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Molecular characterization and analysis of drug resistance-associated protein enolase 2 of *Eimeria tenella*

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ABSTRACT

Eimeria tenella, an intestinal parasite, has brought huge economic losses to the poultry industry. The prevalence and severity of the development of drug resistance has increased the challenge of coccidiosis control. We previously identified the enolase 2 of *E. tenella* (*Et*ENO2) was differentially expressed in drug-sensitive (DS) and drug-resistant strains using RNA-seq. In this study, the expression of *Et*ENO2 in diclazuril-resistant (DZR), maduramicin-resistant (MRR), and salinomycin-resistant (SMR) strains was analyzed by quantitative real-time PCR (qRT-PCR) and western blots. *Et*ENO2 was highly expressed in several drug-resistant strains compared with the DS strain. The qRT-PCR showed that the transcription level of *Et*ENO2 in the field-isolated resistant strains was upregulated compared with the DS strain. The enzyme activity results indicated that the catalytic activity of *Et*ENO2 in the drug-resistant strains was higher than in the DS strain. In addition, qRT-PCR and western blots showed that the expression level of *Et*ENO2 was higher in second generation merozoites (SM) and unsporulated oocysts (UO) than that in sporozoites (SZ) and sporulated oocysts (SO). Immunofluorescence localization revealed that *Et*ENO2 was distributed throughout SZ and SM and on the surface of the parasites. After the SZ invasion DF-1 cells, it was also observed on the parasitophorous vacuole membrane. Our secretion experiments found that *Et*ENO2 could be secreted outside the SZ. This study indicated that *Et*ENO2 might be related to the interaction between *E. tenella* and host cells and be involved in the development of *E. tenella* resistance to some anticoccidial drugs.

1. Introduction

Coccidiosis is a serious epidemic parasitic disease affecting the intestinal tract of chickens. It is caused by one or more parasite of the *Eimeria* spp and is one of the most harmful diseases in intensive chicken farming (McDonald and Shirley, 2009). Chicken coccidiosis causes huge economic losses to the poultry industry every year, with an estimated global economic loss of over £10.4 billion (Blake DP et al., 2020). In addition, *Eimeria* spp infection can lead to an intestinal microflora disorder, affecting the structure and diversity of the microbial communities and the transmission of food-borne zoonotic pathogens (Blake DP et al., 2021). *Eimeria tenella*, *Eimeria acervulina*, and *Eimeria maxima* are the most common and pathogenic species among several *Eimeria* spp., and *E. tenella* is the model species for this study of coccidiosis.

The control of coccidiosis relies mainly on anticoccidial drugs, including synthetic drugs (produced by chemical synthesis) and ionophorous polyether (ionophore) drugs (Min et al., 2004; Peek and Landman, 2011). Chemically synthesized drugs include diclazuril, nicarbazine, probenazine and other chemical synthetic drugs (Song et al., 2000). In general, chemically synthesized drugs work mainly by inhibiting the biochemical pathways of the parasite. Diclazuril has the lowest drug concentration among the anticoccidial drugs currently used. Since it has an excellent anti-coccidiosis effect, it is widely used to prevent and control coccidiosis. Its structure is similar to a nucleoside. Therefore, researchers believe it affects nucleic acid synthesis in coccidia, leading to reproductive disorders by preventing further differentiation of the nucleus in schizonts and microgametocytes (Vanparijs et al., 1989; McDougald et al., 1990). Maduramicin and salinomycin belong to the ionophorous polyether drugs. These drugs change the permeability of the parasite's cell membrane, affecting ion

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exchange. The resulting sharp increase in the intracellular osmotic pressure leads to a large amount of water entering the parasite, causing its cells to expand and rupture (Chapman and Jeffers, 2014). In addition, a study found that these drugs could also affect merozoites by disrupting internal organelles and cell boundaries (Mehlhorn et al., 1983).

In 1954, Waletzky and Neal (1954) first identified a sulfonamide-resistant strain of *E. tenella*. With advancing research, researchers have also found multidrug-resistant strains (Stephen et al., 1997). The development of drug resistance of *Eimeria* has resulted in poor or even ineffective anticoccidial effects of many drugs. Even with different application strategies, the lack of drugs available in chicken farms has become one of the main obstacles in the current prevention and control of coccidiosis.

Previous studies have shown that drug resistance arises in different ways depending on the mechanism of the drug action. With the development of biotechnology, researchers have found differences in genes and proteins between drug-resistant and drug-sensitive (DS) strains of *E. tenella*. Chen et al. (2008) obtained the differentially expressed genes between the two drug-resistant strains (monensin-resistant strain, maduramicin-resistant strain) and DS strain by cDNA microarray technology. Thabet et al. (2017) found 25 proteins were upregulated in the monensin-resistant strains of *E. tenella* drug-resistant strains of *E. tenella* isolated from the field compared to DS strain by LC-MS/MS. Although researchers have speculated and studied the causes and mechanisms of drug resistance of *E. tenella*, the molecular mechanism of drug resistance has not been clarified, and the target genes controlling drug resistance have not been found.

To further study the molecular mechanisms of drug resistance, diclazuril-resistant (DZR) and maduramicin-resistant (MRR) strains of *E. tenella* with the same genetic background were induced from the DS strain in our laboratory. The obtained DZR strain was completely resistant to 1.2 ppm diclazuril and completely sensitive to other drugs, while the MRR strain was completely resistant to 7.0 ppm maduramicin and completely sensitive to other drugs (Han et al., 2004). Then, the differentially expressed genes between the resistant (DZR and MRR) and sensitive strains of *E. tenella* were obtained by transcriptome sequencing analysis. We found that enolase 2 of *E. tenella* (*Et*ENO2) was significantly upregulated in the two drug-resistant strains (Xie et al., 2020).

Enolase is an important catalytic enzyme that promotes the conversion between phosphoenolpyruvate and phosphoglycerate in glycolysis, playing an important role in cellular energy metabolism (Peshavaria and Day, 1991). In addition to catalyzing glycolysis, enolase also participates in the invasion and transfer of a variety of pathogenic microorganisms to the host, and can be used as a vaccine candidate factor and drug target. Studies on *Plasmodium falciparum* enolase (*Pf*ENO) revealed that *Pf*ENO might play a role in invasion, food vacuole formation, and transcription (Bhowmick et al., 2009). However, there are few reports on the study of enolase in *E. tenella*, especially its relationship with drug resistance.

In this study, *Et*ENO2 was cloned, characterized, and its biological function in the DS strain was investigated. Importantly, the differential expression of *Et*ENO2 at the transcription and protein levels in DS and different drug-resistant strains was compared, providing a basis for the further study of drug resistance mechanisms.

2. Materials and methods

2.1. Animals and parasites

The birds used in this study were provided by a local farm (Shanghai, China), and the New Zealand rabbits were obtained from Jiagan Biology Company (Shanghai, China). All experimental animals were raised in an environment without coccidia.

The DS strain of *E. tenella* was isolated from a farm in Shanghai (Resource Number CAAS21111601) and maintained in our laboratory (Huang et al., 1993). The DS strain was sensitive to the anticoccidial drugs diclazuril, maduramicin, and salinomycin. Coccidia-free 2-week--old chickens were used for propagating the passages as previously described (Tomley, 1997). Our laboratory induced the DZR, MRR, and salinomycin-resistant (SMR) strains from low to high concentrations in the DS strain by the concentration gradient method (Han et al., 2004; Wang et al., 2019). They were fully resistant only to 1.2 ppm diclazuril, 7.0 ppm maduramicin, and 60 ppm salinomycin, respectively. Coccidia-free 2-week-old chickens were also used for breeding, and the corresponding diclazuril, maduramicin, or salinomycin were added to the feed 2 days before inoculating with *E. tenella*.

We collected and purified unsporulated oocysts (UO) using standard procedures, and sporulated oocysts (SO) were formed after oxidation of UO at an appropriate temperature (Han et al., 2010). Sporozoites (SZ) were collected from purified SO in vitro (Miska et al., 2004). One hundred and 12 h after inoculation, the second-generation merozoites (SM) were collected and purified from the cecum of infected chickens (Zhou et al., 2010).

Wild diclazuril-resistant strains (D4, D5, D7, and D9) were isolated from the field by the single-oocyst method (Khalafalla and Daugschies, 2010), and resistance to 1.0 ppm diclazuril was demonstrated by drug sensitivity experiments (unpublished).

2.2. Cloning and sequencing of EtENO2

Total RNA was extracted from *E. tenella* SO using TRIzol reagent (TaKaRa, Tokyo, Japan). The quantity and quality of total RNA were evaluated using a Biospectrometer (Eppendorf, Hamburg, Germany) and 1% agarose gel electrophoresis. A reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) and Oligo (dT) primers were used to reverse transcribe RNA into complementary DNA (cDNA). The cDNA was then used as a template for further amplification.

Specific primers with *EcoR* I and *Xho* I restriction sites (underlined) were designed according to the ORF sequence (ToxoDB Accession Number: ETH_00024910). They were as follows: forward primer, 5'-GCGGAATTCATGTGGGGCCAAGCTGAGGCTCAGCAG-3′ and reverse primer, 5'-GCGCTCGAGCTAGTTGGAGGGGTTTCGGAAGTTCTC-3′. *Et*ENO2 was amplified by the polymerase chain reaction (PCR) using the first strand of the cDNA of the SO as the template. PCR products were analyzed and purified by 1% agarose gel electrophoresis (Qiagen, Dusseldorf, Germany) and subcloned into the pGEM-T-easy vector (Promega, Madison, WI). Positive clones for recombinant plasmids were then sequenced by Sangon Biotech (Shanghai, China) to confirm the sequence accuracy.

2.3. Sequence analysis of EtENO2

The full-length cDNA sequence of *Et*ENO2 was analyzed using BLAST programs from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). The deduced theoretical isoelectric point and molecular mass were obtained using ProtParam tools (http://www.expasy.org/tools/protparam.html). Protein motifs, signal peptide sequences, and transmembrane (TM) regions of *Et*ENO2 were predicted using Motif scan (http://hits.isb-sib.ch/cgi-bin/motif_scan), SignalP (http://www.cbs.dtu.dk/services/SignalP/), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively.

2.4. Quantitative real-time PCR (qRT-PCR)

The mRNA expression profiles of *Et*ENO2 from UO, SO, SZ, and SM of the DS strain were determined by quantitative real-time PCR (qRT-PCR). The qRT-PCR was performed using the SYBR1 Green I dye method. Total RNA from the four life stages were extracted according to the above method, and then the genomic DNA was removed by RNeasy Mini Kits (Qiagen). The cDNA was synthesized from 2 μg of total RNA by SuperScript II reverse transcriptase (Invitrogen) and random primers. The *Et*ENO2 primers used for qRT-PCR were 5'-CGGCCTTCAGCACCCCCTTG-

3′ (sense) and 5'-CAAGTCCCGCTGCTGCTGCT-3′ (antisense). Meanwhile, the 18S rRNA housekeeping gene (GenBank accession number: EF122251) from *E. tenella* was used as an internal control (Jiang et al., 2012), and the primers were 5'- TGTAGTGGAGTCTTGGTGATTC-3′ (sense) and 5'-CCTGCTGCC TTCCTTAGATG-3′ (antisense). Reactions were in triplicate, and experiments were repeated three times. The relative expression of *Et*ENO2 was measured using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

We also used qRT-PCR to compare the transcriptional level of *Et*ENO2 in SO of the DS strain and different drug-resistant strains, including DZR, MRR, and SMR strains. The MRR strains resistant to different concentrations of maduramicin (3 and 5 ppm) and the DZR strains resistant to different concentrations of diclazuril (0.2, 0.5, 0.8, and 1.0 ppm) were compared with the DS strain. In addition, qRT-PCR was also used to detect the transcription level of *Et*ENO2 in field DZR strains (D4, D5, D7, and D9) obtained from the wild.

2.5. Expression and purification of recombinant EtENO2

The correctly sequenced recombinant plasmids pGEM-T-*Et*ENO2 and expression vector pGEX-4T-1(Novagen, Darmstadt, Germany) were double digested with *EcoR* I and *Xho* I and then ligated to construct the recombinant expression plasmid pGEX-4T-*Et*ENO2. The recombinant plasmid was identified by sequencing. The identified recombinant plasmid was transformed into *E. coli* BL21 (DE3) (Tiangen, Beijing, China), and expression of the recombinant protein was induced by 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA). After sonication, the subcellular distribution of the recombinant protein was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The recombinant *Et*ENO2 (r*Et*ENO2) protein was purified from SDS-PAGE gel bands (Richard, 2009). The quality of the purified r*Et*ENO2 protein was identified by 12% SDS-PAGE, and the concentration of purified r*Et*ENO2 was determined using the BCA protein assay kit (Beyotime, Haimen, China).

2.6. Preparation of anti-rEtENO2 polyclonal serum

The purified r*Et*ENO2 protein was emulsified with Freund's complete adjuvant (Sigma-Aldrich) in a ratio of 1:1. The emulsified protein was first used to immunize New Zealand white rabbits (2-month-old) by subcutaneous injection at a dose of 200 μg protein per rabbit. Two weeks later, the same protein was emulsified with Freund's incomplete adjuvant (Sigma-Aldrich) for five booster immunizations, each one week apart. Antiserum against r*Et*ENO2 was collected one week after the final immunization. The serum collected before the first immunization was used as a negative control.

2.7. Western blot analysis

Parasites at different developmental stages (UO, SO, SZ, SM) of the DS strain and SO of the DS and drug-resistant (DZR, MRR, and SMR) strains were suspended in phosphate-buffered saline (PBS) and lysed on ice by ultrasonication. Total protein concentration was quantified using a BCA protein assay kit (Beyotime). The same amount of parasite proteins (20 μg) were individually subjected to 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% (w/v) skimmed milk and incubated with r*Et*ENO2-immunized rabbit sera (1:100 dilution) or mouse anti-α-tubulin monoclonal antibody (1:2000 dilution, Beyotime) as primary antibody. Mouse monoclonal anti-α-tubulin (Beyotime) was used as an internal reference. IRDye® 680CW goat anti-rabbit IgG or IRDye® 680RD donkey anti-mouse IgG (1:10,000 dilution) (LI-COR Biosciences, Lincoln, NE, USA) was subsequently used as the secondary antibody. Starting with the blocking step, the membranes were washed five times with PBS containing 0.5% Tween-20 (PBST) after each step. Finally, the strips were scanned using an Odyssey® Infrared Imaging System (LI-COR Biosciences).

The purified r*Et*ENO2 was used for western blots, with infected chicken sera, healthy chicken sera, and GST monoclonal antibody as first antibodies to analyze reactogenicity.

2.8. Immunolocalization of EtENO2 in parasites

Fresh SZ and SM were transferred to glass slides and air-dried, then fixed on the glass slides using 4% paraformaldehyde. The fixed parasites were infiltrated with 1% Triton X-100 and blocked with 2% (w/v) bovine serum albumin. Slides were incubated successively with r*Et*ENO2-immunized rabbit sera (1:100 dilution) and healthy rabbit IgG as the primary antibody and goat anti-rabbit IgG fluorescein isothiocyanate-conjugated antibody (1:500 dilution; Sigma-Aldrich) as the secondary antibody. The nuclei were then stained by 4,6-diamidino-2-phenylindole (DAPI) (Beyotime). After each of the above steps, the slides were gently cleaned four times with PBST. Finally, the slides were placed on a glass slide with 50 μL Fluoromount aqueous mounting medium (Sigma-Aldrich) and observed under a fluorescence microscope (Olympus, Tokyo, Japan) and a laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

In addition, clean cell slides were put into 6-well plates. DF-1 cells (2×10^5 cells per well) were inoculated into each well and cultured overnight in an incubator. The freshly purified SZ (6×10^5 cells per well) were then added to the 6-well plate to invade the cells. The slides were collected at 0.5, 12, 48, and 62 h post-infection and were synchronously treated and observed using the same methods described above.

2.9. Secretion assay of EtENO2

The secretion assay was performed as reported by Péroval et al. (2006), and a reported secreted protein, *E. tenella* microneme-2 protein (*Et*MIC2), was used as a positive control and *E. tenella* tubulin alpha chain (*Et*TUBA), was used as a negative control (Tomley et al., 1996). Briefly, freshly isolated SZ (3×10^6) were resuspended in 100 μL PBS or Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Paisley, UK) and incubated at 41 °C or 4 °C for 2 h. After centrifugation, the supernatant containing the excretory–secretory antigens (ESA) were detected by western blot as described above. The rabbit anti-r*Et*ENO2, anti--r*Et*MIC2, or anti-r*Et*TUBA polyclonal antibody (1:200 dilution) was used as the primary antibody, and the IRDye® 680CW goat anti-rabbit IgG (1:10,000 dilution) was used as the secondary antibody.

2.10. Enzyme activity

The activity of *Et*ENO2 in DS, DZR, and MRR strains was determined using the enolase activity assay kit (Sigma-Aldrich) by spectrophotometry according to the manufacturer's instructions. Freshly purified SO (1×10^7) of different strains were dissolved by sonication on ice. The protein concentration was determined using the BCA protein assay kit (Beyotime). Then, 50 μL of reaction buffer was added into each well and mixed with the substrate before 50 μL DS, DZR, or MRR protein of the same concentration (0.5 mg/mL) was added. The results were observed at 450 nm. The experiments were repeated three times.

2.11. Statistical analysis

All data were analyzed using SPSS statistical software for Windows version 22 (SPSS, Chicago, IL, USA). Duncan's test was used to test the differences between groups by one-way analysis of variance. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Cloning and sequence analysis of EtENO2

The ORF sequence of the *Et*ENO2 gene was amplified with the first strand of cDNA of SO of the *E. tenella* DS strain as the template, and a 1560-bp product was obtained. BLAST analysis showed that the sequence displayed 100% homology with *E. tenella* enolase 2 (GenBank number: XM_013373897.1), indicating that the ENO2 gene of *E. tenella* was successfully cloned. Nucleotide sequence analysis showed that the gene encoded a polypeptide of 519 amino acids with a theoretical isoelectric point of 5.58 and a predicted molecular mass of approximately 53.6 kDa. The amino acid sequence had 94%, 75%, and 72% identity with the putative ENO2 from *E. necatrix* (XP_013435662.1), *E. brunetti* (CDJ49758.1), *and E. maxima* (XP_013332868.1), respectively. The amino acid sequence displayed 73% identity with *Besnoitia besnoiti* ENO2 (XP_029219568.1), 71% identity with *Toxoplasma gondii* ENO2 (XP_002365578.1), and 71% identity with *Plasmodium falciparum* ENO2 (XP_001347440.1). The above results indicated that this protein was conserved in protozoa.

The predicted motif structure indicated that *Et*ENO2 contained four casein kinase II phosphorylation sites (residues 174–177, 258–261, 375–378, and 435–438), ten N-myristoylation sites (residues 3–8, 74–79, 82–87, 104–109, 113–118, 136–141, 236–241, 282–287, 452–457, and 473–478), two protein kinase C phosphorylation sites (residues 456–458, and 487–489), a tyrosine kinase phosphorylation site (residues 125–132), an enolase N-terminal domain (residues 77–213), a Tim barrel domain at the C-terminal of enolase (residues 223–518), and a PAP/25A-related domain (residues331–389) (Fig. 1). Bioinformatics analysis showed that the protein had no signal peptide or transmembrane domains.

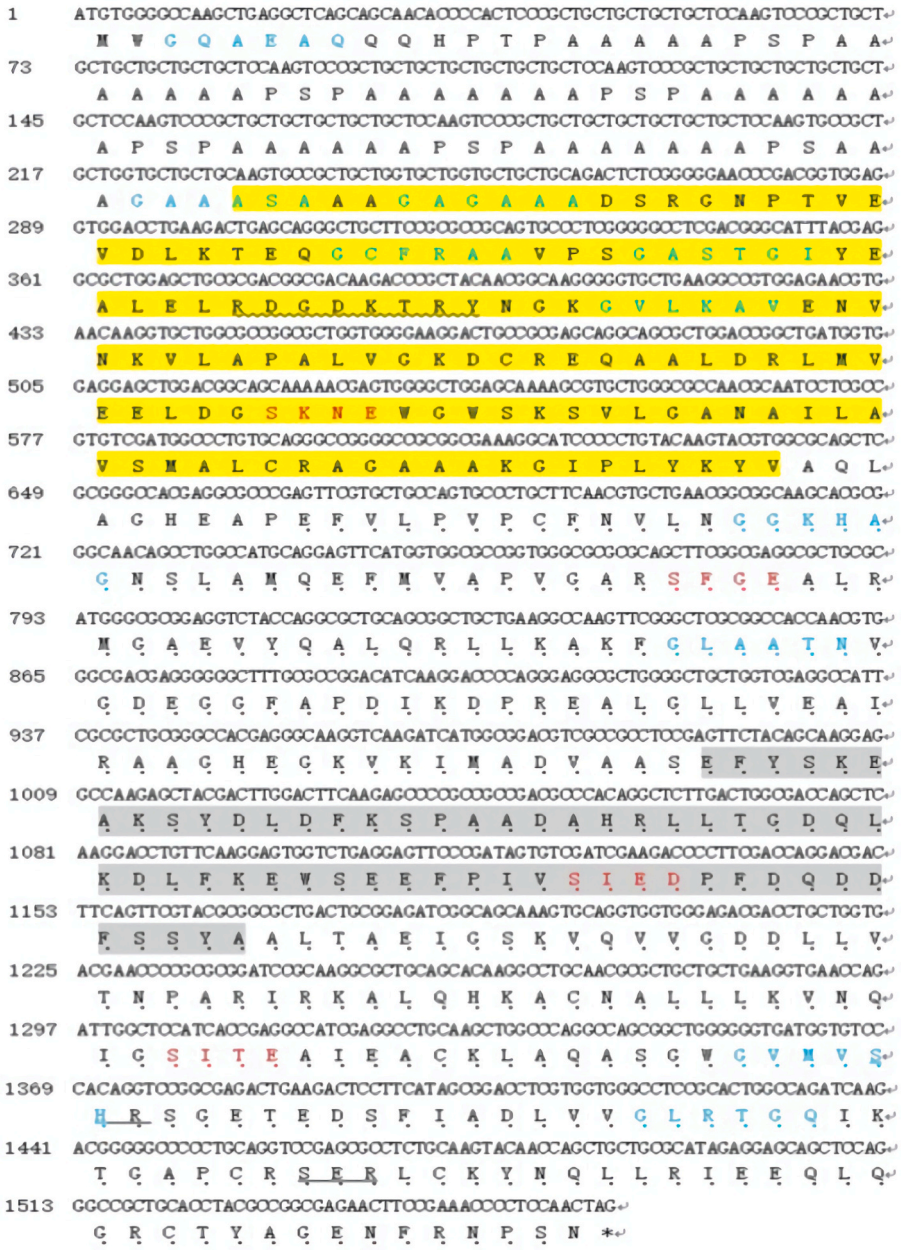

Fig. 1. Bioinformatic analysis of *Et*ENO2. Red: Casein kinase II phosphorylation site; blue: N-myristoylation site; uderline: Protein kinase C phosphorylation site; wavy underline: Tyrosine kinase phosphorylation site; yellow: Enolase, N-terminal domain; black spots: Enolase, C-terminal TIM barrel domain; grey: PAP/25A associated domain; *: Stop codon. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Expression and purification of recombinant EtENO2

The r*Et*ENO2 was expressed with a GST-tag in *E. coli* BL21 as the fusion protein. SDS-PAGE analysis showed that r*Et*ENO2 was expressed mainly in the form of inclusion bodies. After purification by grinding the SDS-PAGE bands, the r*Et*ENO2 protein band (molecular weight of approximately 79.6 kDa) fused to the GST-tag (26 kDa) was detected as expected (Fig. 2A).

Western blots indicated that r*Et*ENO2 could be recognized by infected chicken sera and anti-GST monoclonal antibody. Healthy chicken sera failed to detect r*Et*ENO2 (Fig. 2B).

3.3. Transcription and translation of EtENO2 at the developmental stages of the DS E. tenella strain

Using 18s rRNA as a control, the mRNA transcription levels of *Et*ENO2 at the developmental stages (UO, SO, SZ, SM) of the *E. tenella* DS strain were detected by qRT-PCR. The results showed that *Et*ENO2 transcription was concentrated mainly in SM and UO, with much lower transcript levels in SO and SZ (Fig. 3A).

The protein levels of *Et*ENO2 in UO, SO, SZ, and SM were determined by western blot. Mouse anti-α-tubulin monoclonal antibody was the control. Western blot showed a higher level of *Et*ENO2 translation in UO and SM and a lower level in SO and SZ (Fig. 3B and C).

3.4. Immunofluorescence localization of EtENO2

Using rabbit anti-r*Et*ENO2 polyclonal antibody, the distribution of *Et*ENO2 in SZ, SM, and different developmental stages after SZ invaded DF-1 cells was analyzed by an indirect immunofluorescence technique. It was observed that *Et*ENO2 was distributed throughout the cytoplasm and on the surface of SZ and SM, except for the refractive body of SZ (Fig. 4B and C). When SZ invaded DF-1 cells to develop into schizonts, the *Et*ENO2 protein remained in most areas of the parasite (Fig. 4D–G). No obvious fluorescence was seen in the negative control group (Fig. 4A). After infection for 48 h, *Et*ENO2 was also observed in the parasitophorous vacuole membrane (PVM). The fluorescence intensity of *Et*ENO2 increased gradually during the further development of the SZ into schizonts in the cell.

3.5. Secretion of EtENO2

To test the secretion of *Et*ENO2, fresh SZ were incubated in PBS or DMEM either at 41 °C or 4 °C. The ESA samples were examined by western blot. The results showed that *Et*ENO2 could be secreted when SZ were incubated in both PBS and DMEM at 41 °C or 4 °C (Fig. 5). Overall, the secretion of *Et*ENO2 was more favorable in DMEM at 41 °C than in other conditions. No protein was identified in the negative control.

3.6. Differences in transcription and protein levels of EtENO2 between sensitive and resistant strains

The mRNA transcription levels of *Et*ENO2 in the SO stage of the resistant (DZR and MRR) strains and the DS strain of *E. tenella* were detected by qRT-PCR. As shown in Fig. 6A, the transcription level of *Et*ENO2 in the DZR and MRR strains was higher than in the DS strain. The difference was significant ($p < 0.001$). These results agreed with the RNA-seq results (Xie et al., 2020).

Similarly, the protein levels of *Et*ENO2 in the SO of the DS, DZR, and MRR strains were determined by western blot using rabbit antiserum against r*Et*ENO2. The results showed that *Et*ENO2 was upregulated in the DZR and MRR strains compared with the DS strain, and the difference was significant in the MRR strain (Fig. 6B and C).

The cDNAs of SO from the DZR strains (0.2 ppm, 0.5 ppm, 0.8 ppm, and 1.0 ppm) and MRR strains (3 ppm and 5 ppm) at different concentrations were extracted as templates, and the relationship between the expression level of *Et*ENO2 and the drug concentration was detected by qRT-PCR. From Fig. 6D and E, we found that with the increasing concentrations of diclazuril and maduramicin, the expression levels of *Et*ENO2 also gradually increased. Compared with the DS strain, the expression levels of *Et*ENO2 in the MRR strains with 3 ppm and 5 ppm and the DZR strains with 0.5 ppm, 0.8 ppm, and 1.0 ppm were significantly increased.

Using the single-oocyst isolation method, we collected SO of the DZR strains in the field and analyzed the transcription level of *Et*ENO2 by qRT-PCR (Fig. 6F). The results showed that compared with the DS strain, the expression levels in the D4, D5, D7, and D9 strains were significantly upregulated. However, there was still a gap compared with the strains fully resistant to diclazuril.

We compared the transcription levels of *Et*ENO2 in the SO of the DS and SMR strains using qRT-PCR. The results showed that compared with the DS strain, the transcription level of *Et*ENO2 in the SMR strain was significantly upregulated (Fig. 7A). Using anti-r*Et*ENO2 serum as the primary antibody and α-tubulin monoclonal antibody as the control, we compared the protein levels of *Et*ENO2 by western blots. It was also found that the translational level of *Et*ENO2 in the SMR strain was significantly upregulated compared with the DS strain (Fig. 7B and C).

3.7. Enzyme activity

The enzyme activities in the DS, DZR, and MRR strains were analyzed and compared using the enolase activity assay kit. The results showed that the enzyme activity of *Et*ENO2 was significantly improved in the SO of the resistant strains compared with the DS strain (Fig. 8).

4. Discussion

The emergence and development of drug resistance of *Eimeria* spp.

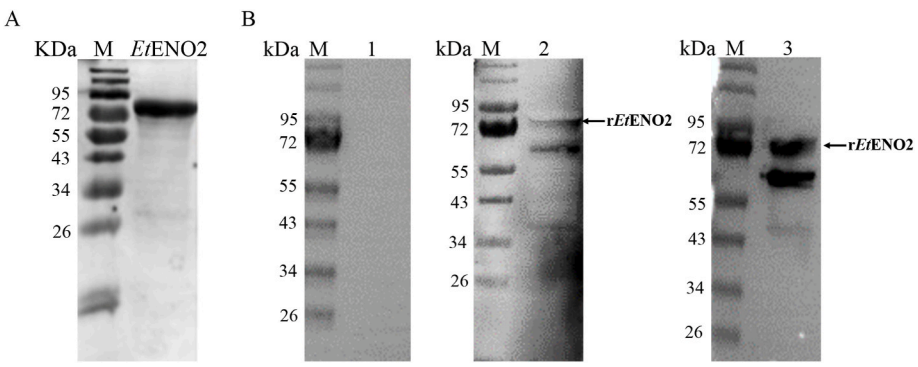

Fig. 2. A: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified r*Et*ENO2. B: Western blotting analysis of r*Et*ENO2. Lane 1, r*Et*ENO2 probed with healthy chicken sera. Lane 2, r*Et*ENO2 probed with monoclonal anti-GST antibody. Lane 3, r*Et*ENO2 probed with infected chicken sera.

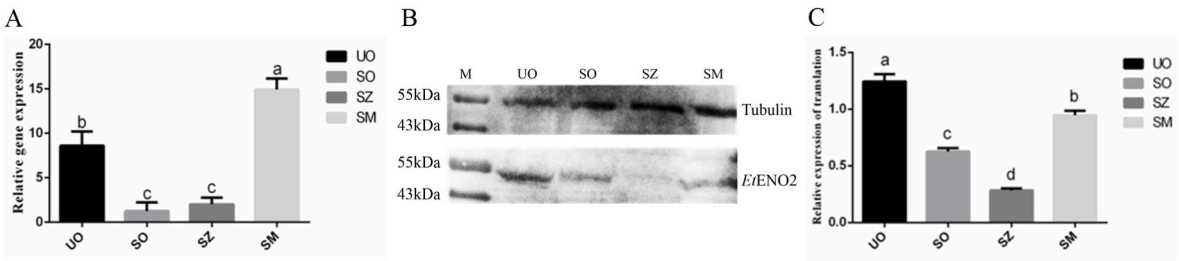

Fig. 3. Quantitative real-time PCR and western blot analysis of *Et*ENO2 expression at different developmental stages of the drug-sensitive strain. UO, unsporulated oocysts; SO, sporulated oocysts; SZ, sporozoites; SM, second-generation merozoites. *Et*ENO2 was recognized by rabbit sera anti-r*Et*ENO2, and anti-α-tubulin antibody was used as the control. (A) Transcription levels of *Et*ENO2. (B–C) Protein levels of *Et*ENO2. The data represent the mean ± SD of triplicate determinations and are representative of three independent experiments. a, b, c, and d indicate significant differences ($p < 0.05$) between groups.

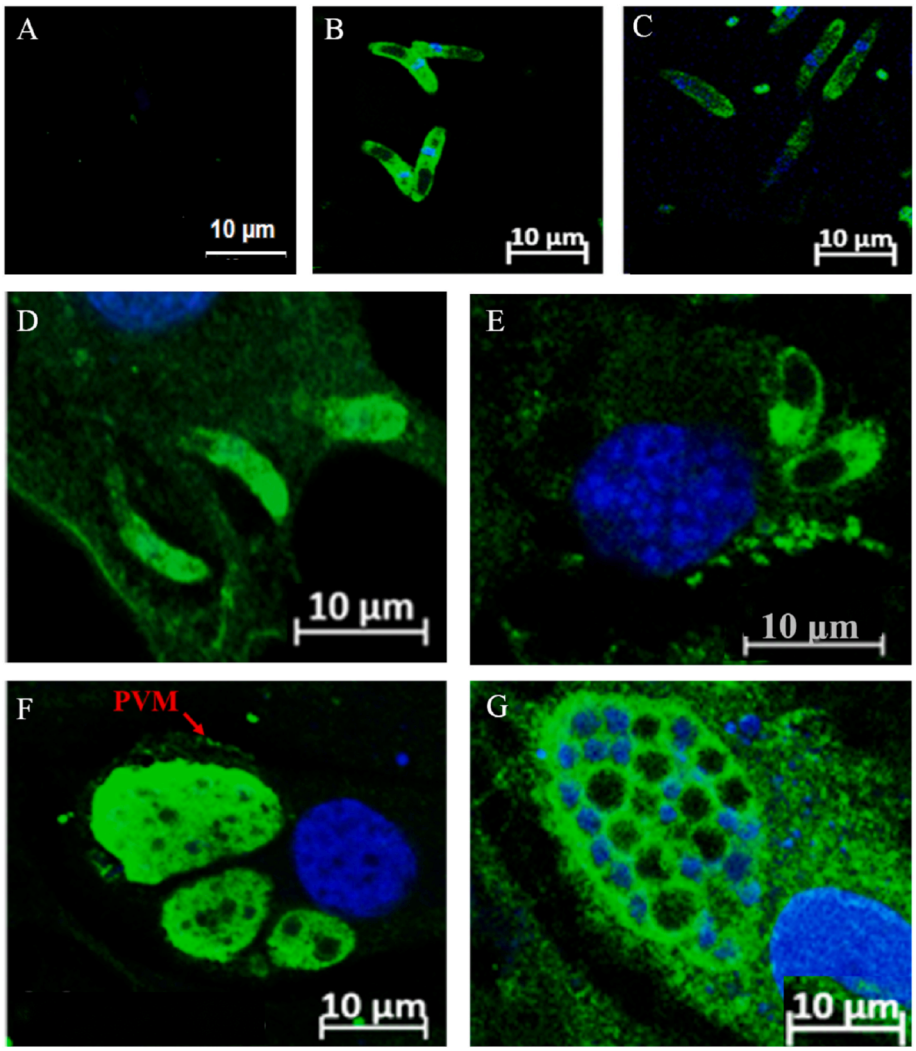

Fig. 4. Localization of *Et*ENO2 at different developmental stages of *E. tenella* using rabbit sera against r*Et*ENO2. (A) Negative control, healthy rabbit IgG as first antibody. (B) Sporozoites in phosphate-buffered saline (PBS). (C) Second-generation merozoites in PBS. Infected DF-1 cells were collected post infection (p.i.) at the indicated times. (D) Immature schizont (iSc) at 30 min p.i. (E) iSc at 12 h p.i. (F) iSc at 48 h p.i. PVM, parasitophorous vacuole membrane. (G) iSc at 62 h p.i.

have caused huge economic losses to the chicken industry, and is the main problem in controlling chicken coccidiosis. We previously found by transcriptome sequencing that *Et*ENO2 is highly expressed in two drug-resistant strains (DZR and MRR). We speculated that it might be related to *E. tenella* resistance to anticoccidial drugs (Xie et al., 2020). Enolase is a key enzyme in the glycolytic pathway, which exists widely in the biological world. An increasing number of studies have shown that enolase is a multifunctional protein that plays a catalytic role in glycolysis, cell differentiation, apoptosis regulation, and gene transcription. Studies on *Leishmania mexicana*, *Schistosoma japonicum*, and *Echinococcus granulosus* have shown that enolase can be used as a drug target or potential vaccine candidate, which is of great significance in controlling the occurrence of parasitic diseases (Vanegas et al., 2007; Gan et al., 2010).

In this study, the enolase 2 gene of *E. tenella* was successfully cloned and characterized. Through BLAST analysis, we found that the obtained

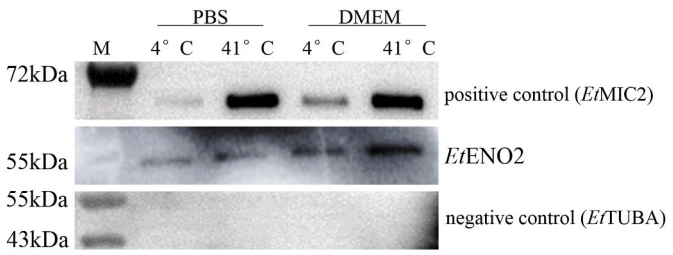

Fig. 5. *Et*ENO2 is a secreted protein. Fresh sporozoites were incubated in PBS or Dulbecco's modified Eagle's medium (DMEM) at either 4 °C or 41 °C for 2 h. After incubation, supernatants containing excretory–secretory antigens (ESA) were harvested and *Et*ENO2 secretion was examined by western blots with rabbit sera against r*Et*ENO2. *Et*MIC2 was used as a positive control. *Et*TUBA was used as a negative control.

sequence was 100% homologous to the published *E. tenella* enolase 2 (GenBank number: XM_013373897.1) and had high identity with ENO2 of other *Eimeria* and protozoa, such as *B. besnoiti*, *T. gondii*, and *P. falciparum*. The structural analysis of the protein showed that it contained multiple functional sites, indicating that it might participate in multiple reactions and play important functions in the life cycle of *E. tenella*.

Western blot and qRT-PCR showed that the expression levels of *Et*ENO2 in the UO and SM stages of *E. tenella* were significantly higher than that in the SO and SZ stages. The metabolic changes caused by hypoxia in the chicken intestine may be one of the reasons for the high expression of *Et*ENO2 in the UO and SM stages. Both stages (UO and SM) develop in the chicken and belong to the endogenous phase of the parasite's life cycle. A study found that anaerobic glycolysis occurs in the intracellular phase of *E. tenella* (Labbé M et al., 2006). We speculate that under hypoxia, to provide energy, the parasite may enhance glycolysis by increasing the gene expression of key enzymes in the glycolytic pathway, such as enolase. A study on *Saccharomyces cerevisiae* found that the expression of glycolytic enzymes changed under hypoxia and enolase responded fastest to hypoxia (Yoshimura et al., 2021). Importantly, the endogenous phase is the main phase of drug action. The high expression of *Et*ENO2 in this phase might also provide the energy for *E. tenella* to resist and excrete the drugs. Wang et al. (2010) found that the expression levels of enolase were significantly different in different developmental stages of *Moniezia expansa*, and the gene expression was significantly positively correlated with the exuberant degree of life activities of the parasite.

Indirect immunofluorescence localization showed that *Et*ENO2 was distributed throughout the cytoplasm and on the surface of *E. tenella*. As a key enzyme in the glycolysis pathway, enolase is generally expressed in large quantities in the cytoplasm. However, it also exists on the surface of many eukaryotic and prokaryotic cells and is even secreted outside the cell (Andrade et al., 2005). The diverse localization of enolase suggests that, in addition to its catalytic role in glycolysis, it may

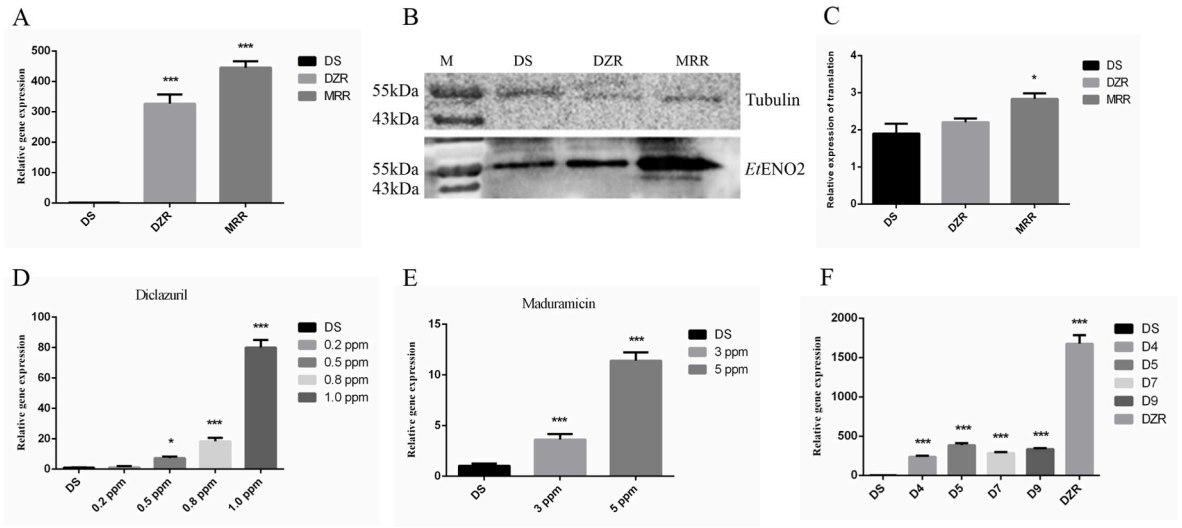

Fig. 6. Quantitative real-time PCR and western blot analysis of *Et*ENO2 expression in different resistant strains in SO. (A) Transcription levels of *Et*ENO2. (B–C) Protein levels of *Et*ENO2. *Et*ENO2 was recognized by rabbit anti-r*Et*ENO2. Anti-α-tubulin antibody was used as a control. (D–E) Transcription levels of *Et*ENO2 at different concentrations of diclazuril-resistant strains and maduramicin-resistant strains. (F) Transcription levels of EtENO2 in field diclazuril-resistant strains DS, drug-sensitive strain; DZR, diclazuril-resistant strain; MRR, maduramicin-resistant strain; D4–D9, four field diclazuril-resistant strains. The data represent the mean ± SD of triplicate determinations and are representative of three independent experiments. *$p < 0.05$; ***$p < 0.001$.

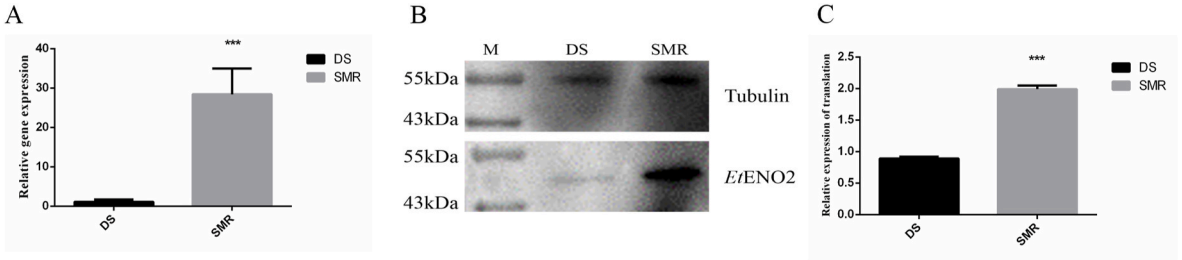

Fig. 7. Transcription and protein levels of *Et*ENO2 of the salamycin-resistant (SMR) strain in SO. DS, drug-sensitive strain; SMR, salamycin-resistant strain. Anti-α-tubulin antibody was used as a control. *Et*ENO2 was recognized by rabbit anti-r*Et*ENO2. (A) Transcription levels of *Et*ENO2 in the SMR strain. (B–C) Protein levels of *Et*ENO2 in the SMR strain. The data represent the mean ± SD of triplicate determinations and are representative of three independent experiments. ***$p < 0.001$.

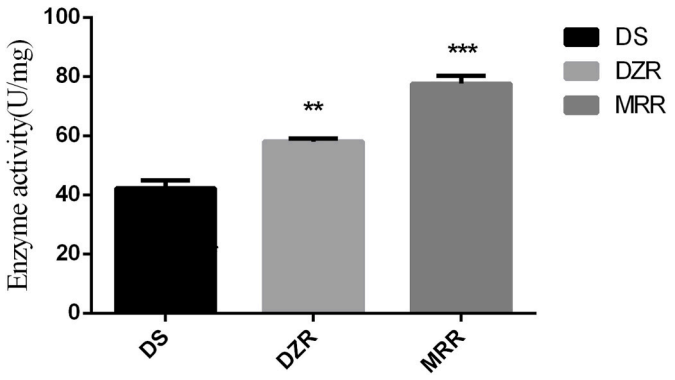

Fig. 8. *Et*ENO2 activity of the SO of the drug-sensitive (DS), diclazuril-resistant (DZR), and maduramicin-resistant (MRR) strains.

participate in many other biological functions. A study on *P. falciparum* found that enolase on the surface of merozoites was involved in red blood cell invasion, enolase in vesicles was involved in food vesicle formation, and enolase in the nucleus played a role in transcription (Bhowmick et al., 2009). During the development of SZ-invading cells into schizonts, the fluorescence intensity of *Et*ENO2 gradually increased, which meant that the expression of *Et*ENO2 protein increased with the growth and development of *E. tenella* in the cells. The previous qRT-PCR and western blot results also found that the expression level of *Et*ENO2 in SM was significantly higher than in SZ. Therefore, we speculated that *Et*ENO2 could play an important function in the growth and reproduction of parasites in cells. Ferguson et al. (2002) pointed out that enolase played an important role in gene regulation during the proliferation and cleavage of *T. gondii*. Some studies have found that most of the enolase of *Angiostrongylus cantonensis* existed in the cytoplasm of the parasite, and it might be involved in regulating the growth and development of the parasite (Zhang et al., 2014). These studies have led us to speculate on the important role of enolase in the growth and development of *E. tenella.*

Using fluorescence localization, we found that *Et*ENO2 was distributed on the PVM and surface of *E. tenella*. The PVM is a key structure formed by invagination of the host cell membrane to protect the parasite against the host cell's antagonistic environment (Daszak, 1999). Therefore, we hypothesized that *Et*ENO2 on the PVM and surface of the parasite might participate in the interaction between parasite and host cell and help parasites evade the host's immune response. However, our invasion experiment found that anti-r*Et*ENO2 antibody could not inhibit the invasion of *E. tenella* SZ (data not shown). A study of *T. gondii* found that fructose-1,6-diphosphate aldolase, another glycolytic pathway enzyme, could bind to cell surface adhesin. However, it did not affect parasite invasion and played a role mainly in energy metabolism (Shen and Sibley, 2014). This suggests that *Et*ENO2 might be involved in the interaction between parasites and host cells and provide energy support but is not involved in host cell invasion. We further speculated that *Et*ENO2 might be secreted. The secretion experiment also confirmed that *Et*ENO2 can be secreted outside the parasite. Avilán et al. (2011) found that enolase existed in the surface-secreted protein of *Leishmania* and might be a receptor for plasminogen. Bernal et al. (2004) found that the recombinant enolase of *Clonorchis sinensis*, *Echinostoma*, and *Leishmania* could specifically bind to the host fibrinogen, participate in the interaction between the parasite and the host, and is also a target molecule of antibody interaction in infection and immunity. These all reflect interactions of enolase in the parasite with the host. Most proteins are synthesized and localized to the cell surface or secreted outside the cell with the help of signal peptides. However, bioinformatics analysis found that *Et*ENO2 protein may not have a signal peptide and transmembrane structure. Some studies have shown that although enolase does not have a signal peptide and transmembrane region, it can still be expressed on the cell surface (Andrade et al., 2005; Agarwal et al., 2008). Studies have found that some secreted proteins lacking signal peptides could reach the cell membrane or extracellular environment through unconventional secretory pathways, such as signal transduction pathways or post-transcriptional modifications (Nickel, 2003). Further studies are needed to determine whether *Et*ENO2 also localizes and plays a role in a similar way.

Many studies have shown that the emergence of drug resistance was related to the differential expression of genes. Similar to *E. tenella*, *Plasmodium* belong to protozoa, and research on their drug resistance has made major breakthroughs. In the case of *P. falciparum*, Yang et al. (2019) found that a mutation in pfkelch13 led to reduced kelch13 protein abundance in *P. falciparum*, decreased hemoglobin catabolism, and reduced activation of artemisinin activation and toxic proteins, and developed resistance to artemisinin. The multidrug resistance transporter 1 gene of *P. falciparum* (pfmdr1) also regulated the sensitivity of parasites to heme-binding drugs by upregulating expression or mutations (Sidhu et al., 2006; Wicht et al., 2020).

In this study, qRT-PCR and western blots showed that *Et*ENO2 was significantly upregulated in the two drug-resistant strains (DZR and MRR) compared with the DS strain. This result is consistent with a previous finding (Xie et al., 2020). We also detected the *Et*ENO2 enzyme activity of each strain, and the results showed that the activity of *Et*ENO2 in the drug-resistant strains was significantly higher than that in the DS strain. Furthermore, we found that the expression of *Et*ENO2 in the DZR and MRR strains increased with increasing drug concentration. The expression level of *Et*ENO2 was also significantly upregulated in our laboratory-induced SMR strain compared with the DS strain. We speculated that the expression of *Et*ENO2 would increase under the long-term action of the drug. Enolase is a key glycolytic enzyme that contributes to the Warburg effect, and studies on enolase and drug resistance have been reported. The study of *Leishmania* also found differential expression of enolase in resistant parasites, suggesting that enolase may protect parasites from oxidative stress by giving them a mechanism to deal with drug stress (Singh and Sundar, 2017). Therefore, we speculated that the highly expressed *Et*ENO2 in drug-resistant strains could be involved in countering drug stress, promoting drug efflux from cells to reduce the effective intracellular drug concentration in cells, and providing energy support. However, this requires further research.

To further verify the expression levels of *Et*ENO2 in different drug-resistant strains, we used qRT-PCR to detect the expression levels of several drug-resistant strains isolated from the field. *Et*ENO2 was significantly upregulated in these wild-type drug-resistant strains compared with the DS strains. However, the expression level of *Et*ENO2 in the field drug-resistant strains had a gap compared with the laboratory-induced DZR strains. We speculated this might be because the wild strains we obtained showed resistance to diclazuril but were not completely resistant to high concentrations of diclazuril. Their different sensitivities to drugs might lead to different expression levels. We suspected that the development of drug resistance and the increased expression of *Et*ENO2 in drug-resistant strains were caused by gene mutations. It has been shown that mutations in genes of drug-resistant *Plasmodium* strains increase their expression (Kasturi et al., 2018). We performed the whole genome resequencing analysis of resistant strains, but found no mutation in *Et*ENO2 (data unpublished). Thus, drug resistance may be caused by other mechanisms, such as mutations in other genes encoding proteins that interact with *Et*ENO2, amino acid substitution, and changes in gene transporters, which require further study.

In addition to ENO2, other glycolytic enzymes have also been associated with drug resistance. Huang et al. (2022) found that the expression of glyceraldehyde-3-phosphate dehydrogenase was significantly upregulated in the resistant strains of *E. tenella* In our previous study, we also found that hexokinase and lactate dehydrogenase were upregulated in MRR compared with DS strain of *E. tenella* (Xie et al., 2020).

Therefore, we hypothesized that some glycolytic pathway enzymes might constitute a drug-resistant phenotype individually or collectively. We speculated that *Et*ENO2 in this study might be related to the drug resistance of *E. tenella*, and the upregulation of *Et*ENO2 expression can be used as an index to detect the drug sensitivity of *E. tenella* in vitro.

Declaration of competing interest

The authors declare that they have no competing interests.

The funding agencies played no role in the design or implementation of the study, analysis or interpretation of the data, or the preparation and submission of the manuscript.

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