

# Protective immunity induced by a DNA vaccine encoding *Eimeria tenella* rhomboid against homologous challenge

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**Abstract** Rhomboid protein in Apicomplexa was associated with the process of host cell invasion. To evaluate the potential of the protein in eliciting protective immunity against challenge, a DNA vaccine pVAX1-Rho encoding *Eimeria tenella* rhomboid was constructed. Recombinant protein was expressed in Hela cells and verified by indirect immunofluorescence and western blotting analysis. In vivo experiments, 1-week-old chickens were randomly divided into three groups. Experimental group of chickens were immunized with DNA vaccines while control group of chickens were injected with pVAX1 plasmid alone or sterile water. Two weeks following the booster dose, all chickens were inoculated orally with  $5 \times 10^4$  sporulated oocysts of *E. tenella*. The host immunity and protective efficacy of this vaccine against *E. tenella* challenge in broilers were evaluated. Results showed that specific antibody, the levels of interleukin-2 (IL-2), interferon- $\gamma$  (IFN- $\gamma$ ), and the percentages

of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte cells were significantly increased in the pVAX1-Rho group. Challenge experiments demonstrated that pVAX1-Rho vaccination could reduce oocyst excretion, decrease cecal lesion, increase body-weight gains and provide chickens with oocysts decrease ratio around 75.8 %. These results suggest that the pVAX1-Rho was able to induce humoral and cellular responses and generate protective immunity against *E. tenella* infection.

## Introduction

*Eimeria* species cause coccidiosis in poultry, resulting in severe economic losses. Currently, drug resistance against most anticoccidials has been observed due to long-term drug use. Moreover, public concerns increase on chemical residues in food and this makes the development of an effective vaccine strategy more urgent (Williams 1999; Allen and Fetterer 2002; Li et al. 2006; Sharman et al. 2010). Unattenuated live vaccines or attenuated live vaccines for coccidiosis control are available in the poultry industry (Irfan et al. 2008). However, these live vaccines are limited by the pathogenicity or the risk of reverting back to a pathogenic form (Sharman et al. 2010). Consequently, research efforts have been invested in the development of anticoccidial subunit or DNA vaccines as an alternative to the live vaccines. Although significant efforts have been made, there is no DNA vaccine available against *Eimeria tenella*. It has been a hot pursuit for a suitable candidate as target for *E. tenella* vaccine. Especially, proteins involved in host cell invasion, including rhomboid protein, are proposed as targets of drug- or vaccine-based control options (O'Donnell and Blackman 2005; Dowse et al. 2008; Muhammad et al. 2010). Experimental data from *T. gondii* have linked rhomboid protein with the

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invasion process. Rhomboid protease cleaved cell surface adhesins during invasion by *Toxoplasma* and *Plasmodium* (Brossier et al. 2005; Dowse and Soldati 2005; Baker et al. 2006; O'Donnell et al. 2006).

In prior works, we have cloned a rhomboid-like cDNA sequence from *E. tenella* (Li et al. 2006; Zheng et al. 2011). The recombinant rhomboid proteins of *E. tenella* expressed in *E. coli*, fowlpox virus, and *Mycobacterium bovis* BCG could elicit immune responses and provide partial protection against *E. tenella* challenge (Li et al. 2012; Yang et al. 2008; Wang et al. 2009).

In the present study, DNA vaccine pVAX1-Rho encoding *E. tenella* rhomboid gene was constructed. Specific antibody, the levels of interleukin-2 (IL-2), interferon- $\gamma$  (IFN- $\gamma$ ), and the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte cells were evaluated in chicken immunized with pVAX1-Rho. The protective efficacy of this DNA vaccine against *E. tenella* challenge was also examined.

## Materials and methods

### Construction of plasmid pVAX1-Rho

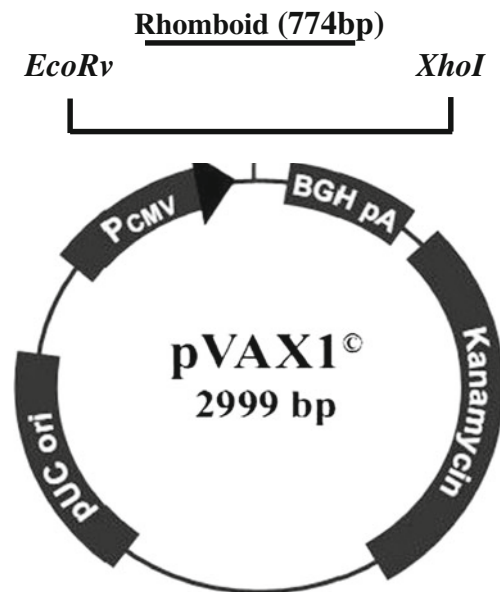
The open reading frame (ORF) of *E. tenella* rhomboid gene was amplified by PCR with genomic DNA as template, using the following primers:

(Forward primer): 5'-CGATATCATGTCCGACATCGAATCCCAGAGAGT-3',

(Reverse primer): 5'-CCGCTCGAGTTATGCGCATCCATGGGCAAAGGA-3', in which the EcoRv and XhoI restriction sites were introduced, respectively. The amplified DNA fragments were inserted into pMD18-T vector (TaKaRa, China) to form a recombinant plasmid pMD-Rho. The rhomboid ORF in pMD-Rho was subcloned into eukaryotic expression vector pVAX1 to form a plasmid pVAX1-Rho as shown in Fig. 1.

### pVAX1-Rho expression in Hela cells

Recombinant plasmid pVAX1-Rho was transfected into Hela cells with lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were fixed with 100 % acetone for 30 min. After washing with PBS-0.1 % Triton-X-100 (PBST) for three times, the cells were blocked in 10 % bovine serum for 2 h and washed again. The cells were then incubated with mouse anti-sporozoites of *E. tenella* polyclonal antibody at 37 °C for



**Fig. 1** Construction of plasmids pVAX1-Rho. *E. tenella* rhomboid fragments were amplified by PCR and inserted into pVAX1 at the restriction sites formed recombinant plasmid pVAX1-Rho

2 h. This polyclonal antibody was prepared as described previously (Zheng et al. 2011). After washing with PBST for three times, the plates were incubated with fluorescein isothiocyanate-labeled goat anti-mouse IgG antibody (1:2,000; Boster Biological Technology, Wuhan, China) at 37 °C for 2 h. After washing, fluorescences were examined under a fluorescence microscope (Carl Zeiss, Germany). Hela cells transfected with pVAX1 served as the negative control.

For western blotting, cells lysates were prepared and verified by SDS-PAGE and transferred to a polyvinylidene difluoride membrane as described previously (Yu et al. 2010). Then mouse anti-sporozoites *E. tenella* polyclonal antibody (1:500) and HRP-labeled goat anti-mouse IgG antibody (1:2,000) were used respectively.

### Vaccination and infection

One-day-old male Leghorn broilers obtained from a commercial breeder (Changchun, China) were reared in a coccidian-free environment. Food and water without anti-coccidia drugs were available ad libitum, and constant light was provided at night during the entire experimental period. At 7 days of age, the chickens were randomly assigned to three groups (20 birds in each group). Experimental group of chickens were intramuscularly injected with pVAX1-Rho (100  $\mu$ g/each) while control group of chickens were injected with pVAX1 plasmid alone or PBS (100  $\mu$ L/each) for three times at 7, 14, and 28 days of age. Two weeks after the final immunization, chickens were challenged with sporulated oocysts of *E. tenella* (Xinjiang strain, provided

by Dr. Xun Suo, Chinese agricultural university) at a dose of  $3 \times 10^4$  per bird.

#### Specific antibody responses

Serum samples were collected from 10 chickens randomly chosen in each group at days 7, 14, 28, and 42 and Rho-specific IgG antibody was examined by ELISA (Li et al. 2012). The optical density (OD) value was read at 490 nm in a microplate reader.

#### Serum levels of IL-2 and IFN- $\gamma$

Serum samples were collected randomly at days 7, 14, 28, and 42. IL-2 and IFN- $\gamma$  were measured using a direct binding ELISA kit specifically designed for chicken (BlueGene Biotechnology Co., Shanghai, China) according to the manufacturer's instructions. The results were described as picograms of IL-2 or IFN- $\gamma$  per 100  $\mu$ l of serum samples.

#### The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells

Chickens selected randomly were killed at day 42 and their spleens were removed aseptically. The splenocytes were incubated with R-phycoerythrin-conjugated mouse anti-chickens CD4 antibody (0.1 mg/ml) and fluorescein-conjugated mouse anti-chickens CD8 $\alpha$  antibody (0.5 mg/ml) (Southern Biotech Associates, Inc) for evaluation of the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> cells (Li et al. 2012). The results were analyzed by flow cytometry.

#### Protective efficacy

The protective efficacy of pVAX1-Rho against *E. tenella* challenge was evaluated according to the cecal lesion scores, the numbers of oocysts, and bodyweight gains (BWG). Weight gain was determined by the body weight of the chickens at the end of the experiments subtracting the body weight at the time of challenge. Cecal lesion scores were determined according to the method of Johnson and Reid (1970). Oocyst counts were expressed by the number of oocysts from 1 g of the caecal content from each chicken

using McMaster's counting technique. Oocyst reduction ratio (ORR) was calculated as follows: ORR (percent) =  $(OC - OV) / OC \times 100$  %. OC = oocyst counts in controls, OV = oocyst counts in vaccinated birds.

#### Statistical analysis

Statistical analysis was performed using SPSS 14.0 software for variance and Duncan's multiple ranges.  $P < 0.05$  was considered statistically significant.

## Results

#### Identification of recombinant plasmid pVAX1-Rho

The ORF fragment of *E. tenella* rhomboid gene was amplified by PCR and inserted into pVAX1 eukaryotic expression vector and the resultant plasmids were named pVAX1-Rho (Fig. 1).

#### Recombinant protein expression in HeLa cells revealed by IFA and western blotting

Fluorescence could be seen in HeLa cells transfected with plasmid pVAX1-Rho 48 h after transfection, but not in cells transfected with pVAX1 vector (Fig. 2). Western blotting analysis showed that HeLa cells transfection with pVAX1-Rho plasmid resulted in the expression of the expected recombinant proteins (rhomboid, 28 kDa), whereas no band was detected from cells transfected with pVAX1 vector (Fig. 3).

#### Evaluation of specific antibody response

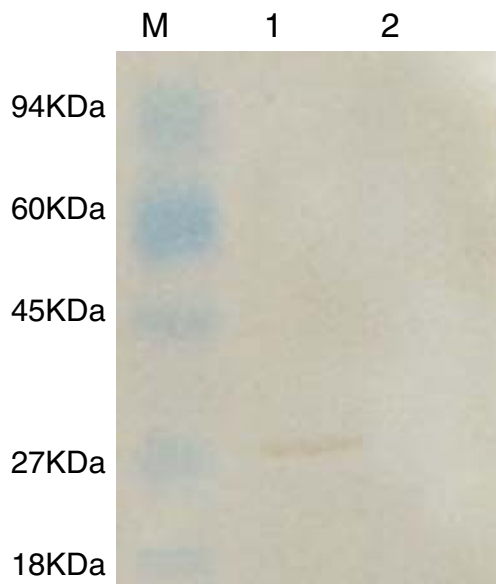
Two weeks after the third immunization, vaccination with plasmid pVAX1-Rho induced specific IgG antibody response. Compared with the PBS group, the differences in mean absorbance values were significant (Fig. 4).

#### Evaluation of IL-2 and IFN- $\gamma$ productions

The average expression level of IL-2 in pVAX1-Rho group was  $271.97 \pm 29.23$  pg/100  $\mu$ l, which was significantly higher

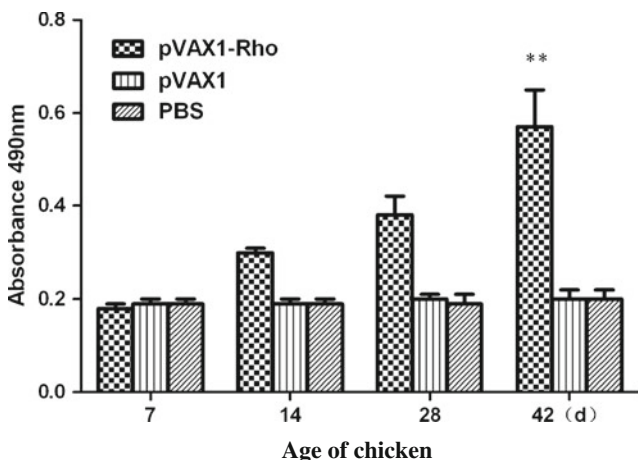
**Fig. 2** Indirect immunofluorescence detection of recombinant rhomboid proteins expression on HeLa cells. HeLa cells were transfected with pVAX1-Rho (a) and vector pVAX1 (b), respectively. Normal HeLa cells were shown in (c)



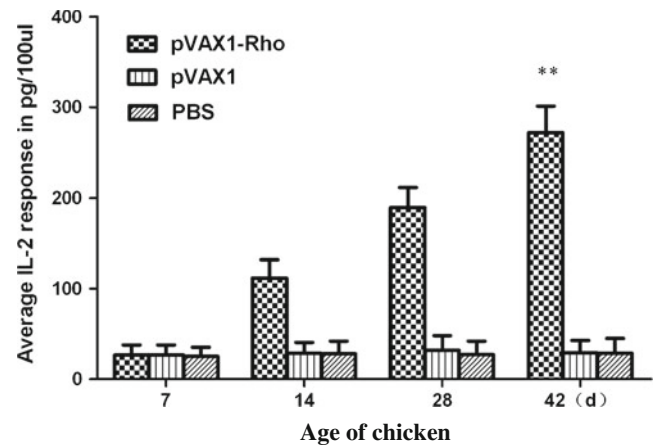


**Fig. 3** Western blotting analysis of recombinant rhomboid proteins expression in HeLa cells. Recombinant *E. tenella* rhomboid (28 kDa) proteins have been expressed in HeLa cells transfection with pVAX1-Rho. The lanes show: M, protein weight marker; lane 1, pVAX1-Rho; lane 2, vector pVAX1

when compared to the PBS group with  $46.41 \pm 18.17$  pg/100  $\mu$ l (Fig. 5). The average expression level of IFN- $\gamma$  in pVAX1-Rho group was  $316.42 \pm 34.23$  pg/100  $\mu$ l, which was a significant increase compared to the PBS group ( $62.28 \pm 23.17$  pg/100  $\mu$ l) Fig. 6.



**Fig. 4** Antibody responses induced by plasmids pVAX1-Rho. Chickens were immunized with plasmids pVAX1-Rho, while control group of chickens were injected with pVAX1 plasmid alone or PBS (100  $\mu$ L/each) for three times at 7, 14, and 28 days of age. Sera were collected, diluted 1:200, and analyzed by ELISA. The asterisks represented significant increase of serum antibody when compared with those of pVAX1 or PBS control (\*\* $P < 0.01$ ; \* $P < 0.05$ )



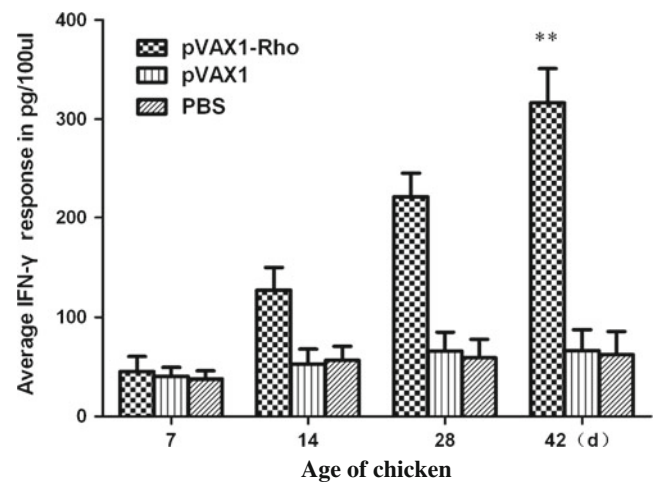
**Fig. 5** Production of IL-2 induced by pVAX1-Rho. Each bar represented mean  $\pm$  S.E. ( $n = 10$ ). The asterisks indicated significant increase in IL-2 expression level compared to the pVAX1 or PBS group (\*\* $P < 0.01$ ; \* $P < 0.05$ )

#### Evaluation of the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes

Two weeks after the third immunization, the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in the pVAX1-Rho group increased significantly when compared with those in PBS group. The data was shown in Table 1.

#### Protective efficacy of pVAX1-Rho vaccination

The number of oocysts, BWG, cecal lesion scores, and oocyst decrease ratio were summarized in Table 2. After challenge,



**Fig. 6** Production of IFN- $\gamma$  induced by pVAX1-Rho. Each bar represented mean  $\pm$  S.E. ( $n = 10$ ). The asterisks indicated significant increase in IFN- $\gamma$  expression level compared to the pVAX1 or PBS group (\*\* $P < 0.01$ ; \* $P < 0.05$ )

**Table 1** The percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from immunized chicken

| Groups    | CD4 <sup>+</sup><br>(%: mean±S.E., n=5) | CD8 <sup>+</sup><br>(%: mean±S.E., n=5) |
|-----------|---|---|
| pVAX1-Rho | 17.38±2.15*                             | 26.42±1.99*                             |
| pVAX1     | 10.54±0.78                              | 21.44±0.80                              |
| PBS       | 9.90±0.32                               | 20.58±0.66                              |

\**P*<0.05, significant compared to PBS and pVAX1

birds immunized with pVAX1-Rho gained bodyweight, had reduced numbers of oocysts, and had significantly decreased cecal lesion compared with chickens in the PBS group. The DNA vaccine pVAX1-Rho could provide chickens with an oocyst decrease ratio around 75.8 %.

## Discussion

To evaluate the potential of *E. tenella* rhomboid as DNA vaccine, recombinant plasmid pVAX1-Rho was constructed and expressed in Hela cells. Compared to control group, pVAX1-Rho vaccination was able to elicit significant specific antibody and cellular immune responses. Birds in pVAX1-Rho group had reduced oocysts output, decreased cecal lesion, as well as increased bodyweight gain against *E. tenella* challenge.

In recent years, significant progress has been made in the identification of DNA vaccine candidates in *E. tenella* which could elicit a protective immune response. DNA pcDNA3-SO7 with a single SO7 gene of *E. tenella* was the first naked DNA to be used to vaccinate chickens (Kopko et al. 2000). Since then, several genes were attempted as DNA vaccine candidates for *E. tenella*, i.e., *E. tenella* SO7 (Kopko et al. 2000; Klotz et al. 2007), *E. tenella* MZ5-7 (Geriletu and Xurihua 2011), *E. tenella* EtMIC2 (Ding et al. 2005), *E. tenella* TA4 (Xu et al. 2008; Song et al. 2009), *E. tenella* refractile body gene (Et1A; Wu et al. 2004), *E. acervulina* 3-1E (Song et al. 2000; Lillehoj et al. 2005; Ma et al. 2011), *E. acervulina* lactate dehydrogenase (LDH) (Song et al. 2010), and *E. acervulina* cSZ-2 (Shah et al. 2010a,b, 2011). Various

eukaryotic expression vectors were used for *Eimeria* DNA vaccines construction, i.e., pVAX1 (Song et al. 2010; Shah et al. 2010a, 2011), pcDNA4.0 (Geriletu and Xurihua 2011), pcDNA3.1 (Wu et al. 2004; Ma et al. 2011), pcDNA3 (Kopko et al. 2000; Klotz et al. 2007), pVR1012 (Klotz et al. 2007), and pBK-CMV (Song et al. 2000). No significant differences were found concerning different vector systems for SO7 such as pcDNA3 and pVR1012 (Klotz et al. 2007). Protective efficacy of the DNA vaccines for *E. tenella* had been identified and could obviously alleviate cecal lesions, bodyweight loss, and reduce oocyst excretion. In the present study, a key host invasion molecule (*E. tenella* rhomboid) as vaccine target and pVAX1 as the eukaryotic expression vector were used. Immune mechanism and protective efficacy of this DNA vaccine pVAX1-Rho were evaluated.

Protective immunity against protozoa within the phylum Apicomplexa (e.g., *Cryptosporidia*, *Eimeria*, *Neospora*, *Plasmodia*, and *Toxoplasma*) is generally CD4<sup>+</sup> T cell dependent and is elicited along the IL-12/IFN- $\gamma$ /iNOS effector axis (Brake 2002). In the present study, the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, IFN- $\gamma$ , and IL-2 cytokines were examined. The increases for the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes of the chickens immunized with pVAX1-Rho indicated that the plasmids could activate cell-mediated immunity. Cytokines synthesized and secreted by leukocytes play important regulatory roles during the immune response against infections. In avian coccidiosis, it was shown that IFN- $\gamma$  is produced by the host mat sites of infection (Rothwell et al. 2000; Yun et al. 2000), and IFN- $\gamma$  release has been used to screen for protective antigens against *E. tenella* infections (Breed et al. 1999). Co-expression of cytokines IL-2 and IFN- $\gamma$  with *Eimeria* antigen could enhance the immune responses of DNA vaccines (Lillehoj et al. 2005; Xu et al. 2008; Song et al. 2009; Shah et al. 2010b; Shah et al. 2011). In this report, IFN- $\gamma$  and IL-2 levels in the serum samples collected from vaccinated birds were significantly higher than those of the control group. However, under certain conditions, antibodies seem to be more important in protection against *Eimeria* infections (Constantinoiu et al. 2008). In the present study, specific IgG antibody against *E. tenella* was elicited in chickens immunized with recombinant plasmids. These

**Table 2** Protective efficacy of DNA vaccine against *E. tenella* challenge

Data are expressed as mean±S.E. (n=10)

\*\**P*<0.01; \**P*<0.05, significant compared to PBS and pVAX1

| Groups           | OPG ( $\times 10^6$ ) | Oocysts decrease ratio (%) | Body weight gain (g) | Lesion scores |
|------------------|-----------------------|----------------------------|----------------------|---------------|
| pVAX1-Rho        | 0.66*                 | 75.8                       | 80.46±6.73*          | 1.29±0.35**   |
| pVAX1            | 1.57                  | 42.5                       | 52.50±4.30           | 3.00±0.64     |
| Positive control | 2.73                  | 0                          | 39.72±5.73           | 3.57±0.55     |
| Negative control | –                     | –                          | 97.68±3.35           | –             |

results suggest that pVAX1-Rho was capable to elicit humoral and cell-mediated immunity in birds.

The protective efficacy of pVAX1-Rho against homologous challenge was also evaluated. As shown from the results, oocysts excretion, and cecal lesions were decreased significantly and the body weight also increased significantly in birds immunized with pVAX1-Rho compared with the control.

In summary, vaccination with an *E. tenella* rhomboid gene involved in host invasion process could be an alternative strategy in the development of an effective anti-*E. tenella* DNA vaccine. Homologues of *E. tenella* rhomboid protease have been identified in *T. gondii*, *Plasmodium* spp., and *Cryptosporidium parvum* (Baker et al. 2006; Trasarti et al. 2007; Sheiner et al. 2008), and results from the present study raises the possibility of using rhomboid protease as a vaccine candidate for apicomplexan parasites.

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