Veterinary Parasitology 244 (2017) 44–53

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

Humoral and cytokine response elicited during immunisation with recombinant Immune Mapped protein-1 (EtIMP-1) and oocysts of *Eimeria tenella*

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ARTICLE INFO

Keywords:
Eimeria tenella
EtIMP-1
Oocyst
Immunisation
Interleukins

ABSTRACT

Eimeria tenella, the causative agent of caecal coccidiosis, is a pathogenic gut dwelling protozoan which can cause severe morbidity and mortality in farmed chickens. Immune mapped protein-1 (IMP-1) has been identified as an anticoccidial vaccine candidate; in the present study allelic polymorphism was assessed across the IMP-1 coding sequence in *E. tenella* isolates from four countries and compared with the UK reference Houghton strain. Nucleotide diversity was low, limited to expansion/contraction of a CAG triplet repeat and five substitutions, three of which were non-synonymous. The EtIMP-1 coding sequence from a cloned Indian *E. tenella* isolate was expressed in *E. coli* and purified as a His-tagged thioredoxin fusion protein. An *in-vivo* vaccination and challenge trial was conducted to test the vaccine potential of recombinant EtIMP-1 (rEtIMP-1) and to compare post-vaccination immune responses of chickens to those stimulated by live oocyst infection. Following challenge, parasite replication measured using quantitative PCR was significantly reduced in chickens that had been vaccinated with rEtIMP-1 (rIC group; 67% reduction compared to UC or unimmunised controls; 79% reduction compared to rTC group or recombinant thioredoxin mock-immunised controls, $p < 0.05$), or the birds vaccinated by infection with oocysts (OC group, 90% compared to unimmunised controls). Chickens vaccinated with oocysts (OC) had significantly higher levels of interferon gamma in their serum post-challenge, compared to rEtIMP-1 vaccinated birds (rIC). Conversely rEtIMP-1 (rIC) vaccinated birds had significantly higher antigen specific serum IgY responses, correlating with higher serum IL-4 (both $p < 0.05$).

1. Introduction

Coccidiosis of domestic chickens (*Gallus gallus domesticus*) is a disease caused by protozoan parasites of the genus *Eimeria*. Seven species are recognized to infect chickens: *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E.mitis*, *E. necatrix*, *E. praecox* and *E. tenella*, (Long, 1973; Shirley, 1979; Shirley et al., 1983), all of which have a worldwide distribution (Clark et al., 2016). *Eimeria tenella* is an important species due to its common occurrence and high pathogenicity (Reid et al., 2014; Kumar et al., 2015). Peak morbidity due to *E. tenella* is commonly observed in chickens between three and seven weeks of age (Urquhart et al., 1996) and broilers reared for meat production in deep litter systems are commonly affected towards the end of their growing period, when mortality can be high (Jatau et al., 2012). The outcome of infection is influenced bythe magnitude of oocyst dose, bird age and genotype, previous exposure and nutritional status. Coccidiosis control currently relies on good flock management and hygiene, chemoprophylaxis and vaccination. Chemoprophylaxis is most commonly employed, but parasite genetic resistance to anticoccidial drugs is widespread and concerns related to drug and chemical residues in meat and eggs are of increasing importance (Young and Craig, 2001). Current coccidiosis vaccines are mostly based on oral infection with controlled doses of wild-type or attenuated parasites, and most vaccines include oocysts of several parasite species to provide broad protection against disease. In some formulations multiple strains of a single species (for example *E. maxima*) are included to combat challenge by antigenically divergent strains (Shirley and Bellatti, 1988).

The commercial use of live anticoccidial vaccines has been limited

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http://dx.doi.org/10.1016/j.vetpar.2017.07.025
Received 5 June 2017; Received in revised form 16 July 2017; Accepted 22 July 2017

by production capacity (virtually all vaccine lines have to be grown and purified from chickens) and the relative cost of the products compared to anticoccidial drugs (Shirley et al., 2007). The constraints on current vaccines encourage development of next generation versions based on the use of adjuvanted recombinant proteins or immunoprotective antigens expressed in appropriate live vector systems (reviewed in Blake and Tomley, 2014). Many antigens including apical membrane antigen-1, several micronemes (MIC) and heat shock proteins (HSP-90 and HSP-70), have been tested experimentally with varying degrees of success (e.g. Peroval et al., 2006; Subramanian et al., 2008; Sathish et al., 2011; Jiang et al., 2012; Sathish et al., 2012; Zhang et al., 2012; and Qi et al., 2013).

Immune mapped protein-1 (IMP-1) was first identified as a novel vaccine antigen for *E. maxima* in a genetic mapping study (Blake et al., 2011), and shown to provide partial immune protection against challenge infection when administered as a recombinant protein. Understanding the nature of the immune response stimulated by vaccination, compared with natural infection, offers scope to improve adjuvant choice and maximize immune protection. IMP-1 orthologues have been identified in other coccidians. Antibodies against NcIMP-1 reduce *Neospora caninum* infection of Vero cells *in-vitro* (Cui et al., 2012a); vaccination with recombinant TgIMP-1 prolongs survival of mice experimentally infected with *Toxoplasma gondii* (Cui et al., 2012b); and recombinant EtIMP-1 (rEtIMP-1) partially protects chickens against *E. tenella* infection (Yin et al., 2013; Yin et al., 2015). If IMP-1 is to be developed further as an anticoccidial vaccine antigen it is important to understand the extent of its naturally occurring polymorphism; allelic diversity has undermined the utility of several anti-parasite vaccine candidates, most notably for malarial parasites such as *Plasmodium falciparum* (Healer et al., 2004). Here, we report comparison of EtIMP-1 sequences generated from parasites collected from three continents and test the immunizing capacity of rEtIMP-1from an Indian variant parasite, comparing the immune responses induced with those stimulated by oral oocyst vaccination.

2. Materials and methods

2.1. Experimental birds and parasite isolates

CARIBRO Vishal broiler chickens were obtained from the Central Avian Research Institute, Izatnagar, Bareilly, India, for passage and amplification of *E. tenella* parasites, collection of sera and experimental vaccination studies. The birds were reared under strict specific pathogen-free conditions in steel cages on raised stands with wired flooring, with a standard feeding and watering regimen without anticoccidial drugs. Faecal trays were placed under each cage floor and cleaned daily. *Eimeria tenella* oocysts used in the study were derived from Indian isolate-1 (Kundu et al., 2013). The ethical review process is described in the Ethical Statement.

2.2. Collection of chicken anti-E. tenella convalescent sera

Six chickens were gavage inoculated with 2500 sporulated oocysts at 19 days of age and two weeks later with 5000 oocysts. A week after the second oocyst infection, blood was collected from the brachial veinand allowed to clot at room temperature for 2 h. Serum was harvested by centrifugation at 1000 g for 10 mins. Harvested serum was used for characterization of rEtIMP-1 by Western Blot.

2.3. RNA isolation and cDNA preparation

Total RNA was extracted from *E. tenella* oocysts using an RNeasy mini kit (Qiagen). Sporulated oocysts numbering 1.5×10^5 were suspended in 50 µl lysis buffer provided with the kit and an equal volume of 0.3-0.5 mm DEPC treated, pre-sterilised glass beads were added. Oocysts were ruptured as described previously for extraction of RNA (Krucken et al., 2008). RNA extracted as per the manufacturer's instruction was eluted in a 50 µl volume. The concentration and purity was checked using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific).Complementary DNA (cDNA) was synthesized from *E. tenella* RNA, using a Revert Aid H minus first strand cDNA synthesis kit (Thermo Scientific), in a 20 µl reaction mixture as described by the manufacturer.

2.4. Polymerase chain reaction (PCR) based amplification, cloning and sequencing of E. tenella immune mapped protein-1(EtIMP-1)

Primers, forward (5′-AAT*GAATTC*TGAGCCTCCTGTCTCTGCTG-3′) and reverse (5′-TTA*CTCGAG*AGTTGCTGCCGCCACATTTC-3′) were used for PCR amplification of an EtIMP-1 1134 bp fragment, incorporating *Eco*RI and *Xho*I restriction sites respectively (shown in italics). PCR amplification was performed in a 25 µl reaction mixture consisting of cDNA 2 µl, Dream Taq green buffer 2.5 µl, 1 µl each of forward and reverse primers (10 pmol μl^{-1}), 10 mM dNTP mix 0.5 µl, Pfu polymerase/Taq polymerase blend 1 µl and nuclease free water to make up the volume. The PCR reaction was carried out under initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min, and final extension at 72 °C for 15 minThe PCR productwas purified using a Minelute PCR purification kit (Qiagen, Germany), ligated into TA vector pTZ57R/T and transformed into DH5α *Escherichia coli* cells using an InsTA cloning kit (ThermoScientific, USA). The *Eco*RI/*Xho*I double digested EtIMP-1/pTZ plasmid and pET32b vector were gel purified and ligated. The ligation reaction was performed with 5X ligation buffer (4 µl), digested vector (50 ng/4 µl), digested PCR product (30 ng/8 µl), T4 DNA ligase (5 Weiss units/1 µl) and nuclease free water (3 µl), in 0.2 ml PCR tubes at 4 °C with overnight incubation. The ligated product was cloned into *E. coli* (Nova Blue strain) using a Transform Aid bacterial transformation kit (Thermo Scientific, USA). The clones were sent for custom sequencing of the insert atthe Department of Biochemistry, University of Delhi, India.

2.5. Bioinformatic analysis

Nucleotide sequences obtained after custom sequencing were searched for similarity using the BLASTn program (nucleotide blast) through the National Center for Biotechnology Information (NCBI;http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using the Megalign program in DNA Star (Laser gene Suite 6.0) software. The protein encoding nucleotide sequences were translated *in-silico* using the Edit Sequence program of DNA Star (Laser gene Suite 6.0) and BLASTp (protein–protein BLAST) was performed. The sequences generated here were compared to the reference *E. tenella* Houghton strain IMP-1 (accession number FN813229), as well as a published sequence derived from a Chinese *E. tenella* isolate (KC215109). Additional EtIMP-1 sequences were extracted from next-generation sequence data generated previously from *E. tenella* isolates collected in the UK and the US (the Weybridge and Wisconsin reference isolates; Reid et al., 2014; Blake et al., 2015). The EtIMP-1 sequence generated here has been deposited in GenBank under the accession number KC758959. The number of nucleotide haplotypes and total nucleotide polymorphism (Pi) using the Jukes-Cantor correction were calculated with DnaSP (version 5.10.01, Librado and Rozas, 2009). Tajima's D, and Fu and Li's D* and F* tests were conducted to test for signatures of selection using DnaSP.

2.6. Expression and purification of recombinant E. tenella IMP-1 (rEtIMP-1)

Recombinant IMP-1 was expressed in BL21pLysS *E. coli* cells as a His-tagged thioredoxin fusion protein which was purified under native conditions. Maximum expression of rEtIMP-1 was achieved by