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PROTOZOOLOGY - ORIGINAL PAPER

Molecular characterization of surface antigen 10 of *Eimeria tenella*

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Abstract

Chicken coccidiosis is caused by the apicomplexan parasite *Eimeria* spp. At present, drug resistance of *Eimeria* is common because of the indiscriminate use of anticoccidial drugs. The gene encoding surface antigen 10 of *Eimeria tenella* (*Et*SAG10) is differentially expressed between drug-resistant and drug-sensitive strains. RNA-seq analysis indicated that this gene was downregulated in strains resistant to maduramicin and diclazuril compared to susceptible strains. *Et*SAG10 DNA sequence alignment revealed that they contained one and ten mutations in MRR and DZR, compared with DS, respectively. A full-length *Et*SAG10 cDNA was successfully cloned and expressed, and the polyclonal antibody was prepared. The transcription and translation levels of *Et*SAG10 were analyzed by quantitative real-time PCR (qPCR) and Western blotting. The localization of *Et*SAG10 in Spz, Mrz, and parasites in the first asexual stage was determined by indirect immunofluorescence. The potential association of *Et*SAG10 with sporozoite invasion of host cells was assessed by invasion inhibition assays. The results showed that *Et*SAG10 had a predicted transmembrane domain at the C-terminal end and a predicted signal peptide at the N-terminal end. *Et*SAG10 was downregulated in drug-resistant strains, which is consistent with the RNA-seq results. The *Et*SAG10 protein was localized to the parasite surface and parasitophorous vacuole membrane. This protein was shown to play a role in the infection of chicken intestine by sporozoites.

Keywords *Eimeria tenella* · Differential expression · *Et*SAG10

Guiling Liu and Shunhai Zhu contributed equally to this work.

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Introduction

Chicken coccidiosis is a parasitic disease caused by the apicomplexan protozoan *Eimeria* spp. and causes considerable economic losses to the poultry industry worldwide. *Eimeria tenella* parasitizes chicken cecal epithelial cells and is one of the most pathogenic species among the seven identified species (Williams 2002). The current control strategies for coccidiosis rely on anticoccidial drugs and live oocyst vaccines (Sun et al. 2014a; Shirley et al. 2005).

However, the extensive use of anticoccidial drugs has resulted in the development of resistance in *Eimeria* spp. And the use of these drugs has been restricted by the emergence of resistance (Chapman 1997). *Eimeria* spp. have been shown to be resistant to all available anticoccidial agents. A Dutch study evaluated various *E. tenella* strains and found that their sensitivity to drugs was reduced over time (Peek and Landman 2003). Several studies investigated drug resistance in *Eimeria* (Jiang et al. 2005; Chen et al. 2008; Thabet et al. 2017). Nonetheless, the underlying molecular mechanisms of resistance are not fully understood. Therefore, exploring the function of resistance-related genes is essential.

Our research group previously used the RNA-seq method to compare differences between maduramicin- and diclazuril-resistant strains and drug-sensitive strains of *E. tenella* and screened for differentially expressed genes and found that the expression of surface antigen 10 of *E. tenella* (*Et*SAG10) was downregulated in two resistant strains (data unpublished). Transcriptome sequencing showed that the log2Ratio (DZR/DS) was reduced by − 1.5 and the log2Ratio (MRR/DS) was reduced by − 2.7.

Therefore, the role of *Et*SAG10 in drug-resistant strains deserves further exploration. More than 80 SAGs are expressed in *E. tenella*. *Et*SAG10 is one of the surface antigen (SAG) subfamily members; all known subfamilies encode signal peptides and addition sites for GPI anchors (Reid et al. 2014). Some SAGs induce pro-inflammatory cytokine responses in *E. tenella* in vitro (Chow et al. 2011). Moreover, SAG genes in *E. tenella* encode single-domain, membrane-bound proteins tethered by GPI anchors to the surface of invasive sporozoites and merozoites (Tabares et al. 2004). *E. tenella* SAG1 binds to mammalian cells (Jahn et al. 2009). Several SAGA subfamily proteins, but not SAGB subfamily proteins, can bind to cultured cells (Reid et al. 2014). The surface antigens of other apicomplexan protozoans such as *Toxoplasma gondii* (*T. gondii*) can be potentially used as diagnostic antigens and vaccines (Leng et al. 2014). TgSAG1 elicited protective responses to *T. gondii* infection (Aosai et al. 1999), and TgSAGs can stimulate host innate and adaptive immune responses through Toll-like receptors (Gazzinelli and Denkers 2006). In addition, SAGs are believed to play an important role in the early recognition of protozoa, adherence to and invasion of host cells, immune regulation, and immune escape)Cardwell and Martinez 2009; Gould et al. 2017; Leal-Sena et al. 2018). The number of studies on SAGs has increased in recent years.

*Et*SAG10 had not been characterized in *E. tenella* to date. In this study, *Et*SAG10 DNA sequences of three strains were aligned by using the Clustal X tool, and the full-length *Et*SAG10 cDNA was cloned and expressed, and its biological functions were determined. The differential expression of SAG10 in drug-resistant and drug-sensitive strains of *E. tenella* was evaluated. This study will lay the foundation for identifying molecular markers of drug resistance.

Materials and methods

Animals, parasites, and cells

Day-old yellow-feathered broiler chickens were purchased from the Shanghai Fengxian District, China, and were reared in a coccidia-free animal facility. New Zealand rabbits were obtained from the Songlian Experimental Animal Farm. BALB/c mice were purchased from the Slack Laboratory Animal Limited Company.

The Shanghai drug-sensitive (DS) strain of *E. tenella* was collected and isolated in a chicken farm in Shanghai, China, in the 1980s, and was kept in our laboratory (Huang et al. 1993). A maduramicin-resistant strain (MRR) and diclazuril-resistant strain (DZR) were induced and maintained in our laboratory (Han et al. 2004). Parasites were propagated by passage through coccidia-free 2-week-old chickens, as described previously, and stored in 2.5% potassium dichromate solution (Tomley 1997). Sporozoites (Spz) were obtained from sporulated oocysts, and unsporulated oocysts (UO) and sporulated oocysts (SO) were obtained as previously described (Shirley 1995; Han et al. 2010). Second-generation merozoites (Mrz) were collected and purified using Percoll density gradient centrifugation (Zhou et al. 2010).

The chicken embryo fibroblast cell line DF-1 was kept in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, USA), 100 U/mL penicillin/streptomycin (Invitrogen), and 2.0 mM L-glutamine (Invitrogen).

Analysis of the DNA sequence of *Et*SAG10 in three strains

Genomic DNA was extracted from sporulated oocysts of two drug-resistant strains and the drug-sensitive strain of *E. tenella* according to the instructions of TIANamp Genomic DNA Kit (Tiangen, China), respectively. The DNA sequence of *Et*SAG10 (ID: ETH_00034975) was amplified using the following primers: 5′-ATGCTGCAGCGGAAGCTACC

ACCCA-3′, 5′-TCATAAAGTCATAATGCCGAACGTC-3′. The 1168-bp amplified DNA sequences were cloned into the pGEM-T easy cloning vector (Promega, Madison, WI, USA) and sent to Sangon Biotech (China) for sequencing. Further, the DNA sequences of the three strains were aligned by using Clustal X tool. The DNA sequences were sequenced and aligned in triplicate.

RNA extraction, cDNA synthesis, and gene cloning

Total RNA was extracted from sporulated oocysts of a drug-sensitive strain of *E. tenella* using Trizol (Invitrogen). The concentration and purity of RNA were determined by measuring the optical density at 260 and 280 nm using an ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). The RNA was treated with DNase I (Invitrogen) and reverse transcribed into cDNA using a SuperScript™ III reverse transcriptase kit (Invitrogen) and Oligo (dT) primers.

The full-length coding region of the *Et*SAG10 gene (ID: ETH_00034975) was amplified from the cDNA of *E. tenella* using the following primers with the BamHI and SalI restriction sites (underlined): 5′-GCGGATCC ATGCTGCAGCGGAA GCTACCACCCA-3′, 5′-GCGTCGACTCATAAAGTCATAA TGCCGAACGTC-3′. The 786-bp amplified DNA fragment was cloned into the pGEM-T easy cloning vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* Top10. The bacteria transformed with recombinant plasmid pGEM-T-*Et*SAG10 was sent to Sangon Biotech (China) for sequencing. The gene sequences and deduced amino acid sequences of *Et*SAG10 were analyzed using BLAST programs from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), the genome sequence of *E. tenella* (http://www.genedb.org/Homepage/Etenella), translation tools from the ExPASy server (http://www.expasy.org/tools/protparam.html), a prediction tool for membrane protein topology and signal peptides (http://octopus.cbr.su.se/index.php), and Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan) (Zhai et al. 2016).

Protein expression and purification

The ORF was excised from recombinant pGEM-T-*Et*SAG10 plasmids by digestion with *Bam* HI and *Sal* I after sequencing and ligated into the prokaryotic expression vector pGEX-4T-2 (Novagen, Germany) digested with the same restriction endonucleases. Recombinant expressed plasmids were extracted from the clones and subjected to DNA sequencing to confirm the sequence and orientation of the inserts. The pGEX-4T-*Et*SAG10 plasmids were expressed using an *E. coli* BL21 (DE3) expression system (Tiangen). Bacteria carrying pGEX-4T-*Et*SAG10 were grown in 200 mL Luria Broth medium containing 50 mg/mL at 37 °C. When the culture growth reached an optical density of 0.6 at 600 nm, protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma-Aldrich, St. Louis, USA) at 37 °C for 8 h. The recombinant bacteria were isolated by centrifugation at 10,000*g* for 15 min, and the pellet was then suspended in 20 mL phosphate-buffered saline (PBS, pH 7.4) and extracted using an ultrasonic processor to release the fusion proteins. Lysates were centrifuged at 10,000*g* for 10 min to separate inclusion bodies and cellular debris from the remaining soluble substances. The pellet was resuspended in 5 mL 1× binding buffer plus 6 M urea. Analysis by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that recombinant *Et*SAG10 (r*Et*SAG10) was expressed in soluble form. The soluble r*Et*SAG10 was purified by using GST-bind resin (Merck, Darmstadt, Germany) according to the manufacturer's instructions. The purified protein was separated on 12% SDS-PAGE and stained with Coomassie Blue. Protein concentration was determined using a BCA protein assay kit (Beyotime, Haimen, China).

Preparation of polyclonal antibodies against *Et*SAG10

Two-month-old rabbits and 6-week-old mice were immunized with 0.2 mg and 0.05 mg of purified r*Et*SAG10 per animal, respectively. r*Et*SAG10 was emulsified with the same volume of Freund's complete adjuvant (Sigma-Aldrich) used at the immunization. After that, experimental animals were injected with r*Et*SAG10 emulsified with the same volume of Freund's incomplete adjuvant (Sigma-Aldrich). After three immunizations, the sera of rabbits and mice were collected. Reactogenicity of r*Et*SAG10 was determined by Western blotting.

Quantitative real-time PCR (qPCR)

The expression profile of *Et*SAG10 mRNA was examined at different developmental stages (UO, SO, Spz, and Mrz) in the DS strain of *E. tenella* and in SO of the strains DS, DZR, and MRR using qPCR. Total RNAs were isolated using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. All samples were treated with deoxyribonuclease I (Invitrogen). Total RNAs (35 μg/reaction) were reverse transcribed into cDNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen) and random primers, respectively. The housekeeping gene 18S rRNA was used as an internal control (Livak and Schmittgen 2001; Kumar et al. 2012; Wang et al. 2016). The qPCR primers for *Et*SAG10 were 5′-TCCAGCACCAGAGGAAGGAGAACTA-3′ (sense) and 5′-TGAAGGCGGGAGTCCAGTGAAGTTT-3′ (antisense). The qPCR primers for 18S rRNA were 5′-TGTA GTGGAGTCTTGGTGATTC-3′ (sense) and 5′-CCTG CTGCCTTCCTTAGATG-3′ (antisense). qPCR was performed using the SYBR® Premix DimerEraser™ (Perfect Real Time) (TaKaRa, Japan) kit in a QuantStudio®5 Real-

Time PCR system. The $2^{-\Delta\Delta Ct}$ method (Geysen et al. 1991; Livak and Schmittgen 2001) was applied for measuring relative changes in gene expression.

Western blotting

Parasites at four developmental stages (UO, SO, Spz, and Mrz) of *E. tenella* DS strain and parasites at the SO stage in strains DS, DZR, and MRR were lysed in RIPA buffer (Beyotime) and Protease Inhibitor Cocktail (Sigma) and centrifuged at 4000*g* for 10 min. The supernatants or purified r*Et*SAG10 were incubated with SDS sample buffer and denatured by heating. The protein samples were separated on a 12% SDS-PAGE gel electrophoresis and blotted onto Immobilon-P transfer membranes (Millipore). The membranes were blocked with phosphate-buffered saline (PBS) containing 5% skimmed milk powder for 2 h at 37 °C, washed three times with PBS for 5 min, and incubated with anti-α-tubulin monoclonal antibody (1:400 dilution), GST-monoclonal antibody, polyclonal anti-rEtSAG10 (1:200 dilution) and anti-sporozoite rabbit serum (1:200 dilution) which observed in our lab (Han et al. 2015) for 2 h at 37 °C. After that, the membranes were washed three times with PBST for 5 min and incubated with IRDye® 680RD donkey anti-mouse IgG and IRDye® 800CW goat anti-rabbit antibody IgG (LI-COR Biosciences, USA, 1:10,000) diluted in PBS for 1 h at 37 °C in the dark and visualized using an Odyssey® Infrared Imaging System (LI-COR Biosciences). Alpha-tubulin (Sigma) was used as a reference protein for normalization.

Localization of *Et*SAG10 by immunofluorescence microscopy

The immunolocalization of *Et*SAG10 at different developmental stages of *E. tenella* was performed as described previously (Peroval et al. 2006; Jiang et al. 2012). To confirm the location of *Et*SAG10 in the parasite, purified sporozoites and second-generation merozoites were transferred to glass slides and air-dried. The chicken embryo fibroblast cell line DF-1 was used for in vitro infection experiments. The cells were cultured in six-well plates (Corning, NY, USA) with Φ25mm Circle Microsope Cover Glass (NEST) and cultured in complete medium [DMEM (Invitrogen) containing 10% FBS, 100 U/mL penicillin/streptomycin] at 37 °C and 5% CO_2 for 10 h. Freshly purified sporozoites were incubated in complete medium for 2 h at 41 °C and were added to the cultured cells. At the indicated post-inoculation time points, infected DF-1 cells were fixed, washed, and transferred to glass slides. The slides were fixed in 2% paraformaldehyde in PBS, air-dried, and permeabilized in 1% Triton X-100 in PBS for 15 min. The slides were blocked with 2% (*w*/*v*) bovine serum albumin in PBS overnight at 4 °C and incubated with polyclonal anti-r*Et*SAG10 diluted 1:100 in PBS for 1 h at 37 °C. The slides were incubated with a 1:500 dilution of goat anti-rabbit IgG fluorescein isothiocyanate-conjugated antibody (Sigma-Aldrich) for 1 h at 37 °C. Nuclei were stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (Beyotime) (10 mg/mL) for 30 min at room temperature. After each step, the slides were washed three times for 10 min with PBS containing 0.05% Tween 20. After that, the slides were mounted using 60 μL Fluoromount Aqueous Mounting Medium (Sigma-Aldrich) and observed under a laser scanning confocal microscope (ZEISS, Germany) and a fluorescence microscope (OLYMPUS, Japan).

Invasion inhibition assays

Invasion inhibition assays were carried out as described previously (Jiang et al. 2012; Wilson et al. 2015). DF-1 cells (2×10^5 cells per well) were cultivated in 24-well plates in complete medium for 12 h at 37 °C and 5% CO_2. Freshly purified sporozoites were labeled using the dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Beyotime Biotechnology), according to the manufacturer's instructions and resuspended in DMEM containing 5% FBS and 500 U/mL penicillin/streptomycin and preincubated with 50 μg/mL, 100 μg/mL, 200 μg/mL, or 400 μg/mL purified anti-r*Et*SAG10 IgG for 2 h at 37 °C. Sporozoites (6.0×10^5 per well) were added to infected DF-1 cells for 12 h at 41 °C and 5% CO_2. The same concentration of rabbit IgG (Sigma-Aldrich, USA) was used as the negative control, and sporozoites incubated without antibodies were used as the positive control. Cells were washed, trypsinized, collected, and detected using a Cytomics FC500 flow cytometer (Beckman Coulter, USA). All assays were performed in triplicate.

Statistical analysis

The relative expression ratio of a target gene was calculated from the PCR efficiency and C_t of the treated sample versus the untreated sample using the $\Delta\Delta C_t$ method. The 18S rRNA gene was used as a reference for normalization. Native rabbit IgG was used as a control in invasion inhibition assays. Statistical analyses were performed using SPSS software version 22.0 (SPSS). Differences between the groups were calculated by one-way analysis of variance, followed by Duncan's multiple range tests, and the differences were considered statistically significant at $P < 0.05$.

Ethics statements

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

Results

*Et*SAG10 DNA sequence analysis of three strains

The 1168-bp full-length DNA sequences of *Et*SAG10 were obtained from three strains (DS, DZR, and MRR) of *E. tenella*, respectively, which included three introns (111 bp to 293 bp, 445 bp to 522 bp, and 899 bp to 994 bp). Three DNA sequences of *Et*SAG10 of three strains were compared by Clustal X tool (Fig. S1). The results showed that ten point mutations were observed in the *Et*SAG10 DNA sequence of DZR at the 247th, 660th, 684th, 707th, 712th, 716th, 721st, 757th, 833th, and 946th position and one point mutation was recorded in the *Et*SAG10 DNA sequence of MRR at the 591th position, compared with DS (Fig. 1). One synonymous mutation in the *Et*SAG10 DNA sequence, CGA to CGC coding for arginine (Arg) amino acid residue at the 591st position, was found in MRR. Three synonymous mutations in the *Et*SAG10 DNA sequence was found in DZR, ACG to ACA coding for threonine (Thr) amino acid residue at the 684th position, CAA to CAG coding for glutamine (Gln) amino acid residue at the 660th position, and TTG to CTG coding for leucine (Leu) amino acid residue at the 757th position. Moreover, five missense mutations in the *Et*SAG10 DNA sequence were found in DZR, CTT to CAT coding for leucine (Leu) amino acid residue changed into histidine (His) amino acid residue at the 707th position, AGA to GGA coding for arginine (Arg) amino acid residue changed into glycine (Gly) amino acid residue at the 712th position, AGG to AAG coding for arginine (Arg) amino acid residue changed into lysine (Lys) amino acid residue at the 716th position, GTG to ATG coding for valine (Val) amino acid residue changed into methionine (Met) amino acid residue at the 721st position, and CCA to CTA coding for proline (Pro) amino acid residue changed into leucine (Leu) amino acid residue at the 833th position.

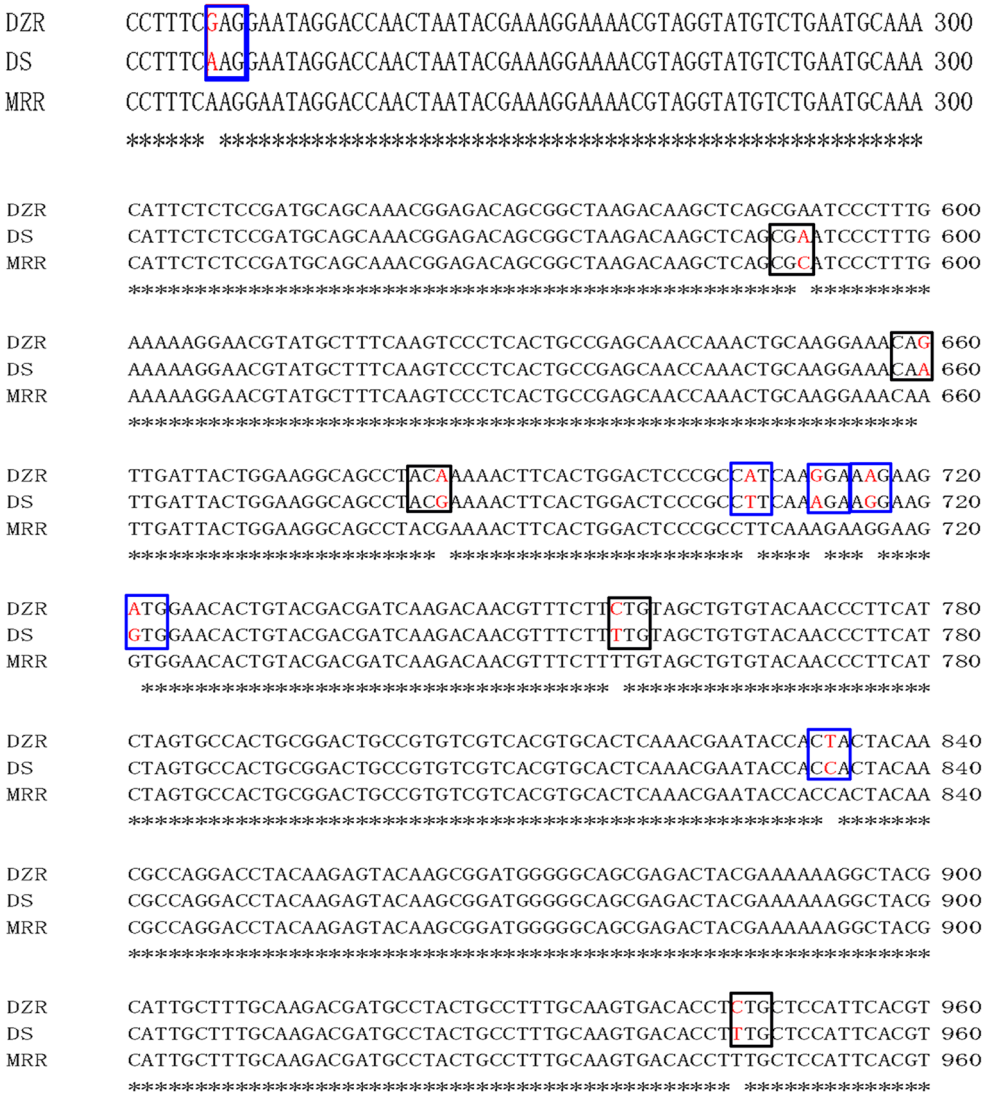

Fig. 1 Nucleotide sequence of the genomic DNA of *Et*SAG10 of DS, DZR, and MRR. The missense mutation is in a blue rectangle. The synonymous mutation is in a black rectangle

Sequence analysis of *Et*SAG10 cDNA

The full-length coding sequence of the *Et*SAG10 gene in the DS strain of *E. tenella* was amplified, cloned, and sequenced, and a 786-bp product was obtained. This sequence presented 99% similarity with *Et*SAG10 previously isolated from *E. tenella* (GenBank: XM_013375905.1). The newly identified gene encodes a putative protein of 261 amino acid residues with a predicted molecular weight of 27.9 kDa, a theoretical isoelectric point 4.7, a predicted transmembrane domain at the C-terminal end, and a predicted signal peptide at the N-terminal end. The analysis of the predicted motif structure indicated that *Et*SAG10 contained two casein kinase II phosphorylation sites (residues 143–146 and 149–152), three N-myristoylation sites (residues 61–66, 139–144, and 197–202), three protein kinase C phosphorylation sites (residues 80–82, 143–145, and 201–203), three N-glycosylation sites (residues 136–139, 156–159, and 182–185), and one threonine-rich domain (residues 177–202) (Fig. S2).

Expression and purification of r*Et*SAG10

E. tenella SAG10 cDNA was amplified and cloned into a pGEM-T easy cloning vector, subcloned into the expression vector pGEX-4T-2, and expressed in *E. coli* BL21 (DE3). The bacterial culture was incubated at 37 °C and induced with 0.5 mM IPTG. The SDS-PAGE results demonstrated that the r*Et*SAG10 was expressed as a soluble protein (Fig. 2a). The experimental size of r*Et*SAG10 agreed with the predicted size above 53.9 kDa (including 26 kDa the GST tag protein). The expressed protein was purified by immobilized nickel affinity chromatography under native conditions. The Western blot results indicated that the purified protein was recognized by the anti-sporozoite rabbit serum and the GST-monoclonal antibody. Native rabbit IgG failed to detect any protein of the expected size of r*Et*SAG10. These results indicated that r*Et*SAG10 has good reactogenicity and that protein r*Et*SAG10 had been degraded into peptides (Fig. 2b).

Transcription and translation of *Et*SAG10 in three *E. tenella* strains

The mRNA levels of *Et*SAG10 were measured in the DS, DZR, and MRR strains by qPCR. mRNA transcription in the DZR and MRR strains was lower than that in the DS strain (Fig. 3a). The presence of *Et*SAG10 in the DS, DZR, and MRR strains was determined by immunoblotting using rabbit antiserum against r*Et*SAG10. Western blot analysis showed that the protein in the DZR and MRR strains was downregulated compared with that of the DS strain (Fig. 3b, c), and these results agree with the RNA-seq results.

Transcription and translation levels of *Et*SAG10 at different developmental stages of the DS strain

The mRNA expression profile of *Et*SAG10 was examined in UO, SO, Spz, and Mrz in the DS strain by qPCR. The *Et*SAG10 mRNA level in SO was higher than that at the other three stages (Fig. 3d). Moreover, the presence of *Et*SAG10 at four developmental stages in the drug-sensitive strain was determined by immunoblotting using rabbit antiserum against r*Et*SAG10. Western blotting detected proteins in SO (two bands of approximately 27.9 kDa and greater than 34 kDa), Spz (two bands of approximately 27.9 kDa and greater than 34 kDa), Mrz (two bands of approximately 27.9 kDa and 34 kDa), and UO (one band of approximately 27.9 kDa). Protein expression was higher in SO and UO than in Spz and Mrz (Fig. 3e, f).

Immunofluorescence localization of *Et*SAG10 in *E. tenella*-infected DF-1 cells, merozoites, and sporozoites

The localization and distribution of *Et*SAG10 protein in Spz, Mrz, and parasites in the first asexual stage were evaluated by immunofluorescence. *Et*SAG10 was predominantly localized to the surface of Spz and Mrz, and green fluorescence

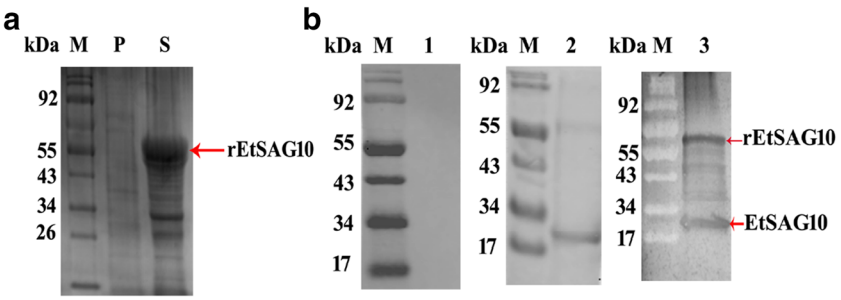

Fig. 2 **a** The solubility analysis r*Et*SAG10, **b** Western blot analysis of purified r*Et*SAG10. Rabbit antiserum against *E. tenella* sporozoite or monoclonal anti-GST antibody was used as the primary antibody. P, pellet; S, supernatant. Lane 1, r*Et*SAG10 was probed with the naive rabbit serum. Lane 2, r*Et*SAG10 was probed with the monoclonal anti-GST antibody. Lane 3, r*Et*SAG10 was probed with the anti-sporozoite rabbit serum

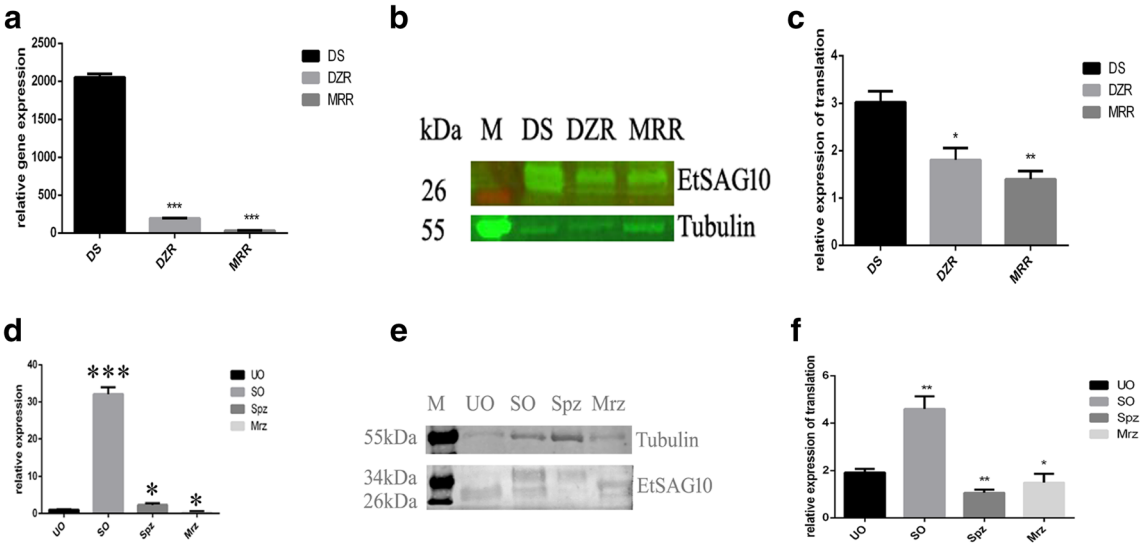

Fig. 3 Transcription and translation levels of *Et*SAG10. DS, drug-sensitive strain; DZR, diclazuril-resistant strain; MRR, maduramicin-resistant strain; UO, unsporulated oocysts; SO, sporulated oocysts; Spz, sporozoites; Mrz, second-generation merozoites. Anti-α-tubulin antibody was used as a loading control. *Et*SAG10 was recognized by rabbit anti-r*Et*SAG10. **a** Transcription level of *Et*SAG10 in DS, DZR, and MRR. **b** and **c** *Et*SAG10 translation level in DS, DZR, and MRR. **d** mRNA level of *Et*SAG10 at different developmental stages in the DS strain of *E. tenella*. **e** and **f** *Et*SAG10 protein expression level at different developmental stages of the DS strain of *E. tenella*. The data represent the mean ± S.D. of triplicate determinations and are representative of three independent experiments

intensity on the surface of Mrz was higher than that on the surface of Spz (Fig. 4a, b). After infection of DF-1 cells by sporozoites for 24 h, *Et*SAG10 was mainly localized to the surface of trophozoites, and green fluorescence intensity was also enhanced at this stage (Fig. 4c). As parasite development progressed in the cells, *Et*SAG10 was localized to the cytoplasm of immature schizonts, and the intensity of *Et*SAG10 staining was increased (Fig. 4d–f). *Et*SAG10 was also detected in the parasitophorous vacuole membrane (PVM) at 60 h (Fig. 4d).

Inhibition of *E. tenella* sporozoite invasion by r*Et*SAG10 polyclonal antibodies

The degree with which the rabbit anti-r*Et*SAG10 inhibited the invasion of DF-1 cells by *E. tenella* sporozoites was determined using invasion inhibition assays. Native rabbit IgG was used as a control. Under experimental conditions, the inhibition rate appeared to increase as the antibody concentration increased. The invasion rate was approximately 60% after pretreatment with 400 μg/mL of the anti-r*Et*SAG10 polyclonal antibody. In contrast, invasion was not significantly inhibited by native rabbit IgG (Fig. 5).

Discussion

At present, *Eimeria* spp. resistance to drugs is widespread; therefore, it is crucial to quickly diagnose resistance in parasites. In this study, we compared the DNA sequences of *Et*SAG10 of three strains (DZR, MRR, and DS). Sequence alignment found that they contained missense mutations and synonymous mutations in DZR and MRR, compared with DS. Previous reports showed that mutations in some genes determining resistance to drugs such as artemisinin and antifolates have been identified in *Plasmodium* (Gregson and Plowe 2005; Mbengue et al. 2015). And it was reported that the mutations were associated with mRNA and protein expression levels (Jia and Zhao 2017). So we speculated that the mutations in two drug-resistant strains may be associated with mRNA and protein expression levels and contribute to the resistance to these two drugs. Moreover, we compared the expression of the *Et*SAG10 gene between drug-resistant and sensitive strains using different methods. qPCR and Western blot analysis demonstrated that the expression of *Et*SAG10 in two resistant strains was downregulated compared with susceptible strains. These results agree with previous findings, wherein surface antigens were downregulated in *E. tenella* MRR (Chen et al. 2008). Similarly, the expression of surface antigens in drug-resistant strains of *Plasmodium falciparum* and *T. gondii* was lower than that in sensitive strains (Doliwa et al. 2013; Antony et al. 2016). The results of these studies and the present study evidenced that drug resistance in *Eimeria* spp. may be related to the downregulation of the expression of SAGs. Therefore, we speculated that the downregulation of *Et*SAG10 was essential for drug resistance in *E. tenella*. A previous study found that the downregulation of the surface antigen CD53 in human neutrophils was the result of the activation of a proteolytic mechanism (Mollinedo et al. 1998), which is consistent with our hypothesis.

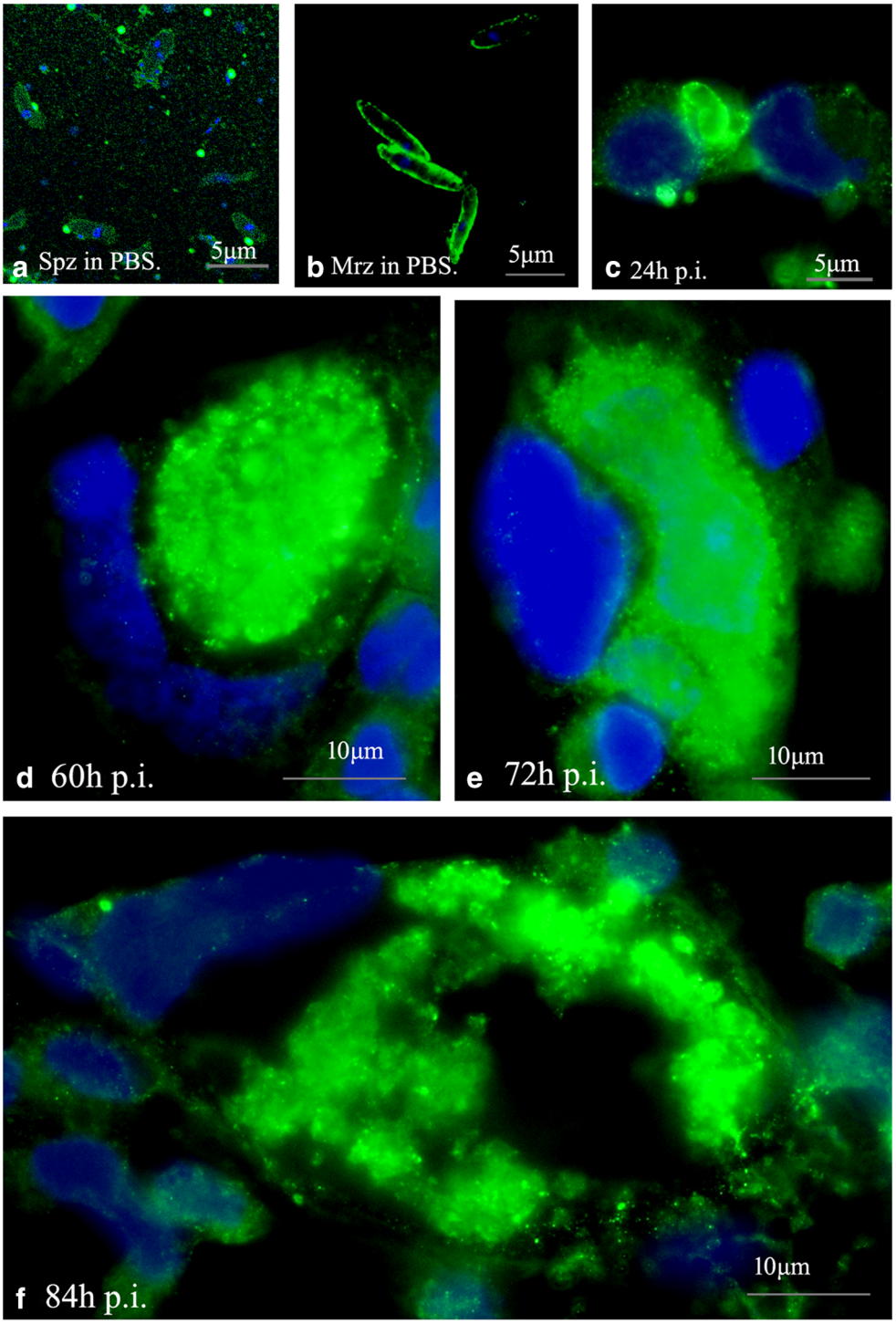

Fig. 4 Indirect immunofluorescence to localize *Et*SAG10 at different developmental stages of *Eimeria tenella* using rabbit sera against r*Et*SAG10. **a** Sporozoites (Spz) in PBS; **b** second-generation merozoites (Mrz) from infected chicken caeca in PBS; **c** trophozoites (Tropho) at 24-h post-infection (pi); **d** immature schizonts at 60 h pi; **e** immature schizonts at 72 h pi; **f** immature schizonts at 84 h pi

The function of *Et*SAG10 was further investigated by determining its expression at different developmental stages of *E. tenella* by qPCR. *Et*SAG10 mRNA expression was higher in SO than the other three stages, and these results agreed with Western blotting results. We hypothesized that this result was related to changes in the environment of SO. For instance, it has been reported that the expression of sHSPs is increased under different stress conditions, including heat, cold, chemical intoxication, and nutritional stress (Montero et al. 2008; Perez-Morales et al. 2009; Liu et al. 2012; Sun et al. 2014b). Nonetheless, compared with qPCR results, Western blot results also showed that *Et*SAG10 protein expression was higher in UO and Mrz; we speculated that the time and location of transcription and translation of eukaryotic genes may be

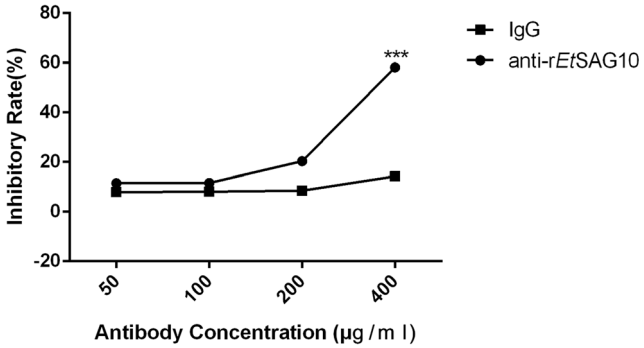

Fig. 5 Inhibition of sporozoite invasion in vitro by anti-r*Et*SAG10. Anti-r*Et*SAG10, rabbit antiserum against recombinant *Et*SAG10 protein; IgG, negative rabbit serum. All assays were performed in triplicate

separated by time and space and that the mRNA might have been degraded when the protein levels peaked at different time points of detection (Ben-Ari et al. 2010). In addition, Western blot analysis revealed two bands in SO, Spz, and Mrz, and the size of one of these bands was larger than the predicted size of 27.9 kDa. A previous study has shown that the amino acid sequence of *Et*SAG10 has a high frequency of change between amino acid residues 140 and 160, which may change the spatial conformation and hydrophilicity of the protein (Qiu et al. 2017). This change in protein size may also be due to post-translational modifications, including acylation, alkylation, and methylation. A study on *T. gondii* found that rhoptry protein 2 and microneme protein 2 had two bands on Western blots, corresponding to a precursor form and an active form (Entzeroth et al. 1998).

Indirect immunofluorescence using an antibody against r*Et*SAG10 showed that the protein was mainly localized to the parasite surface. Furthermore, *Et*SAG10 expression was higher in Mrz than Spz, and this result was consistent with the Western blot results. *Et*SAG10 expression was increased as parasite development progressed in DF-1 cells. Parasite surface proteins are particularly interesting because of their potential role in resisting the external environment and adapting to new environments. For instance, the trypomastigote small surface antigen (TSSA) plays a critical role in the infectivity and differentiation of *T. cruzi* trypomastigotes and the phenotypic variability of parasite strains (Camara et al. 2017). Moreover, the expression of a parasite surface antigen may cause a protective immune response in the host (Tabares et al. 2004; Chow et al. 2011; Petitdidier et al. 2016). Major changes in gene expression of surface antigen 10 in MRR and DZR may be involved in pathogenesis and immune evasion. The immune evasion mechanism of parasites involves (1) constantly changing the antigenicity of the parasite, (2) reducing the host immune response, and (3) avoiding or resisting the site of immune attack (Saito et al. 2017; Yam and Preiser 2017; Belachew 2018). In addition, *Et*SAG10 was detected in the PVM. The parasitophorous vacuole is crucial to protect the parasite against the host cell environment, and the PVM is a major route of communication between the intracellular parasite and the host cell by allowing the exchange of metabolites between them (Entzeroth et al. 1998; Beyer et al. 2002). Therefore, we hypothesized that *Et*SAG10 might escape the host immune response to protect the parasite against the intracellular environment.

*Et*SAG10 might also be involved in host cell invasion because invasion inhibition assays indicated that pretreatment with the anti-r*Et*SAG10 polyclonal antibody reduced the capacity of sporozoites to invade DF-1 cells in vitro. In a previous study, TSSA served as an anchor for invading parasites (Canepa et al. 2012). *Et*SAG10 is one of the SAG family members. Proteins from all SAG subfamilies contain signal peptides and GPI anchors (Reid et al. 2014). These GPI-anchored proteins are present at several developmental stages in *T. gondii*, *Plasmodium*, *Cryptosporidium*, and *Neospora*, and are essential for the adhesion of parasites to host cells (Lekutis et al. 2001; Leal-Sena et al. 2018). The present results supported these findings. However, the changes in surface antigens upon drug-stimulated coccidial invasion of host cells need to be better explored.

Conclusions

In this study, a full-length *Et*SAG10 cDNA from *E. tenella* was cloned, expressed, and characterized. The mRNA level was higher in sporulated oocysts than at other developmental stages. The present results suggested that *Et*SAG10 may be important in pathogenesis and immune evasion and may play a fundamental role in the invasion of host cells by sporozoites in vitro. Importantly, the difference between drug-resistant and sensitive strains may be related to *E. tenella* drug resistance to maduramicin and diclazuril, and this differential expression and mutations may provide a basis for seeking a marker of coccidial resistance. However, the function of this molecule needs to be further explored.

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