Diclazuril-Induced Expression of CDK-Related Kinase 2 in the Second-Generation Merozoites of *Eimeria Tenella*

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Abstract

Background Coccidiosis caused by *Eimeria tenella* infection, directly or indirectly leads to great loss to poultry industry. With the emergence of drug-resistance in chicken coccidia, it is imperative to develop new drugs. Cyclin-dependent kinases (CDKs) regulate cell cycle progression in numerous organisms by acting as key molecular switches.

Results: In the present study, a diclazuril anticoccidiosis animal model was established and CDK-related kinase 2 (CRK2) in the second-generation merozoite of *E. tenella* (*Et*CRK2) gene was amplified through reverse transcription-polymerase chain reaction (RT-PCR) and expressed in *Escherichia coli* Rosetta (DE3). Purified recombinant protein was used for antiserum preparation. Subsequently, *Et*CRK2 transcription and translation levels were detected through quantitative real-time PCR and Western blot analysis, respectively. The localization of *Et*CRK2 in merozoites was examined via immunofluorescence techniques. Results showed that the mRNA and protein expression levels of *Et*CRK2 decreased in the infected/diclazuril group compared with those in the infected/control group. In addition, immunofluorescence analysis showed that *Et*CRK2 was localized in the cytoplasm of merozoites. The fluorescence intensity of *Et*CRK2 in the infected/diclazuril group was significantly weaker than that in the infected/control group.

Conclusions: This study demonstrated that the anticoccidial drug diclazuril against *E. tenella* by affecting the expression pattern of *Et*CRK2 molecule, and *Et*CRK2 may be used as a candidate target for new drug development.

Background

Eimeria tenella is an obligate intracellular parasite that must invade chicken cecal epithelial cells to keep survive and complete the lifecycle. This parasite causes a serious form of coccidiosis and huge economic losses to the poultry community [1]. The prevention and control of coccidiosis mainly rely on ionophores and synthetic chemicals. However, coccidial resistance is induced by the long-term use of anticoccidial drugs [2]. Therefore, new drug targets and control strategies must be sought.

E. tenella has a complex lifecycle that involves an asexual stage (sporogony and schizogamy) and sexual stage (gametogony) [3]. During schizogamy, the *E. tenella* proliferates within host cells at a high division rate [4], this process is similar to cell cycles in other eukaryotes regulated by cyclin-dependent kinase (CDKs) [5]. CDKs are a kind of serine/threonine kinases that regulate the activity of substrate proteins through phosphorylation [6, 7], which regulates cell cycle progression, proliferation, and differentiation and modulate transcription [8–11]. CDK2 plays a pivotal role in regulating G1/S and S/G2 transitions during the cell cycle [12, 13]. Several CDK-like kinases have been identified and characterized in parasitic protozoans. *Toxoplasma gondii* CRK1 (TgCRK1), TgCRK2, TgCRK4 and TgCRK6 are essential for tachyzoite replication and growth [14]. *Plasmodium falciparum* CDK-related kinase 3 (PfCRK-3) fulfills a key role in the intraerythrocytic development of *P. falciparum* [15]. MA Speranca, et al. [16] study

showed that plasmodial CRK2 gene expression product can interfere with the progress of the well-conserved eukaryotic cell cycle. *Theileria annulata* CRK3 may be a regulator in gene transcription [17]. CDK2 is involved in differentiation of *Giardia lamblia* into cysts [8]. CDK-related kinase 2 (*Et*CRK2) is only CDK that has been confirmed in *E. tenella.* It is expressed during the asexual and sexual states of *E. tenella* development [5, 18].

Diclazuril, a classic anticoccidial drug, significantly induces merozoites apoptosis and effectively decreases the number of merozoites and alleviates the damage in the cecum induced by *E. tenella* [19–24]. Here, we reported the changes in *Et*CRK2 in response to diclazuril treatment against *E. tenella* infection. In addition, the expression levels of *Et*CRK2 mRNA and protein and the spatial location of *Et*CRK2 were observed in the present study.

Results

Cloning of the EtCRK2 gene

The 1015 bp *Et*CRK2 gene was amplified from the second-generation merozoites and cloned into pMD-19T vector. A DNA fragment of *Et*CRK2 ORF amplified from pMD-19-*Et*CRK2 was subcloned into pET-28a (+). The recombinant plasmid pET-28a-*Et*CRK2 was analyzed through PCR with pET-28a (+) vector universal primers, and the product length is in according with ORF 891 bp plus vector sequence 360 bp (Fig. 1). At the same time, the insertion 891 bp ORF was also indicated by *Eco*R I and *Hin*d III enzyme digestion (Fig. 2) and sequenced for 100% correctness.

Expression of recombinant EtCRK2 protein and polyclonal preparation

The expressed of the r*Et*CRK2 protein was induced at 37 °C with 0.5 mM IPTG for 4 h, to get to high expression and separated through SDS-PAGE (Fig. 3). The theoretical molecular weight r*Et*CRK2 protein was approximately 37.67 kDa. Purified r*Et*CRK2 protein (Fig. 4) was used antibody preparation. The antiserum has a specific binding with the recombinant protein (Fig. 5) and the titer of antibody against r*Et*CRK2 exceeded 1:512K (Fig. 6).

Expression of EtCRK2 mRNA

Fig. 7 showed that compared with that in the infected/control group, the mRNA expression level of *Et*CRK2 in the infected/diclazuril group was downregulated by 42.3% ($P < 0.01$).

Western blot analysis

As shown in Fig. 8, the *Et*CRK2 protein expression level in the infected/diclazuril group was downregulated by 59.32% ($P < 0.01$) relative to that in the infected/control group.

Immunofluorescence analysis

The subcellular location of *Et*CRK2 in the second-generation merozoite was visualized through immunofluorescence microscopy. As shown in Fig. 9, *Et*CRK2 was widely distributed in the cytoplasm of merozoites. The green fluorescence in the merozoites of the infected/diclazuril group was darker than that in the merozoites of the infected/control group.

Discussion

Coccidiosis caused by the apicomplexan parasites of the genus *Eimeria* induces high economic problem by lowering poultry productivity and performance [25]. *E. tenella* is one of the most virulent species of *Eimeria*, which mainly infects chicken cecum. During second-generation schizogony, large amounts of released second-generation merozoites reinvasion other uninfected cecal epithelial cells. Therefore, inhibiting the reproductive process of second-generation merozoites may be an effective strategy to control coccidiosis.

E. tenella utilize complex and distinctive mechanisms to regulate their replicative cycles [14]. CDKs participates in mediating the cell proliferation and division of the parasite [7, 26–28]. In *Plasmodium berghei*, CRK2 mRNA is expressed richly in gametocytes and mosquito endophase; this characteristic suggests that CRK2 protein has a key role throughout the life cycle [29]. In the schizont stage, the inhibition of *P. falciparum* Cdc2-related kinase-1(PfCRK-1) protein interferes with the growth of *P. falciparum* [30], and the depletion of *Plasmodium*-specific kinase PfCRK4 result in the complete blockage of nuclear division and intensively inhibits DNA replication [31]. The downregulation of *P. falciparum* MO15-related protein kinase (PfMRK), Cdc2-related kinase PfPK5, *P. falciparum* CRK3 and other cyclins during dormancy arrests parasite progression at the G1 phase, and halts DNA synthesis [32]. The inhibition of CDK7 kinase causes transcript dysregulation in *T. gondii* [33]. P Mitra, et al. [34] reported that *T. gondii* CRK9 regulates RNA polymerase II by phosphorylating the *T. gondii* riboflavin binding protein 1 carboxyl-terminal domain. The loss of cytoplasmic TgCRK2 results in *T. gondii* cell cycle G1 phase arrest [14]. CDK2 participates in intraerythrocytic development of *Babesia bovis* [35]. These results suggest that CDKs are important molecular switches that contribute to cell cycle progression in parasite [30]. In this study, the levels of *Et*CRK2 mRNA and protein in the infected/diclazuril group was decreased relative to those the infected/control group. The results indicate that diclazuril blocks merozoites in the G1 phase by interfering with *Et*CRK2 pathway, thus preventing the normal reproductive cycle of *E. tenella* and subsequently decreasing the number of second-generation merozoites [19].

The spatial distribution of proteins is also involved in the performance of protein function. The localization of *T. annulata* CRK2 protein located in the parasite nuclei upregulated transiently in mid-merogony. This phenomenon suggests that TaCRK2 is likely to coordinate the parasite's [17]. The protozoan *G. lamblia* CDK2 localized in the cytoplasm participate in the cytoplasm participates in the increase in cyst formation and shows increased expression during encystation [8]. The members of *P. falciparum* CDK-like kinase (CLK) family, PfCLk-1 and PfCLK-2, are primarily localized in the nucleus, and the further dispersion of PfCLK-2 into the cytoplasm, indicates that PfCLKs participate in gene regulation via the post-transcriptional modification of mRNA in the malaria blood stage [36]. In the present study,

*Et*CRK2 was found to be localized in the cytoplasm of second-generation merozoite. Under diclazuril treatment, the fluorescence intensity of *Et*CDK2 decrease. This phenomenon is consistent with the determined expression levels. These results implied that *Et*CRK2 play an essential role in the asexual life cycle of *E. tenella*, indicating that agents targeting CRK2 may be potential therapeutic strategies against coccidiosis.

Conclusion

The results in the present study demonstrate for the first time that the diclazuril likely downregulate the mRNA and protein expression of *Et*CRK2 and disturb the cell cycle of the second-generation merozoites. This action may be an aspect of the molecular mechanism of the anticoccidial action of diclazuril.

Methods

Inoculum and Drug

Ocysts of *E. tenella* (Luoyang strain) were passaged before inoculation. Diclazuril (>99%) (Shanghai Veterinary Research Institute, CAAS, China) was supplied at a dose of 1 mg/kg in broiler feed.

Chickens and Treatment

1-day-old male Chinese Yellow broiler chickens were obtained from Gonghua Commercial Hatchery, Luoyang, China. The chickens were kept on wire-floored batteries for 14 days under coccidiosis-free conditions. On the 14^{th} days, healthy 90 chickens with similar weight were randomly divided into two groups of 45 with 3 biological replicates of 15 in each group: (1) Chickens that were challenged with *E. tenella* sporulated oocysts and that received commercial diet without drugs were designated as the infected/control group. (2) Chickens that were challenged with *E. tenella* sporulated oocysts and treated with 1 mg/kg diclazuril in feed from 96 h to 120 h were designated as the infected/diclazuril group. Inoculation was performed with a dose of 8×10^4 sporulated oocysts/chicken on days 14. 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

At 120 h post inoculation, chickens killed by CO_2 asphyxiation, cecal tissues from randomly selected 10 chickens pooled in each replicate were used for preparation of the second-generation merozoites in accordance with our previously described method [19-21]. The collected merozoites samples were subjected for RNA preparation, Western blot and immunofluorescence assay, respectively. Thus, there were three samples in each group for each experiment.

Total RNA Preparation and cDNA Synthesis

In accordance with the manufacturer's procedure, the total RNA of merozoites was extracted with TRIzol® Reagent (Invitrogen, USA), and cDNA was synthesized by using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing).

Amplification of EtCRK2 Gene

Based the sequence form GenBank: AY508221.1, specific primers P1: 5'-AAGGGACTTACGGAGTGGTTTA-3' and P2: 5'-TGAATTTACGTGAATATGTTGG-3' were used to amplify *Et*CRK2 gene including an open reading frame (ORF) from a cDNA template. Amplified fragments were separated through 1% agarose gel electrophoresis and isolated using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (Takara, Beijing) following the manufacturer's instructions. The purified amplifying fragments were ligated into the pMD-19T vector (Takara, Beijing), and then transformed into *Escherichia coli* strain DH5α (Takara, Beijing). The recombinant clone of pMD-19-*Et*CRK2 was identified through polymerase chain reaction (PCR) and sequenced by Sangon Biotech (Shanghai) Co., Ltd. The positive plasmid was named pMD-19-*Et*CRK2.

Prokaryotic Expression and Purification of EtCRK2

Using positive pMD-19-*Et*CRK2 as template, the ORF of *Et*CRK2 was amplified using primers containing *Eco*R I (P2-F: 5'-CGGGAATTCATGGAGCGCTACAAGA-3') or *Hin*d III (P2-R: 5'-TCTTGTAGCGCTCCATGAATTCCCG-3') restriction sites. Amplification products and pET-28a (+) were digested by *Eco*R I and *Hin*d III prior to ligation. The recombinant plasmid pET-28a-*Et*CRK2 was transformed into *E. coli* Rosetta (DE3) competent cells that were induced at 37 °C with 0.5 mM IPTG. Recombinant *Et*CRK2 (r*Et*CRK2) protein was purified through Ni-NTA His Bind Resin affinity chromatography (Novagen, Germany) and then analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Polyclonal Antibody Preparation

To produce polyclonal antibody, purified r*Et*CRK2 protein was used as antigen in the following immune procedures. r*Et*CRK2 protein emulsified with the same volume of Freund's complete adjuvant (Sigma-Aldrich) was injected into New Zealand rabbits at a dose of 500 mg/rabbit. After 2 weeks, purified r*Et*CRK2 protein emulsified with Freund's incomplete adjuvant (Sigma-Aldrich) was injected to the rabbits at a dose of 500 mg/rabbit for secondary immunization. Then, at the interval of a 2-week hiatus, the third and fourth immunizations were performed separately. Ten days after the last immunization, serum samples were collected and determined by indirect enzyme linked immunosorbent assay (ELISA) [37]. Briefly, 500 ng/well recombinant protein was used to coat the 96-well plates at 4 °C overnight, the antiserum were diluted with PBS at 1:2K, 1:4K, 1:8K, 1:16K, 1:32K, 1:64K, 1:128K, 1:256K, 1:512K, 1:1024K, 1:2048K, and then incubated with antigens at 37 °C for 2 hours. After washing by TBST, Horse Radish Peroxidase (HRP)-conjugated goat anti-rabbit IgG were added to all microwells at 37 °C for 2 hours. After washing, the substrate solution tetra-methyl ben-zidine (TMB) was added and the microplate was read at 450 nm in a microplate reader (Multiskan FC, Thermo Scientific, USA). Preimmunization

rabbit serum and PBS were used as the control. The specificity of antibody was determined by Western blot, briefly, 25 ng recombinant protein was used for loading for SDS-PAGE, preimmunization rabbit serum and antiserum was used as the primary antibodies followed by HRP conjugated goat anti-rabbit IgG (Biolab, Beijing). HRP activity was revealed by enhanced chemiluminescence system using BeyoECL Plus substrate (P0018S, Beyotime Biotechnology, China)

EtCRK2 mRNA Expression Analysis

The mRNA expression level of *Et*CRK2 was quantified through real-time PCR using the CFX96 touch real-time PCR system (Bio-Rad, America) and TB Green®*Premix Ex Taq*™ GC (Perfect Real Time) (Takara, Beijing). Each reaction was performed in triplicate, and the entire experiment was carried out in triplicate. *E. tenella* 18S rRNA was used as the control. The primer sequences are shown in Table 1. Relative mRNA expression was determined by using the $^{\Delta\Delta}$Ct method.

Western Blot Assay

Purified merozoites were treated with RIPA lysis buffer (Beyotime, Shanghai) for Western blot analysis and determined by BCA Protein Assay Kit (Cwbio, Beijing) for concentration assessment. Pyrolysis products were dissolved in SDS-PAGE sample buffer (Beyotime, Shanghai), heated at 96 °C for 5 min, separated on 12% SDS-PAGE, and then electrotransferred to a polyvinylidene difluoride membrane (Membrane Solutions, USA). The membrane was detected with rabbit antiserum against *Et*CRK2 as the primary antibodies or anti-β-tubulin monoclonal antibody (1:1000 dilution, K200059 M, Solarbio, China), followed by HRP conjugated goat anti-rabbit IgG (Biolab, Beijing). HRP activity was revealed by enhanced chemiluminescence system using BeyoECL Plus substrate (P0018S, Beyotime Biotechnology, China) and Image J Software. The independent experiments were performed in triplicate.

Immunofluorescence Test

In accordance with our previously described method with minor modifications [22], the merozoites were prepared into smears and fixed with 4% paraformaldehyde. After washing three times with PBS, the merozoites were permeabilized with 1% Triton X-100 (Sangon-Biotech, Shanghai) and blocked with 2% BSA-PBS. Rabbit antiserum against *Et*CRK2 (1:2000 dilution) was used as the primary antibody, and FITC-conjugated goat anti-rabbit IgG (Servicebio, Wuhan) with 1:100 dilution was used as the second antibody. Finally, the merozoites were stained with 4',6'-diamidino-2-phenylindole (DAPI) (Boster, China). Images were visualized with a confocal laser scanning microscope (LSM 800, ZEISS) at an $l_{ex/em}$ of 492 nm/520 nm and 358 nm/461 nm, respectively.

Statistical Analysis

Data were expressed as mean ± standard deviation. Student's *t* test was used for statistical analyses. Values of $P < 0.05$ and $P < 0.01$ were considered significant.

Abbreviations

CDK
Cyclin-dependent kinases; CRK:CDK-related kinase; PCR:Polymerase chain reaction; bp:base pair; ORF:Open reading frame; IPTG:Isopropyl-b-D-thiogalactopyranoside; SDS-PAGE:Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kDa:Kilodalton; ELISA:indirect enzyme linked immunosorbent assay. PBS:Phosphate buffer saline; TBST:Tris-Buffered Saline containing 0.05% Tween 20; TMB:Tetramethylbenzidine; BSA:Bovine serum albumin; FITC:Fluoresceine isothiocyanate; *E. tenella*:*Eimeria tenella*; *T. gondii*:*Toxoplasma gondii*; *P. falciparum*:*Plasmodium falciparum*; *P. berghei*:*Plasmodium berghei.*

Declarations

Ethics approval and consent to participate

Animals housing and care complied with the Guide for Ethical Review of laboratory animal welfare, China (GB/T 35892-2018). The study was approved by the Institutional Animal Care and Use Committee of Henan University of Science and Technology.

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the findings of this article are included within the article.

Competing interests

No potential conflict of interest was reported by the authors.

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Authors' contributions

BZ: Software, Validation, Formal analysis, Data Curation, Methodology, Visualization, Writing - Review & Editing, Funding acquisition. HD: Writing - Original Draf, Visualization, Investigation. JY, JC, HG and ET: Investigation. All authors read and approved the final version of the manuscript.

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Tables

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 rRNA	ATCGCAGTTGGTTCTTTTGG CCTGCTGCCTTCCTTAGATG	248–417	170	U67121
*Et*CRK2	CTAGACGACTGCCGACCTTC GGGTTCCGTCTCTGCTTATG	524–746	223	AY508221.1

Figures

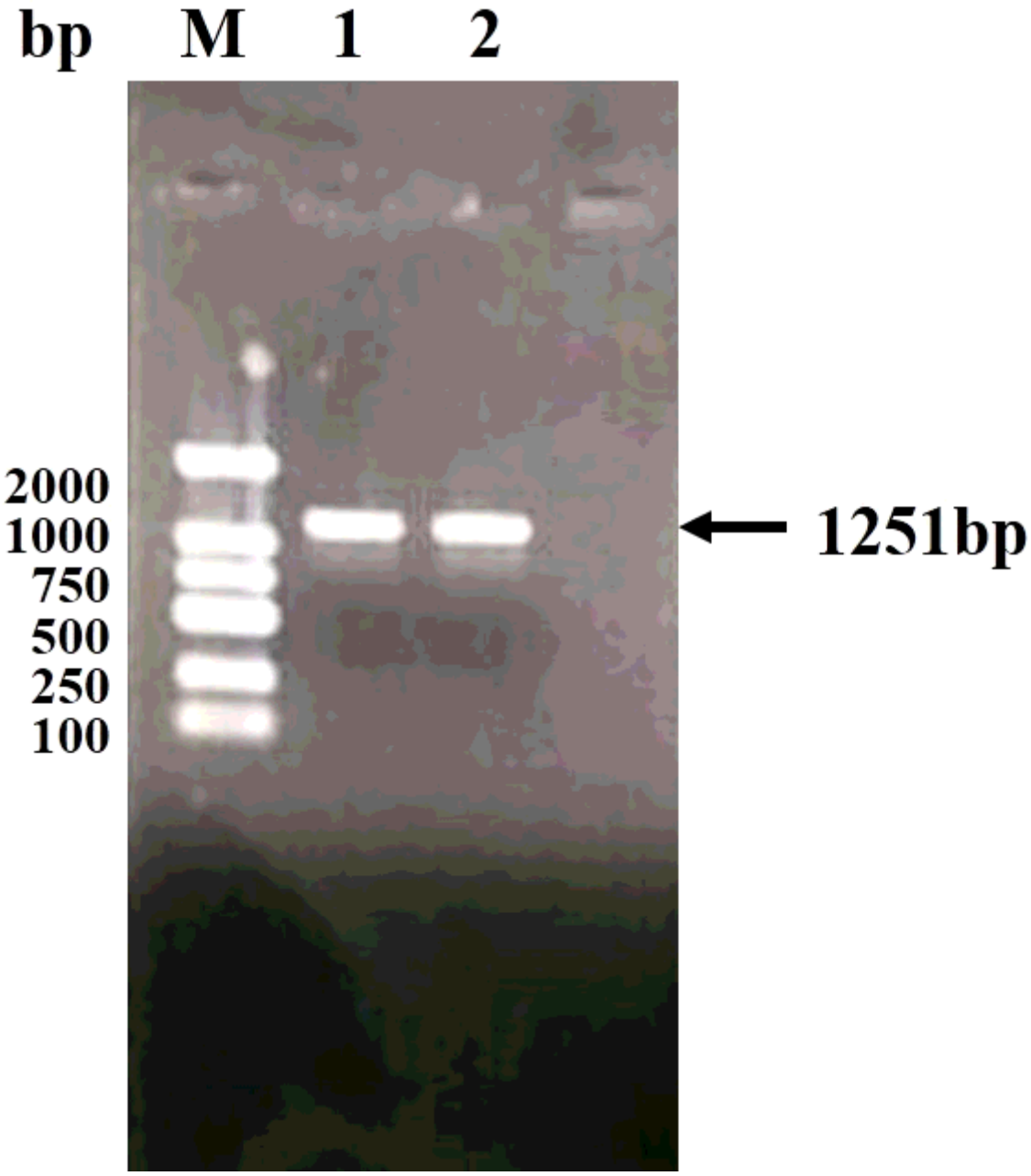

Figure 1

Agarose gel electrophoresis of EtCRK2 gene ORF. M, DL2000 DNA Marker; 1and 2, PCR amplification products via pET-28a (+) vector universal primers.

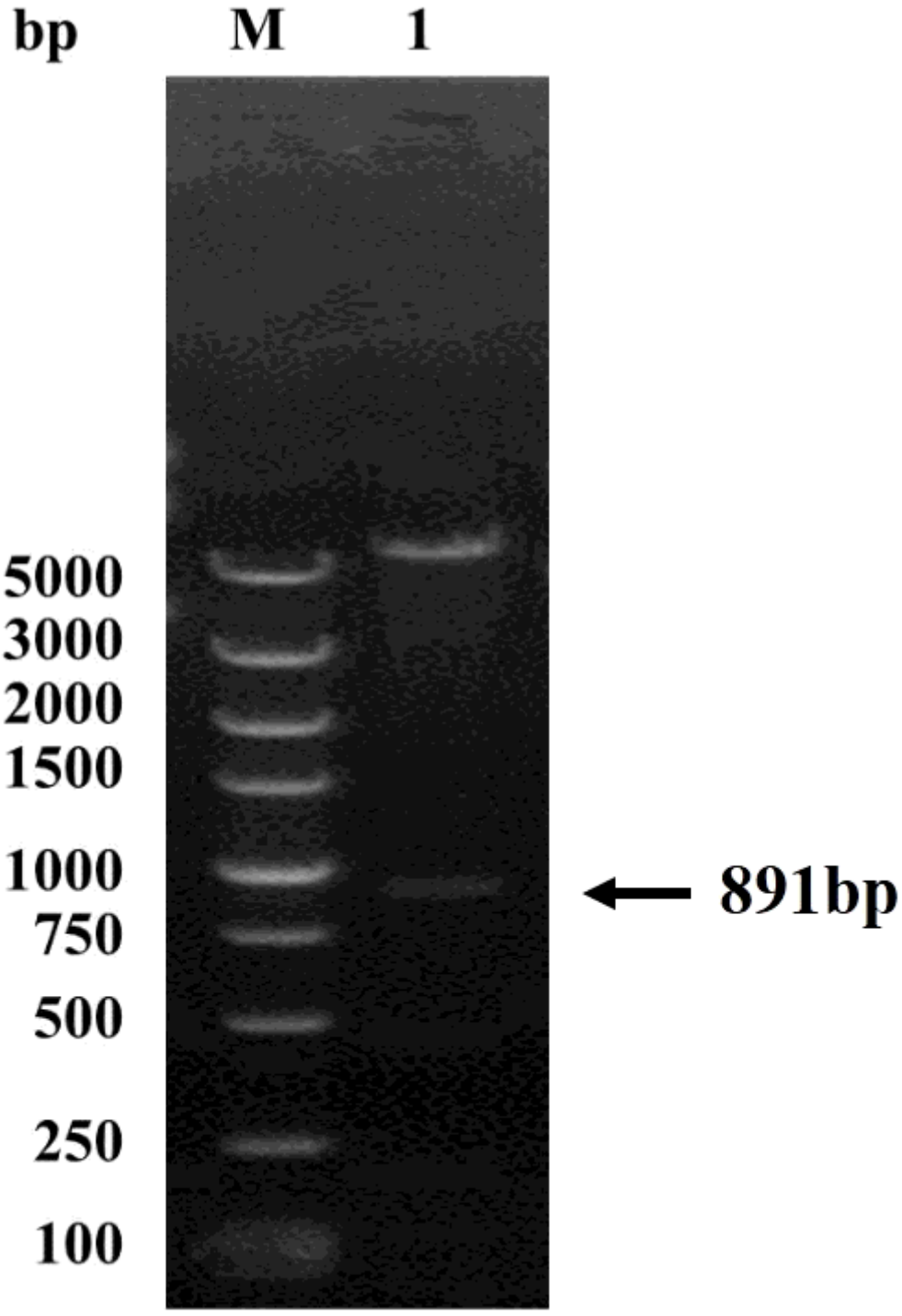

Figure 2

Restriction digestion analysis. M, DL5000 DNA Marker; 1, Plasmid pET-28a-EtCRK2 digested by EcoR I and Hind III.

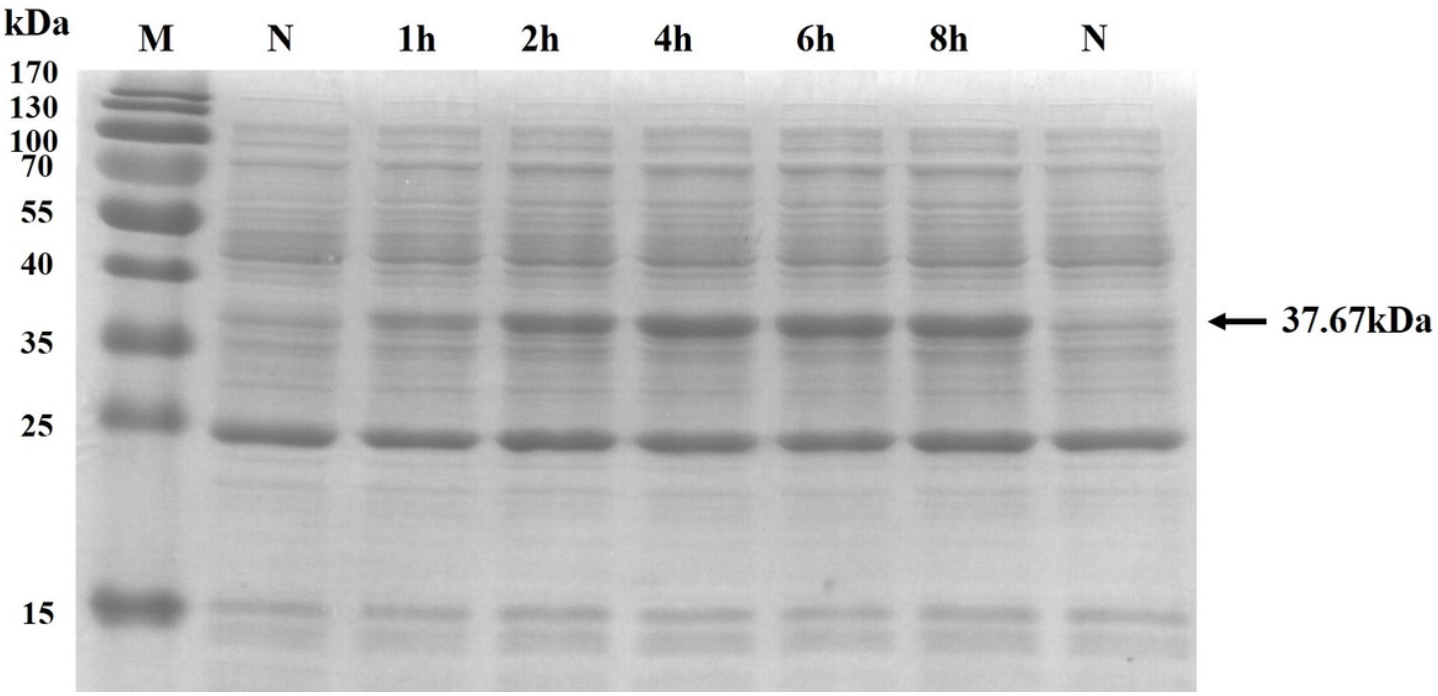

Figure 3

Induced expression of EtCRK2 fusion protein. M, Protein molecular weight Marker; pET-28a-EtCRK2 were induced expression of 1 h, 2 h, 4 h, 6 h and 8 h, respectively; N, empty pET-28a were induced expression.

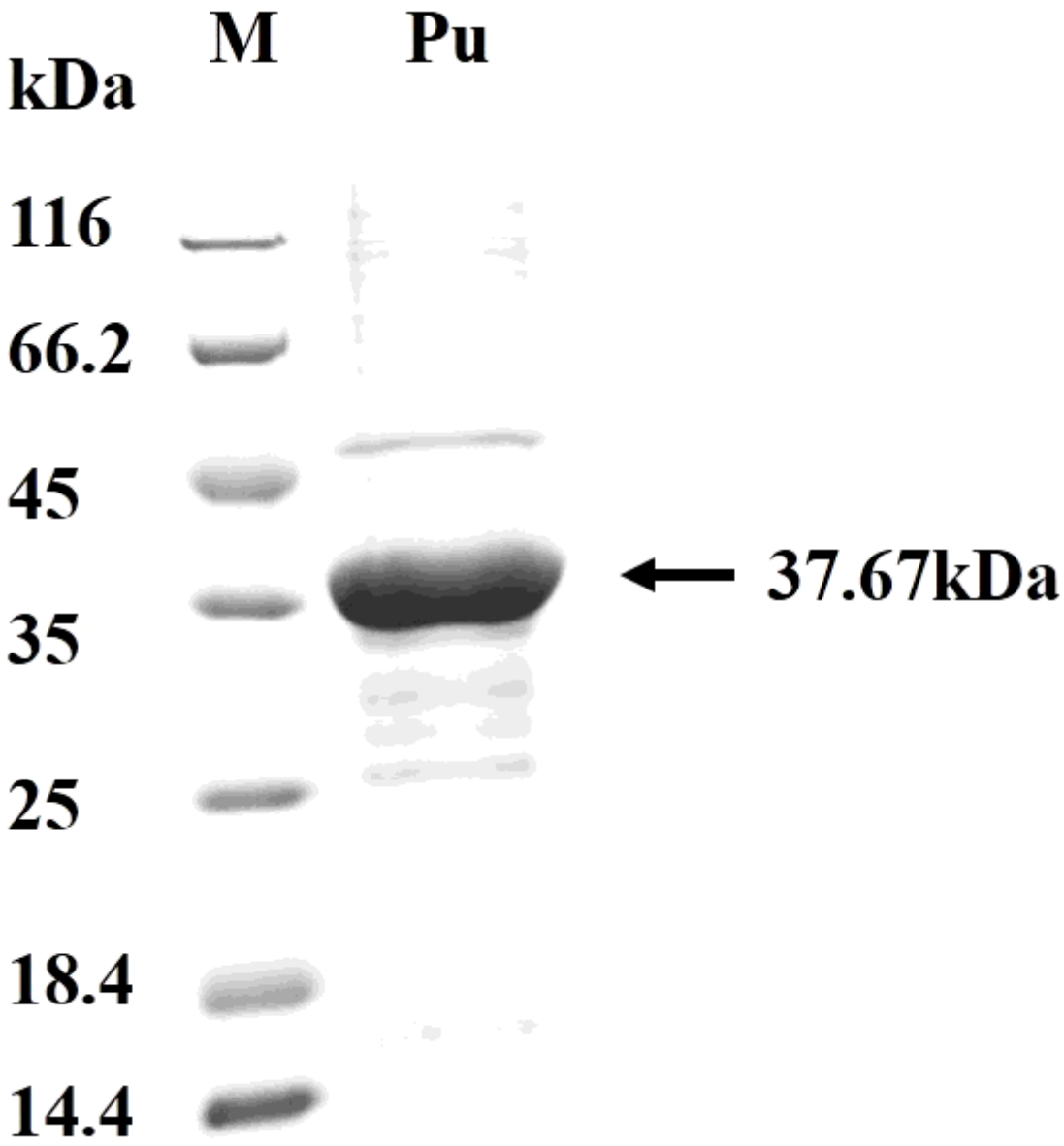

Figure 4

Purification of rEtCRK2 protein. M, Protein molecular weight Marker; Pu, purified rEtCRK2 protein.

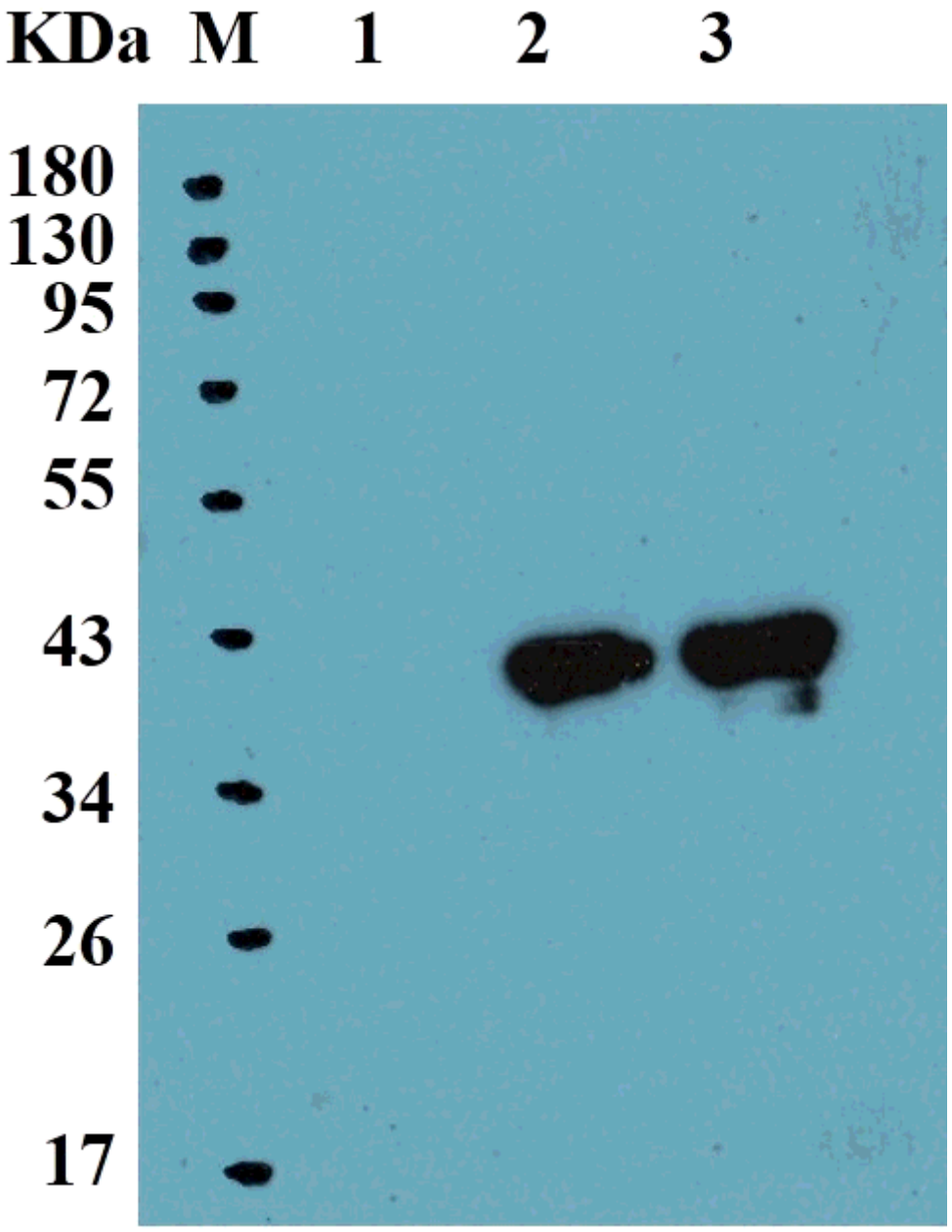

Figure 5

Rabbit-anti-EtCRK2 serum specific binding analysis by Western blot. M, Protein molecular weight Marker; 1, Pre-immunization serum as the first antibody; 2 and 3, antiserum as the first antibody.

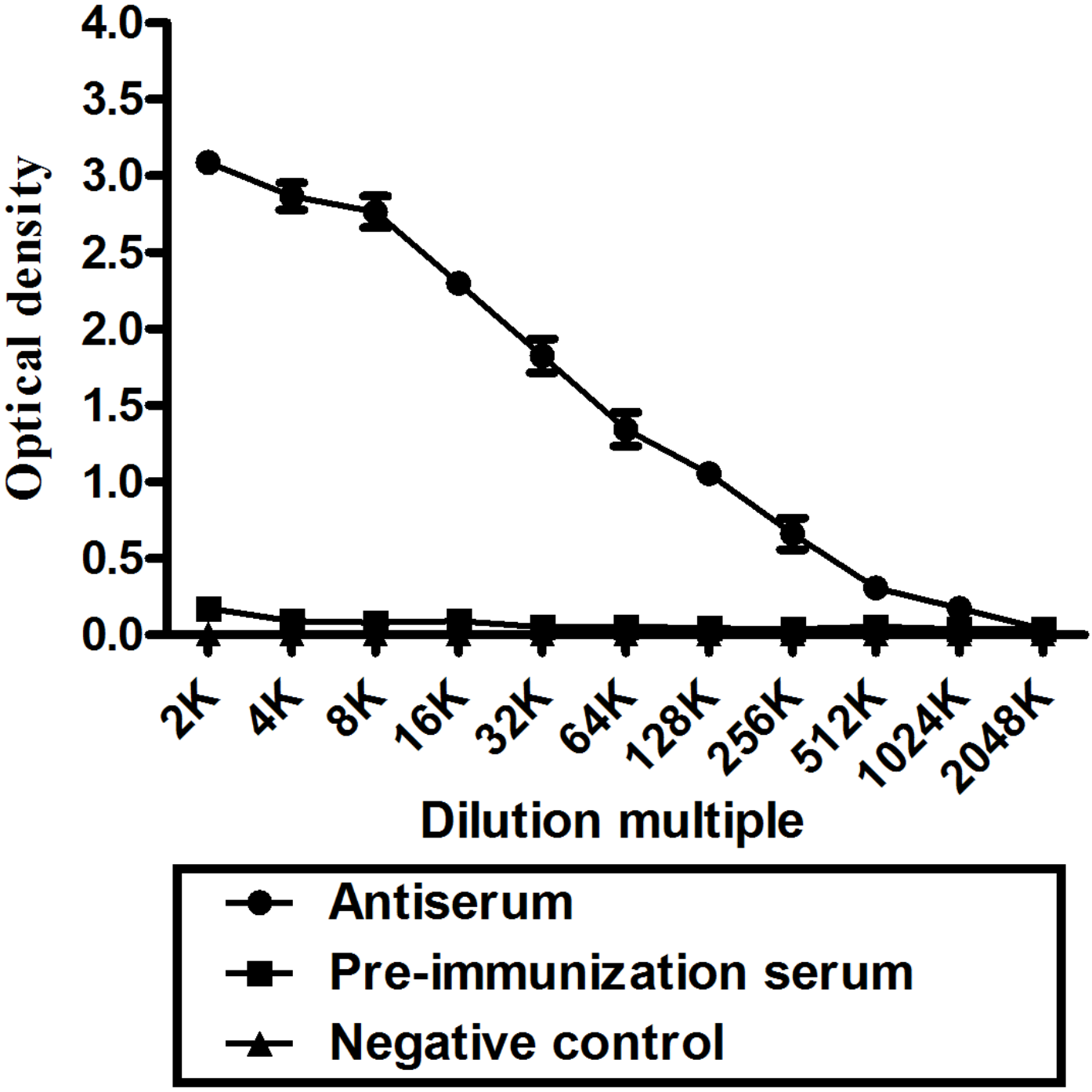

Figure 6

Serum anti-EtCRK2 antibody levels measured by ELISA

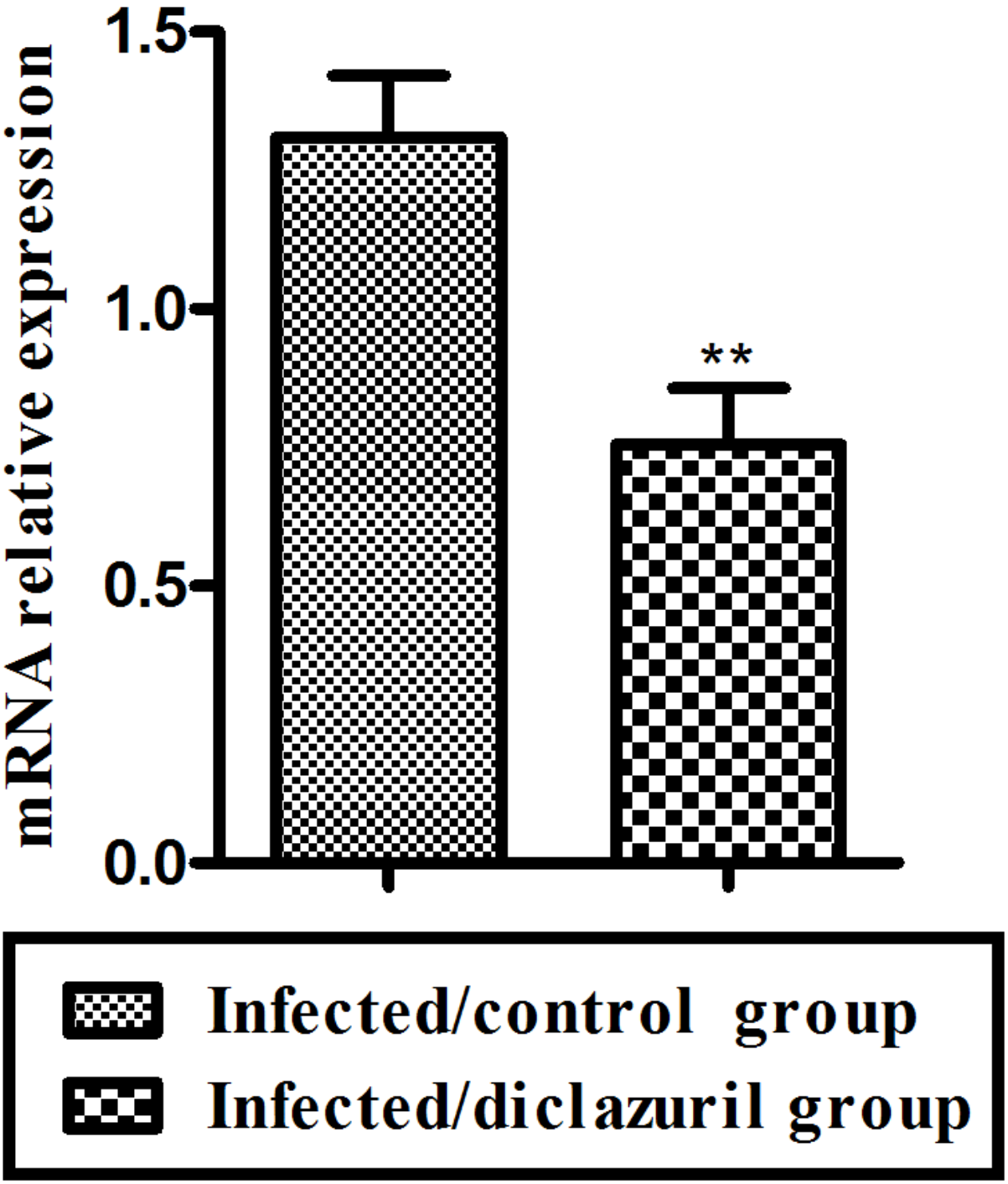

Figure 7

The mRNA relative expression level of EtCRK2

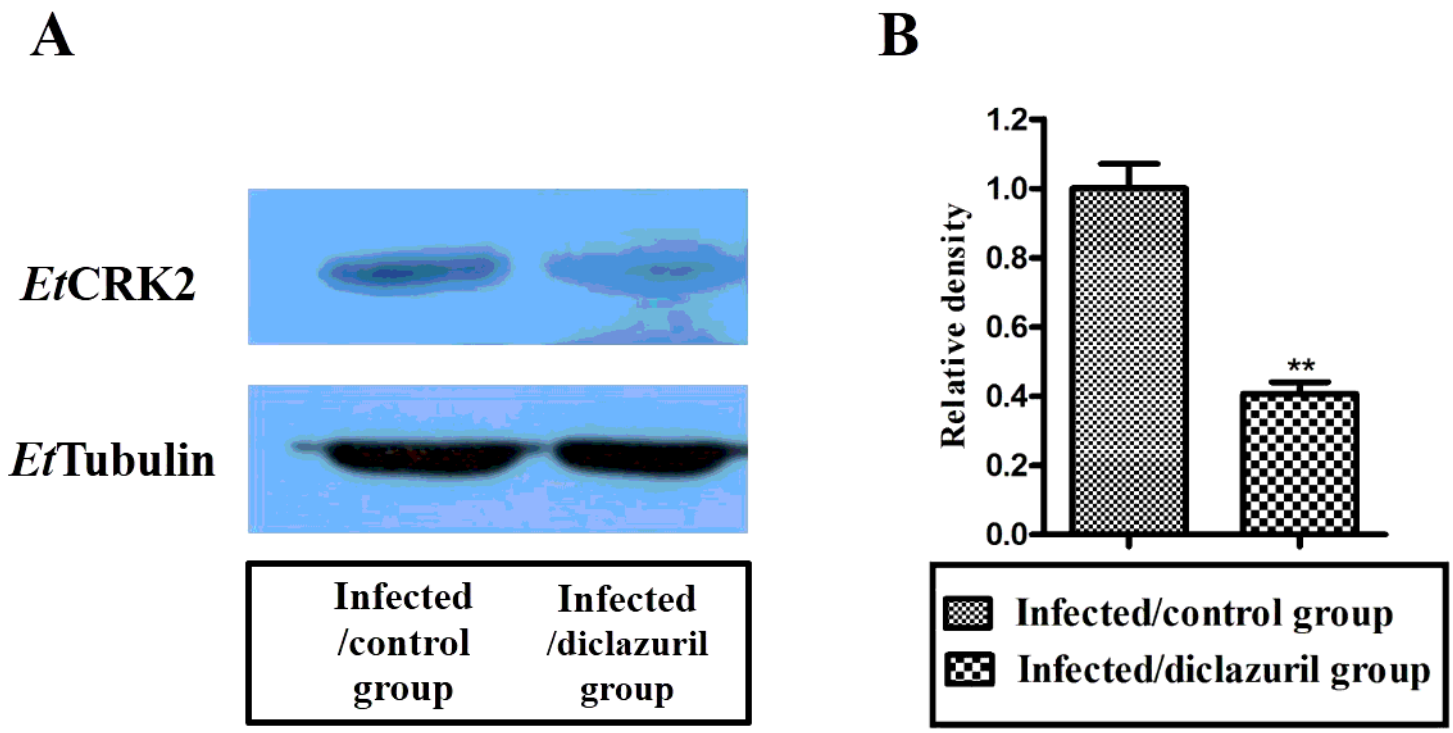

Figure 8

Western blot analysis of EtCRK2 protein expression. A, Western blot electrophoretic pattern of EtCRK2 and EtTubulin. B, EtCRK2 relative density. ** P 0.01 compared with the infected/control group.

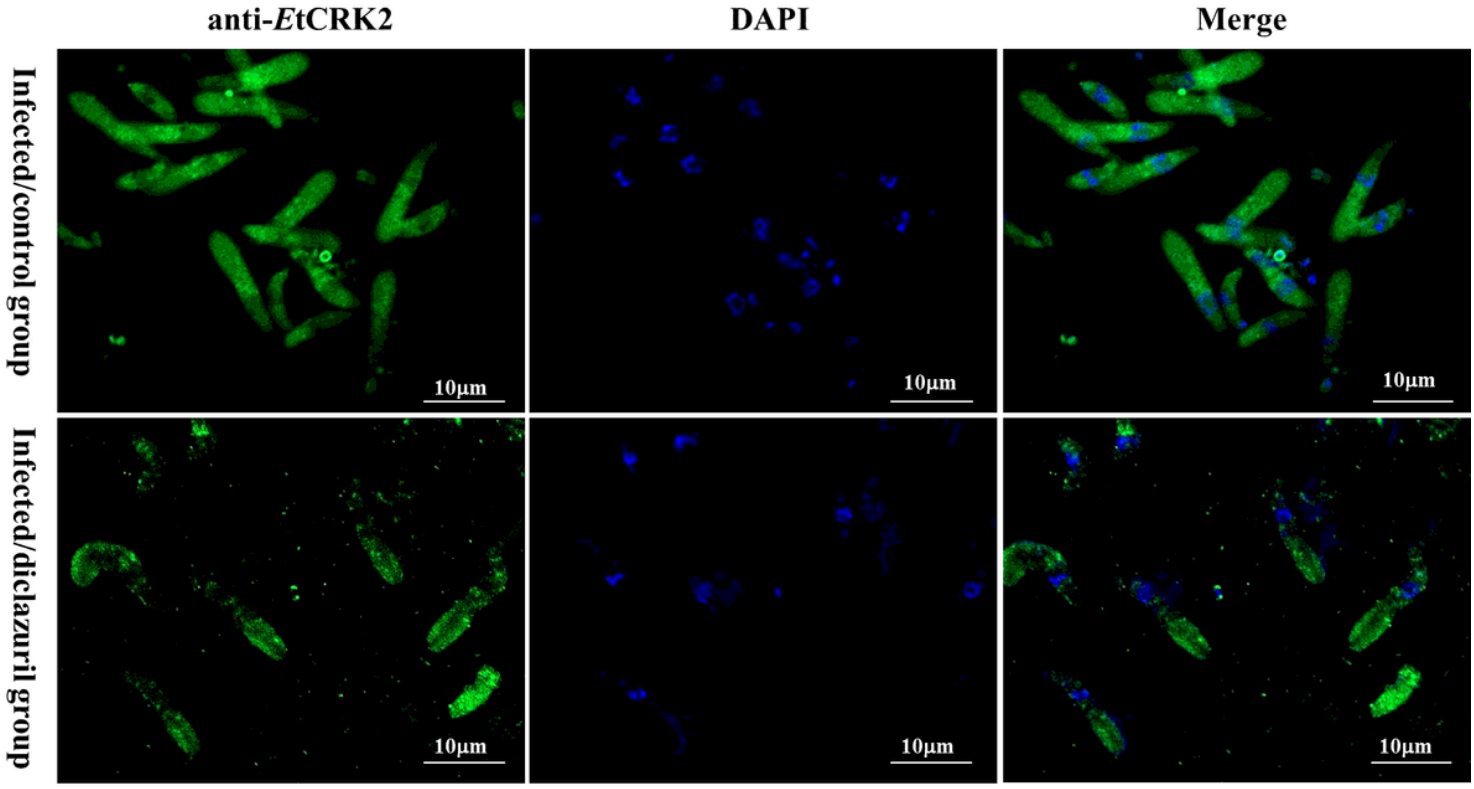

Figure 9

Localizations of EtCRK2 in second-generation merozoites. EtCRK2 is widely distributed to the cytoplasm in merozoite. Compared with the infected/control group, the EtCRK2 immunostaining staining was significantly weaker in the merozoite of the infected/diclazuril group.

Supplementary Files

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