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

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## 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  $p<0.05$ ).

**Key Words:** *Eimeria tenella*, EtIMP-1, oocyst, immunisation, interleukins

## 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 by the 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 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-1 from 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 2,500 sporulated oocysts at 19 days of age and two weeks later with 5,000 oocysts. A week after the second oocyst infection, blood was collected from the brachial vein and allowed to clot at room temperature for 2 hours. Serum was harvested by centrifugation at 1,000g 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  $1.5 \times 10^5$  were suspended in 50  $\mu$ l 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  $\mu$ l 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 RevertAid 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'-AATGAATTCTGAGCCTCCTGTCTCTGCTG-3') and reverse (5'-TTACTCGAGAGTTGCTGCCGCCACATTTC-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<sup>-1</sup>), 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 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min, and final extension at 72°C for 15 min. The PCR product was 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 (50ng/ 4µl), 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 at the 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 induction of bacterial culture at optical density (OD) of 0.6 with 0.6 mM final concentration Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). Each gram of harvested cell pellet was lysed by incubating in buffer (75mM phosphate, 500mM NaCl, 20mM imidazole, pH 8.0) containing 1% w/v tritonX-100, followed by sonication on ice. Supernatant from the lysate after centrifugation was passed through 1ml volume of pre-equilibrated Ni-NTA super flow resin (Qiagen, USA). The column was washed with wash buffer (75 mM phosphate, 500mM NaCl, 40mM imidazole, pH 8.0) to remove unbound proteins. Resin bound rEtIMP-1 was eluted in 0.5 ml fractions with elution buffer (75 mM phosphate, 500mM NaCl, 500mM imidazole, pH 8.0). The concentration of eluted protein was estimated using the Bradford assay method (Bradford assay kit, Amresco, USA).

## **2.7. SDS PAGE and Western blotting**

Expressed recombinant protein was resolved on 12 % SDS-PAGE mini gels (Bio-Rad mini protean vertical electrophoresis system, as described by Laemmli, 1970). The rEtIMP-1 expressed from 1134 bp nucleotides predicted 378 amino acid residues. Purified proteins resolved on 12% SDS-PAGE gel were transferred on to nitrocellulose membrane, pore size 0.45 $\mu$ m (Thermo Scientific, USA) using a Bio-Rad mini trans-blot system, with a constant power supply of 100V for 1 hour. Expression of the recombinant protein was confirmed by probing the blotted proteins with a 1:1,000 dilution of Ni-NTA HRP conjugate (Qiagen), as per the protocol provided by the manufacturer. The specificity of rEtIMP-1 was confirmed by immunoblot using anti-*E. tenella* convalescent serum.

## **2.8. Expression and purification of recombinant thioredoxin (TRX)**

His-tagged thioredoxin was expressed and purified alone to be used for mock immunisation. The plasmid pET32b+ was used to transform *E. coli* BL21pLysS (DE3) cells in a manner analogous to that carried out for expression of rEtIMP-1. Protein concentration was estimated by the Bradford method.

## **2.9. Immunisation trial**

Forty eight chickens were reared under specific pathogen free conditions from day of hatch (day 0) to 7 days of age in steel cages on raised stands with wired flooring. Pre-immunisation serum was collected from each bird from the brachial vein at 7 days of age. Thereafter, chickens were separated into four groups of ten birds and one group of eight birds, including groups immunised using recombinant EtIMP-1 (rIC), recombinant thioredoxin (rTC, vector control), live oocyst exposure (OC), birds left unimmunised and challenged (UC) and birds unimmunised and unchallenged (UU). Primary immunisation was administered on day 7, using 50 µg of protein (rIC or rTC), adjuvanted with TiterMax Gold (Sigma, USA) by the intramuscular (IM) route using thigh muscle or oral gavage with 1,000 sporulated oocysts of *E. tenella* Indian isolate-1 (OC; Table 1). Booster immunisation was administered on day 21, using 50 µg of protein (rIC or rTC), adjuvanted with Freund's Incomplete Adjuvant by the IM route in thigh muscle or oral gavage with 2,000 sporulated oocysts of *E. tenella* Indian isolate-1 (OC; Table 1). Groups, rIC, rTC, OC and UC were challenged by oral inoculation of 5,000 sporulated *E. tenella* Indian isolate-1 oocysts on day 32. Group details, adjuvants, immunisation and challenge schedule for each group is depicted in Table 1.

## **2.10. Circulating IgY quantification using ELISA (Enzyme Linked Immunosorbent Assay)**

ELISA was used to measure antibody (IgY) response against rEtIMP-1 post immunisation and post challenge in sera harvested from whole blood obtained by brachial vein puncture. Serum samples were harvested on the following days: 7 day old chickens (pre-immunisation); 14 day old chickens (7 days post-primary immunisation, PI); 21 day old chickens (14 days PI); 28 day old chickens (7 days post-booster immunisation, PB) and 37 day old chickens (5 days post-challenge, PC). Circulating IgY were estimated by ELISA after laboratory standardisation of antigen and serum concentration by chequer board titration. Antigen concentration of 2.5 ng  $\mu\text{l}^{-1}$  and serum dilution of 1:100 was used for ELISA.

#### **2.11. Quantitative PCR (qPCR) based estimation of *E. tenella* load**

Birds (eight each from rIC, rTC, OC and UC groups) were sacrificed 5.5 days post challenge (experimental day 37.5) by cervical dislocation after chloroform anesthesia. The right caecum from each bird was removed, gently rinsed with sterile PBS, pH 7.4 to remove the caecal contents, placed in sterile 50 ml centrifuge tubes containing 10 ml RNA later (Qiagen), processed as recommended by the manufacturer and stored at  $-80^{\circ}\text{C}$  until further processing for DNA extraction. Post thawing, caeca were homogenized using a tissue homogenizer (Ika, Ultra Purax, Germany) and DNA was extracted using a QIAamp DNA mini kit (Qiagen), as per the protocol provided by the manufacturer. Primers TENF (5'-AGCAGCAGCTGCCTCTCATTGACC-3') and TENR (5'-CAGAGAGTCGCCGTCACAGT-3') were used for the quantification of total *E. tenella* genomes, with TBPF (5'-TAGCCCGATGATGCCGTAT-3') and TBPR (5'-GTTCCCTGTGTCGCTTGC-3') targeting a fragment of the host (chicken) TATA binding protein coding sequence used as a reference target for standardisation of parasite genome numbers against host genome numbers (Nolan *et al.*, 2015). Quantitative PCR was performed in 20  $\mu\text{l}$  reactions on

a Stratagene MX3050P real time thermal cycler. Each reaction mixture was prepared using Sso Fast Eva Green supermix (10µl), forward and reverse primers (500nmol/ 1µl each), template DNA (1µl) and nuclease free water. The amplification conditions were standardized as initial denaturation 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing and extension at 60°C for 10 sec. Quantification of *E. tenella* genome numbers in caecal samples were carried as per the procedure of Nolan *et al.* (2015).

#### **2.12. Determination of serum cytokine levels (IL-4, IL-10 and IFN-γ) by ELISA**

The quantity of circulating interleukin-4 (IL-4), interleukin 10 (IL-10) and interferon gamma (IFN-γ) was estimated in pre-immunisation, post-immunisation and post-challenge sera. Interleukin assay kits based on sandwich ELISA (for IFN-γ) or competitive ELISA (for IL-4 and IL10) supplied by Blue Gene, Shanghai (China) were used as described by the manufacturer.

#### **2.13. Statistical analysis**

IBM SPSS Statistics 20.0 (IBM Corp., 2011) was used for statistical analysis of experimental data generated during the study. Analysis of variance (ANOVA) supplemented by Duncan's post hoc test was used to analyse anti-EtIMP-1 IgY levels (OD<sub>450</sub> values of ELISA), and normalised genome copy numbers of parasites. Post interleukin ELISA assay four parameter logistic curve (4PL) standard curves were prepared using the OD<sub>450</sub> values of the standards versus their respective concentrations. The OD<sub>450</sub> values of each sample were interpolated in the 4PL curve to obtain absolute values of interleukins in pg ml<sup>-1</sup>. These values were used in statistical analysis with ANOVA (Duncan's post hoc) test.

### **3. Results**

#### **3.1. Amplification, sequencing and bioinformatics analysis of EtIMP-1**

PCR amplification and sequencing of the partial EtIMP-1 coding sequence from the Indian *E. tenella* isolate 1 resulted in a 1,146 bp product (GenBank accession number KC758959). Sequence similarity calculated by BLASTn against the NCBI non-redundant database revealed greatest similarity with EtIMP-1 from a Chinese isolate (KC215109) and EtIMP-1, from the Houghton strain, UK (FN813229), with 99.9% and 97.4% similarity. Alignment of the sequences identified 30 additional nucleotides in the Asian (Chinese and Indian) isolates as compared to European and North American samples (Houghton, Weybridge and Wisconsin strains), with an expansion/contraction of a partially degenerate CAG repeat (Figure 1). The extra nucleotides add 10 amino acids to the Indian and Chinese isolate IMP-1 sequences in the peptide chain (QQQEQQQQEQ). Additionally, five single nucleotide polymorphisms (SNPs) were detected, three of which incurred non-synonymous substitutions (Figure 1, Table 2). Sequence comparison revealed low nucleotide diversity ( $Pi[JC] = 0.00305$ ), although four alleles were detected with only those from China and India found to be identical (Table 2). Calculation of Tajima's D, and Fu & Li's D\* and F\* statistics failed to detect any statistically significant signatures of selection, although the sample size may have been limiting.

#### **3.2. Recombinant EtIMP-1 production**

Recombinant EtIMP-1, fused to thioredoxin, was expressed under native conditions and resolved on SDS PAGE (Supplementary Figure 1). The protein had a predicted length of 382 amino acid residues and predicted molecular weight of 40.53 kDa using EXPASY (Gasteiger *et al.*, 2003). Molecular weight of the expressed and purified protein as estimated using Syngene software, after

capturing the image of the gel in a Syngene gel documentation unit, was approximately 60.5 kDa. This increase was due to co-expression with a thioredoxin and two hexa-histidine tags from the vector. BL21pLysS cells were used rather than BL21 cells, because the latter produced leaky expression. Recombinant cells expressing rEtIMP-1 were lysed within 30 min of incubation at room temperature with lysis buffer. The recombinant protein was detected within the supernatant (soluble fraction) when resolved on SDS-PAGE, indicating that the protein rEtIMP-1 was soluble when expressed in the pET32 system. The polyhistidine tag on the recombinant protein was identified by Ni-NTA HRP conjugate (Figure 2). The recombinant protein reacted with convalescent sera (Figure 2).

### **3.3 Effect of rEtIMP-1 immunisation on caecal parasite burden**

The impact of rEtIMP-1 or live parasite immunisation on subsequent *E. tenella* replication was assessed using qPCR to determine the number of parasite genomes 132 hours post-challenge, normalized by comparison to the number of host genomes (Figure 3). Significant reductions ( $p < 0.05$ ) in parasite burden of 78% and 67% was observed in the group rIC (rEtIMP-1) ( $14.14 \pm 5.2$ ) when compared to the TC and UC control groups ( $68.47 \pm 16.1$  and  $42.92 \pm 11.4$ ), respectively. Live oocyst immunisation (the OC group) resulted in ~ 90% reduction in parasite burden ( $4.09 \pm 2.7$ ) compared to the UC group ( $42.92 \pm 11.4$ ).

### **3.4. Antibody (IgY) response and interleukin response against EtIMP-1 after oocyst or rEtIMP-1 immunisation**

Immunoglobulin (IgY) response to oral oocyst or recombinant protein immunisation is presented in Figure 4. The highest anti-EtIMP-1 IgY responses were observed in the rIC (rEtIMP-1)

immunised group ( $p<0.05$ ). Post immunisation (PI) and post challenge sera of the mock-immunised rTC group also had increased antibody levels, reflecting inclusion of thioredoxin in the rEtIMP-1 antigen used in the ELISA assay, however these were significantly lower than the rIC group. Significant antibody responses were also observed against EtIMP-1 in the OC group after primary and booster immunisations compared to the UC group, although levels always remained lower than the rIC and rTC groups.

Comparison of interleukin responses between groups and week-wise (within groups) was assessed by generating four parameter logistic (4PL) curves for all three interleukin standards tested from XLSTAT analyses of OD<sub>450</sub> values of standards against their concentration. The formulae for deducing the sample OD values of each interleukin were derived from their respective 4PL curves and tabulated (Supplementary table).

The IL-4 response to immunisation and challenge is presented in Figure 5A. IL-4 was detected in all groups tested prior to immunisation (7 day old chickens) with no significant differences between the groups. By day 7 PI (14 day old chickens) IL-4 levels were significantly elevated in all groups compared to day 0 PI with the OC group having the highest value ( $p<0.05$ ), no significant difference between rTC and UC groups, and rIC being significantly lower ( $p<0.05$ ). At day 14 PI (21 day old chicken), IL-4 levels in OC were similar to UC and rTC groups and significantly lower to the rIC group ( $p<0.05$ ). Post booster (at 28 days of age), IL-4 levels were significantly higher in rIC, OC and rTC groups compared to the previous week; however they remained significantly highest in the rIC group. Five days post challenge at 35 day age, no increment in IL-4 was observed in any of the groups, but higher levels of IL-4 persisted in the rIC group, albeit at a lower level than at pre-challenge. No change in circulating IL-4 was observed in

UC chickens (unimmunised and challenged) compared to UU chicken (unimmunised and unchallenged) on 35<sup>th</sup> day of the study (5 days post-challenge).

The IL-10 responses to immunisation and challenge are presented in Figure 5B. The most significant IL-10 responses were observed in birds of the rIC and rTC groups at days 7 and 14 PI (14 and 21 days age). Post booster and post challenge IL-10 levels in rTC and rIC groups persisted but at significantly lower levels compared to earlier times. In OC birds IL-10 levels were significantly high after 14 days PI (21 days age) and then decreased after booster and challenge. Serum IL-10 levels in UC were significantly higher than UU birds at 05 days post challenge.

IFN $\gamma$  responses to immunisation and challenge are presented in Figure 5C. IFN $\gamma$  levels increased in all groups by day 14, with significant increases for the oocyst (OC) and rEtIMP-1 (rIC) immunised groups compared to the unimmunised. After the booster immunisation, serum IFN $\gamma$  increased further in the rIC and OC groups, most notably in the OC which became significantly higher than rIC and a small increase in the rTC. Post challenge, IFN $\gamma$  was significantly increased in UC chicken compared to UU birds, but reduced in rIC birds.

#### **4. Discussion**

Immune mapped protein-1 (IMP-1) was first described as an anticoccidial vaccine candidate in *E. maxima* (Blake et al., 2011). Subsequently, immunisation trials with recombinant IMP-1 have also been reported for *E. tenella* (Yin et al., 2014, 2015). If IMP-1 is to be developed as an anticoccidial vaccine candidate it is important to understand features of its biology including the occurrence and extent of naturally occurring allelic diversity, and the nature of the immune responses stimulated by its use in vaccination.



Here, we have assessed IMP-1 allelic diversity by comparison of coding sequences derived from *E. tenella* isolates originating from China, India, the USA and the UK. Analysis revealed very low nucleotide diversity, restricted to the expansion/contraction of a degenerate 'CAG' repeat motif and five SNPs. Similarly low levels of polymorphism have been described for other anticoccidial vaccine candidates such as apical membrane antigen 1 (AMA1; Blake et al., 2015), enhancing the prospect of using such antigens in a future recombinant vaccine.

The effect of vaccination using recombinant rEtIMP-1 protein was compared with immunoprotection induced by oral infection with sporulated oocysts. Following challenge infection, chickens vaccinated with rEtIMP-1 were found to have a 67% reduction in caecal parasite genome numbers determined using qPCR compared to unimmunised chickens, and a 79% reduction compared to birds mock immunised with recombinant thioredoxin. Previously, Yin *et al.* (2013 and 2014) have reported a 60-66% reduction in oocyst output following immunisation with whole rEtIMP-1 or a C-terminal derivative of EtIMP-1. This EtIMP-1 was derived from a Chinese *E. tenella* isolate and the recombinant protein was adjuvanted with Freund's complete adjuvant. Adjuvants related to Freund's complete have commonly been associated with a bias towards Th1-type responses (Shibaki and Katz, 2001; Stills, 2005). Here, TiterMax Gold was used as the adjuvant for the first immunisation in the present study given reports of a more balanced Th1/Th2 response (Stephenson et al., 2014), followed by Freund's incomplete adjuvant for subsequent immunisations. Freund's incomplete adjuvant has been associated with stronger Th2-type responses (Shibaki and Katz, 2001; Stills, 2005). In the present study, rEtIMP-1 was derived from an Indian isolate of *E. tenella*. The EtIMP-1 sequences of the Chinese and Indian isolates were identical (Figure 1 and Table 2). Immune protection, though partial in the present studies, was estimated based on the reduction in number of *E. tenella* genomes in the caecum, following

the protocol established by Nolan *et al.* (2015). The reduction in parasite replication was similar to that observed by Yin *et al.* (2013 and 2014), where effects of immunisation were estimated based on reduction in faecal oocyst output.

*Eimeria* infections induce specific antibody responses that are detected in peripheral blood circulation (IgM and IgY), intestinal mucosal secretions (IgM and IgA) and biliary secretions (IgA) (reviewed by Yun *et al.*, 2000b). There is a dichotomy in opinion regarding the importance of antibodies or humoral immune responses in protection against *Eimeria* infection. Most studies and reports identify a minor or negligible role for antibodies in protection against natural coccidiosis in poultry (Lillehoj, 1987; Rose, 1987). However, Belli *et al.* (2009), Wallach (2010) and Constantinou *et al.* (2011), have reported a protective role for immunoglobulins against *Eimeria* infections. Nguyen *et al.* (2004) described protection against challenge infection of *E. tenella* and *E. acervulina* after feeding a dietary supplement of IgY powder prepared from hyperimmune sera of chickens raised against antigen 3-1E (profilin). In this study, we found that immunisation with rEtIMP-1 produced a significantly strong IgY response post-immunisation and post-booster, likely enhanced by the use of Freund's incomplete adjuvant for the booster. Low immunizing doses of 1,000 and 2,000 *E. tenella* oocysts also induced a detectable primary IgY response and an increased anamnestic IgY response, post booster. Smith *et al.* (1993) reported a similar IgY profile, with a small increase after primary infection, followed by a rapid increase after challenge.

In the recent past many researchers have undertaken immunisation with a variety of recombinant proteins and observed the effects on interleukin responses in spleen or caecal IELs by RT-qPCR or ELISA. Interleukins are cytokines or signaling molecules secreted by various cell types of the immune system. These molecules play an important role in chemotaxis, clonal proliferation, as

mediators in inflammation, angiogenesis and many other cellular mechanisms. IELs have been found to express high levels of Th1 related cytokines like IFN $\gamma$ , along with the Th2 cytokine IL-4 and IL-10 during coccidiosis (Cornelissen *et al.*, 2009). Serum IL-4 in chickens immunised with rEtIMP-1 (rIC) was higher post booster than after primary immunisation. Post challenge a decrease in serum IL-4 was observed. Vaccination with various recombinant proteins has shown higher levels of IL-4 transcripts in other studies (Hoan *et al.* 2014). Oocyst immunisation showed a significant rise in IL-4 after primary dose but post-booster responses were lower in comparison to the rEtIMP-1 vaccination. Hong *et al.* (2006) observed a slight increase in IL-4 transcription after primary challenge and a subsequent decrease after secondary challenge when using a higher oocyst dose of 20,000. Post booster serum IL-4 levels in rIC birds were significantly higher than the OC group, possibly influenced by the switch to Freund's incomplete adjuvant (Stephenson *et al.*, 2014). High levels of circulating IL-4 after booster and challenge in rIC birds may have influenced the high serum antibody levels which increased even after challenge. On the contrary, IL-4 expression was reduced after re-infection (booster) and challenge in OC birds and may have contributed to the fall in antibody levels post challenge. Changing the adjuvant used during boosting could enhance the Th1 response further, and might consequentially increase the magnitude of the anti-*Eimeria* response (Chapman *et al.*, 2013).

IL-10 is the master regulator of Th1 and Th2 responses and mediates its effect directly on T cells, B cells, antigen presenting cells and NK cells (Ding *et al.*, 2003; Mocellin *et al.*, 2003; Couper *et al.*, 2008). It is a potent inhibitor of pro-inflammatory cytokines and T helper 1 responses such as IFN $\gamma$ , IL-1 $\beta$ , TNF $\alpha$ , IL-12 and nitric oxide production, as well as suppressing expression of MHC class II molecules (Fiorentino *et al.*, 1991; Gazzinelli *et al.*, 1992; Aste-Amezaga *et al.*, 1998; Rothwell *et al.*, 2004; Wu *et al.*, 2016). In the present study it was found that serum IL-10 levels

increased after immunisation with rEtIMP-1 and oocysts. However, the levels in the recombinant protein immunised group was higher than the oocyst immunised chickens. Wu *et al.* (2016), reported that serum IL-10 increases by fifth day post *E. tenella* infection. Estimation of serum IL-10 in the present study was estimated on day 7 post infection and thus it is likely that peak production was missed after the primary immunisation. Post booster serum IL-10 decreased in both groups. Post challenge an increase in serum IL-10 was observed among all groups except the unchallenged and unimmunised birds (UU). Hong *et al.* (2006) observed a decrease in levels of IL-10 mRNA transcripts during *E. tenella* re-infection. Low IL-10 in the OC group post booster is indicative of a low Th2 response and high Th1 (IFN $\gamma$ ) activity.

IFN $\gamma$  has an important mediatory role in resistance during primary *E. tenella* infection in chickens (Zhang *et al.*, 1995a, b). Recombinant IFN $\gamma$  inhibited the development of *E. tenella* sporozoites within the cells but did not block the invasion process during *in-vitro* studies (Lillehoj and Choi, 1998). Yun *et al.* (2000a) and Laurent *et al.* (2001) demonstrated upregulation of IFN $\gamma$  transcripts in the caeca after *E. tenella* infection. In the present study a significant increase in IFN $\gamma$  response of a similar magnitude was observed in rEtIMP-1 and oocyst immunised groups by 7 days post immunisation. Post booster serum IFN $\gamma$  increased greatly in oocyst immunised birds but only slightly in rEtIMP-1 immunised chickens. Hong *et al.* (2006) observed elevation of IFN $\gamma$  transcripts in caecal IELs during primary *E. tenella* infection. It was observed that oocyst immunisation predominantly elicited a strong Th1 response in the form of IFN $\gamma$  in the later part of immunisation studies (booster). However, in the recombinant IMP immunised birds there was a strong IL-4 response as well, indicating a Th2 response, confirmed by higher IgY levels. Serum IFN $\gamma$  levels did not increase following challenge of the oocyst immunised group, as described before in previously infected chickens (Yun *et al.*, 2000a). Significant IFN $\gamma$  increases were

detected after challenge of the rTC and UC groups, both of which were exposed to *E. tenella* for the first time, although a reduction was detected in the recombinant IMP-1 immunised chickens. Such a result was unexpected and may reflect immunoprotection induced by an adjuvanted recombinant protein through a mechanism that is not the same as that induced by oocyst infection. From this study we conclude that recombinant EtIMP-1 has the potential to induce immune-protection against challenge with a homologous *E. tenella* isolate. Since the first description of IMP-1 from *E. maxima* by Blake *et al.* (2011) orthologues have been identified and reported from several other apicomplexans, including EtIMP-1 from *E. tenella* (Yin *et al.*, 2013), NcIMP-1 from *N. caninum* (Cui *et al.*, 2012a), TgIMP-1 from *T. gondii* (Cui *et al.*, 2012b) and PfIMP-1 from *Plasmodium falciparum* (Benjamin *et al.*, 2015). It has been hypothesized that the protein may play a role in host cell invasion, although recent studies using IMP-1 knockout *T. gondii* (Jia *et al.*, 2016) suggest that, for this species, there is no essential role in cell invasion. While the function of IMP-1 is yet to be deciphered, the present and previous vaccination studies have indicated its potential as a target vaccine candidate for immunoprophylaxis against apicomplexan pathogens of domestic livestock and poultry. While it remains possible that antigenic diversity may influence efficacy against heterologous challenge, the low level of amino acid diversity reported here complements that reported previously for EtAMA-1 (Blake *et al.*, 2015) and supports the relevance of recombinant vaccination. Protection observed during homologous immunisation studies with multiple antigens such as EtMIC-1 and EtMIC-2 by Satish *et al.* (2011 and 2012) revealed that better results can be achieved by dual antigen immunisation. Inclusion of multiple antigens may help prevent immune-escape of variant strains and slow immune-selection for vaccine resistant genotypes.

## **Ethics Statement**

All animal studies were carried out within the Experimental Animal Shed of the Division of Parasitology, Indian Veterinary Research Institute. Prior approval for experimental trials on chickens was obtained from the Institutional Animal Ethics Committee, IVRI (registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forest and Climate Change, Government of India), reference F.1-53/2012-13-J.D. (Res) dated 10.09.2013. Permission for use of recombinant proteins in the experiment was obtained from the Institutional Biosafety Committee, IVRI, reference F.12-8/2002-J.D. (R) dated 28.12.2012, as per the norms of Department of Biotechnology, Government of India.

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## **Authors' contribution**

The work done was part of a PhD thesis submitted by the first author (KK) with direct supervision from PSB. RG, SK and MM contributed to the experimental immunisation and laboratory studies. KK led preparation of the manuscript. PSB, RG, FMT and DPB contributed to experimental planning and preparation of the manuscript.

## **Conflict of interest**

The authors declare that there is no conflict of interest what so ever in relation to publication of the manuscript or financial implications.

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Fig. 1. Comparison of *Eimeria tenella* immune mapped protein (IMP-1) coding sequences derived from parasites collected in three different continents including isolates from India and China, as well as the reference Houghton, Wisconsin and Weybridge strains. (A) targeted nucleotide and (B) whole allele amino acid sequences. Polymorphisms are highlighted in the open boxes.

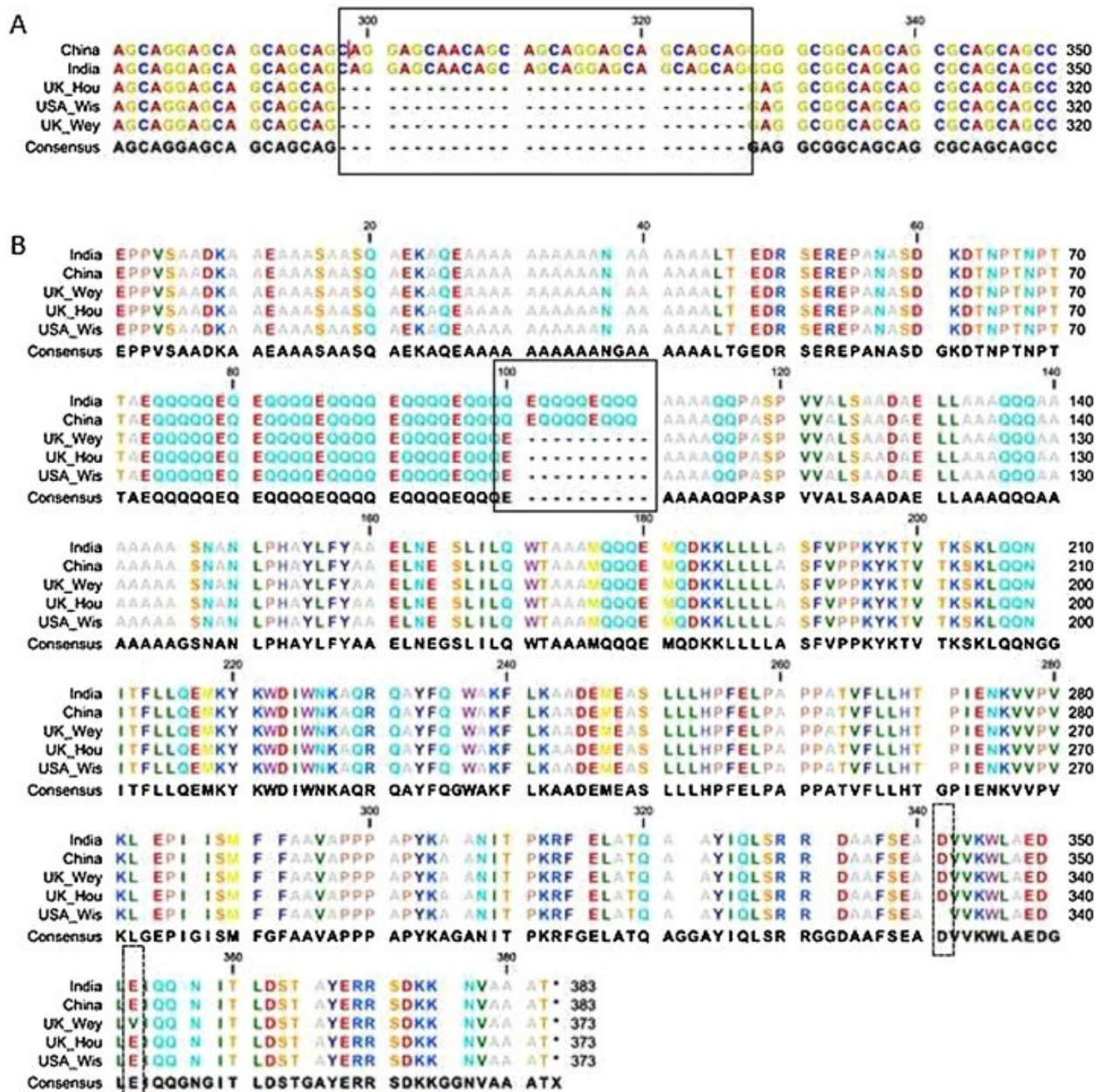


Fig. 2. Production, confirmation and characterization of recombinant EtIMP-1rEtIMP-1 blotting with Ni-NTA HRP conjugate (Lane 1) and 1:20 dilution of convalescent chicken serum (Lane 2).

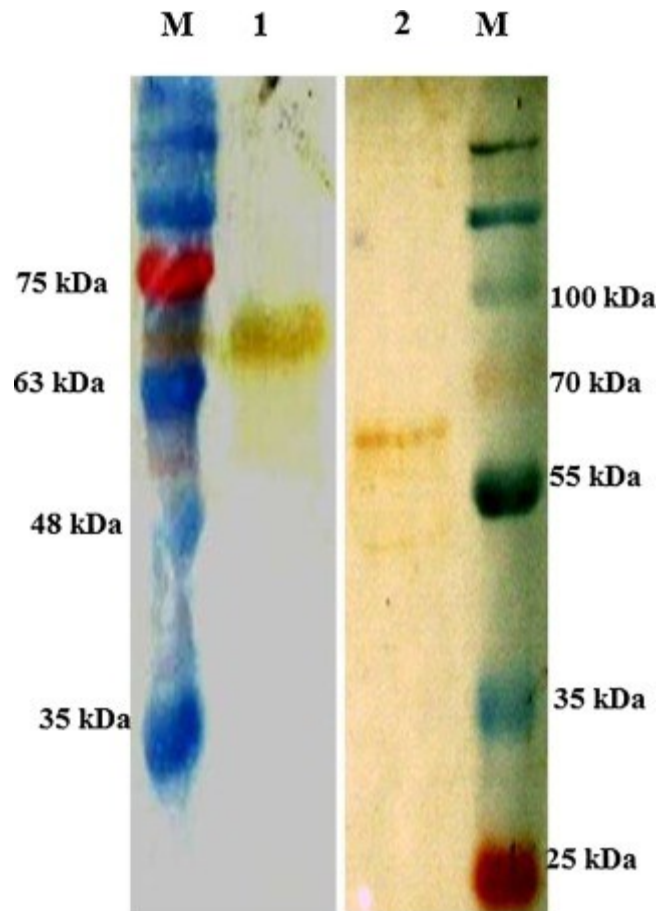


Fig. 3. Mean number of *Eimeria tenella* genomes in caecal tissue estimated by qPCR at 5.5 days post-challenge (n = 10). rIC- rEtIMP-1 immunized, rTC- Mock immunized with recombinant thioredoxin, OC- Immunized with *E. tenella* Indian isolate-1 sporulated oocysts, UC- Unimmunized (injected with sterile normal saline). All groups were challenged with 5000 sporulated oocysts of *E. tenella* Indian isolate-1. *Eimeria tenella* were not detected in the UU (unimmunized, unchallenged) control group. Bars annotated using different letters were significantly different ( $p < 0.05$ ).

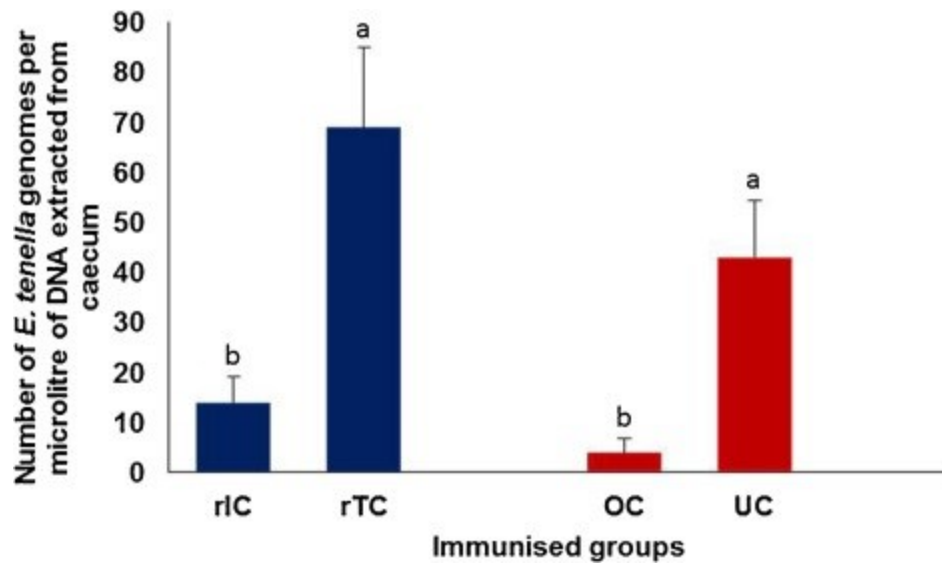


Fig. 4. Anti-EtIMP-1 IgY response in immunized and challenged chickens detected by antigen specific-ELISA. rIC- Immunized with rEtIMP-1, rTC- Mock immunized with recombinant thioredoxin, OC- Immunized with *E. tenella* Indian isolate-1 sporulated oocysts, UC- Unimmunized. All groups were challenged with 5000 sporulated oocysts of *E. tenella* Indian isolate-1. The unimmunized, unchallenged control (UU) is not shown. Different superscripts in small letters (a, b, c, d, e) indicate significant differences in serum anti- EtIMP-1 IgY values within each group over time. Different superscripts in capital letters (A, B, C, D, E) indicate significant changes in serum IgY values between groups for the same day. All values were estimated by ANOVA (Duncan's post hoc) at  $p \leq 0.05$ .

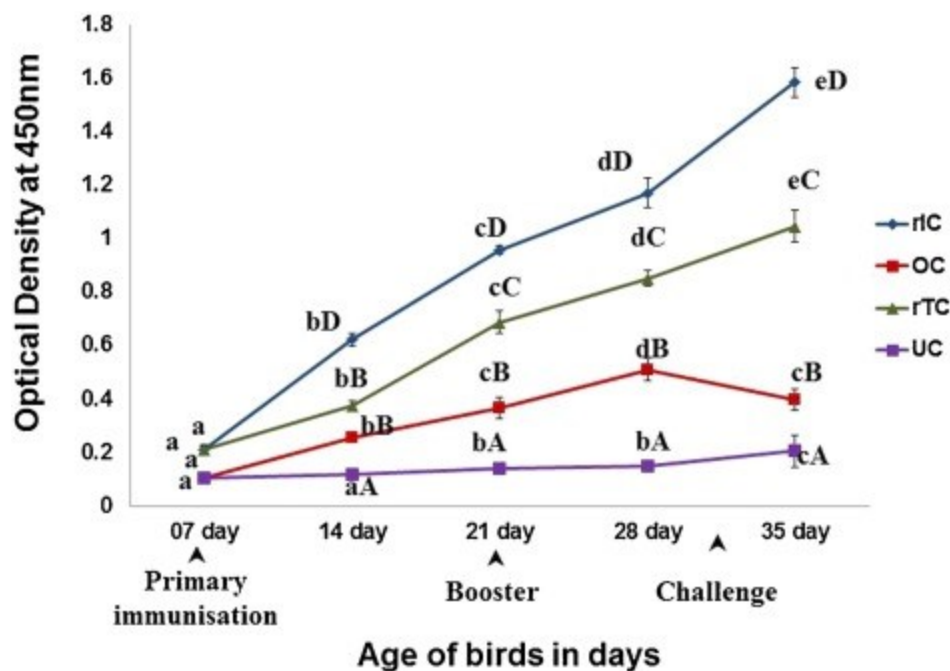


Fig. 5. Interleukin response to immunization and *Eimeria tenella* challenge (A) Interleukin-4 (IL-4) (B) Interleukin-10 (IL-10) and (C) Interferon gamma (IFN $\gamma$ ). rIC- Immunized with rEtIMP-1, rTC- Mock immunized with recombinant thioredoxin, OC- Immunized with sporulated oocysts, UC- Unimmunized challenged control, UU- Unimmunized, unchallenged control (only tested on day 35). All groups except UU were challenged with 5000 sporulated oocysts of *E. tenella* Indian isolate-1. Different superscripts in small letters (a, b, c, d, e) indicate significant differences in interleukin within a group, over time, and different superscripts in capital letters (A, B, C, D, E) indicate significant difference between groups for the same day. All values were estimated by ANOVA (Duncan's post hoc) at  $p \leq 0.05$ .

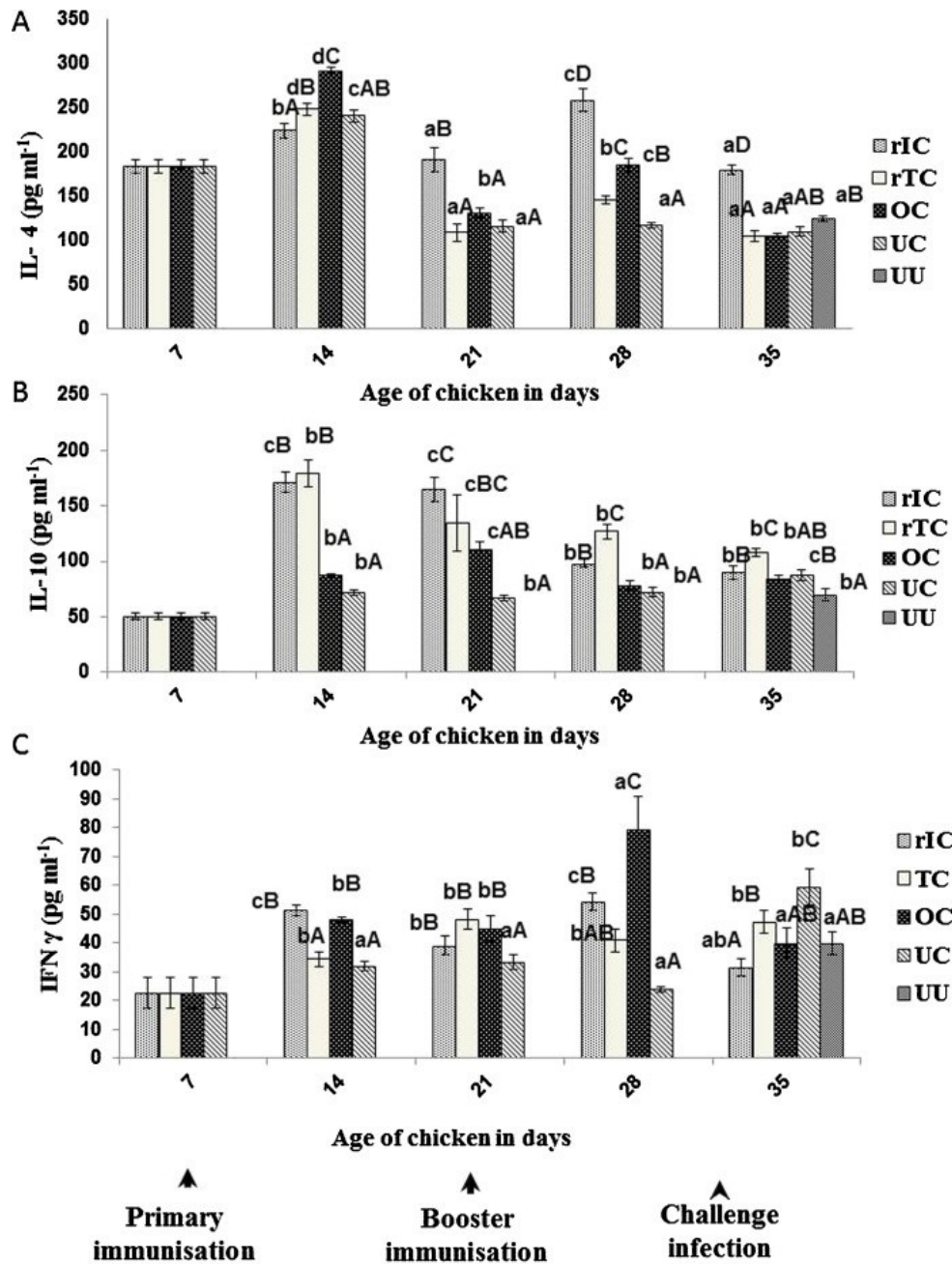




Table 1. Details of immunization schedule employed in the vaccination/challenge study: Group rIC- immunized with rEtIMP-1; Group rTC- mock immunized with TRX; Group OC- orally immunized with oocysts; Group UC- un-immunized; Groups rIC, rTC, OC and UC challenged with *E. tenella* Indian isolate 1. Group UU- un-immunized and unchallenged. I.M\* – Intramuscular unmarked, FIA\*\*- Freund's Incomplete Adjuvant, P.O\*\*\*- per os (Oral gavage).

Groups	No. of birds	Immunization - Dose and Route		Challenge - (Dose and Route)
		8 day old chicken	21 day old chicken	32 day old
Group rIC	10	rEtIMP-1 50 µg I.M route* Adjuvant: TiterMax gold	rEtIMP-1 50 µg ml <sup>-1</sup> I.M route Adjuvant: FIA**(booster)	5000 oocysts <i>E. tenella</i> Indian isolate 1, P.O.
Group rTC	10	rTRX 50 µg I.M route* Adjuvant: TiterMax gold	TRX 50 µg ml <sup>-1</sup> I.M route* Adjuvant: FIA(booster)	5000 oocysts of <i>E. tenella</i> Indian isolate 1, P.O
Group OC	10	1000 oocysts <i>E. tenella</i> Indian isolate 1, P.O***	2000 oocysts <i>E. tenella</i> Indian isolate 1, P.O***(booster)	5000 oocysts <i>E. tenella</i> Indian isolate 1, P.O.
Group UC	10	Sterile normal saline via IM route	Sterile normal saline via IM route	5000 oocysts <i>E. tenella</i> Indian isolate 1, P.O.
Group UU	8	Sterile normal saline via IM route	Sterile normal saline via IM route	-

Table 2. *Eimeria tenella* IMP-1 allelic diversity. Polymorphic nucleotides detected in the EtIMP-1 coding sequences derived from five isolates and the impact on amino acid identity. NS = non-synonymous substitution, S = synonymous substitution.

Alignment Position (bp)	UK	UK	US	China	India	Outcome	Amino acid switch
	Houghton	Weybridge	Wisconsin	Beijing			
329	A	A	A	G	G	NS	Gly-Glu
384	C	C	T	C	C	S	Asp-Asp
618	G	A	G	G	G	S	Gln-Gln
1022	A	A	G	A	A	NS	Asp-Gly
1055	A	T	A	A	A	NS	Glu-Val