Veterinary Immunology and Immunopathology 162 (2014) 117–121

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

# Protective immunity against *Eimeria tenella* infection in chickens induced by immunization with a recombinant C-terminal derivative of EtIMP1

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ARTICLE INFO

*Article history:*
Received 16 July 2014
Received in revised form 17 October 2014
Accepted 21 October 2014

*Keywords:*
*Eimeria tenella*
Immune mapped protein1
Coccidiosis
Vaccine

ABSTRACT

Immune mapped protein-1 (IMP1) is a new protective protein in apicomplexan parasites, and exits in *Eimeria tenella*. Cloning and sequence analysis has predicted the antigen to be a novel membrane protein of apicomplexan parasites. In order to assess the immunogenicity of EtIMP1, a C-terminal derivative of EtIMP1 was expressed in a bacterial host system and was used to immunize chickens. The protective efficacy against a homologous challenge was evaluated by body weight gains, lesion scores and fecal oocyst shedding. The results showed that the subunit vaccine can improve weight gains, reduced cecal pathology and lower oocyst fecal shedding compared with non immunized controls. The results suggested that the C-terminal derivative of EtIMP1 might be considered as a candidate in the development of subunit vaccines against *Eimeria* infection.



## 1. Introduction

Coccidiosis in chickens is caused by seven species of apicomplexan parasites belonging to the genus *Eimeria*. These infectious diseases lead to major economic losses worldwide in poultry industries (Shirley et al., 2005; Williams, 2002). Chemotherapeutic treatment remains the most cost effective means of controlling the disease, however, the increase of resistant parasite populations and increasing public pressure to limit the use of chemicals in animal feed underlines the need to find alternative methods (Blake et al., 2011). And one of the most effective methods in the management of infectious diseases in veterinary practice is through the induction of protective immunity by vaccination.

Immune mapped protein-1 (IMP1) is a newly discovered protein in *Eimeria maxima*, and has been demonstrated to be immunogenic and confer protection against *E. maxima* challenge in chickens (Blake et al., 2011). Recently, IMP1 has also been identified as immunoprotective antigens from other apicomplexan parasites, such as *Toxoplasma* and *Neospora* (Cui et al., 2012a,b). And the IMP1 gene was also identified to be a vaccine candidate against *Eimeria tenella* infections (Blake et al., 2011). In our previous study, the EtIMP1 gene has been cloned and its protein has been expressed in *Escherichia coli* cells, with further

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http://dx.doi.org/10.1016/j.vetimm.2014.10.009
0165-2427/

identification and investigation of protective effect as a vaccine candidate (Yin et al., 2013c). Immunization of birds with the recombinant EtIMP1 protein reduced the oocyst output by 60%, comparable with the protective effects of other antigens, such as EtMIC1, EtMIC11 and profilin (Lillehoj et al., 2005; Sathish et al., 2012; Subramanian et al., 2008; Yin et al., 2013c).

Bioinformatic analysis of EtIMP1 revealed that it may be a disordered protein, the N-terminal part (amino acid residues 1–160) contains most of the predicted disordered regions, and the C-terminal part (residues 161–397) was predicted to be a flavin mononucleotide (FMN)-dependent reductase (Yin et al., 2013c). In the present study, we hypothesized that the C-terminal derivative of EtIMP1 may be the immunodominant region of the protein thus providing similar or greater immune protection of chickens from *Eimeria* infection compared with the full length of EtIMP1. To test our hypothesis, we expressed EtIMP1-C and EtIMP1, investigated the immunogenicity of them and evaluated the protective efficacy of the recombinant proteins against *E. tenella* infection in chickens.

## 2. Materials and methods

### 2.1. Chickens and parasites

One-day-old male Hy-Line variety brown layer chickens were purchased from Fuzhou Hy-Line variety brown Poultry Breeding. They were housed in isolators and given feed and water ad libitum. The *E. tenella* (XJ strain) was maintained and propagated in coccidia-free, 2- to 3-wk-old AA broilers in our laboratory (Huang et al., 2011; Yin et al., 2013a). Oocysts were collected from feces of chickens 6 to 9 d postinfection and were isolated, purified, and sporulated as described previously (Long et al., 1976; Yin et al., 2013c).

### 2.2. Cloning and expression of a recombinant C-terminal fragment of EtIMP1

The C-terminal 711 bp fragment of EtIMP1 (amino acid residues 161–397) was amplified by PCR using the following primers with introduced EcoRI and XhoI sites (underlined): 5′-GAATTCATGGGAAGCAACGCGAACCTG-3′ and 5′-CTCGAGAGTTGCTGCC GCCACATTTC-3′. PCR was performed with the following program: 4 min at 94 °C; 30 cycles of 40 s at 94 °C, 40 s at 55 °C and 1 min at 72 °C, and 72 °C for 10 min. After EcoR I/XhoI digestion, the amplified fragment was ligated into the EcoR I and XhoI sites of the expression vector pET-28a (Novagen, Germany) and the recombinant plasmid was propagated by transformation into *E. coli* strain DH5-a. Then the vectors were transformed into *E. coli* (BL21) for protein expression. The transformed *E. coli* was grown overnight to mid-log phase, induced with1.0 mM of IPTG for 6 h at 37 °C, collected by centrifugation, and disrupted by sonication on ice. The recombinant protein EtIMP1-C was purified using the Hi-Trap metal chelating column (GE Healthcare, USA) according to the manufacturer's instructions. Final purity was confirmed by SDS-PAGE and Western blotting with mouse anti-$His_6$ monoclonal antibody (Transgen, Beijing, China) at a 1:2000 dilution.

### 2.3. Vaccination and parasite challenge infection

To test whether EtIMP1-C vaccination confers effective protection against *E. tenella* infection, two-week-old male Hy-Line variety brown layer chickens were randomly divided into five groups, each group consist of 30 birds. The first two groups were immunized intramuscularly in the breast with 100 μg recombinant EtIMP1 emulsified in Freund's complete adjuvant (FCA) (Group 1), and 100 μg EtIMP1-C in FCA (Group 2). The challenged control group (Group 3) and unchallenged control group (Group 4) were injected with PBS. The final group was immunized intramuscularly with 100 μl FCA (Group 5) as the adjuvants controls. Fourteen days after the primary immunization, birds in each group were boosted with the same dose as the primary immunization. Fourteen days after the final immunization, birds in Groups 1, 2, 3, and 5 were challenged with 5000 virulent *E. tenella* oocysts. Sera (10 birds per group) were collected 14 days after the final immunization and stored at −20 °C for further analysis.

At 5 days post-challenge, cecal lesions (10 birds per group) were scored on a graded scale from 0 (none) to 4 (high) in a blinded fashion by two independent examiners as previously described (Johnson and Reid, 1970). Body weights were measured at 0 and 8 days post-challenge (10 birds per group). For the determination of fecal oocyst shedding, 10 birds per group were placed in oocyst collection cages separately and fecal samples were collected between 6 and 8 dpi. Oocyst shedding per bird was determined using McMaster egg counting chamber (Lee et al., 2007; Sun et al., 2014).

### 2.4. Determination of anti-IMP1 antibodies

Chicken IgG was detected by ELISA as previously described (Cui et al., 2012a; Huang et al., 2011). Briefly, 96-well microtiter plates were coated with the recombinant EtIMP1 (4 μg/ml) in 50 mM carbonate buffer (pH 9.6) overnight at 4 °C and blocked for 1 h at 37 °C with 5% milk powder (Difco™skim milk, BD) in PBST (PBS containing 0.05% Tween 20). After washing with PBST, sera were added in a dilution of 1:100 and incubated for 1 h at 37 °C. Antigen specific antibodies were detected using rabbit anti-chicken IgG conjugated to horse-radish peroxidase ($1:10^4$ dilution). The ELISA was developed using TMB and $H_2O_2$ as substrates, and optical density was read at 450 nm (A450) with an ELISA reader (Bio-TekEL 680, USA).

### 2.5. IFN-γ ELISPOT assay

Chicken IFN-γ was detected by ELISPOT assay as previously described (Ariaans et al., 2008; Yin et al., 2013b; Yin et al., 2013c). Briefly, 14 days after the final immunization, chicken peripheral blood mononuclear cells (PBMC) (10 birds per group) were used to determine the levels of IFN-γ secretion. ELISPOT 96-well plates (Multiscreen Assay System, Millipore, USA) were coated with 5 μg/ml mouse-anti-Chichen IFN-γ capture antibody (Biosource