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Eukaryotic elongation factor 2 is involved in the anticoccidial action of diclazuril in the second-generation merozoites of *Eimeria tenella*

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ABSTRACT

Eimeria tenella, an obligate intracellular parasite, can actively invade the cecal epithelial cells of chickens and cause severe enteric disease. Eukaryotic elongation factor 2 (eEF2) plays a major role in protein synthesis and cell survival. This study aims to explore the exact mechanisms underlying diclazuril inhibition in second-generation merozoites of *E. tenella*. The eEF2 cDNA of the second-generation merozoites of *E. tenella* (*Et*EF2) was cloned by reverse transcriptase polymerase chain reaction and rapid amplification of cDNA ends. Diclazuril-induced expression profiles of *Et*EF2 were also analyzed. The cloned full-length cDNA (2893 bp) of the *Et*EF2 nucleotide sequence encompassed a 2499 bp open reading frame (ORF) that encoded a polypeptide of 832 residues with an estimated molecular mass of 93.12 kDa and a theoretical isoelectric point of 5.99. The *Et*EF2 nucleotide sequence was submitted to the GenBank database with the accession number KF188423. The *Et*EF2 protein sequence shared 99 % homology with the eEF2 sequence of *Toxoplasma gondii* (GenBank XP_002367778.1). The GTPase activity domain and ADP-ribosylation domain were conserved signature sequences of the eEF2 gene family. The changes in the transcriptional and translational levels of *Et*EF2 were detected through quantitative real-time PCR and Western blot analyses. The mRNA expression level of *Et*EF2 was 2.706 fold increases and the protein level of *Et*EF2 was increased 67.31 % under diclazuril treatment. In addition, the localization of *Et*EF2 was investigated through immunofluorescence assay. Experimental results demonstrated that *Et*EF2 was distributed primarily in the cytoplasm of second-generation merozoites, and its fluorescence intensity was enhanced after diclazuril treatment. These findings indicated that *Et*EF2 may have an important role in understanding the signaling mechanism underlying the anticoccidial action of diclazuril and could be a promising target for novel drug exploration.

1. Introduction

Translation, as an essential process for protein synthesis, could rapidly alter protein production by cells and thus reduces time and energy consumption for new mRNA transcription (Rennie, 2005; Taha et al., 2013). Translation involves three steps, namely, initiation, elongation, and termination, and each step requires specific factors and energy investment for high regulation (Holcik and Sonenberg, 2005; Richter and Klann, 2009). The elongation phase includes two protein factors, namely, eukaryotic elongation factor (eEF) 1 and eEF2 (Shi et al., 2018). As a recruiter, eEF1 guides new aminoacyl-tRNA to its position at the ribosomal aminoacyl-site (Vislovukh et al., 2013). eEF2 drives peptidyl-tRNA from the aminoacyl site to the peptidyl site on the ribosome during protein synthesis by catalyzing GTP hydrolysis-dependent translocation (Liu and Proud, 2016; Susorov et al., 2018). eEF2 is not only a necessary factor for protein synthesis (Watanabe et al., 2003), but also cell survival, such as cellular damage repair (Wang et al., 2011), and cell proliferation (Watanabe et al., 2003; Malavé and Forney., 2004) signal transduction (McCamphill et al., 2017), apoptosis (Holcik and Sonenberg, 2005).

Infection with *Eimeria tenella*, an apicomplexan parasite, causes serious coccidiosis in chickens and results in appetite loss, malnutrition, weight loss, anemia, hematodiarrhea, and even death (Marugan-Hernandez et al., 2016). *E. tenella* must actively invade cecal epithelial cells to complete its normal life history because it is an obligate intracellular parasite (Liu et al., 2016). *E. tenella* has a complex life cycle that includes exogenous and endogenous stages (Zhou et al., 2010a). In the endogenous stage of *E. tenella*, the large number of merozoites

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released from lysed host cells must actively reinvade other new cells to continue their development and eventually cause invasive injury to the cecal tissue (Han et al., 2015; Chen et al., 2018). Current conventional control strategies for coccidiosis mainly depend on chemical anticoccidial drugs (Tian et al., 2017). However, long-term use of drugs leads to production of drug-resistant parasites. Thus, new anticoccidial strategies are urgently needed. Diclazuril is a benzene/acetonitrile anticoccidial agent that has been proven to be effective against *E. tenella* in the asexual and sexual stages (El-Banna et al., 2005; Nodeh et al., 2008; Shen et al., 2012; Zhou et al., 2013). However, the underlying mechanism of diclazuril inhibiting second-generation merozoites remains unclear. Screening the exact targets of diclazuril against *E. tenella* could provide a basis for anticoccidian drug development.

Differential gene expression of *E. tenella* eEF2 (*Et*EF2) was screened in our previous study by constructing a diclazuril anticoccidial cDNA library through the suppression of subtractive hybridization (data not shown). The cDNA of the eEF2 gene was cloned from a variety of multicellular eukaryotic organisms (Kohno et al., 1986; Oleinikov et al., 1989; Rapp et al., 1989; Kim et al., 1993; Satyamoorthy and Howe, 1997; Wang et al., 2011). However, the sequence profile and expression characteristics of *Et*EF2 have been rarely reported. In the present study, the complete sequence of *Et*EF2 was cloned, and its molecular composition, phylogenetic properties, and diclazuril-induced expression patterns were analyzed. Results will establish a foundation for understanding the molecular mechanisms of *Et*EF2 in the invasion of *E. tenella* and exploring the mechanism of the anticoccidian action of diclazuril.

2. Materials and methods

2.1. Preparation of inoculum

The Luoyang strain of *E. tenella* oocysts were propagated, harvested, cleaned, sporulated, and maintained in 2.5 % potassium dichromate ($K_2Cr_2O_7$) solution in accordance with the standard processes (Zhou et al., 2010b). Sporulated oocysts were rinsed with distilled water before inoculation and counted using a cytometer.

2.2. Reagents

Diclazuril (> 99 %) was provided by Shanghai Veterinary Research Institute, Chinese Academy of Agriculture Sciences and administered at a dose of 1 mg/kg in chicken feed.

2.3. Chickens and treatment

A total of 300 day-old male Hy-Line Variety Brown layer chickens were purchased from Luoyang Yukou Poultry Industry Co., Ltd (Luoyang, China). The chickens were maintained in a standard animal house. The experimental scheme was approved by the Ethics Committee of the Faculty of Veterinary Medicine and strictly conformed to the guidelines of the Institutional Animal Care and Use Committee of China.

As our report previously (Zhou et al., 2010a), one hundred eighty 14-day-old chickens were selected according to their weight and randomly numbered. Then group by drawing lots. It was randomly divided into two groups ($n = 90$), control group and diclazuril group, with three replicates per group ($n = 30$). Therein, control group (fed with normal diet) and diclazuril group (fed with 1 mg/kg of diclazuril diet continuously from 96 h to 120 h after inoculation). All chickens were orally inoculated with 8×10^4 *E. tenella* sporulated oocysts.

2.4. Extraction of second-generation merozoites

At 120 h post inoculation, cecal tissues from 15 randomly selected chichens pooled in each replicate were used for preparation of the second-generation merozoites as previously described (Zhou et al., 2010c, 2013). Briefly, merozoites were harvested through hyaluronidase digestion at 37 °C for 90 min, erythrocyte lysis [erythrocyte lysis buffer: 0.155 mol/L NH_4Cl, 0.01 mol/L $KHCO_3$, 0.01 mmol/L EDTA, (pH7.4)] at 4 °C for 10 min with intermittent agitation, and Percoll density gradient centrifugation at 2200 *g* for 15 min. The collected merozoites sample was subjected for RNA preparation, Western blot and immunofluorescence assay, respectively. Thus, there were three samples in each group for each experiment.

2.5. Extraction and purification of total RNA and synthesis of cDNA

Total RNA was extracted from second-generation merozoites by using TRIzol™ Reagent (15596026, Invitrogen, USA) in accordance with the manufacturer's instructions. The prepared total RNA samples were treated with RNase-free DNase I (40 U/mg, 9089, Takara, China) and purified with the RNeasy Mini Kit (74106, Qiagen, Germany) following the manufacturer's specifications. cDNA was synthesized from purified total RNA by using SuperScript™ II Reverse Transcriptase (18064071, Invitrogen, USA) and pd $(N)_6$ random hexamer primers. The cDNA was stored at −20 °C for use.

2.6. Molecular cloning of EtEF2 cDNA

The full-length cDNA sequence of *Et*EF2 was synthesized with SMARTer RACE cDNA Amplification Kit (634923, Clontech, USA) following the manufacturer's instructions and in accordance with the expressed sequence tag (EST) sequence of 554 bp obtained in our laboratory. In brief, 3′-RACE-ready cDNA was obtained, and each product was amplified by TaKaRa LA Taq HS (RR02MA, Takara, China). The specific primer for the 3′-RACE (GSP, 5′- ATGATGGAGCGGGCGAGCA GGAGTT-3′) was designed and synthesized based on the known EST sequence. The 3′-RACE cDNA fragments were combined with pMD19-T Vector (3271, Takara, China) and sequenced. The overlapping sequences of the 3′-RACE products were compared and assembled by applying Clustal W2 software (http://www.ebi.ac.uk/Tools/clustalw2/). The full-length cDNA sequence of *Et*EF2 was amplified through reverse transcriptase polymerase chain reaction (RT-PCR) with primers P1 (5′-ATGGTGAACTTTTCAGTGGATC-3′) and P2 (5′-TACAGCTTGTC GTAGTAGTGG-3′) by using first-strand cDNA. The PCR product was purified, inserted into the pMD19-T vector, transformed into *Escherichia coli* strain DH5α, and then sequenced. The positive plasmid was designated as pMD19-*Et*EF2.

2.7. Bioinformatics and molecular evolution analyses

The ORF and deduced amino acid sequences were obtained via the translate tool (https://web.expasy.org/translate/). The amino acid composition, hydrophobicity, theoretical molecular weight, and isoelectric point of the obtained sequences were analyzed by applying the ProtParam tool (http://cn.expasy.org/tools/protparam.html). Signal peptide sequences were detected by using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The conserved domain of *Et*EF2 was identified with CDD software (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict transmembrane regions. Structural domains were analyzed by utilizing Motif_scan software (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and homologous proteins were searched with BLASTp software (http://www.ncbi.nlm.nih.gov/BLAST/). CLUSTAL X 1.83 and MEGA 3.1 were applied to construct a phylogenetic tree of *Et*EF2 and other eEF2 sequences from GenBank (Table 1). Tree topology was evaluated with 1000 replication bootstraps.

2.8. Construction of the EtEF2 expression vector

The ORF of *Et*EF2 was amplified through PCR using the pMD-*Et*EF2

Table 1
GenBank accession numbers for EF2 amino acid sequences.

Abbreviation[a]	Species	Protein	Homology	Accession Number[b]
Et23	*Eimeria tenella*	elongation factor 2	100.00 %	KF188423
En99	*Eimeria necatrix*	elongation factor 2	98.91 %	CDJ69599.1
Ea41	*Eimeria acervulina*	elongation factor 2	98.44 %	CDI82841.1
Em23	*Eimeria maxima*	elongation factor 2	98.20 %	CDJ56123.1
Eb72	*Eimeria brunetti*	elongation factor 2	98.29 %	CDJ46472.1
Ep34	*Eimeria praecox*	elongation factor 2	93.64 %	CDI86234.1
Em94	*Eimeria mitis*	elongation factor 2	94.45 %	CDJ31294.1
Tg78	*Toxoplasma gondii*	elongation factor 2	84.13 %	XP_002367778.1
Nc68	*Neospora caninum*	elongation factor 2	83.89 %	XP_003882268.1
To15	*Theileria orientalis*	elongation factor 2	75.86 %	BAM39015.1
Bb59	*Babesia bovis*	elongation factor 2	76.23 %	XP_001608959.1
Ch02	*Cryptosporidium hominis*	elongation factor 2	76.80 %	XP_668002.1
Pv78	*Plasmodium vivax*	elongation factor 2	73.80 %	XP_001615878.1
Gn20	*Gregarina niphandrodes*	elongation factor 2	68.74 %	EZG63620.1
Ac89	*Acanthamoeba castellanii*	elongation factor 2	67.26 %	XP_004337289.1
Hs52	*Homo sapiens*	elongation factor 2	63.18 %	NP_001952.1
Mm33	*Mus musculus*	elongation factor 2	62.72 %	NP_031933.1
Rn41	*Rattus norvegicus*	elongation factor 2	62.72 %	NP_058941.1
Bt89	*Bos taurus*	elongation factor 2	62.83 %	NP_001068589.1
At38	*Arabidopsis thaliana*	elongation factor 2	62.28 %	AEE33338.1
Dm60	*Drosophila melanogaster*	elongation factor 2	64.10 %	P13060.4
Gg99	*Gallus gallus*	elongation factor 2	63.07 %	NP_990699.1

[a] Abbreviation used in alignment and/or phylogentic tree (Letters represent species initials, Numbers represent the last two digits of the Accession Number).
[b] Sequences were obtained by BLASTp from the nr database (GenBank).

plasmid as a template and specific primers P3 (5′-CTT**GAATTC**ATGGT GAACTTTTCAGTGGATC-3′) and P4 (5′-AGT**CTCGAG**TTACAGCTTGTC GTAGTAGTGG-3′) with *EcoR* I (1040S, Takara, China) and *Xho* I (1094S, Takara, China) sites at the 5′ and 3′ ends of the fragments, respectively. The PCR-amplified fragments were purified using gel extraction kits (9762, Takara, China), inserted into pET-28a vectors digested by restriction enzymes *EcoR* I and *Xho* I with T4 DNA ligase (D7006, Beyotime, China) at 25 °C, and cloned into *E. coli* strain DH5α. The positive plasmid (pET-28a-*Et*EF2) was identified by sequencing and subsequently transformed into *E. coli* strain DBL21 (DE3). Isopropyl-D-1-thiogalactopyranoside (IPTG) was used to induce *Et*EF2expression. His•Bind Purification® Kit (70239-3, Novagen, USA) was used to enrich and purify the recombinant *Et*EF2 (r*Et*EF2). The purified r*Et*EF2 was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.9. Preparation of polyclonal antibodies against EtEF2

Polyclonal antibodies were prepared with 5-week-old female BALB/c mice. The purified r*Et*EF2 protein was used as the antigen. Mice were injected with 100 μg of r*Et*EF2 emulsified in Freund complete adjuvant (344289, Sigma, USA) after 2 weeks and re-enhanced twice with 100 μg of r*Et*EF2 emulsified in Freund incomplete adjuvant (344291, Sigma, USA) at 2-week intervals. One week after the last immunization, serum was collected for determination of the antibody titer of polyclonal anti-*Et*EF2 through enzyme-linked immunosorbent assay.

2.10. Quantitative real-time PCR analysis

cDNA was applied as a template and the housekeeping gene 18S rRNA of *E. tenella* was used as the control (Zhou et al., 2010c). The mRNA level of the *Et*EF2 gene was quantified through quantitative real-time PCR (QRT-PCR) by using the RG-3000A real-time PCR system (RoterGene, USA) and TransStart Green qPCR SuperMix (AQ101-01, TransGen Biotech, China). The sequences of the primers are reported in Table 2. The specificity of amplification was confirmed by agarose gels with ethidium bromide and direct sequencing of the PCR products. Each reaction was performed in triplicate, and the entire experiment was carried out in triplicate.

2.11. Western blot analysis

Second-generation merozoites were suspended in phosphate-buffered solution (PBS) and lysed through ultrasonic method. The lysate of the total protein was quantified, loaded onto SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. Tubulin of *E. tenella* (*Et*Tubulin) was the control to normalize the *Et*EF2 protein expression. The membranes were blocked with 2 % bovine serum albumin (BSA, A8020, Solarbio, China) and incubated with *Et*EF2 antibody (1:800 dilution) or anti-β-tubulin monoclonal antibody (1:1000 dilution, K200059 M, Solarbio, China). The strips were subsequently treated with horseradish peroxidase-conjugated goat antimouse IgG (1:5000 dilution, CW0102, Cwbio, China). Peroxidase activity was detected with eECL Western Blot Kit (CW0049S, Cwbio, China).

2.12. Immunofluorescence assay

Fresh second-generation merozoites were rinsed with PBS, fixed on glass coverslips with 4 % paraformaldehyde, permeabilized with 1 % Triton X-100, and blocked with 2 % BSA. The merozoites were washed with PBS and incubated with *Et*EF2 antibody (1:100 dilution) followed by goat antimouse IgG conjugated with FITC (1:400 dilution, GB22301, Servicebio, China). The slides were incubated with 4, 6-diamino-2-phenyl indole (AR1176, Bosterbio, USA) and antifade mounting medium (AR1109, Bosterbio, USA) for examination under confocal laser scanning microscope (LSM800, Carl Zeiss AG).

2.13. Statistical analysis

All results were presented as mean ± standard deviation. Student's *t* test was performed for comparison of differences. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Cloning and analysis of the EtEF2 sequence

The 3′-RACE sequence of 2504 bp was amplified in accordance with the 554 bp EST sequence. A full-length *Et*EF2 DNA sequence of 2893 bp was obtained after splicing, assembly, and analysis with Clustal W2.

Table 2
Primer sequences with their corresponding PCR product size and position.

Gene	Primers(5′→3′)	Primer locations	Product (base pairs)	Genbank Accession No.
18S rRNA	F: ATCGCAGTTGGTTCTTTTGG R: CCTGCTGCCTTCCTTAGATG	248–417	170	U67121
*Et*EF2	F: TCGGTTGATGGTGTCTGTGT R: GACATTGCGCTCAAAAGTCA	512–667	156	KF188423

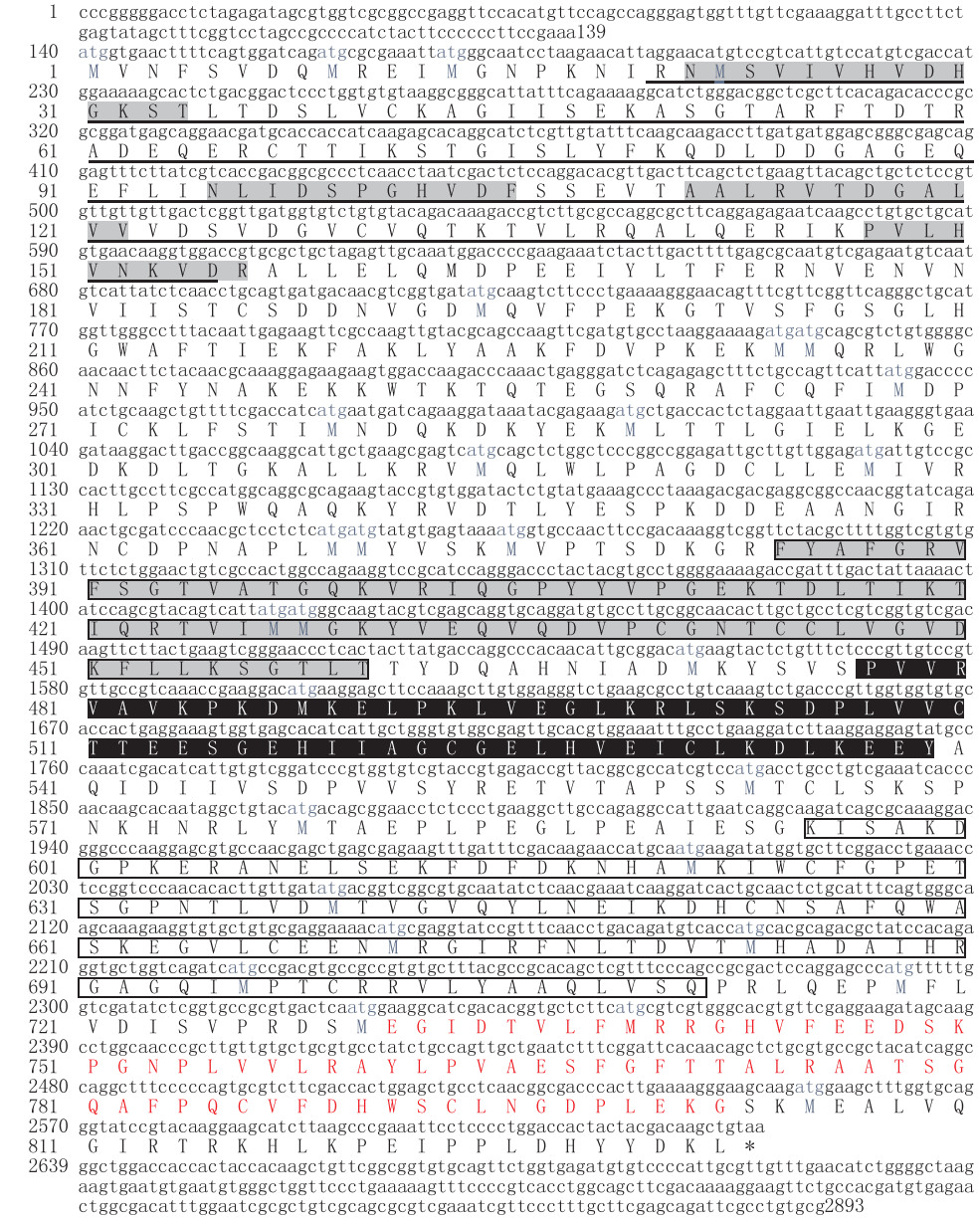

Fig. 1. Nucleotide and predicted amino acid sequences of *Et*EF2. GTP-binding *Et*EF2 signature motif sequences were shaded *gray with black lettering*; EFTU domain were shaded *gray with black box*; GTPase activity domains was *underline*; EFG-II domain was shaded *black with white lettering*; EFG-IV domain was shaded *black box*; EFG-III and EFG-V domains were marked with *red lettering*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The full-length *Et*EF2 sequence contained an ORF of 2499 bp and shared 99.81 % homology with the *E. tenella* (Houghton strain) genome sequence, which encodes a polypeptide of 832 residues with an estimated molecular mass of 93.12 kDa and a theoretical inoelectric point of 5.99. The motif scan indicated that the protein contained four GTP-binding elongation factors (aa 21–34, aa 95–105, aa 111–122, and aa 147–156), an EFTU domain (aa 384–460), and a GTPase activity domain (aa 20–155). EFG-II (aa 477–539), EFG-III/V (aa 731–802), and EFG-IV (aa 595–711) domains were also present in the sequence (Fig. 1). The deduced amino acid sequence lacked a signal peptide and transmembrane region.

Fig. 2 shows the multiple alignments of the deduced *Et*EF2 amino

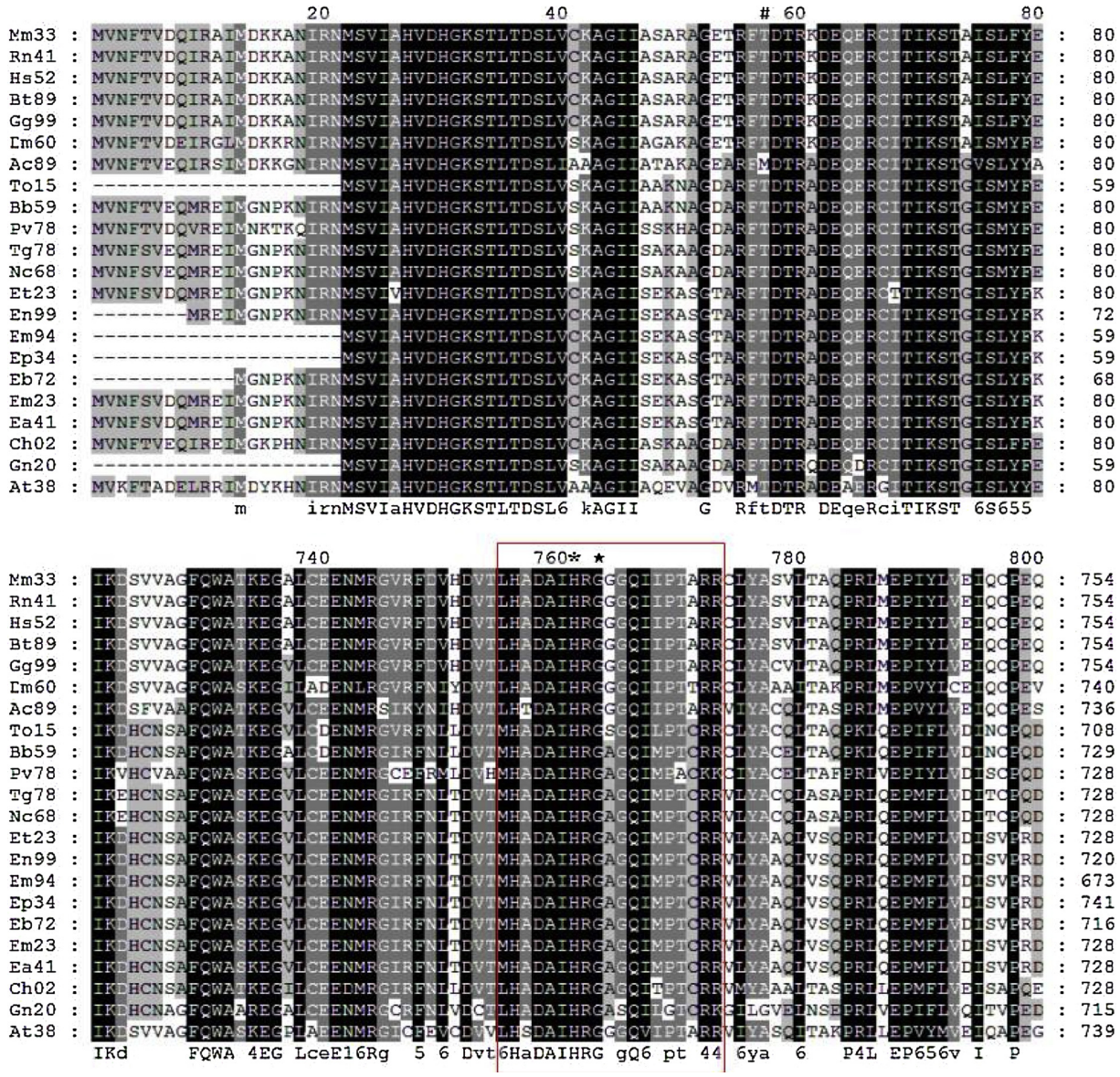

Fig. 2. Multiple alignments of the *Et*EF2 amino acid sequence with eEF2 sequences from other species. The conserved threonine residue (57) was shown with black hashes (#). The ADP-ribosylation domain was highlighted with red box. The conserved histidine residue was marked with black spark (*), and the conserved glycine residue required for ADP-ribosylation was marked with black star (★).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

acid sequence and other members of the eEF2 protein family mentioned in Table 1. Conserved Thr-57, histidine residue, and glycine residue were observed in the predicted amino acid sequence of *Et*EF2. Meanwhile, the deduced *Et*EF2 amino acid sequence had 99 % homology with the eEF2 ortholog of *Toxoplasma gondii* (GenBank: XP_002367778.1). The constructed phylogenetic tree indicated that *Et*EF2 clustered with protozoal eEF2 (Fig. 3).

3.2. Recombinant protein expression of EtEF2

The r*Et*EF2 protein was expressed with a His_6-tagged in *E. coli* BL21 (DE3) cells. The bacterial culture was incubated at 27 °C and induced with 0.1 mM IPTG. The SDS-PAGE results demonstrated that the r*Et*EF2 protein (molecular weight of approximately 97.97 kDa) was successively expressed and mainly existed in the soluble fraction of bacterial lysates (Fig. 4).

3.3. QRT-PCR analysis of EtEF2 mRNA expression

The mRNA expression level of *Et*EF2 is illustrated in Fig. 5. The mRNA expression level of *Et*EF2 in the diclazuril group was 2.706 fold increases ($p < 0.01$) relative to that in the control group.

3.4. Western blot of analysis EtEF2 protein expression

As shown in Fig. 6, *Et*EF2 showed significant protein imprint expression (Fig. 6a), and its protein expression levels in the diclazuril group was significantly increased by 67.31 % ($p < 0.01$) (Fig. 6b) relative to those in the control group.

3.5. Immunolocalization of EtEF2 in second-generation merozoites

As shown in Fig. 7, *Et*EF2 protein was dominantly concentrated in the cytoplasm of the second-generation merozoites. The fluorescence intensity of *Et*EF2 in the diclazuril group was enhanced compared with

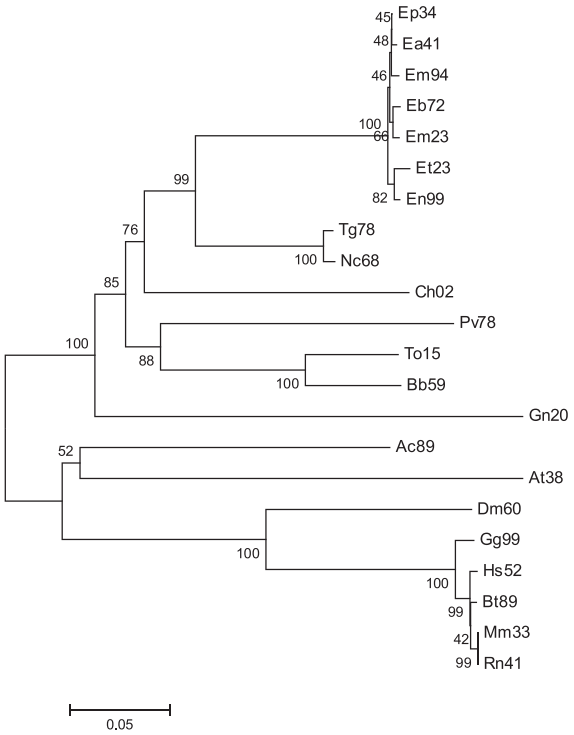

Fig. 3. A phylogenetic tree was constructed to compare the relatedness of the *Et*EF2 with other eEF2.

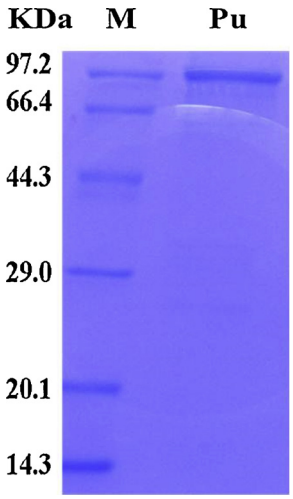

Fig. 4. *Et*EF2 fusion protein purification by Ni^{2+} affinity chromatography. M, Protein molecular weight Marker (Low); Pu, purified product.

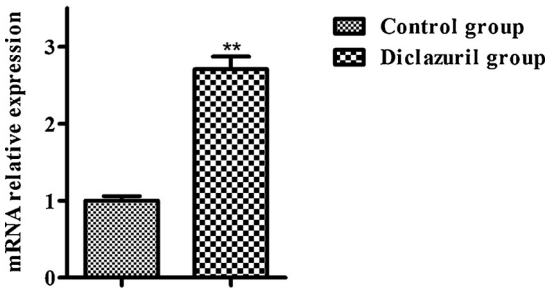

Fig. 5. QRT-PCR determination of the mRNA expression level of *Et*EF2 in second-generation merozoites (N = 3). The results are presented as the mean ± standard deviation values of independent experiments performed in triplicate. $^{**}P < 0.01$ indicated statistically significant differences.

that in the control group.

4. Discussion

eEF2 has an indispensable role as a key factor in polypeptide elongation for maintaining eukaryotic metabolism and regulating cell proliferation and differentiation during eukaryotic protein synthesis (Malavé and Forney, 2004; Qiu et al., 2008). *E. tenalla* is an obligate intracellular parasite of the cecum and is responsible for coccidiosis. Host cell invasion is a prerequisite for the establishment and maintenance of infection which rely on the secretion of various invasion-related proteins (Marugan-Hernandez et al., 2017). These proteins include surface antigens (Ramly et al., 2013), apical membrane antigens (Jiang et al., 2012), microneme proteins (Yan et al., 2018), and rhoptry proteins (Wang et al., 2019). The syntheses of these proteins are related to the participation of eEF2. The full-length *Et*EF2 cDNA sequences obtained in the present study displayed high sequence homology and structural similarity with known eEF2 genes from multicellular and unicellular eukaryotic organisms. A GTPase activity domain and four elongation factor GTP binding domains were identified in the deduced *Et*EF2 amino acid sequence. The *Et*EF2 sequence contains the major physiological phosphorylation site Thr-57 (Thr-56 in mammals and Thr-57 in yeast) which is involved in the elongation rates (Wang et al., 1998). Conserved EFG-II, EFG-III, EFG-IV, and EFG-V domains were also found in the sequence. Cloning and analysis of *Et*EF2 gene can lay the foundation for the study of eEF2 function, which will provide theoretical basis for the exploration of the mechanism underlying the ability of *E. tenella* to invade host cells and development of efficient and safe anticoccidian agents.

Organisms have a certain ability to respond to physiological and environmental stresses during evolution (Wang et al., 2011). The common characteristics of numerous responses are the selective regulation of gene transcription and translation (Rattan, 1996; Holcik and Sonenberg, 2005). eEF2 regulates protein synthesis by constantly changing its expression in cells (Lim and Kim, 2007). The activity and expression of eEF2 change under different experimental conditions, such as temperature fluctuations, nutrient limitation, oxidative stress, hypoxia and exposure to various drugs or toxins (Malavé and Forney, 2004; Wang et al., 2011; Su et al., 2013). Environmental stress can also induce eEF2 expression, which in turn regulates protein synthesis to adapt to the new situation (Ayala et al., 1996; Galliea et al., 1998; Chen et al., 2000; Sans et al., 2004). Wang et al. (2011) reported that eEF2 transcripts increased in shrimps challenged by pH and cadmium stress. The mRNA and protein expression levels of EF2 in *Monochamus alternatus* were up-regulated under treatment with different types of insecticides (Luo and Lin, 2014). In the present study, the high expression of *Et*EF2 in *E. tenella* under diclazuril treatment may be a cooperative adaption to translation activity. Such a high level of translation may compensate for the down-regulation of the expression of invasion-related proteins caused by diclazuril (Zhou et al., 2010a, 2010c), which may be one of the response measures of parasites to the effect of drugs.

Inappropriately high levels of elongation activity may result in missense errors or premature termination (Browne and Proud, 2002). In this study, after diclazuril treatment, the constitutively expression of *Et*EF2 was increased at a high level, which probably promoted ribosomes attachment to mRNA, increased elongation efficiency, and resulted in errors of protein synthesis or premature termination. Translation must consume a massive amount of metabolic energy, of which the vast majority is used in elongation (Browne and Proud, 2002). The increased expression of *Et*EF2 induced by diclazuril likely enhances the rate of protein synthesis, thereby including the consumption of a large amount of amino acids and energy in *E. tenella*. This may be related to the structural changes and number decrease of *E. tenella* induced by diclazuril as our report previously (Zhou et al., 2010b, 2013).

In addition to expression regulation, cell localization is also a magic space regulator to protein function. Only when the protein is in a

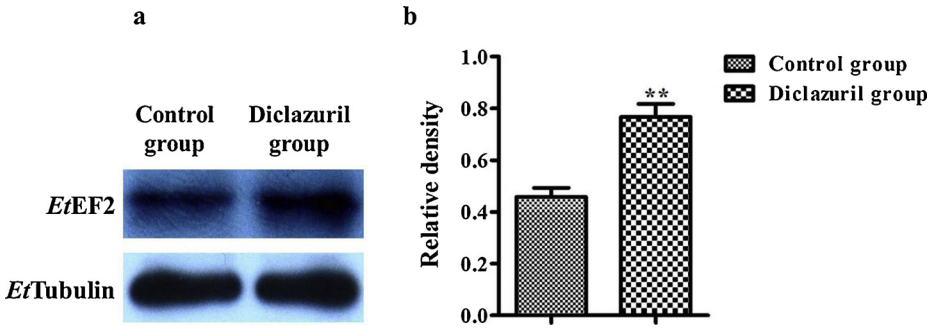

Fig. 6. Western blot analysis of the expression of *Et*EF2. (a) Western blot electrophoretic pattern. (b) *Et*EF2 relative expression levels were presented as the mean ± standard deviation values of independent experiments performed in triplicate (N = 3). $^{**}P < 0.01$ indicated statistically significant differences.

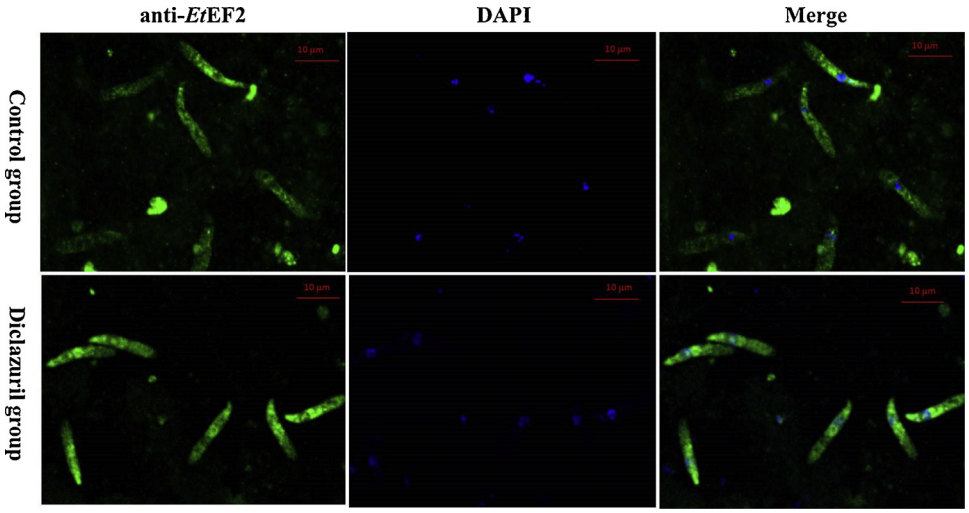

Fig. 7. Immunofluorescence localization of *Et*EF2 in second-generation merozoites. *Et*EF2 was widely distributed throughout the cytoplasm of merozoite cells. The green fluorescence seems a bit brighter in the diclazuril group compared to the control group.

specific position could normal executive function be achieved. Otherwise the organism would suffer from functional deficiency or disorder (Yu et al., 2006; Horton et al., 2007; Imai and Nakai, 2010; Hung and Link, 2011). In the present study, the localization of *Et*EF2 in merozoites was determined through immunofluorescence analysis. *Et*EF2 was dominantly dispersed in the cytoplasm of merozoites. The green fluorescence intensity of *Et*EF2 in the diclazuril group was brighter compared with that in the control group. This finding indicated that a large number of *Et*EF2 in the cytoplasm of *E. tenella* participate in the synthesis of invasion-related proteins for *E. tenella* to cope with the change of survival circumstance. This result is consistent with the outcome of the Western blot analysis.

In conclusion, a full-length *Et*EF2 cDNA sequence from the second-generation merozoites of *E. tenella* was cloned, expressed, and characterized. The up-regulation of *Et*EF2 mRNA and protein expression levels after diclazuril treatment may result in the consumption of a large amount of amino acids and energy and eventually decreased the number of merozoites (Zhou et al., 2010a). Given the important functions of *Et*EF2 in *E. tenella* development, the detailed functions and regulatory mechanism of the participation of *Et*EF2 in host-cell invasion by *E. tenella* require further investigation. *Et*EF2 is essential for cell survival and growth, and its distinct biochemical and structural features may provide novel targets for development of effective additives to prevent and mitigate coccidiosis in the poultry industry.

Declaration of Competing Interest

The experimental scheme was approved by the Ethics Committee of the Faculty of Veterinary Medicine and strictly conformed to the guidelines of the Institutional Animal Care and Use Committee of China.

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