

Effects of diclazuril on the expression of enolase in second-generation merozoites of *Eimeria tenella*

Bian-hua Zhou,^{*,1} Hai-yan Ding,^{*} Jing-yun Yang,^{*} Jun Chai,[†] Hong-wei Guo,[‡] and Hong-wei Wang^{*}

^{*}College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471000, Henan, People's Republic of China; [†]School of Information Technology and Urban Construction, Luoyang Polytechnic, Luoyang 471934, Henan, People's Republic of China; and [‡]College of Animal Science & Technology, Henan University of Animal Husbandry and Economy, Zhengzhou 450046, Henan, People's Republic of China

ABSTRACT *Eimeria tenella* is an obligate intracellular parasite of the chicken cecum; it brings huge economic loss to the chicken industry. Enolase is a multifunctional glycolytic enzyme involved in many processes of parasites, such as infection and migration. In this study, the effect of diclazuril on the expression of enolase in second-generation merozoites of *E. tenella* (*EtENO*) was reported. The prokaryotic expression plasmid pET-28a-*EtENO* was constructed and transformed into *Escherichia coli* BL21 (DE3). Then, it was subjected to expression under the induction of isopropyl- β -D-1-thiogalactopyranoside. The expressed products were identified and purified. The purified *EtENO* protein was used for antibody preparation. The *EtENO* mRNA

and protein expression levels were analyzed via real-time PCR and Western blotting. Localization of *EtENO* on the merozoites was examined by immunofluorescence technique. The mRNA and protein expression levels of *EtENO* were decreased by 36.3 and 40.36%, respectively, by diclazuril treatment. *EtENO* distributed in the surface, cytoplasm, and nucleus of the infected/control group. With diclazuril treatment, it was significantly reduced in the surface and cytoplasm and even disappeared in the nucleus of the infected/diclazuril group. These observations suggested that *EtENO* may play an important role in mechanism of diclazuril anticoccidial action and be a potential drug target for the intervention with *E. tenella* infection.

Key words: enolase, *Eimeria tenella*, diclazuril, glycolysis, drug target

2020 Poultry Science 99:6402–6409

<https://doi.org/10.1016/j.psj.2020.09.068>

INTRODUCTION

Obligate intracellular apicomplexan parasites cause humans and animal diseases (Fernández et al., 2012; Marugan-Hernandez et al., 2017). This phylum contains various parasitic protists, including zoonotic pathogens such as *Plasmodium*, *Cryptosporidium*, and *Toxoplasma* (Yang and Arrizabalaga, 2017) and pathogens (e.g., *Babesia*, *Theileria* and *Eimeria*) that exclusively infect livestock and poultry (Marugan-Hernandez et al., 2017). Among them, the latter apicomplexan parasites cause a serious effect on animal health and production. Coccidiosis is caused by *Eimeria* spp. infection in chicken intestines. *Eimeria* spp. has 7 species; of which, the most pathogenic is *Eimeria tenella*. These species

cause cecal coccidiosis (Suprihati and Yunus, 2018; Wang et al., 2019). *E. tenella* infects the chicken cecum, leading to weight loss, malabsorption, hemorrhage, cecal microenvironment disorder, and even death (Fernández et al., 2012; Marugan-Hernandez et al., 2017; Jia et al., 2020; Zhou et al., 2020). Anticoccidial drugs were widely used for the prevention and control of coccidiosis. Complete effective chemotherapeutic agents to control coccidiosis are few owing to the increasing problem of drug resistances. Thus, discovering and selecting a suitable drug target is essential for the development of new coccidiostats.

The life cycle of *E. tenella* consists of sporogony, schizogony, and gametogony stages. *E. tenella* requires extra-cellular invasive stages for cecal cell and intracellular proliferation (Labbé et al., 2006). To finish its life cycle, *E. tenella* must adjust its metabolism to the different living conditions. Energy metabolism is a necessary process of biological survival (Mi et al., 2017), and the energy source of *E. tenella* is largely dependent on anaerobic energy by glycolysis (Denton et al., 1996). Enolase, as a key enzyme in the glycolysis pathway, catalyzes the reversible

© 2020 Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received June 13, 2020.

Accepted September 23, 2020.

¹Corresponding author: zhoubh@haust.edu.cn

interconversion of 2-phospho-d-glycerate to phosphoenolpyruvate (Mi et al., 2017). In addition, enolase is a highly conserved and multifunctional protein in prokaryotes and eukaryotes, with a wide range of additional functions beyond its classical role in glycolysis (Liu et al., 2016; Mi et al., 2017).

On the cell surface of certain pathogens, enolase acts as a plasminogen receptor (Arce-Fonseca et al., 2018). For example, in *Leishmania Mexicana*, antienolase antibodies inhibited up to 60% of plasminogen binding on live parasites (Vanegas et al., 2007), and the enolase from *Taenia solium* was found capable of binding plasminogen and participating in parasite invasion together with other plasminogen-binding proteins (Ayón-Núñez et al., 2018b). Furthermore, enolase is involved in host cell invasion through pathogenic microorganisms. In *Plasmodium falciparum*, neutralization of enolase on the cell surface of merozoites and ookinetes may inhibit the host cell invasions at erythrocyte and transmission stages (Dutta et al., 2018). Enolase also acts as a candidate antigen of immune diagnosis in parasites, such as *Toxoplasma gondii* (Jiang et al., 2016) and *T. solium* (Ponce et al., 2018). In *E. tenella*, enolase (*EtENO*) was involved in adaptation of the metabolism to the intracellular anaerobic development (Labbé et al., 2006), and it was identified as an immunogenic protein in second-generation merozoites (Liu et al., 2009).

Diclazuril is an effective phenylacetone nitrile anticoccidial drug that has long been used to control coccidiosis caused by *E. tenella* (Zhou et al., 2019). Here, *EtENO* gene was cloned and expressed in *Escherichia coli* competent cells. In addition, the mRNA and protein expression levels of *EtENO* were identified using diclazuril treatment, and the spatial position of *EtENO* was observed through immunofluorescence.

MATERIALS AND METHODS

Inoculum Preparation

Luoyang strain *E. tenella* oocysts were provided by Veterinary Pharmacology Laboratory in Henan University of Science and Technology. Oocysts were propagated, isolated, sporulated, and placed in 2.5% $K_2Cr_2O_7$ solution. Before inoculation, the $K_2Cr_2O_7$ was removed via repeated centrifugation. The precipitated sporulated oocysts were diluted with distilled water.

Diclazuril

Diclazuril (>99%, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences) was administered to chickens through the diet at a dose of 1 mg/kg.

Experimental Animals and Treatment

One-day-old male Chinese yellow broiler chickens were purchased from a commercial hatchery of Luoyang, China. The chickens were kept in wire-floored cages,

housed in an oocyst-free environment, allowed free drinking water, and fed with a diet without any anticoccidial drug.

On day 12, 120 chickens were randomly divided into 2 groups ($n = 60$), with 3 biological replicates per group ($n = 20$). 1) In the infected/control group, the chickens were inoculated with a dose of 8×10^4 sporulated oocysts/chicken by oral infection and administered with normal feed without any anticoccidial drugs. 2) In the infected/diclazuril group, the chickens were inoculated with a dose of 8×10^4 sporulated oocysts/chicken by oral infection and administered with 1 mg/kg diclazuril at 96 h to 120 h after inoculation. The experimental scheme conformed strictly to the guidelines of the Institutional Animal Care and Use Committee (No. 201) of Henan University of Science and Technology (Luoyang, Henan, China).

Preparation of the Second-Generation Merozoites

Second-generation merozoites of *E. tenella* were obtained from chicken cecal tissues as previously described (Zhou et al., 2010a, 2012; Zhou et al., 2010a; Li et al., 2019). In brief, the chickens were euthanized 120 h after infection, and the parasitized caeca were incubated with hyaluronidase (Sigma) at 37°C for 60 min. The crude preparation of merozoites were filtrated and isolated from erythrocytes via lysis (0.155 mol/L NH_4Cl , 0.01 mol/L $KHCO_3$, 0.01 mmol/L EDTA, pH = 7.4) at 4°C for 10 min. After centrifugation was performed, the merozoite pellet was resuspended in 30% Percoll (Pharmacia) with PBS. Five volumes of this merozoite solution was layered gently onto one volume of high-density 50% Percoll with PBS and centrifuged at 2,200 g for 15 min. The lower aqueous layer was carefully collected and washed with PBS.

Preparation of Total RNA and cDNA

Total RNA of the second-generation merozoites was extracted using TRIzol reagent (Ambion, Shanghai) in accordance with the manufacturer's instructions. The purity and concentration of total RNA were measured by 1% agarose gel electrophoresis and Nanodrop 2000c spectrophotometer (Thermo scientific). cDNA was synthesized from the purified total RNA by using Easy-Script One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing).

Cloning of EtENO Gene

With cDNA as template, specific primers (P1-F: 5'-AGCCGACAGTCCCAGCGAAGATG-3', P1-R: 5'-AGTCTGTGGACAAATCGTGGGCA-3') was used to amplify the open reading frame gene fragment of *EtENO* via PCR at a 55°C annealing temperature. Amplified fragments were purified by electrophoresis and isolated using the Agarose Gel DNA Extraction

Table 1. Primer sequences with their corresponding PCR product size and position.

Gene	Primers (5' → 3')	Primer locations	Product (base pairs)	GenBank accession no.
18S RNA	ATCGCAGTTGGTCTTTTGG CCTGCTGCCTTCCTTAGATG	248-417	170	U67121
<i>EtENO</i>	AACCAGATTGGCTCCATCAC GCAGCAGCTGGTTGTACTIONG	1095-1296	202	AF353515.1

Abbreviation: *EtENO*, enolase of *Eimeria tenella*.

Kit (Takara, Beijing) following the manufacturer's instructions.

After fragment isolation, the purified PCR products were inserted into the pMD-19T Vector (Takara, Beijing). The recombinant cloned of pMD-19-*EtENO* was transformed into *E. coli* DH5 α competent cells (Takara, Beijing). The positive recombinant clone was sequenced by Shanghai Sangon Biotech Co. Ltd. The positive recombinant plasmids were extracted in accordance with the instructions of the TaKaRa MiniBEST Plasmid Purification Kit, version 4.0 (Takara, Beijing).

Expression and Purification of Recombinant *EtENO* Protein

With the positive recombinant plasmid (pMD-19-*EtENO*) as the template, the DNA fragments corresponding to the open reading frame of *EtENO* were amplified via PCR. The specific primers were as follows: P2-F: 5'-TGTGAATTCATGGTGGCCATAGTCGAG-3' and P2-R: 5'-CGTAAGCTTCTAGTTGGAGGGGTTTCG-

3' with *Eco*RI and *Hind*III restriction sites. The PCR products and pET-28a vector were double digestion by *Eco*RI and *Hind*III before ligation reaction. The recombinant expression plasmid pET-28a-*EtENO* was transformed into *E. coli* BL21 (DE3) competent cells (Biomed, Beijing). The bacteria containing the recombinant plasmid pET-28a-*EtENO* were induced with 0.5 mmol isopropyl- β -D-1-thiogalactopyranoside (IPTG) at 37°C to express the recombinant proteins. The supernatant of lysate was resolved on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The soluble recombinant *EtENO* (**rEtENO**) protein was enriched and purified under native conditions by using a His-tag Protein Purification Kit (Beyotime, Shanghai) following the manufacturer's instructions.

Polyclonal Antibody Production

The purified r*EtENO* proteins were used as antigen for the generation of polyclonal anti-*EtENO* sera. New Zealand white rabbits were immunized via subcutaneous

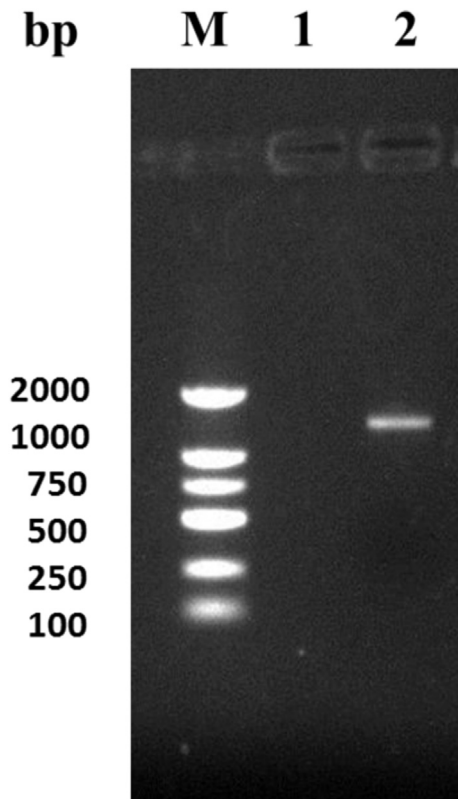


Figure 1. Agarose gel of *EtEno* RT-PCR products of 1,427 bp fragment of *Eimeria tenella*. Abbreviations: M, DL2000 DNA Marker; 1, Blank control; 2, PCR product; *EtENO*, enolase of *Eimeria tenella*.

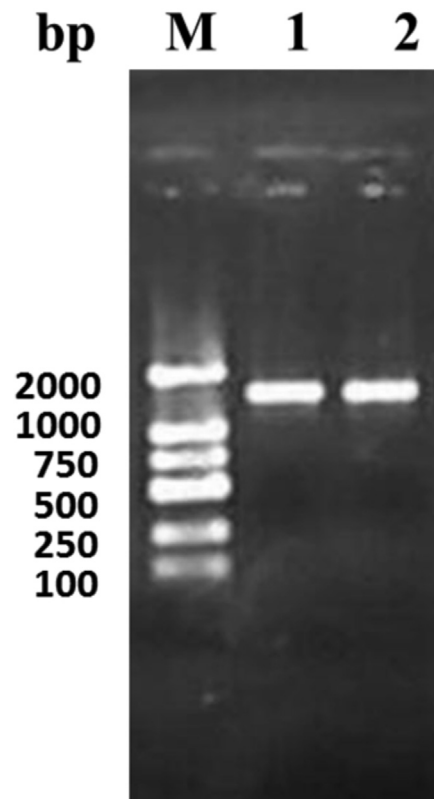


Figure 2. Identification of recombinant plasmid pMD-19-*EtENO* by PCR amplification. Abbreviations: M, DL2000 DNA Marker; 1 and 2, amplification by specified primer; *EtENO*, enolase of *Eimeria tenella*.

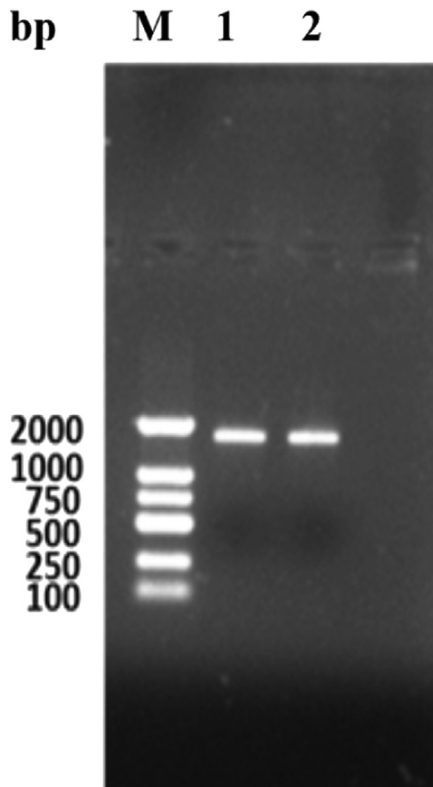


Figure 3. Agarose gel electrophoresis of pET-28a-*EtENO* PCR products. M, DL2000 DNA Marker; 1 and 2, PCR products.

injection of r*EtENO* protein emulsified with Freund's complete adjuvant (Sigma-Aldrich) at 500 µg/rabbit. Two weeks later, r*EtENO* protein emulsified with Freund's incomplete adjuvant (Sigma-Aldrich) was injected into rabbits as a secondary immunization. Subsequently, 2 booster immunizations were administered at an interval of 2 wk. Ten days after the final immunization, *EtENO* polyclonal anti-*EtENO* sera were collected, and the titers of polyclonal anti-*EtENO* sera were evaluated using ELISA.

Real-Time PCR Determination of *EtENO* mRNA Level

The mRNA expression level of *EtENO* was quantified by the CFX96 Touch real-time PCR system (Bio-Rad) and TB Green *Premix Ex Taq* GC (Perfect Real Time) (Takara, Beijing). The 18S rRNA of *E. tenella* acted as the control (Zhou et al., 2010c). The sequences of the primers are reported on Table 1. Each reaction was performed in triplicate, and the entire experiment was carried out in triplicate.

Western Blotting

For Western blotting, the purified merozoites were lysed using RIPA Lysis Buffer (Beyotime, Shanghai) and determined using the BCA protein assay kit (CWBI, Beijing) for concentration quantification. Equal amounts of protein samples in the infected/

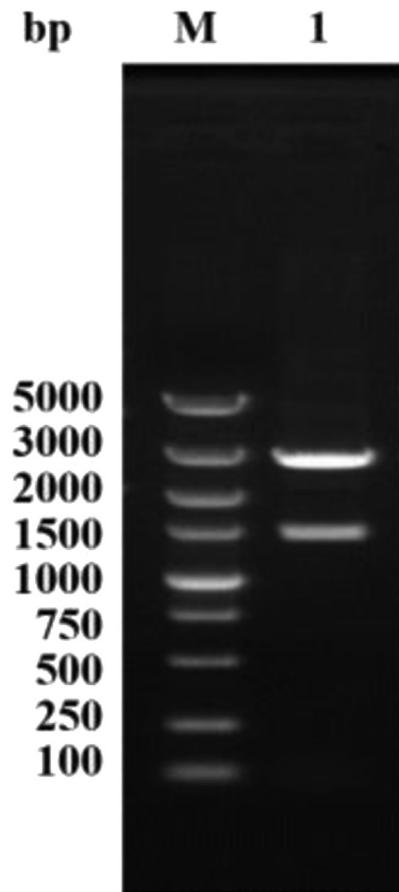


Figure 4. Agarose gel electrophoresis of *EcoR* I/*Hind* III restriction enzyme assay of recombinant expression plasmid pET-28a-*EtENO*. Abbreviations: M, DL2000 DNA Marker; 1, Enzymatic products; *EtENO*, enolase of *Eimeria tenella*.

control and infected/diclazuril groups were separated on 12% SDS-PAGE and subsequently transferred to a polyvinylidene fluoride membrane (Membrane Solutions). The separated proteins were detected using polyclonal rabbit anti-*EtENO* sera as the primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Solarbio, Beijing) as the secondary antibody. Horseradish peroxidase activity was revealed using enhanced chemiluminescence.

Immunofluorescence

The merozoites were resuspended in PBS, smeared on glass coverslips, and fixed with 4% paraformaldehyde (Servicebio, Wuhan) at room temperature. Then, they were permeabilized with 1% TritonX-100 (Sangon Biotech, Shanghai) and blocked with 2% BSA-PBS at 4°C overnight. Subsequently, the merozoites were subjected to incubation with polyclonal rabbit anti-*EtENO* sera (1:1000 dilution) for 1 h at 37°C and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Servicebio, Wuhan) (1:200 dilution) at 37°C in the dark for 1 h. After redyeing with 4',6'-diamidino-2-phenylindole (Boster, China) at room temperature for 30 min was conducted, 50 µL antifade mounting medium (Boster, China) was applied to close the coverslip before

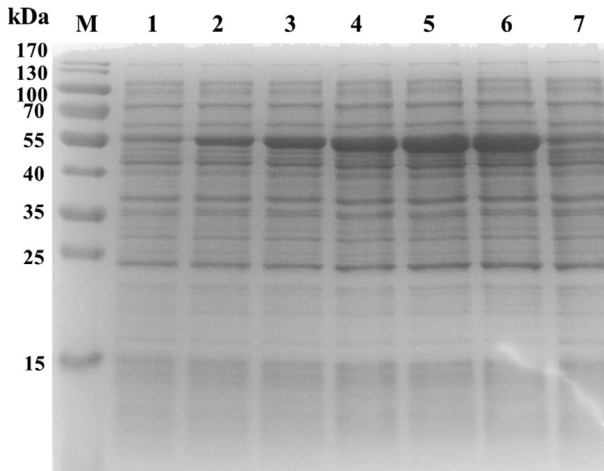


Figure 5. Expression of *EtENO* in *Escherichia coli* BL21 (DE3). M, Protein molecular weight Marker; 1~6, pET-28a-*EtENO* induced the expression of 0 h, 1 h, 2 h, 4 h, 6 h, and 8 h; 7, pET-28a-*EtENO* cultured for 8 h without induce. Abbreviation: *EtENO*, enolase of *Eimeria tenella*.

examination under a confocal laser scanning microscope (LSM 800, ZEISS).

Statistical Analysis

The data were expressed as the means \pm SD. Statistics were performed using SPSS (SPSS, version 21.0; IBM). Differences were considered to be statically significant when $P < 0.05$.

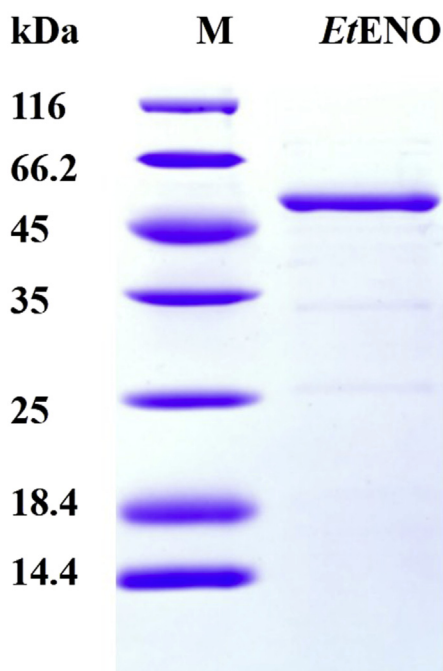


Figure 6. SDS-PAGE of purified r*EtENO* protein. Abbreviation: r*EtENO*, recombinant enolase of *Eimeria tenella*.

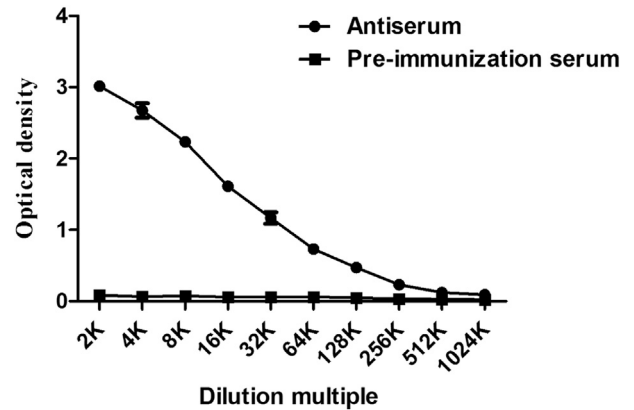


Figure 7. Antiserum *EtENO* antibody levels tested by ELISA. Abbreviation: *EtENO*, enolase of *Eimeria tenella*.

RESULTS

Cloning and Amplification the *EtENO* Gene

As shown in Figure 1, 1,417-bp *EtENO* gene amplification products were obtained. The purified PCR products were cloned into the pMD-19-T vector and analyzed via electrophoresis (Figure 2).

EtENO Protein Expression

The recombinant expression plasmid pET-28a-*EtENO* was identified via electrophoresis (Figure 3). pET-28a-*EtENO* was double digested using *EcoR* I/*Hind* III (Figure 4). The r*EtENO* protein in *E. coli* BL21 (DE3) was analyzed using SDS-PAGE (Figure 5). The results showed that it was solvable fusion protein with theoretical molecular weight of 51.97 kDa (Figure 6).

Anti-*EtENO* Polyclonal Serum Titer Assay

As shown in Figure 7, with preimmunization serum as negative control, no reaction was found when dilution of

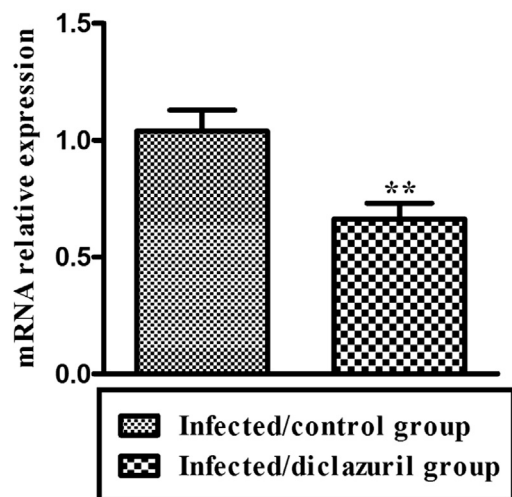


Figure 8. Quantitative real-time PCR analyze the mRNA relative expression level of *EtENO* in second-generation merozoite of *E. tenella*. Abbreviation: *EtENO*, enolase of *Eimeria tenella*.

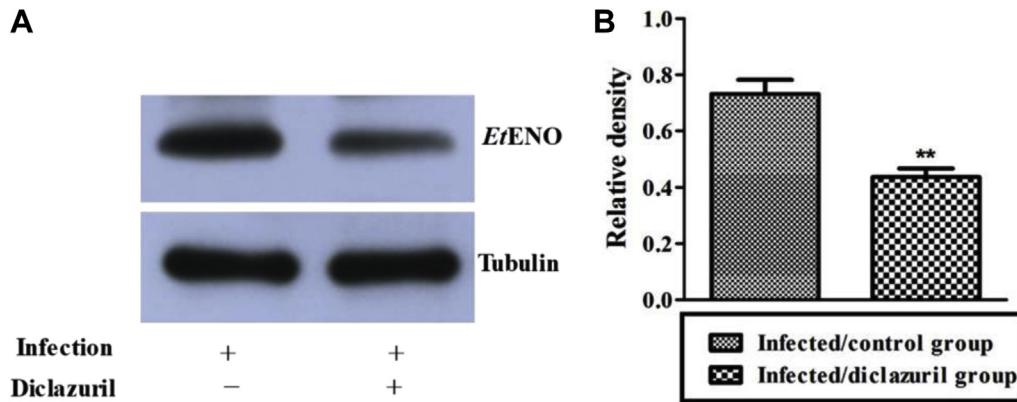


Figure 9. Western blot analysis of the expression of *EtENO*. (A) Western blot electrophoretic pattern. (B) *EtENO* relative expression levels. ** $P < 0.01$ indicated statistically significant differences. Abbreviation: *EtENO*, enolase of *Eimeria tenella*.

serum was more than 1:2,000. The titer of anti-*EtENO* serum was more than 1:128,000. The optical density was higher than 0.5.

Expression of *EtENO* mRNA

The *EtENO* mRNA expression in the infected/diclazuril group was significantly decreased by 36.3% compared with that in the infected/control group (Figure 8, $P < 0.01$).

Western Blotting

As shown in Figure 9A, *EtENO* exhibited obvious protein imprint expression (Figure 9A). The protein expression in the infected/diclazuril group was decreased by

40.36% ($P < 0.01$) (Figure 9B) compared with that in the infected/control group.

Localization of *EtENO* in Second-Generation Merozoites

The results of immunofluorescence analysis showed that considerable *EtENO* immunostaining (green fluorescence) appeared to be at the surface, cytoplasm, and nucleus of the second-generation merozoites in the infected/control group. In the infected/diclazuril group, the *EtENO* immunostaining was significantly lessened in the surface and cytoplasm and even disappeared in the nucleus compared with that in the infected/control group (Figure 10).

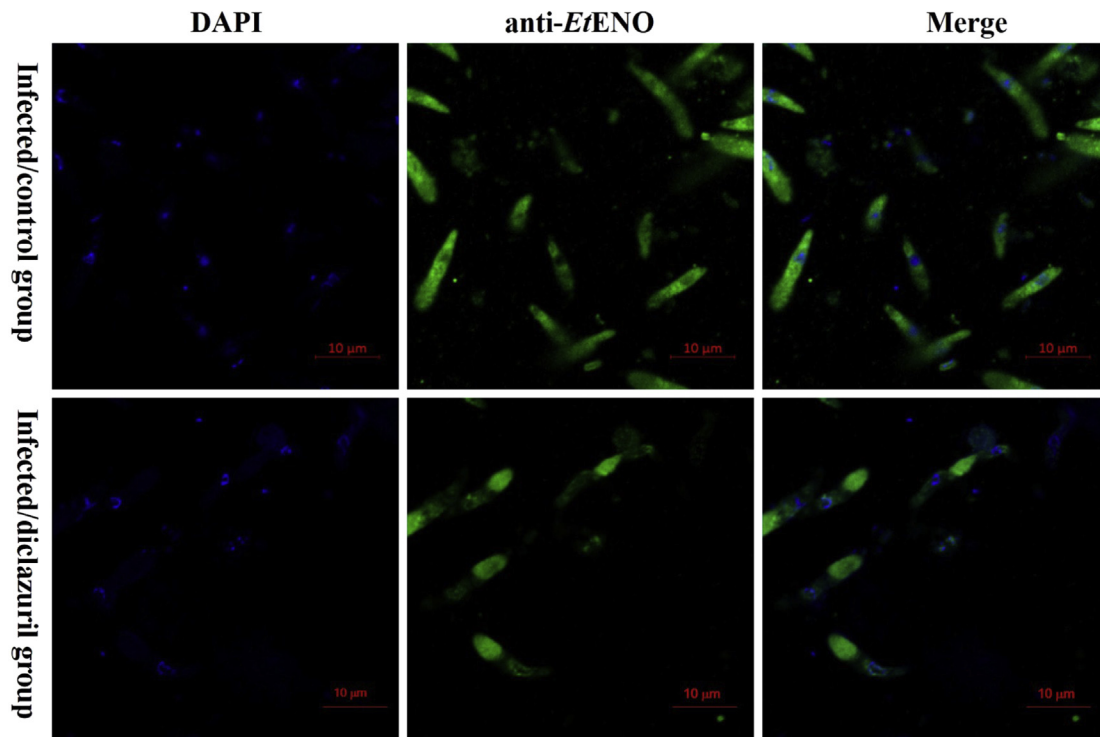


Figure 10. Immunolocalization of *EtENO* in second-generation merozoites. DAPI staining (blue) was used to detect parasite nuclei. Merge was an overlay of anti-*EtENO* and DAPI staining. Abbreviation: *EtENO*, enolase of *Eimeria tenella*.

DISCUSSION

The related proteins involved in host cell invasion and energy metabolism played an important role in the infection and survival of *E. tenella* in chicken cecum cells. Studies showed that enolase is a multifunctional protein; it is not only a glycolytic enzyme but also a participant in many important processes, such as invasion (Avilán et al., 2011), immunoprotection (Liu et al., 2017), gene regulation (Liu et al., 2016), development, and reproduction (Ji et al., 2016). Studying the *EtENO* gene could provide a theoretical basis for exploring the mechanism of infection and invasion of host cells by *E. tenella*. In addition, it could provide some potential therapeutic strategies for the control of *E. tenella*.

E. tenella uses anaerobic glycolysis during schizogony. The proteins involved in ATP production may produce energy for invasion and requirement by invasive merozoites (Lal et al., 2009). As a key enzyme in glycolysis and gluconeogenesis, enolase catalyzes the reversible dehydration of 2-phospho-d-glycerate to phosphoenolpyruvate in the presence of magnesium ions. In the present study, the *EtENO* mRNA and protein expression levels were reduced after diclazuril treatment. Thus, diclazuril may affect the expression of *EtENO*, thereby affecting the energy required by *E. tenella* invasion to cecum cells through glycolysis. Furthermore, diclazuril reduced the number of second-generation merozoites of *E. tenella* and alleviated the cecal damage (Zhou et al., 2010b; Tian et al., 2014), which was also associated with the downregulation of the expression of *EtENO*, subsequently reducing the energy access of *E. tenella*.

In addition to energy supply, enolase participates in other functions of the parasites through spatial location. Enolase is involved in the invasion of parasites as a plasminogen-binding protein (Figueiredo et al., 2015; Aguayo-Ortiz et al., 2017; Ayón-Núñez et al., 2018a, 2018b). Labbé et al. (2006) reported that *EtENO* was partially observed inside the nucleus of sporozoites and schizonts, thus suggesting an involvement in the control of gene regulation. Liu et al. (2016) reported that *EtENO* mainly appeared at the apical end of merozoites, indicating that enolase may participate in the parasite invasion process. In the present study, *EtENO* was observed at the surface and cytoplasm and inside the nucleus of merozoites, suggesting that surface-associated *EtENO* may also participate in the attachment and invasion process of *E. tenella*. The *EtENO* at the cytoplasm mainly provides energy through glycolysis process for the survival of *E. tenella*. With diclazuril treatment, the localization of *EtENO* in nucleus of merozoites almost disappear in the infected/diclazuril group, indicating that *EtENO* may be involved in gene regulation in the proliferation of *E. tenella*.

Furthermore, studies have shown that enolase as a promising vaccine candidate against parasite disease, such as Chagas disease (Arce-Fonseca et al., 2018), trichomonosis (Mirasol-Meléndez et al., 2018), and hydatid disease (Pourseif et al., 2019). The enolase from in *T.*

solium metacestode showed a potential use in the immunodiagnosis for porcine cysticercosis (Ponce et al., 2018). In *Trypanosoma cruzi*, enolase was proposed as a key protein essential for the survival of the parasite and has been used as a drug development target (Valera-Vera et al., 2020). In summary, diclazuril could downregulate *EtENO* expression in merozoites. In view of the role of enolase in parasite infection, immunoregulation, and energy metabolism, *EtENO* is likely to be a potential drug target for the prevention and control of *E. tenella* infection.

ACKNOWLEDGMENTS

This research was funded by National Natural Science Foundation of China (Grants Nos. 31101855 and 31472238) and Natural Science Foundation of Henan (Grant No. 202300410120). This funding body provided financial support only and did not have any involvement in the study design, collection, analysis, and interpretation of data.

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

- Aguayo-Ortiz, R., P. Meza-Cervantez, R. Castillo, A. Hernández-Campos, L. Dominguez, and L. Yépez-Mulia. 2017. Insights into the *Giardia intestinalis* enolase and human plasminogen interaction. *Mol. Biosyst.* 13:2015–2023.
- Arce-Fonseca, M., M. C. González-Vázquez, O. Rodríguez-Morales, V. Graullera-Rivera, A. Aranda-Fraustro, P. A. Reyes, A. Carabarin-Lima, and J. L. Rosales-Encina. 2018. Recombinant enolase of *Trypanosoma cruzi* as a novel vaccine candidate against chagas disease in a mouse model of acute infection. *J. Immunol. Res.* 2018:8964085.
- Avilán, L., M. Gualdrón-López, W. Quiñones, L. González-González, V. Hannaert, P. A. Michels, and J. L. Concepción. 2011. Enolase: a key player in the metabolism and a probable virulence factor of trypanosomatid parasites-perspectives for its use as a therapeutic target. *Enzyme Res.* 2011:932549.
- Ayón-Núñez, D. A., G. Fragoso, R. J. Bobes, and J. P. Lacleste. 2018a. Plasminogen-binding proteins as an evasion mechanism of the host's innate immunity in infectious diseases. *Biosci. Rep.* 38. BSR20180705.
- Ayón-Núñez, D. A., G. Fragoso, C. Espitia, M. García-Varela, X. Soberón, G. Rosas, J. P. Lacleste, and R. J. Bobes. 2018b. Identification and characterization of *Taenia solium* enolase as a plasminogen-binding protein. *Acta Trop.* 182:69–79.
- Denton, H., S. M. Brown, C. W. Roberts, J. Alexander, V. McDonald, K. W. Thong, and G. H. Coombs. 1996. Comparison of the phosphofructokinase and pyruvate kinase activities of *Cryptosporidium parvum*, *Eimeria tenella* and *Toxoplasma gondii*. *Mol. Biochem. Parasitol.* 76:23–29.
- Dutta, S., A. Tewari, C. Balaji, R. Verma, A. Moitra, M. Yadav, P. Agrawal, D. Sahal, and G. K. Jarori. 2018. Strain-transcending neutralization of malaria parasite by antibodies against *Plasmodium falciparum* enolase. *Malar. J.* 17:304.
- Fernández, M. L. S., K. K. Engels, F. Bender, M. Gassel, R. J. Marhöfer, J. C. Mottram, and P. M. Selzer. 2012. High-throughput screening with the *Eimeria tenella* CDC2-related kinase2/cyclin complex EtCRK2/EtCYC3a. *Microbiology* 158:2262–2271.
- Figueiredo, B. C., A. A. Da'dara, S. C. Oliveira, and P. J. Skelly. 2015. Schistosomes enhance plasminogen activation: the role of tegumental enolase. *PLoS Pathog.* 11:e1005335.

- Ji, H., J. Wang, J. Guo, Y. Li, S. Lian, W. Guo, H. Yang, F. Kong, L. Zhen, L. Guo, and Y. Liu. 2016. Progress in the biological function of alpha-enolase. *Anim. Nutr.* 2:12–17.
- Jia, L., B. Zhou, H. Wang, F. Yang, G. Wang, and T. Kong. 2020. Cytokine gene expression and IgA production in the spleen of chickens infected with *Eimeria tenella*, India. *J. Anim. Res.* <https://doi.org/10.18805/ijar.B-1188>.
- Jiang, W., J. X. Xue, Y. C. Liu, T. Li, X. G. Han, S. H. Wang, Y. J. Chen, J. Qi, S. Q. Yu, and Q. Wang. 2016. Identification and characterization of an immunogenic antigen, enolase 2, among excretory/secretory antigens (ESA) of *Toxoplasma gondii*. *Protein Expr. Purif.* 127:88–97.
- Labbé, M., M. Péroval, C. Bourdieu, F. Girard-Misguich, and P. Péry. 2006. *Eimeria tenella* enolase and pyruvate kinase: a likely role in glycolysis and in others functions. *Int. J. Parasitol.* 36:1443–1452.
- Lal, K., E. Bromley, R. Oakes, J. H. Prieto, S. J. Sanderson, D. Kurian, L. Hunt, J.R. Yates, 3rd, J. M. Wastling, R. E. Sinden, and F. M. Tomley. 2009. Proteomic comparison of four *Eimeria tenella* life-cycle stages: unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. *Proteomics* 9:4566–4576.
- Li, X. Y., L. L. Liu, M. Zhang, L. F. Zhang, X. Y. Wang, M. Wang, K. Y. Zhang, Y. C. Liu, C. M. Wang, F. Q. Xue, and C. Z. Fei. 2019. Proteomic analysis of the second-generation merozoites of *Eimeria tenella* under nitroimidazole and ethanamide stress. *Parasit. Vectors* 12:592.
- Liu, L. L., Z. G. Chen, R. S. Mi, K. Y. Zhang, Y. C. Liu, W. Jiang, C. Z. Fei, F. Q. Xue, and T. Li. 2016. Effect of Acetamizuril on enolase in second-generation merozoites of *Eimeria tenella*. *Vet. Parasitol.* 215:88–91.
- Liu, L., L. Xu, F. Yan, R. Yan, X. Song, and X. Li. 2009. Immunoproteomic analysis of the second-generation merozoite proteins of *Eimeria tenella*. *Vet. Parasitol.* 164:173–182.
- Liu, X., C. Zheng, X. Gao, J. Chen, and K. Zheng. 2017. Complete molecular and immunoprotective characterization of *Babesia microti* enolase. *Front. Microbiol.* 8:622.
- Marugan-Hernandez, V., E. Long, D. Blake, C. Crouch, and F. Tomley. 2017. *Eimeria tenella* protein trafficking: differential regulation of secretion versus surface tethering during the life cycle. *Sci. Rep.* 7:4557.
- Mi, R., X. Yang, Y. Huang, L. Cheng, K. Lu, X. Han, and Z. Chen. 2017. Immunolocalization and enzyme activity analysis of *Cryptosporidium parvum* enolase. *Parasit. Vectors* 10:273.
- Mirasol-Meléndez, E., L. G. Brieba, C. Díaz-Quezada, M. López-Hidalgo, E. E. Figueroa-Angulo, L. Ávila-González, R. Arroyo-Verástegui, and C. G. Benítez-Cardoza. 2018. Characterization of multiple enolase genes from *Trichomonas vaginalis*. Potential novel targets for drug and vaccine design. *Parasitol. Int.* 67:444–453.
- Ponce, R., N. León-Janampa, R. H. Gilman, R. Liendo, E. Roncal, S. Luis, S. Quiñones-García, Z. Silverstein, H. H. García, A. Gonzales, P. Sheen, M. Zimic, and M. J. Pajuelo. 2018. A novel enolase from *Taenia solium* metacestodes and its evaluation as an immunodiagnostic antigen for porcine cysticercosis. *Exp. Parasitol.* 191:44–54.
- Pourseif, M. M., M. Yousefpour, M. Aminianfar, G. Moghaddam, and A. Nematollahi. 2019. A multi-method and structure-based in silico vaccine designing against *Echinococcus granulosus* through investigating enolase protein. *Bioimpacts* 9:131–144.
- Suprihati, E., and M. Yunus. 2018. Evaluation of the antigenicity and immunogenicity of *Eimeria tenella* by reproductive index and histopathological changes of cecal coccidiosis virulent live vaccing in broiler chickens. *Afr. J. Infect. Dis.* 12:104–110.
- Tian, E. J., B. H. Zhou, X. Y. Wang, J. Zhao, W. Deng, and H. W. Wang. 2014. Effect of diclazuril on intestinal morphology and SIgA expression in chicken infected with *Eimeria tenella*. *Parasitol. Res.* 113:4057–4064.
- Valera-Vera, E. A., M. Sayé, C. Reigada, M. R. Miranda, and C. A. Pereira. 2020. In silico repositioning of etidronate as a potential inhibitor of the *Trypanosoma cruzi* enolase. *J. Mol. Graph. Model.* 95:107506.
- Vanegas, G., W. Quiñones, C. Carrasco-López, J. L. Concepción, F. Albericio, and L. Avilán. 2007. Enolase as a plasminogen binding protein in *Leishmania mexicana*. *Parasitol. Res.* 101:1511–1516.
- Wang, X., W. Zou, H. Yu, Y. Lin, G. Dai, T. Zhang, G. Zhang, K. Xie, J. Wang, and H. Shi. 2019. RNA sequencing analysis of chicken cecum tissues following *Eimeria tenella* infection in vivo. *Genes (Basel)* 10:420.
- Yang, C., and G. Arrizabalaga. 2017. The serine/threonine phosphatases of apicomplexan parasites. *Mol. Microbiol.* 106:1–21.
- Zhou, B. H., L. S. Jia, H. W. Guo, H. Y. Ding, J. Y. Yang, and H. W. Wang. 2019. Eukaryotic elongation factor 2 is involved in the anticoccidial action of diclazuril in the second-generation merozoites of *Eimeria tenella*. *Vet. Parasitol.* 276:108991.
- Zhou, B. H., L. S. Jia, S. S. Wei, H. Y. Ding, J. Y. Yang, and H. W. Wang. 2020. Effects of *Eimeria tenella* infection on the barrier damage and microbiota diversity of chicken cecum. *Poult. Sci.* 99:1297–1305.
- Zhou, B. H., X. J. Shen, H. W. Wang, T. Li, and F. Q. Xue. 2012. Receptor for activated C kinase ortholog of second-generation merozoite in *Eimeria tenella*: clone, characterization, and diclazuril-induced mRNA expression. *Parasitol. Res.* 111:1447–1455.
- Zhou, B. H., H. W. Wang, X. Y. Wang, L. F. Zhang, K. Y. Zhang, and F. Q. Xue. 2010b. *Eimeria tenella*: effects of diclazuril treatment on microneme genes expression in second-generation merozoites and pathological changes of caeca in parasitized chickens. *Exp. Parasitol.* 125:264–270.
- Zhou, B., H. Wang, F. Xue, X. Wang, C. Fei, M. Wang, T. Zhang, X. Yao, and P. He. 2010a. Effects of diclazuril on apoptosis and mitochondrial transmembrane potential in second-generation merozoites of *Eimeria tenella*. *Vet. Parasitol.* 168:217–222.
- Zhou, B. H., H. W. Wang, F. Q. Xue, X. Y. Wang, F. K. Yang, M. M. Ban, R. X. Xin, and C. C. Wang. 2010c. Actin-depolymerizing factor of second-generation merozoite in *Eimeria tenella*: clone, prokaryotic expression, and diclazuril-induced mRNA expression. *Parasitol. Res.* 106:571–576.