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Research paper

Efficacy of a potential DNA vaccine encoding *Cryptosporidium baileyi* rhomboid protein against homologous challenge in chickens

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ABSTRACT

The parasite *Cryptosporidium baileyi* can infect the larynx, trachea, bursa and cloaca of poultry, causing high mortality during severe infection and leading to substantial economic losses of the poultry industry. The rhomboid protein is very important in *Cryptosporidium* infection. In this study, a nucleic acid based vaccine candidate pEGFP-CbROM was constructed. After orally challenging with *C. baileyi* oocysts, the corresponding immune responses induced were analyzed and the immunoprotective effect evaluated in chickens. Obtained results revealed that this nucleic acid based vaccine could induce antibody responses and peripheral blood T lymphocytes proliferation significantly ($P < 0.05$), while the peripheral blood B lymphocyte proliferation increased significantly ($P < 0.05$) only at a high dose of 100 μg of pEGFP-CbROM, compared with the PBS control group. After *C. baileyi* infection, the duration of oocysts shedding was shortened by 2 days in the 100 μg pEGFP-CbROM group, and the rate of reduction could reach to around 71.3%. While no significant difference in body weight gain was observed among the immunized groups ($P > 0.05$), the differences between the immunized and the non-immunized groups were found to be significant ($P < 0.05$). Our data provides a useful basis for further work in cryptosporidiosis prevention and treatment.

1. Introduction

Cryptosporidium, which belongs to the Apicomplexa phylum, resides in the epithelial cells of gastrointestinal and respiratory tract. Cryptosporidiosis is an important zoonotic parasitic disease caused by *Cryptosporidium*, which is widespread worldwide and induces persistent diarrhea or malnutrition as the main clinical manifestations. In immunocompromised individuals it may even progress to a life-threatening disease (Navin et al., 1999).

Cryptosporidium can infect more than 30 avian species worldwide (Ng et al., 2006). At present, three recognized effective species found in poultry are *Cryptosporidium baileyi*, *Cryptosporidium meleagridis* and *Cryptosporidium galli* (Current et al., 1986; Pavlásek, 2001; Pavlasek, 1994, 1999; Ryan et al., 2003). Among them, *C. baileyi* is the second kind of avian *Cryptosporidium* species found in broilers (Current et al., 1986), following *C. meleagridis* known to infect humans (Slavin, 1955; Xiao and Ryan, 2004). Because of its unique life history and morphological characteristics, *C. baileyi* is considered to be a new effective species (Current et al., 1986). In addition, *C. baileyi*, which mainly parasitizes in larynx, trachea, bursa and the cloaca of hosts, can infect chicken, duck, goose, turkey, quail, ostrich and other poultry. It is the dominant species causing avian cryptosporidiosis, which leads to respiratory tract and digestive tract diseases, resulting in a decline in the performance of animal production, a high mortality rate during severe infection and economic loss of the poultry industry to some extent (Morgan et al., 2001; Siddiki and Masuduzzaman, 2009).

Currently, there is neither effective treatment nor resultful prevention measures for cryptosporidiosis (Cabada and White, 2010). Thus, it is of great importance to explore the structural and functional features of proper antigens as candidate targets to effectively control this disease. Efforts to develop specific antibodies or valid vaccines become a new research direction in this field (Mead, 2010). With the rapid development of molecular biology together with its wide application in parasitology in recent years, cloning, identification and expression of *Cryptosporidium* vaccine candidate genes have made great progress. About 30 potential vaccine candidate molecules of *Cryptosporidium* have been reported, mainly targeting the sporozoite surface antigens, microneme protein, heat shock protein and oocyst wall protein, including CSL, CPS-500, CP15/45/60, CP23, P30 and ROMs (Bouzid et al., 2013).

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In recent years, the rhomboid protein has been investigated in the apicomplexan parasites, such as *Plasmodium* (Baker et al., 2006), *Toxoplasma gondii* (Brossier et al., 2005), *Eimeria tenella* (Li et al., 2006), *Cryptosporidium parvum* and *Cryptosporidium andersoni* (Wu et al., 2009). It is mainly involved in hydrolyzing adhesins during merozoites movement and invasion (Santos et al., 2012), a process closely related to parasitic adhesion and invasion to host cells (Zhou et al., 2004). Among the apicomplexan protozoa, the rhomboid protein was first characterized in *T. gondii* (Brossier et al., 2005). Different rhomboid proteins exhibit diverse characteristics and subcellular localizations, and their expression levels vary in different developmental stages of life history (Brossier et al., 2005; Dowse and Soldati, 2005). It is indicated that the rhomboid protein also exerts important effects in other stages of *Cryptosporidium*, except for its role in the invasion process. *C. parvum* rhomboid protein (CpROM) was first reported by Trasarti et al. (2007) where immunofluorescence test revealed that the protein showed irregular distribution in sporozoites, mainly concentrated in the rear end of sporozoites, while almost non-detectable at the top. Taken together, the results reveal that the rhomboid protein is a feasible vaccine candidate for prevention and treatment of cryptosporidiosis.

A nucleic acid vaccine carrying *C. baileyi rhomboid* gene named pEGFP-CbROM was prepared in this study. The immune responses induced by this nucleic acid vaccine were observed and the immunoprotective effect evaluated after challenging with *C. baileyi* oocysts orally. Our observations provide important insights and fuel new ideas for the prevention and cure of avian cryptosporidiosis.

2. Materials and methods

2.1. Parasites

C. baileyi ZJ strain was isolated in Hangzhou, China. The oocysts were propagated in 3 days old chickens, harvested from the feces, kept in 2.5% potassium dichromate and stored at 4 °C for a maximum of 2 months before use. To prepare for the infection challenge, *C. baileyi* oocysts were purified through discontinuous sucrose density gradient centrifugation (Arrowood and Sterling, 1987), washed with PBS 3 times and centrifuged at 1500*g* for 10 min at 4 °C. The pellet was resuspended and kept for the following experiments.

2.2. Preparation of C. baileyi antigen and genomic DNA

The purified oocysts of *C. baileyi* were pretreated with commercial bleach (Riggs and Perryman, 1987), suspended in PBS supplemented with a cocktail of protease inhibitors (Beyotime Biotechnology), subjected to five freeze/thaw cycles (liquid nitrogen/37 °C), and further processed by ultrasound sonication (300 W, 15 min). After centrifugation at 12,000*g* for 15 min, the supernatant was collected as the antigen solution for subsequent experiment. *Genomic DNA* was extracted from freshly sporulated oocysts after freeze-thaw by a Universal Genomic DNA Extraction Kit (TaKaRa, China) following manufacturer's instructions.

2.3. Experimental chickens

One-day-old male MeiLing native chickens purchased from a commercial hatchery (Zhejiang, China) were fed in *Cryptosporidium*-free environment and supplied with complete diet and clean water. All animals used in the experiment were treated humanely according to the guidelines of the Center of Experimental Animals, Zhejiang University.

2.4. Construction of the eukaryotic expression vector pEGFP-CbROM plasmid

Two pairs of primers for the complete CbROM open reading frame (ORF) were designed as in Table S1, where the *Sac I* and *Kpn I* restriction sites were introduced respectively in the forward and reverse nested primers (underlined). Primers were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd., and the corresponding gene fragment was obtained by nested-PCR amplification with DNA as the template.

The amplified DNA fragment was inserted into pMD18-T Simple Vector (TaKaRa, China), and then subcloned into eukaryotic expression vector pEGFP-C2 via *Sac I* and *Kpn I* digestion to yield the resultant plasmid pEGFP-CbROM. After verification by PCR amplification of the target gene CbROM and restriction digestion, positive clones were confirmed by sequencing.

2.5. pEGFP-CbROM expression in vitro

CEF cells in the logarithmic phase were seeded into 6-well flat bottom microtiter plate (Costar) in 500 μl RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and cultured 24 h with 5% CO_2. Recombinant plasmid pEGFP-CbROM was transfected into CEF cells with lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. After 48 h post-transfection, cells were fixed with 4% paraformaldehyde and stained with DAPI. Fluorescence was examined under a confocal microscopy. CEF cells transfected with the pEGFP-C2 empty vector served as the negative control.

2.6. Western-blot analysis

For western blotting, samples from cells lysates were prepared, separated by SDS-PAGE electrophoresis and transferred to a polyvinylidene difluoride membrane as described previously (Yu et al., 2010). GFP-Tag mouse monoclonal antibody (Roche) diluted 1:3000 in 5% skimmed milk and HRP-labeled goat anti-mouse IgG antibody (Bioword technology) diluted 1:6000 in 5% skimmed milk were used respectively. Signals were detected using chemiluminescent EMSA kit (Thermo Fisher Scientific).

2.7. Immunization protocol and challenge infection by C. baileyi oocysts

Three days old chickens were divided randomly into six groups with 20 chickens in each group (Table S2). Chickens in groups A and B were intramuscularly injected with PBS (100 μl per bird), while chickens in groups C and D-F were given the empty vector pEGFP-C2 (100 μg per bird) and pEGFP-CbROM (25 μg, 50 μg and 100 μg per bird) respectively, using the same protocol. All groups were vaccinated again with the respective components and dosages at days 10 and 20 after the first immunization. Ten days after the third immunization, namely, at the age of 33-day-old, the chickens in groups B–F were challenged orally with 10^6 *C. baileyi* oocysts per bird.

2.8. Enzyme linked immunosorbent assay

Serum samples of 5 chickens chosen randomly in each group were collected from the wing vein at days 0, 10, 20 and 30 post-immunization (PI). Specific antibody responses against *C. baileyi* were determined by enzyme linked immunosorbent assay (ELISA). Briefly, 96-well flat-bottom microtiter plates were coated with 10 μg/ml of crude *C. baileyi* antigens (diluted with 0.1 M carbonate buffer, pH 9.6) at 100 μl per well, and incubated overnight at 4 °C after kept in 37 °C for 1 h. The plates were washed with

PBST (PBS containing 0.05% Tween-20), and then blocked with 5% skimmed milk in PBS at 37 °C for 1 h, followed by incubation with sera diluted at 1:100 in 5% skimmed milk at 37 °C for 2 h. After washing, the plates were incubated with HRP-labeled goat anti-mouse IgG antibody (Sigma) (1:10,000) at 37 °C for 1 h and washed again. Subsequently, substrate solution containing 4 mg *O*-phenylene diamine (OPD) (Sigma) and 30% H_2O_2 in 10 ml 0.1 M citrate-phosphate (pH 5.0) were added 100 μl per well. The reaction was stopped by 15 mins incubation upon adding 50 μl 2 M H_2SO_4 and optical density (OD) at 492 nm was measured using a microplate reader. All samples were run in duplicates.

2.9. Peripheral blood lymphocyte proliferation assay

Peripheral blood was collected randomly from 5 chickens per group from the wing vein at 10 days after the third immunization. Peripheral blood lymphocytes were isolated with lymphocyte separation medium and adjusted to 1.0×10^7 cells/ml in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. For lymphocyte proliferation assay, 96-well flat-bottomed plates were used. Cells prepared as mentioned above were loaded (50 μl/well) to culture plates, cultured at 37° C in 5% CO_2 with 50 μl ConA or LPS (20 μg/ml) for 48 h. MTT 5 mg/ml (Sigma) solution was added to the plates (10 μl per well) and incubated for a further 4 h. The reaction was stopped by the addition of 100 μl dimethyl sulfoxide (DMSO) and the optical density was measured by a microplate reader at 570 nm (OD570). Each sample was analyzed in quadruplicate.

2.10. Evaluation of protective efficacy of DNA vaccine against C. baileyi

The protective efficacy of the DNA vaccine was measured according to the oocyst output and average body weight gains (BWG). In order to evaluate the oocyst output, feces from each group were collected separately on every other day post challenge until day 27 and the numbers of *C. baileyi* oocysts per gram (OPG) feces were counted using haemocytometer counting technique. Besides, the weight of each chicken at the age 3, 33 and 60 was recorded and the corresponding BWG (BWG 1 = the body weight before challenge – body weight before first vaccination; BWG 2 = the body weight after challenge – body weight before challenge) were calculated. Finally, all chickens were euthanized at day 30 post infection.

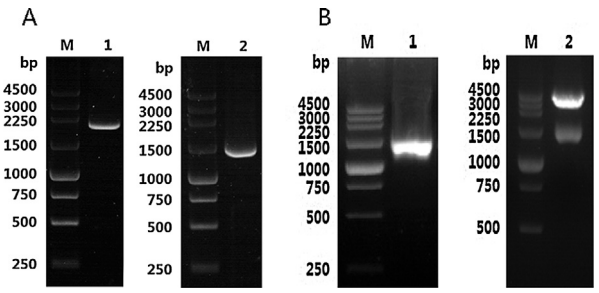

Fig. 1. Amplification of the rhomboid gene from DNA of *C. baileyi* and identification of the recombinant plasmid pEGFP-CbROM. (A) Amplification of the rhomboid gene from DNA of *C. baileyi* by Nested-PCR. M: 250 bp DNA Ladder; 1: the primary PCR products; 2: the secondary PCR products. (B) Identification of the recombinant plasmid pEGFP-CbROM by PCR and restriction enzymes digestion. M: 250 bp DNA Ladder; 1: PCR products of pEGFP-CbROM; 2: pEGFP-CbROM digested by Kpn I/Sac I.

2.11. Statistical analyses

The data were analyzed statistically by one-way ANOVA of variance using GraphPad Prism 5 software. $P < 0.05$ was considered a significant difference.

3. Results

3.1. Nested-PCR amplification and construction of recombinant plasmid pEGFP-CbROM

The full-length CbROM ORF fragment amplified by Nested-PCR with DNA as the template corresponded well to the expected size of 1422 bp (Fig. 1A) and it was cloned successfully into the eukaryotic expression vector pEGFP-C2, giving the resultant plasmid pEGFP-CbROM. The clones were verified by PCR and restriction endonuclease *Sac I*/*Kpn I* digestion (Fig. 1B).

The CbROM fragment was sequenced by Shanghai Biotechnology Co., Ltd. and the nucleotide sequence homology to the CbROM gene sequences of *C. parvum* (**XM_628467.1**), *C. hominis* (**XM_661677.1**) and *C. muris* (**002139608.1**) obtained in GenBank were 63.6%, 63.9% and 36.5% respectively. Amino acid sequence homology to the Romboid protein sequences of *C. parvum* (**XP_628469.1**), *C. hominis* (**XP_666769.1**) and *C. muris* (**XP_002139644.1**) obtained in GenBank were 60.3%, 66.8% and 51.1% respectively.

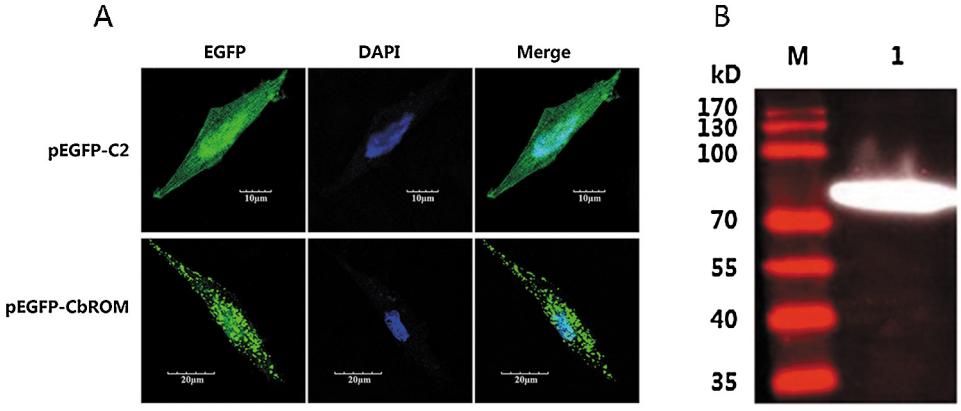

Fig. 2. Detection of protein expression of the recombinant plasmid pEGFP-CbROM in the CEF cells. (A) Detection of CbROM protein expression in CEF cells by confocal microscopy. CEF cells transfected with pEGFP-CbROM and CEF cells transfected with pEGFP. At 48 h post-transfection, CEF cells transfected with pEGFP showing uniformly distributed green fluorescence, while green fluorescence in CEF cells transfected with pEGFP-CbROM are dispersed. (B) Western-Blot analysis of the fusion protein EGFP-CbROM using GFP-Tag mouse monoclonal antibodies. M: Protein Marker; 1: cell culture supernatant from EGFP-CbROM transfected CEF cells after 48 h expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

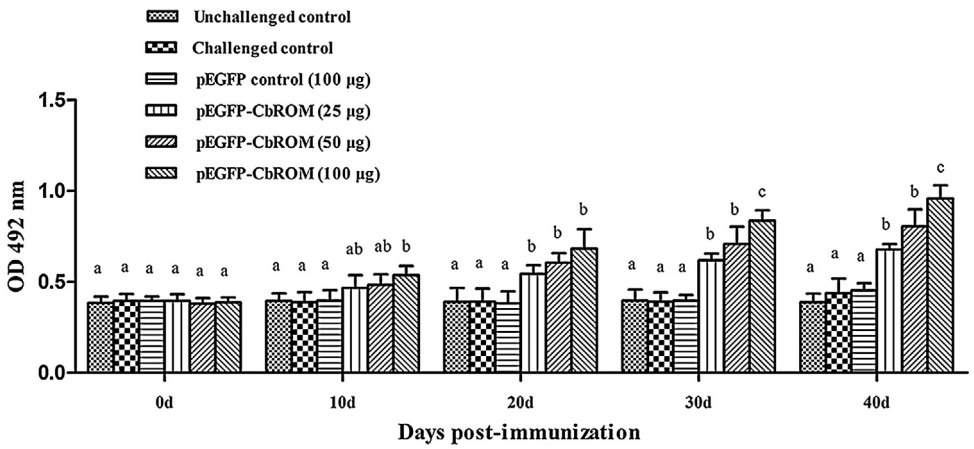

Fig. 3. Humoral immune responses by pEGFP-CbROM DNA vaccination. Chickens were immunized with different doses of pEGFP-CbROM, pEGFP vector, and PBS under the same conditions. Serum samples from 5 randomly chosen chicken in each group were checked for specific anti-*C. baileyi* oocysts antibodies by indirect ELISA. A492 of sera diluted 1:100 is shown. Each bar represents the mean OD (±S.E., n = 5). Bars not sharing the indicated letters (a–c) at the same time point are significantly different ($P < 0.05$).

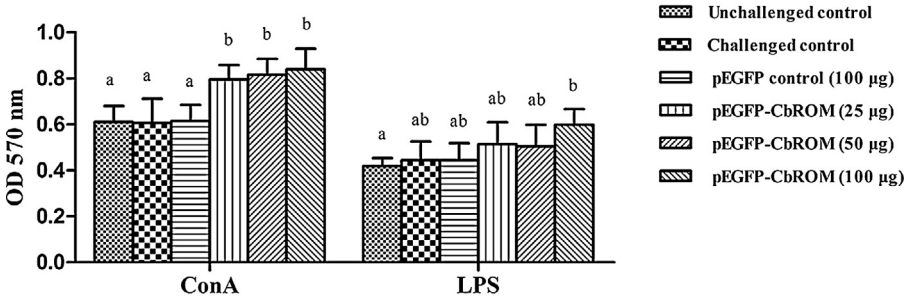

Fig. 4. Analysis of the proliferation of peripheral blood lymphocyte from chickens immunized with pEGFP-CbROM. The mean OD570 values of stimulated lymphocytes measured by MTT (n = 5). Result was detected at 570 nm using an ELISA reader. Bars not sharing the indicated letters (a and b) at the same time point are significantly different ($P < 0.05$).

3.2. CbROM protein expression in CEF cells

At 48 h post-transfection, dispersed green fluorescence could be seen in CEF cells transfected with pEGFP-CbROM, while green fluorescence was uniformly distributed in cells transfected with pEGFP-C2 (Fig. 2A). The results indicated that recombinant CbROM protein was expressed successfully in CEF cells.

3.3. Western-blot analysis of CbROM systhesized in vitro

Western blot analysis showed that CEF cells transfected with the recombinant plasmid successfully expressed the protein pEGFP-CbROM, in accordance with the expected size 79 kDa (Fig. 2B), using GFP-Tag mouse monoclonal antibody and HRP-labeled goat anti-mouse IgG.

3.4. Evaluation of specific antibody responses

The results of ELISA performed on sera samples obtained from both immunized and control chickens are shown in Fig. 3. The differences of antibody responses between the pEGFP-CbROM groups and the control groups immunized with pEGFP or PBS were significant ($P < 0.05$), but no significant difference was observed among the groups immunized with gradient dosages of pEGFP-CbROM at 10 days and 20 days PI ($P > 0.05$). In the plasmid pEGFP-CbROM vaccinated groups, the OD values of specific IgG increased with immunization frequency, and peaked at 30 days PI, compared with the pEGFP-C2 and PBS control groups. Ten days after the third immunization, antibody response was observed to be significantly higher in chickens immunized with the highest dose of pEGFP-CbROM (100 μg) compared to the other groups ($P < 0.05$). Vaccination with plasmid pEGFP-CbROM could induce a specific antibody response.

3.5. Evaluation of lymphocytes proliferation

Cell-mediated immunity patterns as indicated by MTT in the immunized and control chickens are depicted in Fig. 4. At 30 days PI, the peripheral blood T lymphocyte proliferation response was significantly higher in the groups vaccinated with recombinant plasmid pEGFP-CbROM as compared to the control groups ($P < 0.05$), while that of peripheral blood B lymphocyte was also found to be increased significantly only in the group vaccinated with high dose of pEGFP-CbROM (100 μg) as compared to the unchallenged control ($P < 0.05$). These results are consistent with the immune responses caused by *Cryptosporidium* infection and this potential DNA vaccine could cause significant cellular immunity, which plays a very important role in the host immune response against *Cryptosporidium* infection.

3.6. Protective efficacy of DNA vaccination against C. baileyi

To address whether the DNA vaccine was able to induce protection against *C. baileyi* infection, chickens were monitored for 27 days after inoculated with 10^6 oocysts by examining the numbers and duration of parasite oocysts excretion. In the uninfected controls, no oocyst shedding was found in their feces samples. The patterns of oocysts shedding were similar in all experimental groups, with peak excretion on the 11–13th day post challenge and steady decline in the following days (Fig. 5). Chickens in pEGFP-

Table 1
Effects of pEGFP-CbROM vaccination on body weight gain of chickens.

Group	3d (g)	33d (g)	60d (g)	Average BWG 1 (g)	Average BWG 2 (g)
Unchallenged control	31.26 ± 1.500[a]	255.0 ± 22.92[a]	490.9 ± 42.23[c]	223.8 ± 23.08[a]	253.9 ± 43.02[b]
Challenged control	31.27 ± 1.836[a]	252.6 ± 17.12[a]	421.7 ± 25.05[a]	221.3 ± 16.51[a]	169.2 ± 30.30[a]
pEGFP control (100 μg)	31.24 ± 1.642[a]	258.9 ± 22.11[a]	420.7 ± 41.67[a]	227.7 ± 21.74[a]	161.7 ± 35.28[a]
pEGFP-CbROM (25 μg)	29.73 ± 1.951[a]	245.0 ± 18.38[a]	455.5 ± 35.15[b]	215.3 ± 18.15[a]	210.5 ± 28.08[b]
pEGFP-CbROM (50 μg)	30.43 ± 2.001[a]	240.5 ± 24.58[a]	464.4 ± 33.76[bc]	210.1 ± 24.76[a]	223.9 ± 30.48[b]
pEGFP-CbROM (100 μg)	31.72 ± 4.085[a]	247.3 ± 26.04[a]	486.3 ± 39.15[bc]	216.6 ± 23.49[a]	238.9 ± 30.45[b]

Values with different superscript letters (a–c) in the same column are significantly different ($P < 0.05$).

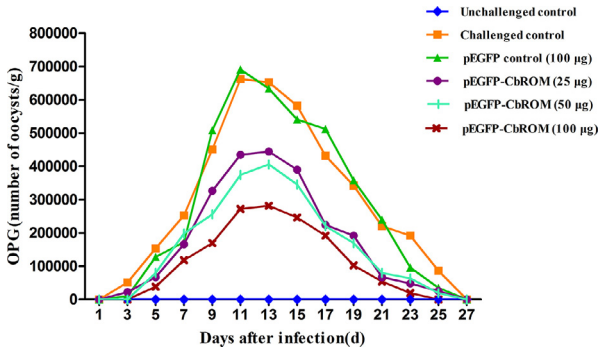

Fig. 5. Regulation of oocysts shedding of chickens after challenging with *C. baileyi* oocysts. All the chickens were infected with 1×10^6 *C. baileyi* oocysts on the 10th days after the third immunization and their oocysts excretion was monitored for 27 days. After challenging with *C. baileyi*, chickens immunized with pEGFP-CbROM showed less oocysts in the feces compared to chickens in controls receiving either PBS or pEGFP-C2. In PBS group, there was no oocysts shedding in their feces. The duration of oocysts shedding became shorter by 2 days in 100 μg pEGFP-CbROM group. Compared with the control group, experimental chicken could partially prevent *C. baileyi* infection.

CbROM experimental groups excreted less oocysts than the groups of pEGFP-C2 and PBS control ($P < 0.05$). Percentage reduction of the 100 μg and 50 μg pEGFP-CbROM group could reach to 71.3% and 53.5% in the level of oocysts shedding, significantly higher than that of the 25 μg group (48.4%) and the challenged controls ($P < 0.05$) (Fig. 5), displaying good protection. From our results, the duration of oocysts shedding was shortened by 2 days in the 100 μg pEGFP-CbROM group (Fig. 5). These suggest that immunization with pEGFP-CbROM could reduce fecal oocysts burden in chickens and partially protect chickens from *C. baileyi* infection.

The body weight gains (BWG) of the chickens in six groups are presented in Table 1. BWG1 shows that no significant difference in body weight was detected among the groups before immunization and at 30 days PI ($P > 0.05$). This means that immune reactions made no obvious effect on the body weight of chickens. BWG2 in all pEGFP-CbROM immunized chickens were higher than that of the challenged control groups ($P < 0.05$). There was no significant difference among the unchallenged group and pEGFP-CbROM immunized groups ($P > 0.05$), especially between the 100 μg pEGFP-CbROM group and the unchallenged group. Percentage reduction of the 25 μg, 50 μg and 100 μg pEGFP-CbROM group in weight gain were respectively 16.7%, 11.5% and 5.4%, significantly lower than that of the challenged controls (the PBS control: 33.4% and the pEGFP control: 36.7%) ($P < 0.05$). These results show that immunization with the pEGFP-CbROM did protect chickens from the *C. baileyi*-induced reduction in body weight gain.

4. Discussion

C. baileyi, the most common avian *Cryptosporidium* spp., has been reported in a wide range of avian hosts. *C. baileyi* frequently causes respiratory cryptosporidiosis, with high morbidity and mortality in birds, especially broiler chickens (Morgan et al., 2001; Sreter and Varga, 2000; Sreter et al., 1995). Despite many attempts, no effective drug or protective vaccine is approved for prevention or treatment of cryptosporidiosis in animals or humans so far. It was hampered due to a lack of systems for continuous culture, complex life history of the parasite, hurdles towards genetic analysis and manipulation and complex antigen composition. This study argued that a nucleic acid vaccine carrying *C. baileyi rhomboid* gene could partially prevent *C. baileyi* infection.

Compared to traditional vaccines, many other advantages offered by nucleic acid vaccine are evident (Kutzler and Weiner, 2008). In addition to its simple preparation, inexpensive cost, convenient storage and transportation (Dhama et al., 2008), it can induce a more comprehensive and longer immune response while the cytotoxic T lymphocytes generated exert a good effect in killing intracellular parasite (Stenger and Modlin, 1998). This type of vaccine avoids the inefficient protective reaction of conventional inactivated vaccine, the risk of resorting mutation of attenuated vaccine, and the high cost and complex preparative process of genetic engineered polypeptide vaccine. DNA vaccine can elicit the best immune effect by intramuscular injection in virtue of 100–1000 times higher absorption and expression efficiency than that through other means (Fynan et al., 1993). Therefore this experiment aims to develop a kind of nucleic acid vaccine and intramuscular injection is selected.

Gene selection is very crucial in the preparation of nucleic acid vaccine. So far, extensive studies have explored the suitability of a variety of sporozoite surface proteins of *Cryptosporidium* as vaccine candidate antigen genes, and their potential in immune protection against *Cryptosporidium* infection (Mead, 2014). In *C. parvum*, efforts on immune protective effect of vaccines based on CP15 (Hong-Xuan et al., 2005; Manque et al., 2011; Roche et al., 2013; Sagodira et al., 1999; Wang et al., 2010), CP23 (Borad et al., 2012; Ehigiator et al., 2007), P2 (Benitez et al., 2011) have been made. Mice immunized with Cp23-DNA acquired partial protection against *C. parvum* infection as shown by the >60% oocyst reduction (Ehigiator et al., 2007). Homologues of *C. baileyi* rhomboid protease have been identified in *T. gondii*, *Plasmodium* spp., *E. tenella*, and *C. parvum* (Baker et al., 2006; Brossier et al., 2005; Li et al., 2006; Trasarti et al., 2007). Rhomboid subunit vaccines and DNA vaccines against *T. gondii* (Li et al., 2012a) and *E. tenella* (Li et al., 2012b; Liu et al., 2013) have been found to induce specific immune response in animals and provide good immune protection effect. These work demonstrated that rhomboid protease is a feasible vaccine candidate for apicomplexan parasites.

In this study, the gene encoding CbROM was amplified and cloned into the expression vector pEGFP-C2. The *in vitro* experiments show that with the help of pEGFP-C2 the exogenous gene *rhomboid* can be effectively expressed in chicken fibroblast. After immunization by intramuscular injection, humoral immune response and cellular immune response in the chickens were analyzed by ELISA and lymphocyte proliferation test respectively. Our results show that serum antibody levels of the recombinant plasmid pEGFP-CbROM immunized group increased significantly compared with the control group ($P < 0.05$), with the highest level

found in the high dose recombinant plasmid (100 μg) immunized group. Stimulated by ConA, the proliferation ability of peripheral blood T lymphocytes of recombinant plasmid pEGFP-CbROM immunized group increased significantly compared with the control group ($P < 0.05$), while the proliferation of peripheral blood B lymphocytes stimulated by LPS increased to a certain extent, while only significant difference observed between high dose (100 μg) immunized group and non-immunized and non-infected groups ($P < 0.05$). This may be due to *Cryptosporidium* being intracellular parasites, cellular immunity plays a very important role in the host immune response against *Cryptosporidium* infection (Heine et al., 1984).

To investigate the protective immunity of recombinant plasmid pEGFP-CbROM against *C. bailey* infection, each bird of the test groups was artificially infected with 10^6 *C. bailey* oocysts 10 days after third immunization. The duration of oocysts shedding was shortened by 2 days in the chicken groups immunized with 100 μg pEGFP-CbROM, and the reduction rate of oocysts shedding could reach up to 71.3%. No significant difference was observed in the weights between the vaccination groups ($P > 0.05$), but a significant difference was found upon comparing with the non-immunized group ($P < 0.05$). No significant difference of body weight between groups was detected ($P > 0.05$) before immunization, showing that experimental animals grouping is sufficiently random and non-bias. A significantly higher BWG2 indicated that vaccination with pEGFP-CbROM did provide protection against *C. baileyi* challenge. According to body weight gains, the best protective immunity was found in the 100 μg recombinant plasmid pEGFP-CbROM immunized group, followed by the 50 μg recombinant plasmid DNA vaccinated group. Although pEGFP-CbROM vaccinated groups produced lower BWG1 than the control groups, the difference was not significant, showing that immune reactions exert no obvious effect on the body weight of experimental animals.

The possibility of cross protection of CbROM vaccine against other species of *Cryptosporidium* and other apicomplexan parasites such as *E. tenella* should be further explored, since studies in gnotobiotic pigs indicated incomplete cross-protection between *C. parvum and C. hominis* (Sheoran et al., 2012). Although complete oocyst infection protection cannot be achieved by current DNA vaccines, our data showed that, high dose (100 μg) CbROM gene recombinant DNA in vaccinated chickens conferred certain protective immunity against *Cryptosporidium* infection, laying the foundation for the development of novel vaccine against *Cryptosporidium*.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2016.05.024.

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