross, Georgia, U.S.A) follows the instruction. Subsequently, the cDNA of lymphocytes was generated using HiScript II Q RT SuperMix (Vazyme, Nanjing, China) for qPCR assay follows the manufacturer's instruction. Specific primers for cytokines of IFN-γ, IL-2, IL-4, IL-10, IL-17, TGF-β and TNF SF15 were designed by NCBI and Primer Ques Tool (IDT). GAPDH gene was designed as internal reference control (Table 2). Meanwhile, amplification efficiencies were evaluated according to the protocol previously reported [22]. qPCR was employed to determine the above mRNA level according to the instruction of ChamQ™ SYBR qPCR Master Mix Kit (Vazyme, Nanjing, China). Determination of cytokines transcription was performed using the 7500 Real Time PCR System (Applied Biosystems, Carlsbad, CA, U.S.A) with a particular program in accordance with the instruction. The fold change of the transcriptional level of cytokine was determined utilizing $2^{-\Delta\Delta CT}$ method compared to the internal reference control gene of GAPDH [23].
Table 2
Primers used for the quantitative real-time PCR

| RNA target | Primers sequence | Accession NO. | Amplification efficiency (%) | Correlation coefficients ($r^2$) |
|------------|------------------|---------------|-----------------------------|---------------------------------|
| GAPDH      | Forward: 5'-GGTGGTGCTAAGCGTTAT-3' | K01458        | 100.74%                     | 0.9917                          |
|            | Reverse: 5'-ACCTCTGTCATCTCTCCACA-3' |               |                             |                                 |
| IL-2       | Forward: 5'-TAACCTGGGACACTGCCATGA-3' | AF000631      | 102.44%                     | 0.9921                          |
|            | Reverse: 5'-GATGATAGAGATGCTCCATAAGCTG-3' |               |                             |                                 |
| IL-4       | Forward: 5'-ACCCAGGGCATCCAGAAG-3' | AJ621735      | 99.09%                      | 0.9936                          |
|            | Reverse: 5'-CAGTGCCGGCAAGAAGTT-3' |               |                             |                                 |
| IL-10      | Forward: 5'-GGAGCTGAGGGTAAGTTGA-3' | AJ621614      | 99.19%                      | 0.9923                          |
|            | Reverse: 5'-GAAGCGCAGCATCTCGACA-3' |               |                             |                                 |
| IL-17      | Forward: 5'-ACCTTCCCATGTCAGAAAT-3' | EF570583      | 100.24%                     | 0.9940                          |
|            | Reverse: 5'-GAGAACTGCCTTGCTAACA-3' |               |                             |                                 |
| IFN-γ      | Forward: 5'-AGCTGACGGTGACCTATTATT-3' | Y07922        | 103.07%                     | 0.9868                          |
|            | Reverse: 5'-GGCTTTTGGCTGGATTC-3' |               |                             |                                 |
| TGF-β      | Forward: 5'-CGGGACGGATGAGAAGA-3' | M31160        | 102.79%                     | 0.9815                          |
|            | Reverse: 5'-CGGCCAGCACTGTAATGAT-3' |               |                             |                                 |
| TNF SF15   | Forward: 5'-GCTTGGCCTTTACCAAGA-3' | NM_001024578  | 100.57%                     | 0.9930                          |
|            | Reverse: 5'-GAAGTGACCTGACATAGA-3' |               |                             |                                 |

*a Amplification efficiency (%) = ($10^{-1/slope}$ - 1) × 100%
2.6.4. Detection of serum IgG antibody level

The blood samples were gathered from the chickens when one week after the primary and booster vaccination. Serum IgG antibody level was determined using indirect ELISA (enzyme-linked immunosorbant assay) [20]. In brief, 0.05 M carbonate buffer was used to dilute the concentration of purified recombinant protein into 10 ng/µL. The 96-well microtiter plate (Corning-Costar NY, U.S.A) was coated with 150 µL of above recombinant protein per well overnight at 4 °C. Subsequently, the plate was blocked with 4.5% non-fat milk powder in PBST, next, incubated with the chicken serum (1:100) and goat anti-chicken IgG antibody (Sigma-Aldrich, Darmstadt, Germany) (1:4500), respectively. Each well was incubated with 3,3,5,5-tetramethylbenzidine (TMB) (Sigma-Aldrich, Darmstadt, Germany) to develop the color. The reaction was stopped with 2 M H$_2$SO$_4$. Finally, a microplate reader (ThermoScientific, Waltham, MA, U.S.A) was utilized to detect the absorbance.

2.7 Protective efficacy evaluation of EmROM2 against homologous species in chickens

As shown in the Table 3, fourteen-day-old chickens, were weighed, next, divided into 8 groups (30 chickens per group) at random. The experimental groups were intramuscularly vaccinated with 200 µg of rEmROM2 and 100 µg of pVAX1-EmROM2, respectively. Meanwhile, vector control groups were injected with 200 µg of pET-32a tag protein and 100 µg of pVAX1 plasmid as the same method as the experimental group separately. The challenged control groups and unchallenged control groups were injected with 200 µL of sterile PBS. When the chickens were 21 days old, a booster vaccination was conducted as described above. At the age of 28 days, all the chickens, with the exception of the unchallenged control groups, were orally challenged with the sporulated oocysts of *E. maxima* (1 × 10$^5$ /chicken) [24]. One week later, weighting and slaughtering the chickens were performed. Finally, the protective efficacy of rEmROM2 and pVAX1-EmROM2 was evaluated based on the average body weight gain, oocyst output, enteric lesion and ACI (anti-coccidial index) [25–28].
Table 3  
Protective efficacy of rEmROM2 and pVAX1-EmROM2 against challenge with *E. maxima*

| Groups               | Average body weight gain (g) | Relative body weight gain (%) | Mean lesion scores | Average OPG(×10^5) | Oocyst decrease ratio (%) | ACI   |
|----------------------|------------------------------|-------------------------------|-------------------|-------------------|--------------------------|-------|
| rEmROM2              | 50.25 ± 6.33^b               | 88.30^b                       | 1.67 ± 0.65^b     | 0.51 ± 0.68^b     | 73.33^b                  | 170.60|
| pET-32a control      | 29.46 ± 11.25^c              | 51.77^c                       | 2.66 ± 0.93^c     | 2.15 ± 0.97^c     | 4.44^c                   | 85.17 |
| Challenged control   | 27.21 ± 8.52^c               | 47.81^c                       | 2.84 ± 0.88^c     | 2.25 ± 0.94^c     | 0^c                      | 79.41 |
| Unchallenged control | 56.91 ± 10.24^a              | 100^a                         | 0 ± 0^a           | 0 ± 0^a           | 100^a                    | 200   |
| pVAX1-EmROM2         | 63.84 ± 8.80^b               | 80.54^b                       | 1.68 ± 0.99^b     | 0.57 ± 0.31^b     | 79.72^b                  | 162.74|
| pVAX1 control        | 38.19 ± 15.39^c              | 48.15^c                       | 2.75 ± 0.62^c     | 2.80 ± 0.16^c     | 0.36^c                   | 80.65 |
| Challenged control   | 39.28 ± 9.72^c               | 49.53^c                       | 2.83 ± 0.72^c     | 2.81 ± 0.13^c     | 0^c                      | 81.23 |
| Unchallenged control | 79.32 ± 9.59^a               | 100^a                         | 0 ± 0^a           | 0 ± 0^a           | 100^a                    | 200   |

Note: there is no significant difference in the same letter (*P* > 0.05), but there is significant difference in the different letters (*P* < 0.05).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the data obtained in accordance with Duncan's multiple range test at a 5% level by using SPSS 20 Data Editor (SPSS Inc., Chicago, IL, U.S.A).

3. Results

3.1. Cloning and expression of the EmROM2 gene

The PCR product of EmROM2 gene was detected by electrophoresis and sequencing analysis. As shown in the Fig. 1, electrophoresis revealed the band of 849 bp, which is equal to the target gene. The sequencing analysis revealed that the EmROM2 gene share 100% similarity in nucleotide sequence with the gene in GenBank (Sequence ID: XM_013480878.1). SDS-PAGE revealed that rEmROM2 was expressed and well purified, showing a band of approximately 49 kDa which is in accordance with the predicted
molecular consist of EmROM2 and pET-32a tag protein (Fig. 2a, lane 4). Western blot assay indicated that rEmROM2 was identified by chicken anti-*E. maxima* serum (Fig. 2b, lane 1).

### 3.2. Construction of recombinant plasmids pET-32a-EmROM2 and pVAX1-EmROM2

Endonuclease digestion and sequencing were performed to verify the constructed recombinant plasmids of pET-32a-EmROM2 and pVAX1-EmROM2. The digestion of pET-32a-EmROM2 with *Bam*H I and *Hind* III generated a band about 849 bp, which is equal to EmROM2 gene, and a larger band of pET-32a vector (Fig. 1b). After digestion with *Bam*H I and *Eco* R I, pVAX1-EmROM2 generated a band about 849 bp of the EmROM2 gene, and a larger band, which is the linearized pVAX1 vector (Fig. 1d). The sequencing analysis also verified the recombinant plasmids.

### 3.3. Transcription and expression detection of recombinant plasmid pVAX1-EmROM2 in vivo

The transcription of pVAX1-EmROM2 in the injected muscles was detected through RT-PCR. As shown in Fig. 3, a band about 849 bp was detected from the muscles injected with pVAX1-EmROM2 by agarose electrophoresis (Fig. 3a, lane 1), indicating the transcription of pVAX1-EmROM2 in vivo. Meanwhile, no band was found from non-injected muscle and pVAX1-injected muscle (Fig. 3a, lanes 2 and 3). Western blot assay was conducted to detect the expression of pVAX1-EmROM2 from the injected muscles. The result revealed a single reaction band in the pVAX1-EmROM2 injected group (Fig. 3b, lane 1), and no band was found in the negative control group (Fig. 3b, lane 2).

### 3.4. Changes of T lymphocytes subsets induced by rEmROM2 and pVAX1-EmROM2

Flow cytometry was performed to analyze the changes in the proportion of CD8⁺/CD3⁺ and CD4⁺/CD3⁺ T lymphocytes from the EmROM2-vaccinated chickens. The results illustrated that, by comparison with control groups, vaccination with rEmROM2 (Fig. 4) and pVAX1-EmROM2 (Fig. 5) obviously upregulated the proportion of CD8⁺ and CD4⁺ T lymphocytes one week after the primary and booster vaccination (*P* < 0.05). No notable differences were found between the pET-32a tag protein control and PBS control (*P* > 0.05), along with the pVAX1 control and PBS control (*P* > 0.05).

### 3.5. Changes of cytokines in transcriptional level induced by rEmROM2 and pVAX1-EmROM2

qPCR was conducted to detect the changes in transcriptional level of IFN-γ, IL-2, IL-4, IL-10, IL-17, TGF-β4 and TNF SF15 cytokine. The rEmROM2-indcued changes in cytokines were shown in Fig. 6. One week after the primary vaccination, the transcriptional level of IFN-γ, IL-2, IL-4, IL-10, TGF-β4 and TNF SF15 was obviously upregulated (*P* < 0.05), while in relation to the control groups, no notable difference was found in the transcriptional level of IL-17 cytokine (*P* > 0.05). However, by comparison with the control groups, the transcriptional level of all cytokines detected was significantly increased one week after the booster
vaccination ($P<0.05$). No notable differences were found between the pET-32a tag protein control group and PBS control group ($P>0.05$). In the pVAX1-EmROM2 vaccinated group (Fig. 7), compared to the control groups, vaccination significantly increased the transcriptional level of all the cytokines detected one week after the primary and booster vaccination ($P<0.05$). Meanwhile, no notable differences were found between the pVAX1 control group and PBS control group ($P>0.05$).

3.6. Changes of serum IgG antibody level induced by rEmROM2 and pVAX1-EmROM2

Specific serum IgG antibody level from vaccinated chickens was detected using indirect ELISA. In the rEmROM2-vaccinated group (Fig. 8) and pVAX1-EmROM2 vaccinated group (Fig. 9), by comparison with the control groups, vaccination significantly increased the serum IgG level from the vaccinated chickens one week after the primary and booster vaccination ($P<0.05$). No notable differences were found between the pET-32a tag protein control and PBS control ($P>0.05$), along with the pVAX1 control and PBS control ($P>0.05$).

3.7. Protective efficacy of rEmROM2 and pVAX1-EmROM2 against E. maxima infection

Protective efficacy of rEmROM2 and pVAX1-EmROM2 was evaluated via challenge with E. maxima (Table 3). Compared to the control groups, vaccination with rEmROM2 and pVAX1-EmROM2 significantly alleviated intestinal lesions, weight loss, and reduced oocyst output ($P<0.05$). No significant differences were observed between control groups ($P>0.05$). Moreover, the ACI values of rEmROM2-vaccinated group and pVAX1-EmROM2-vaccinated group were 170.60 and 162.74 respectively, indicating partial protection against E. maxima infection.

4. Discussion

Avian coccidiosis is a serious intestinal disease, which causes severe economic loss to the poultry industry. Vaccination with subunit or DNA vaccines is a promising alternative strategy of disease control compared to chemical prophylaxis and live vaccination [6, 8]. In recent years, couples of antigens of E. maxima were tested as candidate antigens for subunit or DNA vaccines and showed promising protective efficacy, such as gam56 and gam82 [29], EmMIC2 and EmMIC7 [30, 31] and some Eimeria common antigens (e.g. GAPDH and 14-3-3) [8, 32]. Since ROMs are involved in the invasion of apicomplexan protozoa, they were considered as a new candidate antigen for developing new-generation vaccine [13, 14]. For example, vaccination with rETRHO1 (recombinant protein of E. tenella rhomboid-like protein) and pVAX1-Rho (a DNA vaccine of E. tenella rhomboid-like protein) elicited humoral and cell-mediated immunity and generated protection against infection by E. tenella in chickens [14, 33]. This study was conducted to evaluate the immunogenicity and protective efficacy of EmROM2 in forms of recombinant protein (rEmROM2) and DNA (pVAX1-EmROM2), the results showed that EmROM2 activated notable humoral and cell-mediated immunity and provided partial protection against E. maxima. These results demonstrated that EmROM2 protein and DNA are effective vaccine candidates against E. maxima.
The prevalent view is that cell-mediated immunity plays a major role, and humoral immunity plays a minor role in the process of immunoprotection against coccidiosis [34–36]. In the present study, the proportion of CD8⁺ and CD4⁺ T lymphocytes was obviously enhanced via vaccination with EmROM2 protein and DNA, which indicated that EmROM2 could induce cellular immune responses. Cytokines partially control and regulate the responses of T cells against coccidiosis. Th1-type cytokines (e.g. IFN-γ and IL-2), which are related to cellular immunity, are regarded to be predominant against coccidiosis [36, 37]. IFN-γ are important in the process of regulating anti-coccidial immune responses, because it can activate the phagocytosis of macrophages and killing effect of NK cells and CTLs [35]. IL-2 can induce the proliferation of T cells in vitro, and increase the proportion of CD8⁺ and CD4⁺ T lymphocytes in peripheral blood when co-delivered with vaccines in vivo[38]. In the present study, the transcriptional level of IFN-γ and IL-2 in splenic lymphocytes from rEmROM2-vaccinated and pVAX1-EmROM2-vaccinated chickens were obviously upregulated in relation to the control groups (P<0.05). Similarly, the transcriptional level of five other major cytokines (IL-4, IL-10, IL-17, TGF-β4 and TNF SF15) was significantly increased via the vaccination. The role of antibodies is controversial, and many reports suggest that antibodies contribute to, but are not fundamental function [39]. In some cases, however, antibodies seem to be involved in protection against coccidiosis [39, 40]. In this study, the level of specific IgG antibody was increased significantly when vaccinated with rEmROM2 and pVAX1-EmROM2. In short, the changes in cytokines and IgG antibody suggested that rEmROM2 and pVAX1-EmROM2 could induce significant cellular and humoral immunity.

In this study, vaccination with EmROM2 protein and DNA significantly alleviated intestinal lesions, body-weight loss, reduced oocyst output caused by infection challenge by E. maxima. Moreover, it induced ACI values of more than 160 (170.60 and 162.74, respectively), showing partial protection against E. maxima infection. However, some methods could be taken to improve its protective efficacy. For example, some novel adjuvants, for instance, plant-derived adjuvants (such as saponins, and lectins) and nanoparticles (e.g. polymeric nanoparticles, inorganic nanoparticles and virus-like particles) were reported could activate or strengthen immune responses [41, 42]. Therefore, protective efficacy induced by vaccination can be augmented through co-administered with these adjuvants. Additionally, optimizing the vaccination route, dose, time and age of primary vaccination in chickens also can improve the protective efficacy of vaccine [43].

5. Conclusions

Rhomboid-like proteases are regarded as a new candidate antigen for developing new-generation vaccine due to their significant role involved in the invasion of apicomplexan protozoa. In this study, the recombinant protein and eukaryotic expression plasmid of EmROM2 were used as subunit vaccine and DNA vaccine. Meanwhile, the immunogenicity and protective efficacy induced by EmROM2 were evaluated. The results demonstrated that vaccination with rEmROM2 and pVAX1-EmROM2 activated notable humoral and cell-mediated immunity and provided partial protection against E. maxima infection, indicating EmROM2 protein and DNA are effective vaccine candidates against E. maxima infection.
**Abbreviations**

ACI: anti-coccidial index; bp: base pairs; BSA: bovine serum albumin; cDNA: complementary DNA; Ct: Cycle threshold; DAB: 3,3′-diaminobenzidine tetrahydrochloride; EmROM2: rhomboid protein 2 of *Eimeria maxima*; *E. maxima*: *Eimeria maxima*; *E. tenella*: *Eimeria tenella*; ELISA: enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; IgG: Immunoglobulin G; kDa: Kilo Dalton; M: mole/L; mRNA: message RNA; OD: optical density; PBS: phosphate buffered saline; PBST: phosphate buffered saline-Tween; PCR: polymerase chain reaction; qPCR: quantitative real-time PCR; RIPA: radio immunoprecipitation assay; rEmROM2: EmROM2 recombinant protein; RT-PCR: reverse transcription-PCR; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMB: 3,3,5,5′-tetramethyl benzidine.

**Declarations**

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**Authors' contributions**

SXK conceived and designed the study. LXR, YRF and XLX helped in the study design and analyzed the data. CYF drafted the manuscript and SXK helped to revise the manuscript. TD contributed to the main experiment. CYF performed the laboratory tests. CSY, DWX, SXT, WMY, CC, LXQ and SJZ contributed to the protective efficacy experiment. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets during the current study available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Animal experiments were approved by the Committee on Experimental Animal Welfare and Ethics of Nanjing Agricultural University (approval number: PAT2020001).

**Consent for publication**
Not applicable.

Competing interests

The authors declare that they have no competing interests.

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