

Eimeria tenella *Eimeria*-specific protein that interacts with apical membrane antigen 1 (*EtAMA1*) is involved in host cell invasion

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Abstract

Background: Avian coccidiosis is a widespread, economically significant disease of poultry, caused by several species of the protozoan parasite *Eimeria*. Among these species, *E. tenella* causes hemorrhagic pathologies and high mortality. These parasites have complex and diverse lifestyles that require the invasion of their host cells. This is mediated by various proteins secreted from apical secretory organelles. Apical membrane antigen 1 (AMA1), which is released from micronemes and is conserved across all apicomplexans, plays a central role in the host cell invasion. In a previous study, some putative *EtAMA1*-interacting proteins of *E. tenella* were screened. In this study, we characterized one putative *EtAMA1*-interacting protein, *E. tenella Eimeria*-specific protein (*EtEsp*).

Methods: Bimolecular fluorescence complementation (BiFC) and glutathione S-transferase (GST) fusion protein pull-down (GST pull-down) were used to confirm the interaction between *EtAMA1* and *EtEsp* *in vivo* and *in vitro*. The expression of *EtEsp* was analyzed in different developmental stages of *E. tenella* with quantitative PCR and western blotting. The secretion of *EtEsp* protein was tested with staurosporine when sporozoites were incubated in complete medium at 41 °C. The localization of *EtEsp* was analyzed with an immunofluorescence assay. An *in vitro* invasion inhibition assay was conducted to assess the ability of antibodies against *EtEsp* to inhibit cell invasion by *E. tenella* sporozoites.

Results: The interaction between *EtAMA1* and *EtEsp* was confirmed with BiFC *in vivo* and by GST pull-down *in vitro*. Our results show that *EtEsp* is differentially expressed during distinct phases of the parasite life cycle. An immunofluorescence analysis showed that the *EtEsp* protein is mainly distributed on the parasite surface, and that the expression of this protein increases during the development of the parasite in the host cells. Using staurosporine, we showed that *EtEsp* is a secreted protein, but not from micronemes. In inhibition tests, a polyclonal anti-*EtEsp* antibody attenuated the capacity of *E. tenella* to invade host cells *in vitro*.

Conclusion: In this study, we show that *EtEsp* interacts with *EtAMA1* and that the protein is secreted protein, but not from micronemes. The protein participates in the sporozoite invasion of host cells and maybe involved in the growth of the parasite in the host. These data have implications for the use of *EtAMA1* or *EtAMA1*-interacting proteins as targets in intervention strategies against avian coccidiosis.

Background

Avian coccidiosis is a widespread, economically significant disease of poultry that results in annual global economic losses of approximately \$2.4 billion, including both production losses and disease prevention and treatment costs [1]. It is an enteric disease caused by several species of the protozoan parasite *Eimeria*, predominantly *E. brunetti*, *E. necatrix*, *E. tenella*, *E. acervulina*, *E. maxima*, *E. mitis*, and *E. praecox* [2]. Of these, *E. tenella* is one of the species that causes hemorrhagic pathologies and high mortality. *Eimeria* belongs to the phylum Apicomplexa, which includes important pathogens of humans and domestic animals, such as the causative agents of malaria (*Plasmodium* spp.), toxoplasmosis

(*Toxoplasma gondii*), babesiosis (*Babesia* spp.), and coccidiosis (*Eimeria* spp.). Most apicomplexans are obligate intracellular parasites and are characterized by their apical complexes of specialized secretory organelles (micronemes, rhoptries, and dense granules) [3]. They use actin-based motility coupled to regulated protein secretion from their apical organelles to actively invade host cells [4]. These parasites have complex and diverse lifestyles that involve the invasion of many different cell types, including erythrocytes, lymphocytes, macrophages, and digestive-tract cells. Despite the diversity of their target host cells, they maintain a highly conserved mechanism for this active invasion process [5].

The host-cell invasion mechanism involves the following steps: attachment, apical reorientation, moving junction formation, and the formation of a protective parasitophorous vacuole. Each invasion step is mediated by various proteins, which are secreted from apical secretory organelles [6]. Apical membrane antigen 1 (AMA1), a type I transmembrane protein, is one of a number of proteins released from micronemes that are conserved across all apicomplexans. It is known to play several important roles during host-cell penetration [7]. For instance, previous reports have shown that antibodies against AMA1 or small specific AMA1-binding peptides inhibit the invasion of host cells by *Toxoplasma*, *E. tenella*, *Babesia*, *Neospora*, and *Plasmodium* [3,8–11]. AMA1 is also a long-standing effective candidate vaccine for some apicomplexans, including *N. caninum*, *T. gondii* and *Plasmodium* [11–13]. In *Toxoplasma* and *Plasmodium*, AMA1 is reportedly involved in apical reorientation [14], host-cell attachment [7, 15], invasion and establishment of the moving-junction [16], and the provision of a signal that initiates intracellular replication [17].

In contrast to the functions of AMA1 in other apicomplexan parasites, there are only a few reports of this conserved protein in *Eimeria*. In a previous *in vitro* study, AMA1 antibodies or specific *EtAMA1*-binding peptides inhibited the invasion of host cells by *E. tenella* sporozoites [10, 18]. *EtAMA1* also partially protected host cells against homologous challenge with *E. tenella* when used as a recombinant protein vaccine and against heterologous challenge with *E. maxima* when the AMA1 protein from *E. maxima* was expressed as a live vectored vaccine [19]. Although AMA1 plays an important role in host-cell invasion by *E. tenella* sporozoites, its precise functions are unknown.

Proteins perform a vast number of cellular functions when they interact with one or multiple binding partners. Protein–protein interactions are essential in the mediation of almost all cellular processes, including replication, transcription, translation, and signal transduction [20]. The biochemical analysis of protein complexes and the identification of their components have been fundamental to our understanding of their biological functions in cells [21].

To understand the precise functions of *EtAMA1* during host-cell invasion, we screened *EtAMA1*-interacting proteins with a yeast two-hybrid system and identified 14 putative *EtAMA1*-interacting proteins in a previous study [22]. *E. tenella Eimeria*-specific protein (*EtEsp*) (GenBank accession number: JZ905773) is one of these putative interacting proteins. In this study, we cloned and characterized *EtEsp*. We systematically analyzed its interaction with *EtAMA1* using bimolecular fluorescence complementation (BiFC) *in vivo* and a glutathione S-transferase (GST) pull-down assay *in vitro*. Our results show that the

EtEsp is secreted protein, but not from micronemes, interacts with *EtAMA1*, and is involved in the invasion of host cells by *E. tenella* sporozoites.

Methods

Parasite collection

E. tenella was obtained from the Key Laboratory of Animal Parasitology of the Ministry of Agriculture, Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Shanghai, China. The parasites were maintained and propagated by passage through coccidia-free, 2-week-old chickens, as previously described [23]. Coccidia-free 14-day-old chickens were inoculated with 1×10^4 sporulated oocysts of *E. tenella*. Unsporulated oocysts (UO) were collected from infected chicken ceca at 7 days postinfection. Sporulated oocysts (SO) were derived from UO that had undergone sporulation in 2% potassium dichromate at a temperature 28–30 °C for 72–120 h, under forced aeration with a suitable pump. When more than 90% of the oocysts had sporulated, the oocysts were collected and purified. The sporozoites (Spz) were purified from cleaned SO with *in vitro* excystation [24]. Second-generation merozoites (sMrz) were isolated from infected chicken ceca at 115 h postinoculation, as described previously [25]. All parasites were collected and frozen in liquid nitrogen.

Chickens and rabbits were fed and used according to a protocol approved by the Animal Care and Use Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

The chicken embryo fibroblast cell line, DF-1, a derivative of the East Lansing Line (ELL-0) (Jiang et al. 2012), was used for BiFC and *in vitro* infection experiments.

Molecular cloning and sequence analysis of *E. tenella*-specific protein

Total RNA was extracted from *E. tenella* sporozoites with TRIzol Reagent (Invitrogen, USA). GeneRacer™ primers (GR5P and GR5N) were provided for the random amplification of PCR ends (RACE) in the GeneRacer™ Kit (Invitrogen) and gene-specific primers (GS5P and GS5N) were designed based on the expressed sequence tag (EST) sequence (GenBank accession number: JZ905773) which is 790 bp in length and contains a poly(A) at the 3' end (Table S). The 5' end of this gene was determined according to the manufacturer's protocol. The PCR-amplified fragment was then ligated into the pGEM-T Easy Vector (Promega, USA) and used to transform competent *Escherichia coli* TOP10 cells. After PCR identification, the plasmid DNA was sequenced. After the resulting sequence was assembled and aligned with the original EST sequence, the full-length cDNA sequence of the gene was determined and submitted to the National Center for Biotechnology Information (NCBI) GenBank (accession number: MN161778). The full-length *EtEsp* cDNA sequence was used in a BLAST search of GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the *E. tenella* genome database

(<http://www.genedb.org/Homepage/Etenella>). The deduced amino acid sequence was obtained with the ORF Finder tool at NCBI. The molecular mass and theoretical isoelectric point were calculated with ProtParam tools (<http://web.expasy.org/protparam/>). The signal peptide sequence was identified with the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>), and transmembrane regions were predicted with the TMHMM server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Protein motifs were scanned with Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Recombinant protein expression and polyclonal anti-r*EtEsp* serum

The *EtEsp* open reading frame (ORF) cDNA was amplified with PCR using primers *EtEsp*-UP and *EtEsp*-LP (Table S), which contained *Bam*HI and *Xho*I restriction sites, respectively. The PCR fragment was then ligated into the prokaryotic expression vector pET28a(+) digested with the same restriction endonucleases, to construct the recombinant expression plasmid pET-*EtEsp*. The recombinant protein His-*EtEsp* (r*EtEsp*) was expressed in *Escherichia coli* BL21 cells at 37 °C with 1 mM isopropyl-thio- α -D-galactoside. The cell pellet was lysed with sonication and digested with 10 μ g/mL lysozyme (Sigma-Aldrich, USA). The lysate was then analyzed with 12% SDS-PAGE to confirm that the recombinant protein was present as a soluble protein or inclusion bodies. r*EtEsp* was purified with His-Bind® Resin (Merck, USA) and its concentration measured with a BCA Protein Assay Kit (Beyotime, China).

Two 2-month-old male rabbits were inoculated with 200 μ g of purified r*EtEsp* emulsified in Freund's complete adjuvant (Sigma-Aldrich). After 14 days, a booster of 200 μ g of purified r*EtEsp* in Freund's incomplete adjuvant (Sigma-Aldrich) was administered, followed by a second and third booster on days 28 and 42. One week after the final booster, the rabbit serum was collected and stored at -20 °C until use.

Analysis of *EtEsp* transcript levels with real-time quantitative PCR (qPCR)

The expression profiles of *EtEsp* mRNA were examined in four developmental stages of *E. tenella* (UO, SO, Spz, and sMrz) with qPCR. cDNA samples were synthesized from DNaseI-treated total RNAs of the *E. tenella* developmental stages using SuperScript™ II Reverse Transcriptase (Invitrogen) and random pd(N)6 primer. The housekeeping gene 18S rRNA was used as the internal control. The primers used to amplify the *EtEsp* cDNA (*EtEsp*-SP and *EtEsp*-AP) and the 18S rRNA gene (18S-SP and 18S-AP) were designed with Primer3 v. 0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Table S). qPCR was performed with the StepOnePlus™ Real-Time PCR System using the SYBR® Premix Ex Taq™ II kit (Takara, Japan). All experiments were performed twice, with separate biological replicates. In each experiment, the reactions were performed in triplicate. A dilution series of cDNA templates of the sporozoites was used to establish standard curves, and all standard curves had correlation coefficients of $R^2 > 0.99$. The comparative $2^{-\Delta\Delta Ct}$ method was used to analyze the relative levels of gene expression.

SDS-PAGE and western blotting

Protein samples were prepared from the four *E. tenella* developmental stages (UO, SO, Spz, and sMrz), and from DF-1 cells transfected with the recombinant plasmids, for western blotting. The protein

concentrations were determined with a BCA Protein Assay Kit (Beyotime,China). The purified *rEtEsp* and protein lysates were separated with SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. Rabbit antiserum (1:100) against sporozoite proteins, previously produced in our laboratory [26], a rabbit anti-*rEtEsp* antibody (1:100), a mouse monoclonal anti- α -tubulin antibody (1:1000) (Sigma-Aldrich), and a monoclonal anti-His antibody (1:1000) were used as the primary antibodies to detect *rEtEsp* or native *EtEsp*. Naïve rabbit serum (1:100) was used as the negative control. IRDye-800CW-labelled goat anti-rabbit IgG antibody (1:25,000) and IRDye-680RD-labeled donkey anti-mouse IgG antibody (1:25,000; LI-Cor, Lincoln, NE, USA) were used as the secondary antibodies. The IRDyes were detected with the Odyssey Infrared Imaging System (LI-Cor).

BiFC assay

The ORF fragments of *EtEsp* and the *EtAMA1* ectodomain, with no stop codon, were amplified from the first-strand cDNA with two pairs of primers (Bf*EtEsp*-UP/Bf*EtEsp*-LP and Bf*EtAMA1*-UP/Bf*EtAMA1*-LP, respectively), which contained *EcoRI* and *BglII* restriction sites (*EtEsp*) or *EcoRI* and *XhoI* restriction sites (*EtAMA1*). The fragments were then digested with the appropriate restriction enzymes and ligated into the pBiFC-VN155 and pBiFC-VC155 vectors digested with the same enzymes, respectively, to construct the recombinant plasmids pBiFC-VN155-*EtEsp* and pBiFC-VC155-*EtAMA1*, respectively. Before the BiFC assay, the uptake of the expression vectors by the cells was confirmed. DF-1 cells were transfected with the recombinant plasmid pBiFC-VN155-*EtEsp* or pBiFC-VC155-*EtAMA1* using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, USA), according to the manufacturer's instructions. At 48 h after transfection, the cells were harvested and the proteins were extracted with RIPA Lysis Buffer (Beyotime). Western blots were probed with rabbit anti-*rEtEsp* antibody and rabbit anti-*rEtAMA1* antibody, which were previously produced in our laboratory [10]. After confirmation that the cells had expressed the two constructs, DF-1 cells were cotransfected with pBiFC-VN155-*EtEsp* and pBiFC-VC155-*EtAMA1*. DF-1 cells were also cotransfected with pBiFC-bJunVN55 (I152L) and pBiFC-bFosVC155 or pBiFC-VC155-*EtAMA1* and pBiFC-VN155 empty vector, or pBiFC-VN155-*EtEsp* and pBiFC-VC155 empty vector as the positive or negative control, respectively. The DF-1 cells were observed with fluorescence microscopy 24 h after transfection with the different constructs.

GST pull-down

To confirm the interaction between *EtAMA1* and *EtEsp317* *in vitro*, a GST pull-down assay was performed with the Pierce™ GST Protein Interaction Pull-Down Kit (Thermo Scientific, USA), according to the manufacturer's instructions. The recombinant plasmid pGEX-6P-*EtAMA1* was previously constructed in our laboratory [10]. The expression of the recombinant protein GST-*EtAMA1* was induced and the protein purified with GST resin for use as the bait protein. The ORF of *EtEsp* was inserted into the pET-28a vector to express the recombinant protein His-*EtEsp* (*rEtEsp*) as the prey protein. GST-*EtAMA1* was incubated with equilibrated glutathione-agarose to immobilize the bait protein. *rEtEsp* was then added to the glutathione-agarose and incubated with the bait protein. The bait and prey proteins were eluted from the glutathione-agarose. *E. coli* BL21 cells were transformed with recombinant plasmid pET-*EtMIC2*,

constructed previously in our laboratory [27], to express the recombinant protein His-*EtMIC2* as the negative control. Another, *rEtEsp* was loaded in an empty glutathione-agarose column as the negative. All the proteins were then resolved with 12% SDS-PAGE and detected with western blotting using the appropriate antibodies, as described above.

Assay of *EtEsp* secretion

Freshly excysted sporozoites (4×10^6) were incubated in 100 μ L of complete medium (CM; Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum [FBS], 100 U/mL penicillin/streptomycin, 2 mM l-glutamine) for 2 h at 41 °C under 5% CO₂ for the secretion experiments. They were then incubated with 5, 10, or 20 μ M staurosporine (Sigma; dissolved in dimethylsulfoxide [DMSO]) or an appropriate volume of carrier DMSO, as described previously [28]. The secretion of *EtMIC2* and *EtGRA* (*TgGRA7* homologous protein) was used as the control. The sporozoites were then pelleted by centrifugation for 10 min at 6000 \times g. The supernatants and sporozoites were recovered and analyzed with western blotting using a rabbit anti-*rEtEsp* antibody and rabbit anti-*rEtMIC2* antibody generated previously in our laboratory [27] and mouse anti-*TgGAR7* antibody which generated previous in other laboratory of our Institute .

Immunofluorescence staining of parasites

Purified differentially developed parasites (Spz, sporocysts [Sporo], and sMrz) were transferred to glass slides and air-dried, as previously described [10, 29]. Purified freshly sporozoites were infected DF-1 cells after incubation in CM for 2 h at 41°C. At different time points after infection, the DF-1 cells were collected, washed, transferred to glass slides, and air-dried. The slides were then fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) and placed in 1% Triton X-100 in PBS for 15 min to increase their permeability. Non permeabilized sporozoites and sporocysts were used as control. The slides were blocked with PBS containing 2% (w/v) bovine serum albumin for overnight at 4 °C. A rabbit anti-*rEtEsp* antibody (1:100) was added and the cells were incubated for 1 h at 37 °C. A 1:500 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) was then added and the cells incubated for 1 h at 37 °C. The cell nuclei were stained by incubation in 10 μ g/mL 4',6-diamidino-2-phenylindole (Beyotime) at room temperature for 10 min. After each step, the slides were washed three times for 10 min each with PBS containing 0.05% Tween 20. The slides were finally mounted with 50 μ L of Fluoromount Aqueous Mounting Medium (Sigma-Aldrich) before observation with a fluorescence microscope (Olympus, Tokyo, Japan). At the same time, we performed the co-localization of *EtEsp* and *EtAMA1* in sporozoites. Purified sporozoites were treated with mouse anti-*rEtAMA1* antibody (1:100) and rabbit anti-*rEtEsp* antibody(1:100), then goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibody(1:500) and goat anti-mouse IgG cyanine (Cy3)-conjugated antibody (Sigma-Aldrich)(1:500) were secondary antibody.

Invasion inhibition assay *in vitro*

The invasion inhibition assay was based on previous reports the invasion of DF-1 cells by *E. tenella* sporozoites. Antibodies were purified with Protein A+G Agarose (Beyotime). DF-1 cells (2×10^5 cells per well) were cultured in 24-well plates (Corning) in CM for 24 h at 37 °C under 5% CO₂. The freshly purified sporozoites were counted and labeled with carboxyfluorescein diacetate succinimidyl ester (Beyotime). The labeled sporozoites were incubated at 37 °C with 50, 100, 200, 300 or 400 µg/mL purified IgG directed against r*E*Ɛsp for 2 h. The same quantity of IgG from naïve rabbit serum (Sigma-Aldrich) was used as the negative control, and an equivalent volume of PBS as the normal control. After they were washed twice with sterile PBS, DF-1 cells (10^5 /well) were infected with the labeled sporozoites (10^5 /well) in 24-well plates and cultured for 16 h at 41 °C under 5% CO₂. The cells were then collected and analyzed with flow cytometry on a Cytomics™ FC 500 (Beckman Coulter, Indianapolis, IN, USA). The controls were uninfected DF-1 cells. The infected cells, uninfected cells, and free sporozoites were gated with the CXP software to count the infected (labeled sporozoites) and uninfected (fluorescence-free) cells. All assays were performed in triplicate. The percentages of infected cells in the presence or absence of an anti-r*E*Ɛsp polyclonal antibody were used to calculate the inhibition rates, as previously described [10].

Results

Cloning and sequence analysis of full-length *E*Ɛsp cDNA

The 1108-bp full-length cDNA of *E*Ɛsp was obtained with RACE. A sequence analysis showed that the full-length cDNA included a 5′-untranslated region (UTR) of 70 bp, a 3′-UTR of 542 bp with a poly(A) tail, and an ORF of 501 bp, which encoded 166 amino acids with a calculated molecular weight of 18.1 kDa and a theoretical isoelectric point of 4.2 (Fig. 1). Analysis of the amino acid sequence showed a signal peptide of 19 amino acids at the N-terminus and no transmembrane region. Searches in the Motif Database and the Conserved Domain Database revealed the presence of one N-glycosylation site, five casein kinase II phosphorylation sites, six N-myristoylation sites, one tyrosine kinase phosphorylation site, one intein DOD-type homing endonuclease domain, and no conserved domains (Fig. 1). A BLAST search of the *E. tenella* genome database showed that the ORF sequence shared 100% sequence identity with ETH_00016590, which encodes an *Eimeria*-specific protein, on supercontig Eth_scaff124: 9216–10141.

The amino acid sequence shared 100% homology with the *E. tenella* *Eimeria*-specific protein (XP_013228647.1) and 92% (152/170) identity with the *E. necatrix* *Eimeria*-specific protein (XP_013435139.1) in NCBI. Therefore, this gene was designated *E*Ɛsp and submitted to NCBI GenBank (GenBank accession no. MN161778). It also shared 68% (106/157) amino acid identity with *E. brunetti* conserved hypothetical protein (CDJ53027.1), 63% (108/172) identity with *E. praecox* conserved hypothetical protein (CDI81636.1), 74% (97/131) identity with *E. maxima* conserved hypothetical protein (XP_013336310.1), and 73% (91/124) identity with *E. acervulina* conserved hypothetical protein (XP_013248166.1). Also, this protein is not found in other apicomplexa parasites. These results show that the protein is conserved in *Eimeria* spp.

Expression and characterization of recombinant *E*Ɛsp

rEtEsp was expressed as a His6-tagged fusion protein. SDS-PAGE showed that *rEtEsp* was mainly present in the soluble fraction of the bacterial lysate. After the purification of *rEtEsp* with Ni-NTA chromatography, a protein of approximately 21 kDa was observed with SDS-PAGE. Because 3 kDa of the fusion protein was derived from the vector, the predicted molecular mass of *EtEsp* was about 18.1 kDa. Western blotting showed that purified *rEtEsp* was recognized by rabbit serum directed against sporozoites and by a monoclonal anti-His6 antibody. Naïve rabbit serum failed to recognize any protein corresponding to the expected size of *rEtEsp* (Fig. 2). These results indicate that *rEtEsp* was recognized specifically by rabbit serum directed against a soluble sporozoite protein and by a monoclonal anti-His antibody.

EtEsp* mRNA and protein expression at different developmental stages of *E. tenella

qPCR was used to analyze the UO, SO, Spz, and sMrz stages of *E. tenella* for the presence of *EtEsp* mRNA. The levels of *EtEsp* mRNA were much higher in the sMrz stage than in the other three stages, and *EtEsp* mRNA was almost undetectable in UO (Fig. 3a).

The expression of *EtEsp* in the four developmental stages was also determined with immunoblotting using rabbit antiserum against *rEtEsp*. A monoclonal anti- α -tubulin antibody was used as the control. Western blotting showed that the anti-*rEtEsp* antibody reacted with a band of approximately 18 kDa in the parasite lysates prepared from the four different developmental stages of *E. tenella*. The expression levels of *EtEsp* were higher in sporozoites than in other three stages (Fig. 3b,c).

Confirmation of the interaction between *EtAMA1* and *EtEsp*

To characterize the interaction between *EtAMA1* and *EtEsp* *in vivo*, a BiFC assay was performed. For the BiFC assay, fragments of the *EtEsp* ORF and the *EtAMA1* ectodomain sequence were cloned into the plasmids pBiFC-VN155 and pBiFC-VC155, respectively, to generate the constructs pBiFC-VN155-*EtEsp* and pBiFC-VC155-*EtAMA1*, respectively. The total proteins were extracted from DF-1 cells transfected separately with one or other construct. Western blotting showed that the two constructs were expressed individually in the DF-1 cells at 48 h after transfection (Fig. 4a). Strong green fluorescence was observed in DF-1 cells 48 h after they were cotransfected with both constructs. Green fluorescence was also observed in the positive control. However, there was no visible fluorescence in the DF-1 cells cotransfected with pBiFC-VC155-*EtAMA1* and pBiFC-VN155 empty vector or pBiFC-VN155-*EtEsp* and pBiFC-VC155 empty vector. These results indicate that *EtEsp* interacts with *EtAMA1* in cells (Fig. 4b).

GST pull-down

To confirm the interaction between *EtAMA1* and *EtEsp* *in vitro*, a GST pull-down assay was performed. GST-*EtAMA1* and His-*EtEsp* were expressed individually in *E. coli* and purified. GST-*EtAMA1* was bound to an equilibrated glutathione-agarose column, and then His-*EtEsp* was added to the column. The proteins bound to the glutathione-agarose, and any nonspecifically bound proteins were removed by elution with buffer. The proteins retained on the column were then eluted and detected with

immunoblotting using anti-*rEtAMA1* and anti-*rEtEsp* antibodies (Fig. 5). The results clearly indicated a direct interaction between the *EtAMA1* and *EtEsp* proteins.

***EtEsp* is not secreted from the microneme**

To examine the secretion of *EtEsp*, sporozoites were incubated in CM at 41 °C. The supernatant containing the excretory–secretory antigens (ESA) from the incubated sporozoites and sporozoites pellets were analyzed with western blotting. Immunoblots of the ESA samples and sporozoites were probed with an anti-*rEtEsp* antibody and showed that *EtEsp* was secreted when the sporozoites were incubated at 41 °C under 5% CO₂ in CM. Rabbit serum raised against the micronemal protein *EtMIC2* was used as the experimental control [27]. To demonstrate whether *EtEsp* secretion is dependent on the micronemal pathway, we added staurosporine to the CM because staurosporine is a protein kinase inhibitor known to specifically inhibit microneme secretion [28]. In the parasites treated with 5, 10, or 20 μM staurosporine, the secretion of *EtEsp* and *EtGRA* into the supernatant was not affected, but the secretion of *EtMIC2* in supernatant was significantly reduced compared with their secretion in the presence of the DMSO solvent only (Fig. 6a,b). These results show that *EtEsp* is a secreted protein, but not a micronemal protein.

Immunolocalization of *EtEsp* at different developmental stages of *E. tenella*

To investigate the localization and distribution of the *EtEsp* protein in different development stages of *E. tenella*, including sporozoites, second-generation merozoites, immature schizonts, and mature schizonts, it was localized with immunofluorescence *in vitro* using an antibody against *rEtEsp*. The *EtEsp* protein was mainly distributed on the surfaces of the permeabilized parasite sporozoites, sporocysts, and second-generation merozoites (Fig. 7a-a, b-a, k). The protein were also mainly located on the surface of non permeabilized sporozoites and sporocysts (Fig. 7a-b, b-b). After incubation in CM for 2 h, the fluorescence increased and mainly localized to the anterior and surface of parasites (Fig. 7c). *EtEsp* protein was also mainly located on the surfaces of parasites 2 h after their invasion of DF-1 cells (Fig. 7d). At 12 h after the sporozoites were added to DF-1 cells, *EtEsp* also localized to the cytoplasm of the sporozoites, except for the refractile body in the posterior section of the parasites, and the intensity of *EtEsp* staining had increased (Fig. 7e). At 24–72 h postinfection, the *EtEsp* protein was uniformly distributed in trophozoites, immature schizonts, and mature schizonts, and the protein's expression had increased (Fig. 7e–j).

Co-localization of *EtAMA1* and *EtEsp*

IFAs were performed to determine the location of *EtAMA1* and *EtEsp*. Purified sporozoites were treated with mouse anti-*rEtAMA1* antibody and rabbit anti-*rEtEsp* antibody. The results showed *EtEsp* was mainly located on the surface of sporozoites, *EtAMA1* was distributed throughout the cytoplasm and the membrane of sporozoites with the exception of refractile bodies (Fig 8).

Anti-*rEtEsp* antibodies inhibit DF-1 cell invasion

To evaluate the effect of the *EtEsp* protein on the invasion of DF-1 cells by *E. tenella* sporozoites, an invasion inhibition assay of sporozoites was performed *in vitro*. When the sporozoites were incubated with purified anti-*rEtEsp* antibody before infection, their capacity to invade the DF-1 cells was significantly reduced. After pretreatment with 50, 100, 200, 300, or 400 µg/mL anti-*rEtEsp* IgG antibody, their invasion of cells was highly significantly reduced compared with that of sporozoites treated with naïve rabbit IgG (negative control) ($P < 0.01$). Under these experimental conditions, an inhibition plateau of 62.9% was reached at an antibody concentration of 300 µg/mL. In a comparative analysis, the same dose of the naïve rabbit serum IgG antibody did not significantly affect invasion (Fig. 9).

Discussion

In this study, we cloned and characterized the *E. tenella* *Eimeria*-specific protein, a putative *EtAMA1*-interacting protein, using a yeast two-hybrid system in our laboratory [22]. Although the yeast two-hybrid system is a widely used and powerful method for identifying the partners of proteins in regulatory complexes and in the analysis of protein–protein interactions [30], the system has several limitations, including the possibility of isolating very large numbers of clones with no biological relevance [31]. Therefore, the interaction between *EtAMA1* and *EtEsp* required validation with an alternative technique, such as a BiFC assay or GST pull-down assay. The BiFC assay is a versatile technique for investigating protein–protein interactions in living systems, and is based on the reconstitution of a fluorescent protein *in vivo* [32]. GST pull-down is amenable to more specific investigations of protein–protein interactions *in vitro*, but relies on purified proteins that may not fully mimic the protein's native conformation or posttranslational modification, which mediate its interactions [33]. Although these assays have some advantages in identifying protein–protein interactions, each also has its drawbacks. Therefore, in many research fields, these methods are often combined to identify the interactions between two proteins [33–35]. In this study, the interaction between *EtAMA1* and *EtEsp* was confirmed with a GST pull-down assay *in vitro* and a BiFC assay *in vivo*. These results indicated that *EtEsp* interacts with *EtAMA1*.

Proteins perform a vast number of cellular functions through their interactions with one or multiple binding partners. Moreover, many protein–protein interactions are regulated by posttranscriptional modification (e.g., phosphorylation) of the protein of interest, and these modifications are induced by exposure to certain circumstances [36]. In this study, an amino acid sequence analysis predicted that *EtEsp* contains one N-glycosylation site, five casein kinase II phosphorylation sites, six N-myristoylation sites, one tyrosine kinase phosphorylation site and one intein DOD-type homing endonuclease domain. Inteins, also called protein introns, are parasitic genetic elements that excise themselves at the protein level by self-splicing, allowing the formation of functional, nondisrupted proteins [37]. These data suggest that its functions may be regulated by posttranslational modification. We supposed that the interaction of *EtAMA1* with *EtEsp* may be regulated by posttranslational modification.

To understand the expression of *EtEsp* in the different developmental stages of parasite, we examined its expression patterns with qPCR and western blotting. Our results indicated that *EtEsp* mRNA levels were higher in second-generation merozoites and sporozoites than in sporulated oocysts or unsporulated

oocysts. But western blotting showed that the expression of *EtEsp* was higher in sporozoites than other developmental stages of *E. tenella*. Immunofluorescent localization showed that the expression of the protein increased with the development of the parasites in DF-1 cells. Previous proteomic and transcriptomic data confirm that *EtAMA* paralogues are tightly stage-regulated [38, 39]. *EtAMA1* is a sporozoite-specific protein involved in the invasion process of sporozoites [10, 19, 38]. While another *EtAMA1* paralogues, *EtAMA2* is a merozoites-specific protein not involved in the parasite invasion. All these finding indicate that *E. tenella* parasites harbour stage-specific AMA proteins that could be relevant during specific phases of the parasite cycle [19]. In this study, *EtEsp* is differentially expressed during the distinct phases of the parasite life cycle, and may be very important in the invasion and development of the parasite life cycle.

Immunofluorescent localization showed that *EtEsp* was located on the surface of the sporozoite and concentrated around the anterior of the parasite during its incubation in CM. However, the protein has no transmembrane region or glycosylphosphatidylinositol (GPI)-anchor sequence, but has a signal peptide, and six N-myristoylation sites. The presence of a signal peptide is necessary for the translocation of proteins from their ribosomal sites of translation into the lumen of the endoplasmic reticulum, from where they are trafficked in the endomembrane system to their final locations within the cell or beyond [40]. We speculated that *EtEsp* also undergoes posttranslational modification according to the amino acid sequence analysis, including phosphorylation, myristoylation, and glycosylation. Among these modifications, myristoylation is the key factor in the membrane localization of signal-transducing proteins [41].

Most surface antigens are involved in the invasion, pathogenesis, and immune evasion of parasites. For example, in *Plasmodium*, merozoite surface proteins are critical for parasite invasion, and represent attractive targets for antibody-based therapies against clinical malaria [42]. We also found that the expression of *EtEsp* increased and that the protein mainly localized on the anterior and surface of the parasites after incubation in CM for 2 h. This suggests that the protein is involved in the sporozoite invasion of host cells. To investigate the function of *EtEsp* in the invasion process, we performed an invasion test *in vitro* and found that polyclonal rabbit anti-r*EtEsp* serum efficiently reduced the sporozoite invasion of cultured DF-1 cells. *EtAMA1* also localized to the anterior of the sporozoites after their invasion of DF-1 cells [10]. Previous reports have shown that monospecific mouse anti-r*EtAMA1* serum or polyclonal rabbit antiserum against r*EtAMA1* also blocked the invasion of host cells *in vitro* [10,19]. In this study, *EtEsp* is involved in invasion as demonstrated by using antibodies raised against *EtEsp* *in vitro*. We tested that *EtEsp* is an interacting protein with *EtAMA1* by using BiFC, GST-pull down and yeast two-hybrid system.. Therefore, we speculated that *EtEsp* mediates sporozoites invasion in host cells by interacting with *EtAMA1*. The exact function of *EtEsp* needs further study.

Previous studies have shown that in *T. gondii* and *Plasmodium*, AMA1 interacts directly with rhoptry neck protein 2 (RON2), which is secreted from the parasite rhoptries and specifically localizes at the moving junction. The RON2–AMA1 interaction is a critical step in the moving-junction-dependent invasion of host cells by apicomplexan parasites [43, 44]. Although the interaction between AMA1 and RON2 has not been

reported in *Eimeria* spp., *E. tenella* is an apicomplexan and AMA1 is conserved in this phylum. Therefore, we inferred that *Et*AMA1 may also interact with *Et*RON2 and specifically localize to the moving junction during the invasion of host cells by *E. tenella*. In this study, we have shown that *Et*Esp is an *Et*AMA1-interacting protein, but whether it localizes to the moving junction during invasion requires further study.

Conclusions

In this study, we have shown that interacts with *Et*AMA1 using a BIFC assay *in vivo* and a GST pull-down assay *in vitro*. Using staurosporine, we showed that *Et*Esp is a secreted protein of sporozoites but not from micronemes. An invasion inhibition assay revealed that an antibody against *rEt*Esp also blocked the parasite invasion of its host cells by more than 62%. These data have implications for the use of *Et*AMA1 or *Et*AMA1-interacting proteins as targets in therapeutic intervention strategies against avian coccidiosis.

Supplementary Information

Additional file 1: Table S. Primers sequence used in this study.

Abbreviations

*Et*AMA1: *Eimeria tenella* apical membrane antigen 1; *Et*Esp: *E. tenella* *Eimeria*-specific protein; BiFC: bimolecular fluorescence complementation; GST pull-down: glutathione S-transferase pull-down; RACE: random amplification of PCR ends; UO: unsporulated oocysts; SO: sporulated oocysts; Spz: sporozoites; sMrz: second-generation merozoites; NCBI: the National Center for Biotechnology Information; ORF: open reading frame; CM: complete medium.

Declarations

Acknowledgments

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Declarations

Ethics approval and consent to participate

The protocol was approved and authorized by the Animal Care and Use Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HYH and HD conceived and designed the study. CL, QPZ, SHZ and QJW performed the experiments. CL, BH and HZZ analyzed the data. CL, QPZ, HXW, SLY, YY and SSL collected parasites. CL, QPZ and HYH wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Primers sequence used in this study

Primer name	Primer sequence
GS5P (5' Primer)	5'-ATGGTCTCGGGCCAGTTCTCGTTCA -3'
GS5N (5' Nested Primer)	5'-GGAGATGAGACCCAGGCGGATGAAA -3'
<i>EtEsp</i> -UP	5'-GCGGATCCATGAAGGGCCTGTTCTTCACCGTCG-3'
<i>EtEsp</i> -LP	5'-GCCTCGAGCGAATCTACTTCAAGAAAAGCCACG-3'
<i>EtEsp</i> -SP	5'-CCCCGACTACCTCAAGTTCCTCAGC -3'
<i>EtEsp</i> -AP	5'-TGGGTCCGTCTCCCCCTCCTTGGTG -3'
18S-SP	5'-TGTAGTGGAGTCTTGGTGATTC-3'
18S-AP	5'-CCTGCTGCCTTCCTTAGATG-3'
<i>BfEtEsp</i> -UP	5'-GCGAATTCGGGGCCACCATGAAGGGCCTGTTCTT-3'
<i>BfEtEsp</i> -LP	5'-GCAGATCTGCTGCTCGCGTTGCCAGCAGAT -3'
<i>BfEtEsp</i> -LP	5'-GCGAATTCGGGGCCACCATGCAGCCGCCCTAT-3'
<i>BfEtAMA1</i> -UP	5'-GCCTCGAGGGTATTCTGGTCCAG-3'
<i>BfEtAMA1</i> -LP	

Figures

GAGCATTGCGTCTTTCTCTGCAGTCATCTGTTTGAATACCTGGTTTCTTTTCCAATTAACAAATCGAAA

ATGAAGGGCCTGTTCTTCAACCGTCGCTTTGGGCGTGCTGTCGCCGTC AATGCCTCAGATGATGGCAGCAACAACAAGATGAAGCCTGAG

M K G L F F T V A L G A A V A V N A S D D G S N N K M K P E

GATTTGAGCGCAATCTTCAACGGTGAGGGCCAGGTTGACTGGTCTGCTTTCTCACCTGAGGCTCTGGGCATGCACCTCAACTACGACGAG

D L S A I F N G E G Q V D W S A F S P E A L G M H L N Y D E

ATGAAGCCAGCAGGAGCTCGACGCCGTCGTCGACGCTTTCATCCGCCTGGGTCTCATCTCCCCCGAGGAGCGCGACTCCTTCGCCGCCTCC

M S Q Q E L D A V V D A F I R L G L I S P E E R D S F A A S

CTGGTGGACCCCATGATGCGCAGCATGCTCCAGACCGTGAACGAGAACTGGCCCGAGACCATGCAGAAGATCATCAAGGACCCCGACTAC

L V D P M M R S M L Q T V N E N W P E T M Q K I I K D P D Y

CTCAAGTTCTCAGCCAGTTCCAGAACAGCATTGATGAGGGCGCCCTGGCTACCATCGTTGGTCTGAAGGAGGGACTGAGCGGCGCCATC

L K F L S Q F Q N S I D E G A L A T I V G L K E G L S G A I

CGCCAGAAGGAGCAGCAGGAGGAGGGATCTGCTGGCAACGGCGAGCAGTAG ATCTGAAAATTCCATGGCACTGTATTGCATTTATGTT

R Q K E Q Q E E G S A G N G E Q *

CATTTTGTTAACGTCGTCACACAGACACTTTTGTCCGGCGTGATGCGCGATTTTGTTCATGCCTAGATCTGTTTTGGGGGGTGCGATG

GCGATATGGTGAAGTTCCTCCGGTCGAGTTTCAAGTGGTGGTGCATGACACTGTTACGGGGGACGCAGATGTCGTCAAACGTGTCA

TTGTTCTTCTGTCCTCCCAACAGCCACCCGCGTGGGTTCTCGTGGGCTGGGGGGGCCCAACTGCTGCCCTCCAGACGAGTTGTGCGCGA

CGGTGGTGGCGGGCTTTTGGCTTCGCCGCTGTTTGTAGCGCCAGCTGCAGTGGCACCCGCGAGGCGACGAA TGTTCCGATACAAACAACGTG

GCTTTTCTTGAAGTAGATTCGCCGTCAGCGCTTTGCAGTCAATGCTTGGCACAGGTTTTATCTGCGCATAACTTTTTACTGGACAACCATC

GATAAGTCATGTGCTAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 1

Nucleotide sequence of the full-length cDNA of EtEsp and the deduced amino acid sequence. Start and stop codons are underlined. One putative intein DOD-type homing endonuclease domain is shown with wavy underlining. A putative N-glycosylation site has a double line. Five putative casein kinase II phosphorylation sites are shown in yellow. Six putative N-myristoylation sites are shown in gray.

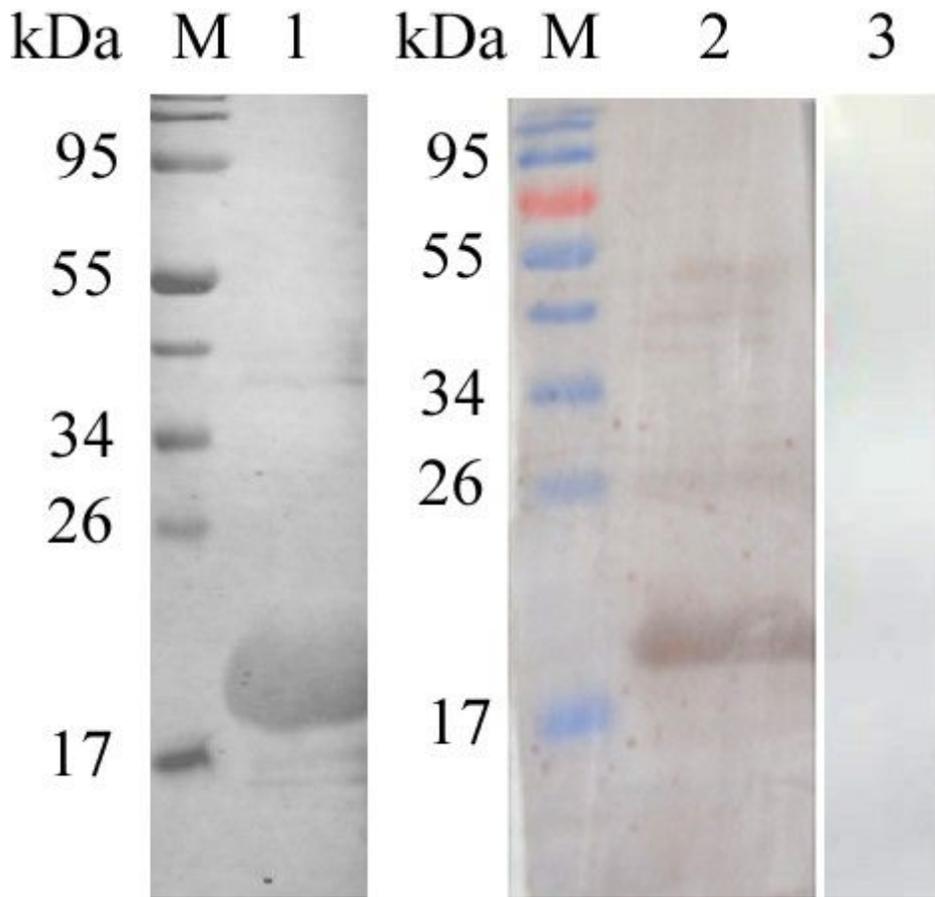


Figure 2

Immunogenicity of rEtEsp. rEtEsp protein was subjected to western blotting. Lane M: protein marker; lane 1: anti-His-tag monoclonal antibody as the primary antibody; lane 2: rabbit serum against sporozoites as the primary antibody; lane 3: naïve rabbit IgG as the primary antibody.

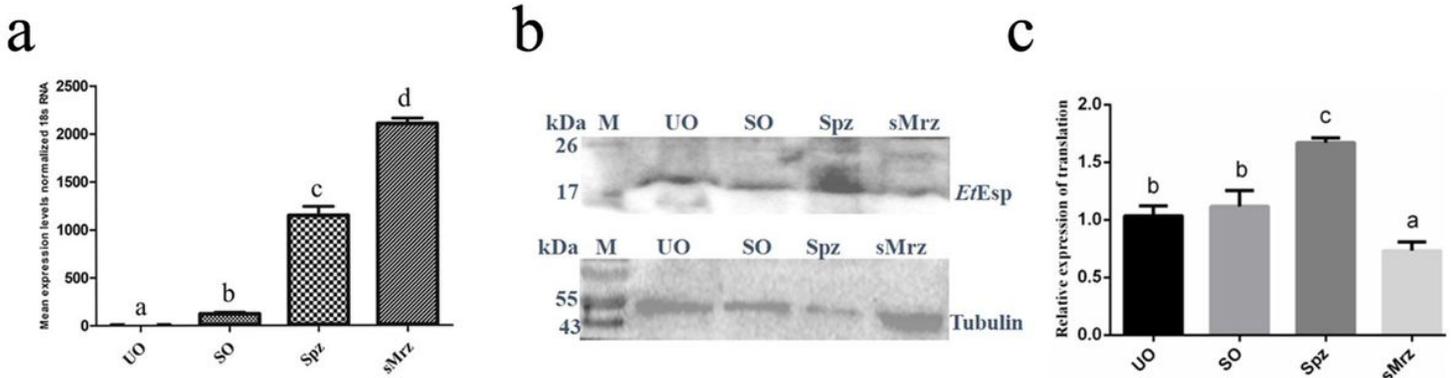


Figure 3

EtEsp expression at different developmental stages of *E. tenella*. UO, unsporulated oocysts; SO, sporulated oocysts; Spz, sporozoites; sMrz, second-generation merozoites. a. qPCR of EtEsp at different developmental stages of *E. tenella*. b. Western blot showing EtEsp at different developmental stages,

probed with rabbit anti-rEtEsp serum or mouse monoclonal anti- α -tubulin antibody. c. The densitometric intensity of western blot images was analyzed using ImageJ software.

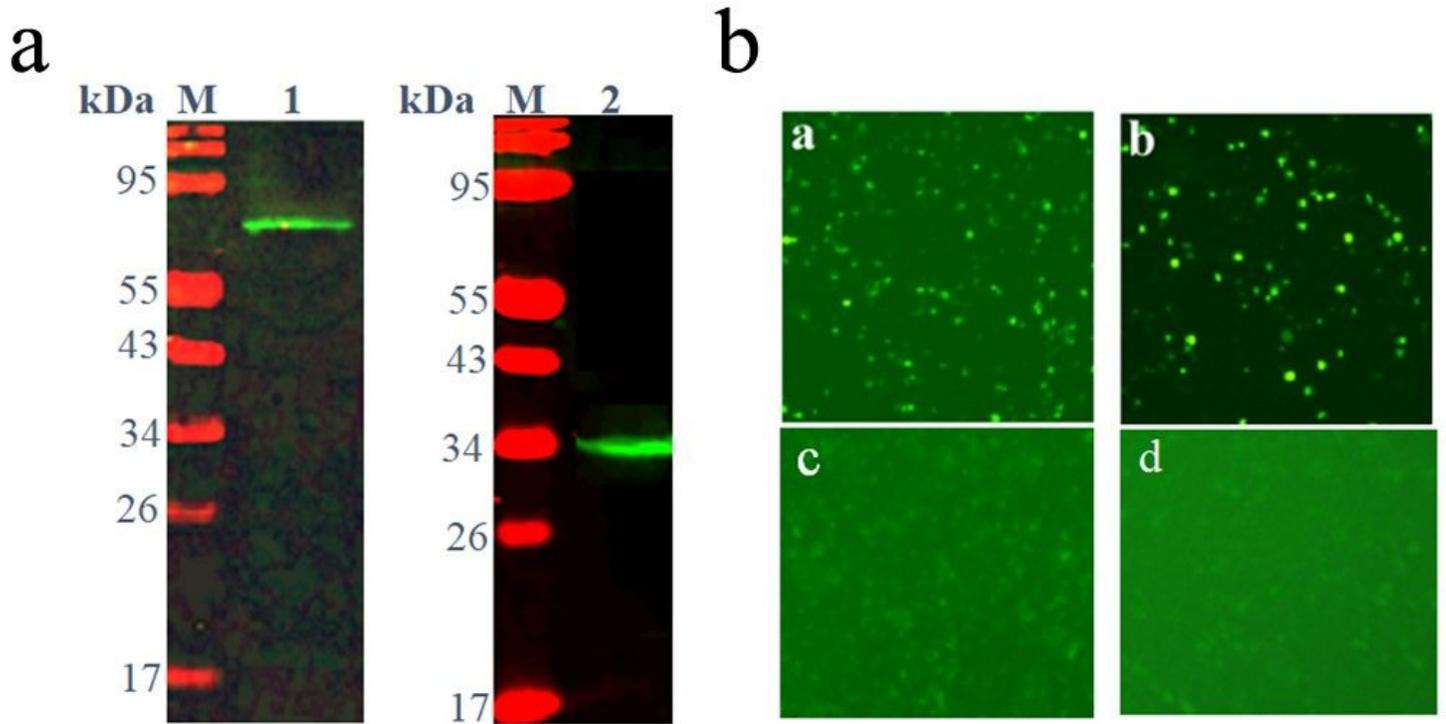


Figure 4

Interaction between EtAMA1 and EtEsp in DF-1 cells assessed with BiFC. a. DF-1 cells were transfected with VC155–EtAMA1 and VN155–EtEsp and the cellular lysates were analyzed with immunoblotting using antisera against EtAMA1 and EtEsp, 1. anti-r EtAMA1 antibody; 2. anti-r EtEsp antibody. b. BiFC was performed. a, DF-1 cells were cotransfected with VC155–EtAMA1 and VN155–EtEsp. b, DF-1 cells were cotransfected with positive controls bFos and bJun. c, DF-1 cells were cotransfected with pBiFC-VC155–EtAMA1 and pBiFC-VN155 empty vector. . d, DF-1 cells were cotransfected with pBiFC-VN155–EtEsp and pBiFC-VC155 empty vector.

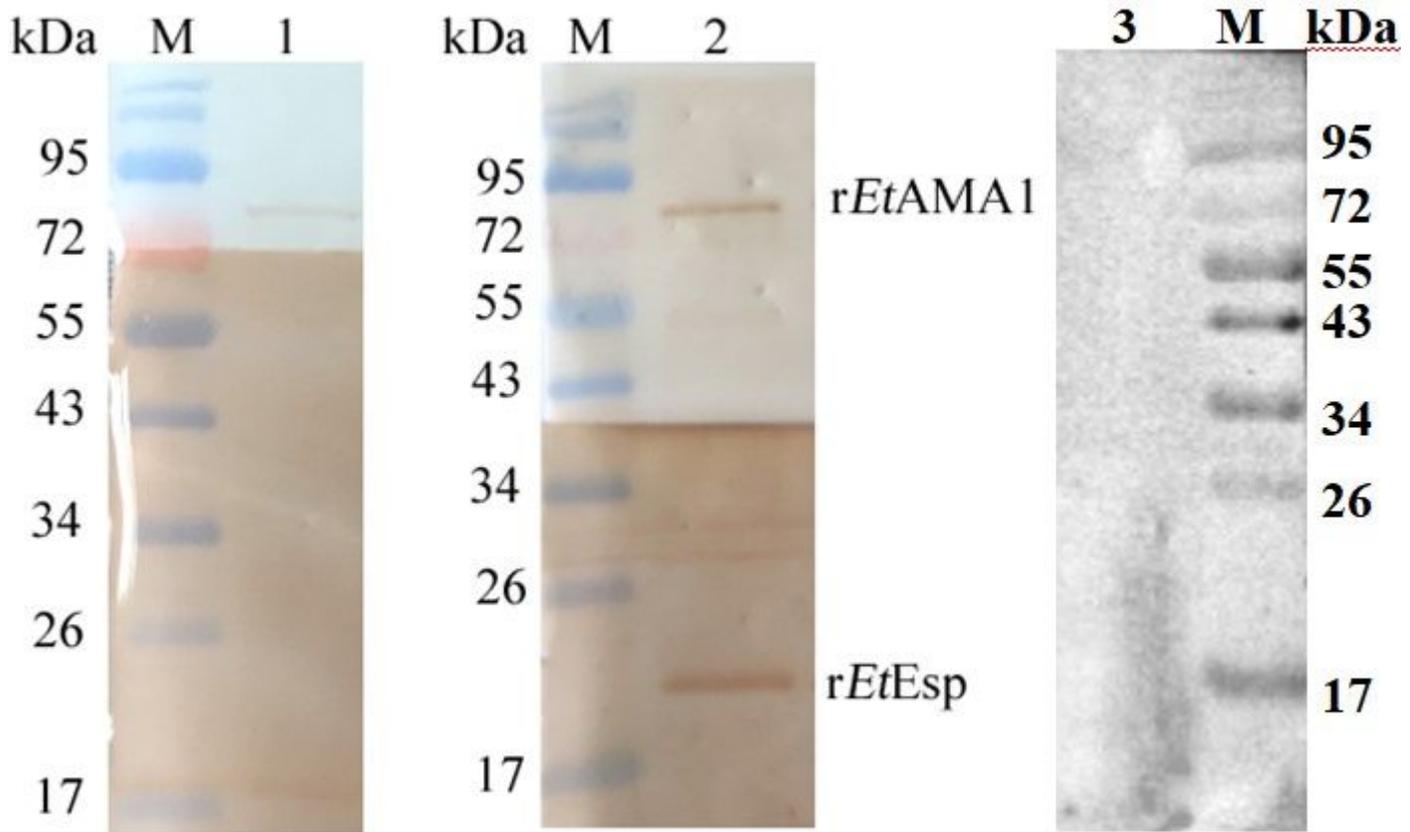


Figure 5

In vitro pull-down assay between EtAMA1 and EtEsp. Lane M: protein marker; lane 1: rEtMIC2 protein incubated with rEtEsp—detected with anti-rEtAMA1 and anti-rEtMIC2 as negative control; lane 2: rEtAMA1 protein incubated with rEtEsp—detected with anti-rEtAMA1 and anti-rEtEsp; lane 3: rEtEsp loaded in an empty glutathione-agarose column, detected with anti-rEtEsp as negative control.

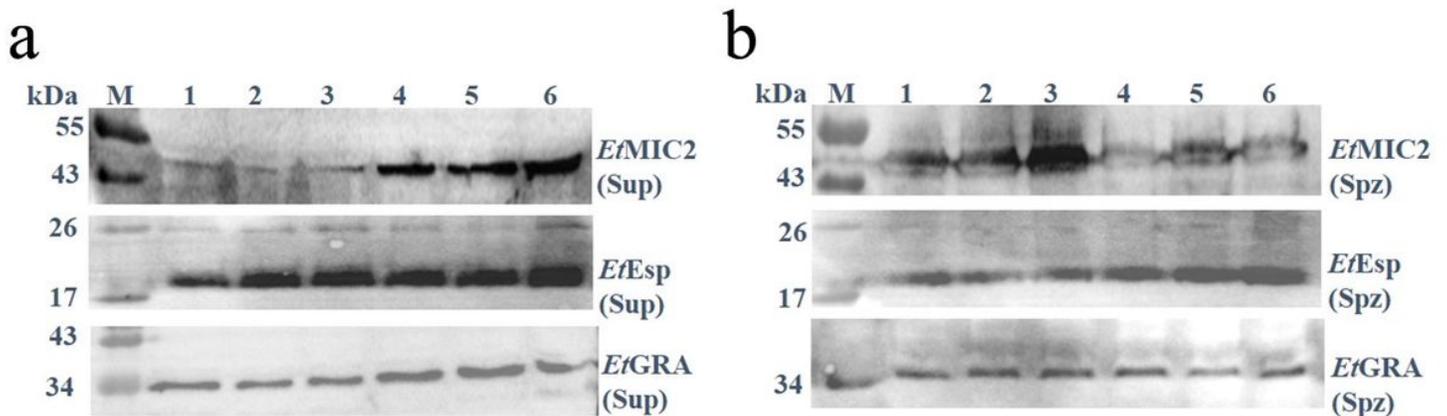


Figure 6

Western blotting analysis of secretion assays (supernatants and sporozoites pellet). (a): the supernatants(Sup); (b): sporozoites pellet(Spz). Lane M: protein marker; lanes 1–3: 5, 10, and 20 μ M staurosporine dissolved in DMSO.; lanes 4–6: volumes of DMSO solvent corresponding to 5, 10, or 20 μ M staurosporine

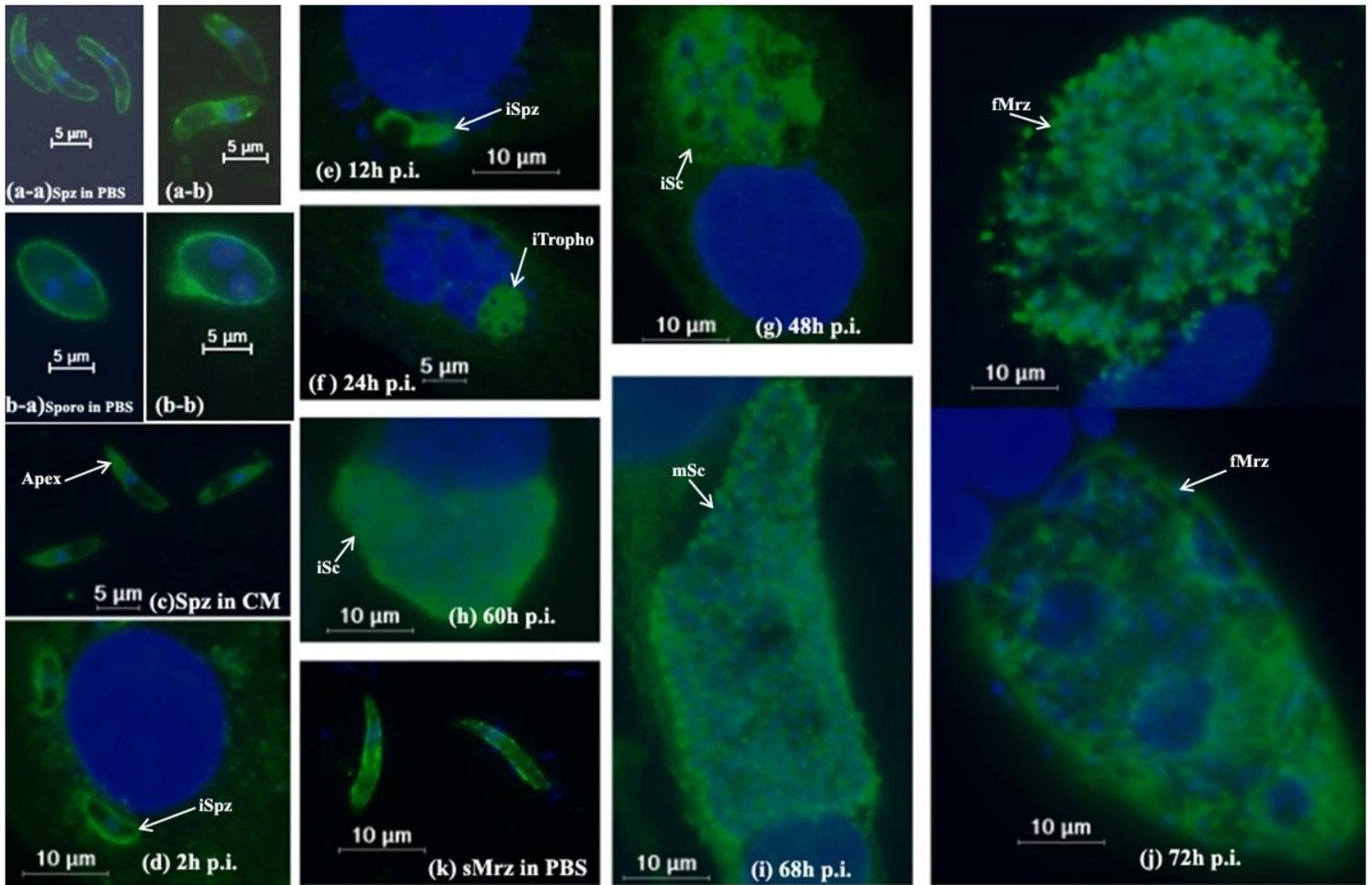


Figure 7

Immunofluorescent localization of EtEsp at different developmental stages of *E. tenella*. Parasites were immunostained with anti-rEtEsp antibody. a, Sporozoites (Spz) were incubated in PBS, a-a permeabilized sporozoites, a-b: non permeabilized sporozoites; b, sporocysts (Sporo) were incubated in PBS, b-a: permeabilized sporocysts, b-b: non permeabilized sporocysts; c, sporozoites (Spz) were incubated in complete medium (CM) for 2 h at 41 °C; d, e, intracellular sporozoites (iSpz) at 2 h and 12 h postinfection, respectively; f, trophozoites (iTropho) at 24 h postinfection; g, h, immature schizonts (iSc) at 48 and 60 h postinfection, respectively; i, mature schizonts (mSc) at 68 h postinfection; j, first-generation merozoites (fMrz) at 72 h postinfection; k, second-generation merozoites (sMrz) in PBS.

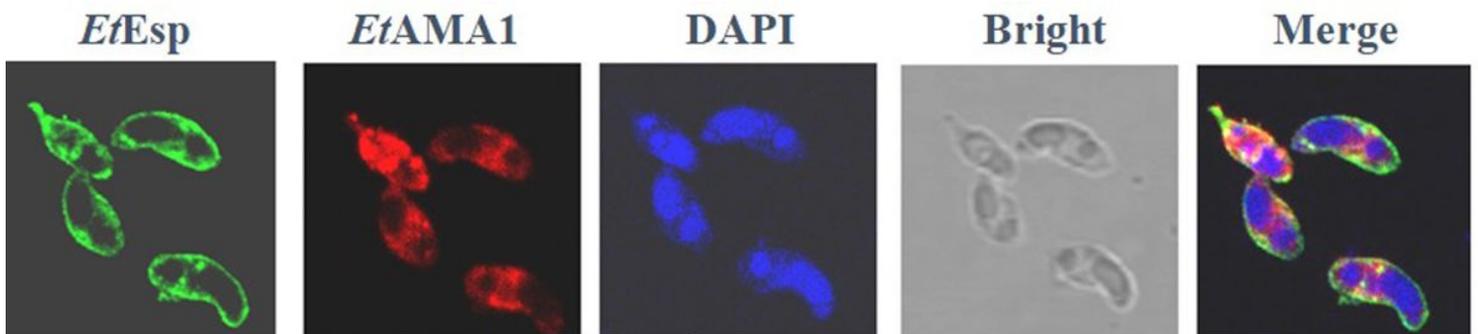


Figure 8

Co-localization of EtEsp and EtAMA1 in sporozoites using mouse anti-rEtAMA1 antibody and rabbit anti-rEtEsp antibody by IFA.

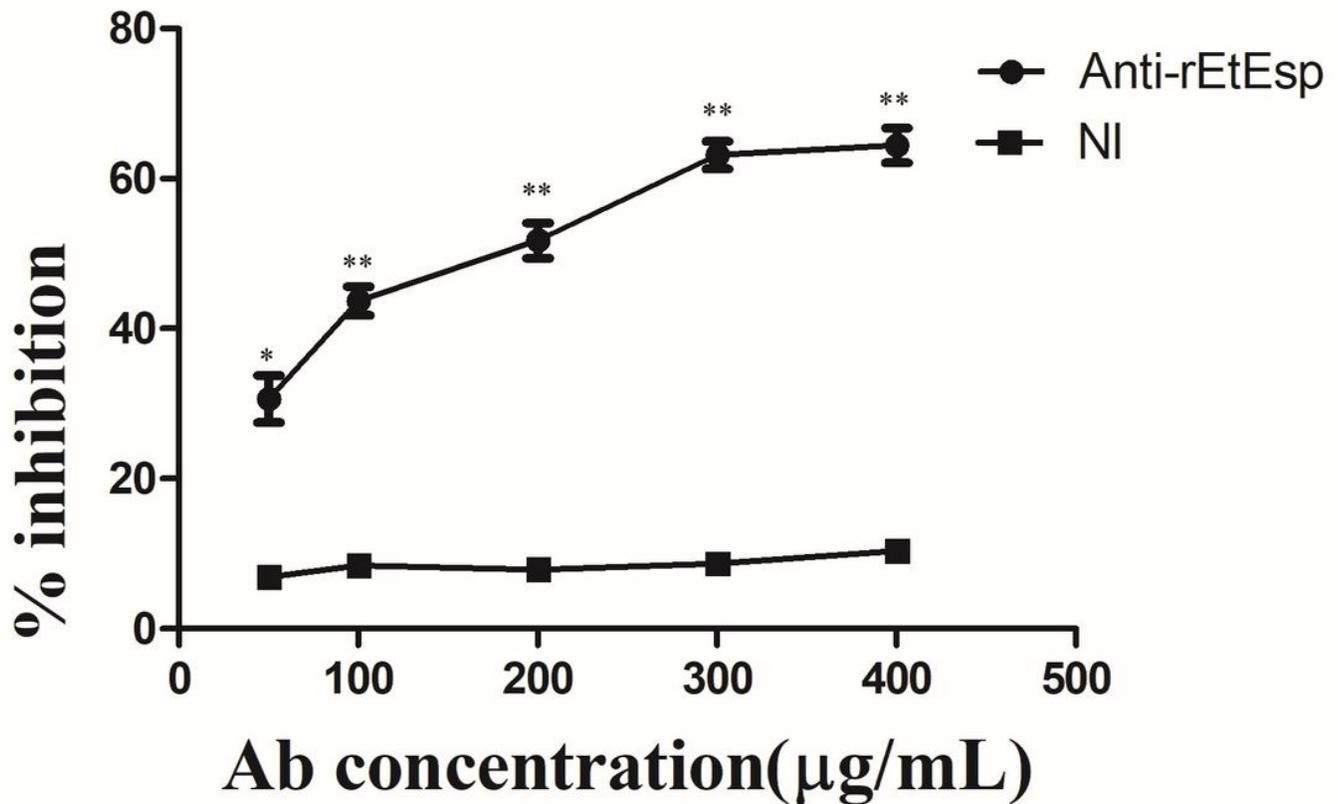


Figure 9

Inhibition of sporozoite invasion in vitro. All the assays were performed in triplicate (anti-rEtEsp, rabbit antiserum generated against recombinant EtESP protein; NI, IgG from naïve rabbit serum). **P < 0.01 for differences between treatment with antibody against rEtEsp or with naïve rabbit serum with the same IgG concentration.

Supplementary Files

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