Research Article



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Molecular characterization and protective efficacy of a new conserved hypothetical protein of *Eimeria tenella*

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Abstract – Eimeria tenella is an obligate intracellular parasite that actively invades cecal epithelial cells of chickens. This parasite encodes a genome of more than 8000 genes. However, more than 70% of the gene models for this species are currently annotated as hypothetical proteins. In this study, a conserved hypothetical protein gene of E. tenella, designated EtCHP18905, was cloned and identified, and its immune protective effects were evaluated. The open reading frame of EtCHP18905 was 1053 bp and encoded a protein of 350 amino acids with a molecular weight of 38.7 kDa. The recombinant EtCHP18905 protein (rEtCHP18905) was expressed in E. coli. Using western blot, the recombinant protein was successfully recognized by anti GST-Tag monoclonal antibody and anti-sporozoites protein rabbit serum. Real-time quantitative PCR analysis revealed that the EtCHP18905 mRNA levels were higher in sporozoites than in unsporulated oocysts, sporulated oocysts and second-generation merozoites. Western blot analysis showed that EtCHP18905 protein expression levels were lower in sporozoites than in other stages. Immunofluorescence analysis indicated that the EtCHP18905 protein was located on the surface of sporozoites and second-generation merozoites. Inhibition experiments showed that the ability of sporozoites to invade host cells was significantly decreased after treatment with the anti-rEtCHP18905 polyclonal antibody. Vaccination with rEtCHP18905 protein was able to significantly decrease mean lesion scores and oocyst outputs as compared to non-vaccinated controls. The results suggest that the rEtCHP18905 protein can induce partial immune protection against infection with E. tenella and could be an effective candidate for the development of new vaccines.

Key words: Eimeria tenella, Conserved hypothetical protein, Characterization, Vaccine, Chicken coccidiosis.

Résumé – Caractérisation moléculaire et efficacité protectrice d'une nouvelle protéine hypothétique conservée d'Eimeria tenella. Eimeria tenella est un parasite intracellulaire obligatoire qui envahit activement les cellules épithéliales du caecum des poulets. Ce parasite code un génome de plus de 8000 gènes. Cependant, plus de 70 % des modèles de gènes de cette espèce sont actuellement annotés en tant que protéines hypothétiques. Dans cette étude, un gène de protéine hypothétique conservé d'E. tenella, désigné par EtCHP18905, a été cloné et identifié, et ses effets immuno-protecteurs ont été évalués. Le cadre de lecture ouvert d'EtCHP18905 était de 1053 pb et codait pour une protéine de 350 acides aminés avec un poids moléculaire de 38,7 kDa. La protéine recombinante EtCHP18905 (rEtCHP18905) a été exprimée dans E. coli. En utilisant le Western blot, la protéine recombinante a été reconnue avec succès par un anticorps monoclonal anti-GST-Tag et un sérum de lapin anti-protéines de sporozoïtes. Une analyse PCR quantitative en temps réel a révélé que les niveaux d'ARNm d'EtCHP18905 étaient plus élevés dans les sporozoïtes que dans les oocystes non sporulés, les oocystes sporulés et les mérozoïtes de deuxième génération. L'analyse par Western blot a montré que les niveaux d'expression de la protéine EtCHP18905 étaient plus faibles dans les sporozoïtes que dans les autres stades. L'analyse par immunofluorescence a indiqué que la protéine EtCHP18905 était localisée à la surface des sporozoïtes et des mérozoïtes de deuxième génération. Des expériences d'inhibition ont montré que la capacité des sporozoïtes à envahir les cellules hôtes était significativement diminuée après le traitement par l'anticorps polyclonal anti-rEtCHP18905. La vaccination avec la protéine rEtCHP18905 a permis de réduire significativement les scores moyens des lésions et les sorties d'oocystes par rapport aux témoins non vaccinés. Les résultats suggèrent que la protéine rEtCHP18905 peut induire une protection immunitaire partielle contre l'infection par E. tenella et pourrait être un candidat efficace pour le développement de nouveaux vaccins.

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Introduction

Avian coccidiosis in the poultry industry is a common disease caused by obligate apicomplexan parasites of the genus *Eimeria*. At present, coccidiosis has historically been controlled by anticoccidial drugs and live vaccines [34]. However, longterm prophylactic drug usage has promoted drug-resistance. As pressure to reduce drug use in poultry production intensifies industry-wide, novel vaccination strategies are needed. Hence, new strategies such as DNA vaccines and subunit vaccines have received widespread attention. Therefore, it is urgent to find novel immunoprotective antigens.

Eimeria tenella is one of the 7 recognized species of Eimeria that infect chickens. More than 8000 genes of E. tenella have been identified throughout the genome [30]. Significant progress has been achieved over the past few several years identifying E. tenella genes involved in development, differentiation, virulence, and susceptibility to therapy. However, the identification of most genes in the genome remains unknown [2, 30]. The genome of the Houghton strain of E. tenella has been sequenced [30]. The data show that more than 70% of gene models are currently annotated as hypothetical proteins in *E. tenella* [2]. These conserved proteins may be important for invasion, development or the E. tenella life cycle. In 2016, Zhai et al. characterized the conserved protein EtCHP559 and studied the function and immunogenicity of EtCHP559 [47]. However, there are many conserved proteins that have neither been studied nor tested for their function.

In the present study, a new conserved hypothetical protein of *E. tenella, Et*CHP18905 (NCBI reference sequence accession number: XP_013231819), was cloned and recombinant protein GST-*Et*CHP18905 (*rEt*CHP18905) was expressed in an *Escherichia coli* BL21 (DE3) expression system. Polyclonal anti-*rEt*CHP18905 antibodies were generated and used to localize *Et*CHP18905 in parasites by immunofluorescence and to assess inhibitory effects in an *in vitro* assay. The results of the present study indicate that *Et*CHP18905 may participate in parasite invasion, growth and development.

Materials and methods

Ethics considerations

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (approval no. SHVRI-SZ-20180106-3), and were conducted in strict compliance following the recommendations outlined in the Guide for the Care and Use of Laboratory Animals.

Animals, parasites and cells

One-day-old Chinese Pudong yellow broilers were obtained from Shanghai Fuji Biological Technology Co., Ltd and reared in steel cages with a wire floor. Animals were provided with water and feed *ad libitum*. The birds were placed in a coccidiafree environment. BALB/c mice were purchased from Shanghai Lingchang Biological Technology Co., Ltd. New Zealand rabbits were obtained from Shanghai SLAC Laboratory Animal Co. Ltd.

Eimeria tenella (CAAS21111601) was obtained from the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The parasites were propagated by inoculating 2-week-old chickens, as previously described [40]. Unsporulated (UO) and sporulated occysts (SO) were obtained and purified using standard procedures [35]. Sporozoites (Spz) were purified *in vitro* from cleaned SO [28]. Second-generation merozoites (Mrz) were collected from the cecal mucosa scraped from the cecum and the cecal contents of chickens at 115 h post inoculation (p.i.) and then purified with Percoll [35].

The chicken fibroblast cell line DF-1 (ATCC CRL-12203) was used for *in vitro* inhibition and immunofluorescence assays [19].

Cloning and sequence analysis of EtCHP18905

Total RNA was extracted from Spz using TRIzol reagent (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. Total RNA was extracted from 2.0×10^7 Spz, counted with a hemocytometer using TRIzol reagent (TaKaRa), according to the manufacturer's protocol. RNA samples were resuspended in diethylpyrocarbonate (DEPC) treated water. Complementary DNA (cDNA) was generated from the total RNA with oligo dT primer and SuperScriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA).

The complete coding region of *Et*CHP18905 (GenBank accession number: XP_013231819) was amplified by PCR. *Et*CHP18905-specific primers (forward primer: 5' – GATGGA-CCGAGACCGTCGCTC – 3'; reverse primer: 5' – GCGCTGT-GGGGGCTCGGGTCG – 3') were used for the PCR assays with the cDNA of Spz as a template. The amplification products were analyzed by 1% agarose gel electrophoresis and purified using a QIAquick[®] Gel Extraction Kit (QIAGEN, Düsseldorf, Germany). The *Et*CHP18905 fragment was subcloned into the pGEM-T-Easy Vector (Promega, Madison, WI, USA) using T4 DNA ligase to construct a recombinant plasmid pGEM-T-*Et*CHP18905. The recombinant plasmid was subjected to DNA sequencing by Sangon (Shanghai, China).

The full-length cDNA sequence was analyzed using a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and the *E. tenella* genome database (http://www.genedb.org/Homepage/Etenella). The molecular mass and theoretical isoelectric point were predicted using the ProtParam tool at the ExPASy server (http://web.expasy.org/protparam/). Signal peptides, transmembrane motifs, and protein motifs were predicted using the computational tools SignalP (http://www.cbs.dtu.dk/services/SignalP/), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and Motif Scan (http://hits.isb-sib. ch/cgi-bin/motif_scan), respectively.

Expression and purification of the recombinant *Et*CHP18905 protein

A 1053-bp fragment of *Et*CHP18905 was amplified from the plasmid pGEM-T-*Et*CHP18905 with the primers: forward primer, 5' - GGAATTCATGGACCGAGACCGTCGCTC - 3';

reverse primer, 5' - GCGTCGACGC TGTGGGGGGCTCG-GGTCG - 3', and ligated into the pGEX-4T-1 vector at the EcoRI and SalI cloning sites (underlined). The recombinant pGEX-4T-EtCHP18905 plasmid was transformed into E. coli BL21 (DE3) cells (Tiangen, Beijing, China). rEtCHP18905 expression in E. coli was induced by addition of 0.8 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma, St Louis, MO, USA) to the bacteria culture after the OD_{600} of the culture reached 0.6 at 37 °C. The bacteria were collected by centrifugation at 8000 $\times g$ for 10 min at 4 °C. The bacteria were lysed by sonication and then the bacteria lysates were analyzed by 12% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The rEtCHP18905 protein was purified by cut SDS polyacrylamide gel [3]. The concentration of the sample was determined using a BCA protein assay kit (Beyotime, Haimen, China). The protein was stored at −20 °C for later analysis.

Generation of anti-r*Et*CHP18905 and anti-sporozoite polyclonal serum

The Spz proteins were prepared using sonication as described by Jiang et al. [19]. To generate polyclonal antibodies, either 50 μ g or 200 μ g of the purified r*Et*CHP18905 protein or the Spz protein was mixed with Freund's complete adjuvant (Sigma) in a 1:1 mixture and injected into six-week-old BALB/c mice or two-month-old New Zealand white rabbits, respectively. After two weeks, mice and rabbits were immunized with the same dose of antigen emulsified with Freund's incomplete adjuvant (Sigma). And then, the rabbits and mice were re-boosted four times at intervals of 1 week. Finally, the polyclonal antibody serum was collected and stored at -80 °C until use. Pre-immune serum was collected from the rabbits' ear vein before immunization for further use as the negative control.

Western blot analysis for rEtCHP18905

In order to verify the expression of rEtCHP18905, the purified rEtCHP18905 protein was separated by 12% SDS-PAGE. All separated proteins were electrically transferred onto polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). The membranes were blocked in phosphate-buffered saline (PBS) containing 5% skimmed milk, washed with PBS containing 0.05% Tween 20 (PBS-T, pH 7.2), and incubated overnight at 4 °C with the anti GST monoclonal antibody (1:2000) (CoWin Biosciences, Beijing, China) and rabbit anti-Spz proteins of E. tenella serum (1:200), respectively. Secondary antibodies, IRDye[®] 800CW Donkey anti-Mouse IgG and IRDye® 800CW Goat anti-Rabbit IgG (1:10,000) (LI-COR, Lincoln, NE, USA) were then applied at 37 °C for 1 h. Naïve rabbit serum (1:200) was used as the negative control. Membranes were scanned with an Odyssey[®] Infrared Imaging System (LI-COR).

*Et*CHP18905 transcript levels in different developmental stages of *E. tenella*

Transcription profiles of *Et*CHP18905 at different developmental stages of *E. tenella* (UO, SO, Spz, and Mrz) were determined using real-time quantitative PCR (qPCR). Total RNAs were isolated by TRIzol reagent (Invitrogen) from UO, SO, Spz, and Mrz. RNA preparations were treated with RNase-free DNase I (Invitrogen) to remove DNA contamination. In brief, 1.0×10^7 purified UO or SO were oscillated and broken in 500 µL of TRIzol with equal volume of 710-1180 µm glass beads (Sigma) for 10 min (4000 rpm). A total of 2.0×10^7 Spz or Mrz were lysed in 500 µL of TRIzol. Total RNAs were precipitated with isopropanol and washed with 75% ethanol and then resuspended in DEPC treated water. cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). qPCR was performed with SYBR1 Green I dye (Takara) on a StepOneTM Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). qPCR primers for EtCHP18905 were: 5' - TCCC-CTCAAGCCCCTCATACAGT - 3' (forward) and 5' - CCA-GCACTAAGTCCACTGAACGC - 3' (reverse). A housekeeping gene of E. tenella, 18S ribosomal RNA, was used as the reference gene and was amplified using the primers 5' – TGTAGTGGAGTCTTGGTGATTC - 3' (forward) and 5' -CCTGCTGCCTTCCTTAGATG -3' (reverse). The reactions for each sample were performed in triplicate, and the experiment was repeated three times. The transcription levels were quantified with the $2^{-\Delta\Delta Ct}$ method [27].

*Et*CHP18905 protein expression in four development stages of *E. tenella*

Total proteins were prepared from four life cycle stages of E. tenella using a commercially available cell-lysis buffer for western blot and immunoprecipitation (Beyotime). Protein concentrations were determined with a BCA protein assay kit (Beyotime). The protein lysate from each sample was separated by SDS-PAGE and transferred to PVDF membrane (Merck Millipore). Membranes were blocked for 2 h with 5% (w/v) skimmed milk powder in PBS, followed by incubation with mouse polyclonal anti-rEtCHP18905 (1:100) and mouse monoclonal anti-\alpha-tubulin (1:5000) at 37 °C for 2 h, respectively. Secondary antibodies, HRP-conjugated Affinipure Goat anti-Mouse IgG (H + L) (1:5000) (Proteintech, Rosemont, IL, USA) were incubated at room temperature for 45 min, and bands were detected using ChemiDoc (Bio-Rad, Hercules, CA, USA). α-tubulin (Sigma) was used as an internal reference for protein extracts at each stage. For comparative quantitative protein expression profile analysis, the resulting images were analyzed by Image J (Rawak Software Inc., Stuttgart, Germany) software.

Localization of *Et*CHP18905 by indirect immunofluorescence

The location of *Et*CHP18905 in Spz, Mrz and parasites invaded DF-1 cells were assessed by indirect immunofluorescence assay (IFA) with anti-r*Et*CHP18905, as previously described [19]. Six-well plates (Corning Inc., Corning NY, USA) precoated with coverslips were seeded with DF-1 cells $(2 \times 10^5$ cells per well). These cells were sequentially cultured in complete medium (CM, DMEM with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin) at 37 °C and 5% CO₂ for 24 h. Freshly cleaned Spz (6 × 10⁵ parasites per

well) were added to invade and develop in the DF-1 cells. The coverslips were collected and washed at 2, 48 and 72 h p.i., respectively. Subsequently, all the coverslips were fixed in 4% paraformaldehyde for 20 min, permeabilized with 1% Triton X-100 in PBS for 15 min, and then blocked with 2% bovine serum albumin in PBS overnight at 4 °C. After washing four times, rabbit anti-rEtCHP18905 polyclonal antibody (1:100 dilution) with PBS was added for incubation at 37 °C for 2 h. Then the goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibody (1:500 dilution) (Sigma) was added, and the samples were incubated at 37 °C for 1 h. Nuclei of parasites and cells were stained with 15 µg/mL 4, 6-diamidino-2-phenylindole (DAPI) (Beyotime) for 30 min at room temperature. At the end of each step, all the coverslips were washed three times in PBS. The coverslips were placed on glass slides using 60 µL of Fluoromount Aqueous Mounting Medium (Sigma) and observed by a laser scanning confocal microscope (Zeiss LSM800 microscope, Carl Zeiss, Germany). Spz were incubated in PBS or CM for 2 h at 41 °C, and air dried on coverslips before fixation. Mrz were also incubated in PBS for observation. Spz or Mrz were evenly smeared onto glass coverslips to localize the protein in Spz and Mrz. After air drying, the coverslips were prepared for immunofluorescence using the method described above.

Invasion inhibition assays

Invasion inhibition assays were performed to investigate whether anti-rEtCHP18905 affects Spz invasion of DF-1 cells [19]. Rabbit IgG against rEtCHP18905 and GST protein were purified using protein A + G agarose (Beyotime) and the concentration of IgG was determined by a BCA Protein Assay Kit (Beyotime). DF-1 cells (3×10^5 cells per well) were cultured in flat-bottomed 24-well plates (Corning) in CM at 37 °C and 5% CO2 for 12 h. Freshly cleaned E. tenella Spz were labeled for 15 min using carboxyfluorescein diacetate succinimidyl ester (CFDA SE) (Invitrogen), according to the manufacturer's protocol. Then, the labeled Spz were incubated with purified rabbit anti-rEtCHP18905 IgG at different concentrations (100, 200, or 300 µg/mL) for 2 h at 37 °C and added to infect DF-1 cells at 41 °C, 5% CO₂ for 12 h. Naïve rabbit serum IgG (Sigma, USA) and rabbit anti-GST IgG were used as the negative control and GST control. Labeled Spz incubated with no antibody were the positive control. The cells were washed, trypsinized, harvested, and analyzed on a flow cytometer (model Cytomics FC500; Beckman Coulter, Brea, CA, USA). All assays were performed in triplicate. The inhibition rate was calculated based on the invasion rate and the positive control [17].

Immunization experimental design

Broilers at 7 days of age were randomly divided into four groups and each group included 12 birds. Broilers were inoculated with a subcutaneous injection of 50 μ g or 100 μ g of purified r*Et*CHP18905 protein emulsified in Montanide ISA 71 adjuvant (Seppic, Puteaux, France) in a 3:7 mixture [18]. The challenged and unchallenged control birds were immunized with PBS emulsified in Montanide ISA 71 adjuvant. A booster

immunization was given one week later with the same amount of components as the first immunization. Subsequently, 7 days after the last immunization, 1×10^4 SO of *E. tenella* were given to all the birds except for the unchallenged control birds. Unchallenged control chickens were given PBS orally.

Evaluation of immune protection

The efficacy of immunization was evaluated by the average body weight gain, mean lesion scores, fecal oocyst output, and percentage reduction of oocyst excretion. Body weight was measured on days 0 and 8 post challenge. Fecal samples were collected daily from days 6 to 8 post challenge. Oocysts per gram of fecal sample were counted using a McMaster chamber [9]. The percentage reduction of oocyst excretion was calculated by the formula: (number of oocysts from the challenged-unvaccinated group – number of oocysts from the challenged-unvaccinated group × 100% [31]. The ceca of each group were collected separately. Intestinal lesions were scored according to the method of Johnson and Reid [20].

Preparation of the serum

The blood of broilers in each group was collected on day 8 post challenge. For the serum IgG, cytokines, sCD4 and sCD8 determination, the sera were separated from isolated blood samples. In brief, the blood samples were incubated at 37 °C for 1 h, and centrifuged at 1000 $\times g$ for 5 min at 4 °C to separate the serum.

Determination of serum antibody levels

The serum IgG against rEtCHP18905 levels were detected by ELISA at day 8 post challenge, as described previously [24]. Briefly, 96-well microtiter plates (Corning) were coated with purified rEtCHP18905 (10 µg/well) and incubated overnight at 4 °C. After three washes with PBS-T, the plate was blocked with PBS containing 1% BSA for 2 h at 37 °C, and then the plate was washed with PBS-T. The plates were incubated with the serum samples diluted 1:25 in PBS (50 μ L/well) for 2 h at 37 °C. After washing five times with PBS-T, the secondary antibodies of HRP-donkey-anti-chicken IgG antibody (50 µL/well) (Sigma) (1:5,000 dilution) was added and incubated for 2 h at 37 °C. The plates were washed five times with PBS-T and developed with 3,3',5,5'-tetramethylbenzidine. Optical densities at 450 nm (OD450) were determined on a microplate spectrophotometer. All assays were performed in triplicate.

Determination of cytokine, sCD4 and sCD8 levels

The immune stimulation effect of r*Et*CHP18905 protein on broilers was measured by ELISA at day 8 post challenge, as previously described [6, 22, 24, 25]. The levels of cytokines, soluble cluster of differentiation 4 (sCD4), soluble cluster of differentiation 8 (sCD8), interferon- γ (IFN- γ), interleukin-10 (IL-10), interleukin-17 (IL-17), and transforming growth factor

Eimeria necatrix	MDRDRRSLYAETDFRSYSG	19
Eimeria mitis	MALETMPAGPIVTVAKRDVSAGRPGSPTGNNLAI	34
Eimeria praecox	MAVETPTVDTVKS	13
Fimeria mavima	MEAAAADVSDDND	13
Eimeria acormilina	MAMETERTEADCR	12
Eimeria_acervurina		10
Elmeria_brunetti	MKGKMPSDIPPVSLIKSLARKERSGV <mark>K</mark> LGSMHSSSGGKGG	40
Consensus		
Eimeria tenella	SGNNYFLLDGCDEEENPCMPELRLSRREF	48
Eimeria necatrix	SGNNYFLLDGCDEEKNPCMPEL	48
Eimeria mitis	SDKELRTEGNSSE, ELPOASDABEERRAR	62
Fimeria prescov	UT FUDDACCDECSUNANNDCCDET DT D	41
Eimeria_praecox	VLERBRAGGREGSVRANNRGGBREERED	11
Elmeria_maxima	VLGQDQFVARPGSLKGNIKGNDRELKLDGISNDDESPQVS	53
Eimeria_acervulina	ALGRECODECPGSANTTICWREELGCOG.SAVEESRKFS	52
Eimeria_brunetti	GDRELRLDGNSSEDAAVSVSDLREERRAR	69
Consensus	r	
Eimeria tenella	RMRHRKSTAGSRGL	62
Fimaria pacatriy	DMDHDVSTAAST CT	62
Eineria necaciix	ATTEND TO CONCLUSION OF THE TOTAL OF	02
Limeria_mitis	AIRINKO1555WGL	10
Eimeria_praecox	ASR	44
Eimeria_maxima	DVREERRGRVSRYKKSVTASWGLGEGVSG	82
Eimeria_acervulina	DFRKERRRRRARYKKSITASWGLGEGVTG	81
Eimeria brunetti	ASRHRRSISSSWGL	83
Consensus		
Fimeria tenalla	NKSUSEDTMDAWTACETTTSLALSISMASTN DSEEKOO	100
Fimaria negativi	NKCUCCTUCANTACETTTCTATCTCMACTM DEFERCT	100
Limeria_necatrix	ANOTIONIVE ANTACIDATE LALDLOWADLN. KSELEQL	100
Limeria_mitis	GLGVIGKMIAVAILGLLLDSISIPIAVFKLHRMCGGRH	114
Eimeria_praecox	RMTGAAILGLILDSMSVPFAVYKLHRTQR	73
Eimeria_maxima	RMTGAAILGLLLLSMSIPFAWYKMHQGQIGR	113
Eimeria acervulina	RMTGVLLLGLULLTFILFIAWYRMHRMCSWR	112
Fimeria brunetti	GEGYSGEMTAVATLELLISIGIPTANLELY. REOGEOO	121
Consensus	111 77	
Consensus	111 V	
Eimeria_tenella	KQQQNTLFAHEPDVRVPSSPSYSASAAYGAGGD	133
Eimeria_necatrix	CCCCNTLPAHEPDVRVPSSPSYRASAAYGTAGD	133
Eimeria mitis	CHPFDLL PMHE PDAAEHPPRHDQDPAAAGGPDAPAQ	150
Eimeria praecox	COPFEVEATHECEAAGGHCCHCCHCCCEPVAFGAPEATLM	113
Fimeria mavima	CYAFDAL PTOGREAGEOHGOCOAAASEETEA TV	146
Eimeria maxima		140
Limeria_acervulina	CHAPGALSHIDQEDQCPGQLQDP11GGVDGF	145
Eimeria_brunetti	CCCLLLEGLGEECCAAADEEEECCLD	147
Consensus		
Eimeria tenella	AELKQEKLEMURNIHRTELSUSLEEINGHURTLOGPENGP	173
Eimeria necatrix	TELTOCKLEMTENTHRTELSTKLTEDNGLURTLOGPENCE	173
Fimaria mitig	DSOFAVOT STUPPT HESPT AT WHICE HOLLI SUPPERIODS	190
Eineria micis	DUD CTUTUUT DIT TO THE CTUCK INTERNET	150
Limeria_praecox	DVADGIKLEVIPALDIALSIKAISDAGDILIVRGALWDS	155
Eimeria_maxima	GHFDAIGVEVICNICATELSIKHTSDHGLILSVRDSRWDS	186
Elmeria_acervulina	GAPDVNKVEAUPNUCAAPAADKHISLHGULLTVRGEDWDA	185
Eimeria_acervulina Eimeria_brunetti	GAPDVNKVFANPNI GAASAATKHISI HELLTVRGEDVDA DSLDTGRLELIRNI HGAFLAFRHIGI FGLLLSVKGPPNDY	185
Eimeria_acervulina Eimeria_brunetti Consensus	GAPDVNKWLAUPN CAASAAFKENSHELL IVRGEDODA DSLDIGRLEINRNHGAGIAURHIGEFGLULSVKGPDODY	185 187
Eimeria_acervulina Eimeria_brunetti Consensus	GAPDVNKVLAPINU GAAGAARKANSUHEUTIVREEDNDA DSLDTGRLINRNUHGAGAARAARKANGUFGUULSVKGPENDY ptlftltdgll w	185 187
Eimeria_acervulina Eimeria_brunetti Consensus	GAPDVNRWIAHFHGABAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	185
Eimeria_acervulina Eimeria_brunetti Consensus Eimeria_tenella	GAPDVRKVGAPENCAAFAAFKDINGGULTVRGEDDLA DSLDTGRUFTERNDHGALAFAAFKDINGGULTVRGEDDLA D t 1 f 1 t d gll w AKEALERSVELULECOEDEETTDEK VETNENNIA	185 187 212
Eimeria_acervulina Eimeria_brunetti Consensus Eimeria_tenella Eimeria_necatrix	GADUNKNAADENUGAADAATKATKAISHAGULTYKGEDDLA DSLDTGRLEIGENUGAADIAATKAISHAGULTYKGEDDLA p t 1 f 1 t d gll w AKEALLRSVDLULECCEDEGEDTDSK.VETNEUNIAWDIA VKEALLRSWDSULESCEDEGEDTDSK.VETNEUNIAWDIA	185 187 212 212
Eimeria_brunetti Consensus Eimeria_tenella Eimeria_necatrix Eimeria_mitis	GAPUVKKVAPINUCAPIALIAPIGUTIVKEEDULA DSLDTGREIERNIHGELENENGUTIVKEEDULA DE 1 fl t d gll w AKEALERSVEIELECOPDETEDTOSK.VETKENIEMTIA VKEALERSVEIEVESPEDEDTOSK.VETKELMIEMTIA SREEFIAALDENAENSESIENTINKSETTELMAAVUTA	185 187 212 212 230
Eimeria_dervulina Eimeria_brunetti Consensus Eimeria_tenella Eimeria_necatrix Eimeria_mitis Eimeria_praecox	GAPUVRKVLAPINUCAAPAATKAINAINAIGULIVKEELMUA DSLUTKKVLAPINUCAAPAATKAINAINAINAINAINAINAINAINAINAINAINAINAINA	185 187 212 212 230 192
Emeria_acervultha Eimeria_brunetti Consensus Eimeria_tenella Eimeria_necatrix Eimeria_mitis Eimeria_praecox Eimeria_maxima	GAPUVKKVAPINICAAPAATATININGULIYKEEDULA DSLDTGREIERNIHGSLEAPHGUTGETLISVKGPEDY D t 1 f 1 t d gll w AKEALERSVDIELECODDETEDTDSK.VETNENIAMUA VKEALERSNDSVLESOPDEDTDSK.VETNENIAMUA SREEFLAALDEVARDHSSIJENTYNKSDTIELNAAVIA VREEFHAALDEVARDHSSIJENTYNKSDTIELNAAVIA	185 187 212 212 230 192 225
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Figure 1. Multiple alignment analysis of *Et*CHP18905 with other *Eimeria* proteins. DNAMAN was used to analyze the deduced protein sequences. The identical amino acids are listed at the bottom. NCBI reference sequence accession numbers: *Eimeria tenella*, XP_013231819, *Eimeria necatrix*, XP_013438465, *Eimeria mitis*, XP_013355934, *Eimeria maxima*, XP_013336337, *Eimeria acervulina*, XP_013251133; GenBank accession numbers: *Eimeria praecox*, CDI76926, *Eimeria brunetti*, CDJ52365.

Consensus

1	AT	GGA	CCG	AGA	CCG	TCG	CTC	CGT	TTA	.CGC	CGA	AAC	CTGA	CTT	CAG	GTC	ATA	CAC	TGG	CAG	TGG	CAA	CAA	TTAT
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	R	М	R	Н	R	Κ	S	Т	А	G	S	R	G	L	Ν	Κ	S	V	S	Е	Р	T	M	R
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505	CC	CGA	ATG	GGG	ACC	CAGC	AAA	GGA	AGC	GCT	GCT	GCG	TTC	AGT	GGA	CTT	AGT	GCT	GGA	GTG	TCA	GCC	GGA	TGAG
	Р	Е	W	G	Р	А	Κ	Е	А	L	L	R	S	V	D	L	V	L	Е	С	Q	Р	D	Е
577	ΤT	TGA	AGA	TAC	AGA	CAG	TAA	AGT	GGA	GAC	TAT	GCG	GCT	GAT	GCT	TGC	GAT	GGA	СТТ	GGC	GTG	тст	TGA	AAAC
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	v	L	K	G	K	L	G	S	D	P	E	L	K	A	A	L	V	K	L	L	L	A	V	R
865	AG	CAG	AGC	AGC	ATC	AAG	TGA	GTA	ССТ	CAG	TGC	тас	тат	тсс	CAC	тат	, GAT	ССТ	GCA	GAG	CGA	000	GAG	CCCC
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Figure 2. Bioinformatic analysis of *Et*CHP18905. The stop codon is indicated with an asterisk. N-myristoylation sites are double underlined. The transmembrane domain is shaded yellow with black lettering. N-glycosylation sites are surrounded by a black box. cAMP- and cGMP-dependent protein kinase phosphorylation sites are shaded black with white lettering. Tyrosine kinase phosphorylation sites is shaded grey with black lettering. Casein kinase II phosphorylation sites are underlined by a wavy line.

(TGF- β 1) were determined with "Chicken Cytokine ELISA Quantization Kits" (CUSABIO, Wuhan, China), according to the manufacturer's instructions.

Statistical analysis

SPSS version 22 (SPSS, Chicago, IL, USA) was used to analyze body weight gain, mean lesion scores, and fecal oocyst output and oocyst reduction ratio. GraphPad Prism version 6.0 (GraphPad, La Jolla, CA, USA) was used to analyze real-time quantitative PCR (qPCR), invasion inhibition, and antibody and cytokine levels. Differences among groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test (p < 0.05 was considered significantly different). The lesion scores were compared by the nonparametric Kruskal– Wallis test.

Results

Characterization of the EtCHP18905 sequence

By analysis of the sequence, the open reading frame (ORF) was 1053 bp and found to encode a protein of 350 amino acids with a predicted molecular mass of 38.7 kDa. Based on BLASTp analysis, the sequence obtained shared 100%, 84.68%, 52.48%,



Figure 3. Expression and purification of *rEt*CHP18905. (A) SDS-PAGE analysis of the *5rEt*CHP18905. Lanes 1, protein marker; 2, negative control (not induced with IPTG); 3, the *rEt*CHP18905 protein with the GST-tag protein of the vector (induced with IPTG for 6 h). (B) Western blot analysis of purified *rEt*CHP18905 protein. Lane 2, protein recognized by an anti GST-Tag monoclonal antibody. (C) Western blot analysis of purified *rEt*CHP18905 protein. Lane 2, protein recognized by rabbit sera against sporozoite, lane 4 incubated with naïve rabbit serum.

40.53%, 44.53%, 39.10%, 41.83% and 47.74% amino acid homology with conserved hypothetical protein (CHP) from E. tenella (NCBI reference sequence accession number: XP_013231819) and *Eimeria necatrix* (XP_013438465), Eimeria mitis (XP_013355934), Eimeria maxima (XP 013336337), Eimeria acervulina (XP_013251133), Eimeria praecox (CDI76926), and Eimeria brunetti (CDJ52365) (Fig. 1), respectively. Analysis of the amino acid sequence showed that a transmembrane domain (amino acid sequence 73–92) was found in the deduced protein, but no signal peptide was detected. Structural module and conservative structure predictions indicated that the protein contains seven casein kinase II phosphorylation sites, four protein kinase C sites, three N-glycosylation sites, three N-myristoylation sites, two cAMP and cGMP-dependent protein kinases phosphorylation sites and a tyrosine kinase phosphorylation site (Fig. 2).

Expression and purification of recombinant *Et*CHP18905

SDS-PAGE analysis showed that the recombinant protein was expressed in *E. coli* BL21 cells successfully and found mainly in the precipitate. After purification by cut SDS polyacrylamide gel, the expected protein band of 64.7 kDa (including with GST tag) (Fig. 3A) was observed in the SDS-acrylamide gel. Western blot analysis indicated that the recombinant protein was recognized by the anti-GST monoclonal antibody (Fig. 3B) and rabbit sera against Spz of *E. tenella* (Fig. 3C, lane 2). Normal rabbit serum failed to detect any protein of the expected molecular weight of r*Et*CHP18905 (Fig. 3C, lane 4).

Transcription of *Et*CHP18905 at different developmental stages of *E. tenella*

qPCR results showed that the levels of *Et*CHP18905 mRNA in UO, SO and Mrz were similar (p > 0.05), which were significantly lower than those in the Spz (p < 0.05) (Fig. 4).



Figure 4. Transcription levels of *Et*CHP18905 in different developmental stages of *E. tenella*. UO, unsporulated oocysts; SO, sporulated oocysts; Spz, sporozoites; Mrz, merozoites. Bars with different letters indicate significantly different expression levels (p < 0.05) and the error bars indicate standard deviations.

*Et*CHP18905 protein expression level in *E. tenella*

Western blot analysis results indicated that *Et*CHP18905 expression levels were higher in the UO and SO than Mrz and Spz stages, and *Et*CHP18905 expression levels were lowest in the Spz stage (Fig. 5).

Localization of *Et*CHP18905 during *in vitro* infection

Indirect immunofluorescence results showed that in Spz incubated with PBS, *Et*CHP18905 was distributed on the surface of Spz (Fig. 6A). *Et*CHP18905 uniformly distributed throughout the cytoplasm in whole Spz after incubation in CM (Fig. 6B). After Spz were added to DF-1 cells for 2 h and 48 h, *Et*CHP18905 was concentrated on the surface of Spz (Figs. 6C and 6D). After infection for 72 h, *Et*CHP18905 was evenly distributed in most areas of the parasite (Fig. 6E). Moreover, *Et*CHP18905 was primarily located on the surface of Mrz (Fig. 6F).



Figure 5. Expression levels of *Et*CHP18905 in different developmental stages of *E. tenella*. (A) Western blot of the internal reference tubulin and *Et*CHP18905 protein. (B) Relative expression levels of the *Et*CHP18905 protein. Bars with different letters indicate significantly different expression levels (p < 0.05) and the error bars indicate standard deviations.



Figure 6. Localization of *Et*CHP18905 in infected DF-1 cells by indirect immunofluorescence. Parasites incubated with anti-*rEt*CHP18905, stained with FITC (green)-conjugated secondary antibodies, and counterstained with DAPI (blue). Infected DF-1 cells were collected at indicated time points post-infection. (A) Sporozoites (Spz) in PBS, pRB, posterior refractile body; (B) Spz in complete medium. Infected DF-1 cells were collected at the indicated time points post-infection (pi); (C) 2 hours pi (hpi); (D) immature schizonts (iSC) 48 hpi; (E) mature schizonts (mSC) 72 hpi; (F) merozoites (Mrz) in PBS.

In vitro invasion inhibition assay

In vitro invasion inhibition assay results showed that the inhibition rate was 28% at an antibody concentration of 300 µg/mL (Fig. 7). Compared with naïve rabbit IgG and GST control groups, the inhibition effect after pretreatment with anti-r*Et*CHP18905 IgG was significant (p < 0.01). In contrast, naïve rabbit sera IgG and GST control groups did not have a significant effect on invasion by *E. tenella* Spz.

Protective efficacy of vaccination on *E. tenella* challenge

Body weight gain, cecal lesion scores, oocyst output and the percentage reduction of oocyst excretion are summarized in Table 1. The results showed that non-immunized challenged control groups exhibited significantly reduced weight gain compared with all immunized groups and the unchallenged control group chickens (p < 0.05) after challenge. The cecal lesion



Figure 7. Inhibition of sporozoite invasion *in vitro* by anti*rEt*CHP18905. Anti-*rEt*CHP18905, rabbit anti-*rEt*CHP18905 IgG; NA, naïve rabbit sera IgG; GST control, rabbit anti-GST IgG. The symbol "*" represents p < 0.05, "**" represents p < 0.01, and "***" represents p < 0.001 for comparison of treatment with anti*rEt*CHP18905 and naïve rabbit sera IgG and anti-GST IgG at the same concentration. The error bars indicate the standard deviation. All assays were performed in triplicate.

scores of chickens immunized with *rEt*CHP18905 were significantly lower than those of the challenged control. The oocyst output was reduced after immunization with *rEt*CHP18905. Chickens immunized with *rEt*CHP18905 presented significantly higher percentage reduction of oocyst excretion compared with the challenged controls (p < 0.05).

IgG titers and cytokine, sCD4 and sCD8 concentrations in sera of immunized chickens

The results in Figure 8A show that serum from chickens immunized with *rEt*CHP18905 had significantly higher levels of IgG antibody (p < 0.001) compared with the challenged control group. sCD4 levels in chickens from the two immunized groups were not significantly higher (p > 0.05) than the challenged control group, but were significantly lower than the unchallenged group (Fig. 8B). No significant differences (p > 0.05) of sCD8 and IL-10 were observed between the immunized and unimmunized-challenged group (Figs. 8C and 8E). The IFN- γ levels in the 100 µg *rEt*CHP18905-immunized group were higher but not significantly than challenged control (Fig. 8D). IL-17 100-µg-immunized groups all showed significantly higher levels (p < 0.05) compared with the unchallenged group (Figs. 8F and 8G).

Discussion

In the present report, a new gene of conserved hypothetical protein from *E. tenella* was cloned and characterized. The 1053 bp ORF was shown to encode a 350 amino acid polypeptide of ~ 38.7 kDa. Sequence analysis showed that the protein had no signal peptide, and amino acids 73–92 formed a transmembrane region. Given this observation, it is speculated that the protein is anchored to the membrane. Bioinformatics analysis predicted that the protein contains seven tyrosine kinase phosphorylation sites, four protein kinase

C sites, three N-glycosylation sites, three N-myristoylation sites, two cAMP and cGMP-dependent proteins kinase phosphorylation sites, and one tyrosine kinase phosphorylation site. These sites and structures indicate that the function of this protein may be regulated by post-translational modifications. The BLASTp results showed that the deduced amino acid sequence of EtCHP18905 was 100% homologous to the E. tenella conserved hypothetical protein (NCBI reference sequence accession number: XP 013231819, GeneID: ETH 00018905) and 84% homologous to the E. necatrix conserved hypothetical protein (NCBI reference sequence accession number: XP_013438465). These results indicate that CHP in E. tenella and CHP in E. necatrix have high homology. Eimeria tenella and E. necatrix are the most pathogenic species among the species represented in the genus Eimeria, which cause severe tissue damage to the host intestine [33]. In addition, the EtCHP18905 protein is a putative interacting protein of the EtCDPK3 that we screened by a yeast two-hybrid system. In this series of experiments, although we were not able to show an interaction between EtCDPK3 and EtCHP using GST pull-down and Co-IP methods (data not shown), we speculated that this protein may be involved in invasion of host cells and development of E. tenella.

The mRNA and protein levels of EtCHP18905 were examined in four different developmental stages. According to qPCR, the EtCHP18905 gene was most prominent in Spz of E. tenella, and only weakly detected in UO and Mrz. These results showed that the EtCHP18905 gene was transcribed predominantly at a distinct phase of the *E. tenella* life cycle. However, western blot showed that protein levels were weakest for EtCHP18905 in SO. A previous study revealed that the ratios between protein and mRNA are mainly determined by translation and protein degradation in a cell [8]. However, the two processes of translation and protein degradation are highly regulated at the overall and gene-specific level [8]. Their study revealed that 15-70% of the variation is explained by posttranscriptional and posttranslation regulation and by measurement errors [5]. Hence, this may explain why the mRNA level of EtCHP18905 is inconsistent with the protein level. However, the specific reasons for this difference need to be investigated further.

The localization of the EtCHP18905 protein in different developmental stages of parasite development was also investigated using an antibody raised against the rEtCHP18905. Indirect immunofluorescence showed that EtCHP18905 was located on the membrane of E. tenella Spz and Mrz stages, which was consistent with the transmembrane protein structure data indicating that the protein was anchored to the membrane. Moreover, immunofluorescence showed that staining was stronger in the Mrz stage. Western blot analysis showed that the expression levels of EtCHP18905 in the Mrz stage was higher than in the Spz stage. However, this result was inconsistent with the results at the transcription level. This may be due to posttranslational modifications [29]. Results from previous studies have suggested that mRNA abundance is a poor indicator of the levels of the corresponding protein [1, 10, 13]. Furthermore, the expression of EtCHP18905 increased after the Spz invaded DF-1 cells for 2 h. Thus, this protein might function in Spz invasion or schizonts evolution. The results of the in vitro experiments confirm this. In vitro invasion inhibition assays using

Group	Average body	Mean lesion scores	Oocyst shedding	Percentage reduction
	weight gains (g)		per bird ($\times 10^7$)	of oocyst excretion (%)
Unchallenged control	$258.62 \pm 70.26^{\circ}$	$0.00 \pm 0.00^{\rm a}$	0.00 ± 0.00^{a}	100^{d}
Challenged control	$180.87 \pm 45.38^{\rm a}$	$3.20 \pm 0.83^{\circ}$	$4.43 \pm 0.99^{\circ}$	$0.00^{\rm a}$
r <i>Et</i> CHP-50 µg	$226.25 \pm 24.47^{\rm b}$	$1.75 \pm 0.95^{\rm b}$	2.10 ± 0.88^{b}	$54.07 \pm 11.76^{\circ}$
r <i>Et</i> CHP-100 μg	$235.25 \pm 23.44^{\rm bc}$	1.20 ± 0.44^{b}	$3.26 \pm 2.47^{\rm bc}$	$31.99 \pm 29.35^{\rm b}$

Table 1. Protective effect of rEtCHP protein on E. tenella infection.

^{a-d} Values with different letters in the same column are significantly different (P < 0.05) according to the ANOVA Duncan test.

polyclonal antibody against r*Et*CHP18905 showed partial blockage of the invasion of Spz into cells. Inhibition of sporozoites was modest at 28%, at antibody concentrations of 300 µg/mL. Likewise, the rate of invasion inhibition increased with the increase of anti-r*Et*CHP18905 IgG concentration. Previous studies have shown that polyclonal antibodies can significantly inhibit Spz invading DF-1 cells [7, 11, 19, 48]. In 2016, Zhai et al. found that rabbit antiserum against r*Et*CHP559 can block invasion of host cells by Spz [47]. The above results suggest that *Et*CHP18905 is related to invasion. This new antigen might be useful for identification of novel vaccine targets, thus improving the knowledge of immunogenic proteins in *E. tenella*.

In previous research, many DNA and recombinant protein vaccines have been reported to induce immuno-protection to live parasite challenge [18, 43, 44]. In the present study, following infection challenge, the body weight gain of nonimmunized chickens was reduced significantly compared with immunized chickens. Moreover, chickens immunized with rEtCHP18905 had significantly lower lesion scores and fecal oocyst output compared to non-immunized birds. Previous studies of Eimeria spp. proteins have shown that a similar effect can be produced after immunization with recombinant protein or recombinant plasmid [25, 26]. The data presented here showed that immunization with rEtCHP18905 could produce partial protection against live E. tenella infection. However, the difference of oocysts output between challenged control groups and the two immunized groups may be under reality. This was probably due to widespread tissue damage and severe hemorrhage that prevents a large number of merozoites from reinfecting intestinal epithelial cells, resulting in a decrease in average oocyst production [12]. It, therefore, appears likely that the differences in lesion score and oocyst output can be caused by merozoite loss [12]. Furthermore, the decrease in oocyst output is not as high as in previous studies [16, 47]. Thus, the effect of rEtCHP18905 in reducing oocyst shedding should be researched further.

Humoral immunity in the immune response against coccidiosis is usually considered to play a minor role [45]. However, as early as 2008, Constantinoiu et al. pointed out that humoral immunity may also contribute to protective immune responses [4]. Their study revealed that chicken infected with an attenuated strain of *E. tenella* mount an antibody response to all lifecycle stages. High levels of antibodies against Spz and Mrz were detected in infected chickens inoculated with SO of *E. tenella* [4]. Moreover, in 1994, Smith also found that antibodies could inhibit parasite development and provide passive immune protection [36]. They found that there was an excellent correlation between antibody titer and protection. Oral infection of hens with *E. maxima* oocysts caused

production of antibodies which were passed into the egg yolk and subsequently to hatchlings. The total number of oocysts excreted in the feces of chicks from eggs has decreased after infection with *E. maxima* oocysts [36]. Huang et al. [16] reported that birds immunized with the *E. maxima* rMIC7 protein and pVAX1-MIC7 exhibited higher IgG concentrations than the PBS and pVAX1 controls. In the present study, the IgG concentrations of the r*Et*CHP18905-immunized chickens were significantly higher than the negative controls. These findings confirmed that in rEtCHP18905-immunized chickens, certain humoral immune responses were induced.

Cell-mediated immunity plays a major role against coccidiosis [39]. The concentration of soluble sCD4 and sCD8 in serum is consistent with the number of CD4+ and CD8+ lymphocytes that produce them [41, 46]. Previous studies reported that levels of sCD4 and sCD8 were higher in experimental groups immunized with rEmSAG and rEmMIC7 compared with the control [16, 26]. However, the results presented here showed that serum sCD4 and sCD8 were not significantly different in immunized chickens compared to non-immunized controls. This suggests that rEtCHP18905 could not stimulate the recruitment of T-cell subpopulations. IFN- γ is reported to be related to protective immune responses to avian coccidiosis [23]. A previous study found that $IFN-\gamma$ concentrations were higher in EmMIC7-vaccinated birds [16]. However, rEtCHP18905 increased the serum concentrations of IFN-y but not significantly compared to the control group. Nevertheless, the relative importance of each cytokine type in inducing immune challenge could not be inferred from these data.

One of the Th2-type cytokines, IL-10 and IL-17 are also involved in immune response to coccidial challenge. IL-10 has been shown to be crucial for control of Eimeria infections [32]. Wu et al. observed that serum IL-10 levels increased on day 5 after infection with E. tenella [42]. However, in this study, there was no significant level of IL-10 detected in groups immunized with rEtCHP18905. Previous reports have shown that the immunization of animals with DNA vaccines produced higher levels of IL-17 [14, 37]. In previous vaccination trials, IL-17 concentrations in the vaccinated groups were significantly higher than those of the unvaccinated groups [16, 26]. In the current study, the concentrations of IL-17 in the immunized groups were also significantly higher than the non-immunized groups. The previous study, and our data, together confirm that IL-17 might be associated with protective immunity to coccidiosis. However, the specific functions of IL-10 and IL-17 in the immunity to coccidiosis requires further investigation.

The Treg-type cytokines TGF- β , produced by Treg cells, have been shown to regulate immunosuppression mechanisms



Figure 8. Levels of IgG (A), sCD4 (B), sCD8 (C), cytokines IFN- γ (D), IL-10 (E), IL-17 (F) and TGF- β 1 (G) in chicken sera were measured using ELISA. Chickens of group *rEt*CHP18905-50 µg and group *rEt*CHP18905-100 µg were immunized with 50 µg or 100 µg of *rEt*CHP18905 protein, respectively. Challenged and unchallenged groups were immunized with PBS and served as controls. The IgG titers and the concentrations of sCD4, sCD8, and cytokines are expressed as Min to Max. (*p < 0.05, **p < 0.01, ***p < 0.001; ns, p > 0.05).

[21]. Two earlier reports showed that TGF- β was significantly higher in chickens immunized with recombinant *Eb*AMA1 [38, 49]. The same result was demonstrated by Liu et al. [26]. Likewise, in the present study, the levels of the TGF- β 1 in the *rEt*CHP18905-immunized groups were significantly higher than the non-immunized group. These findings together confirm that TGF- β 1 may function in coccidiosis-induced immune pathways.

In summary, Th2-type cytokines can down-regulate the expression levels of Th1-type cytokines and regulate the immune response [15]. In the present study, IL-17 and TGF- β 1 levels increased following immunization. In contrast, sCD4, sCD8 and IL-10 did not increase significantly. Moreover, higher IgG concentrations were detected in the *Et*CHP18905 vaccinated chickens. Thus, *Et*CHP18905 might be a supplementary candidate, alongside other proteins that stimulate cellular immunity for the development of new vaccines to combat *E. tenella* infection in chickens.

Conclusions

In this study, *Et*CHP18905 was amplified, expressed and characterized. Its location on Spz and Mrz was determined. Anti-*rEt*CHP18905 antibodies could reduce the rate of Spz invasion. The results of animal immune protection assays indicated that vaccination with *rEt*CHP18905 was capable of eliciting both humoral immunity and cell-mediated immunity, providing moderate protective immunity against *E. tenella*. However, the exact roles of *Et*CHP18905 in coccidial infections require further investigation.

Competing interest

The authors declare that they have no competing interests.

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