Prokaryotic expression and in vitro activity of Ancylostoma ceylanicum calreticulin

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Research Article

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Abstract

Ancylostoma ceylanicum is a zoonotic soil-derived nematode that parasitizes the intestine of humans and animals (dogs and cats), causing malnutrition and iron-deficiency anemia of the host. Studies have shown that the parasite can regulate or block the host's immune response by secreting calreticulin. However, no data are available on *A. ceylanicum* calreticulin. To study the biological function of *A. ceylanicum* calreticulin (*Ace*-CRT), we amplified the cDNA encoding *Ace*-CRT and constructed the prokaryotic expression vector pET28-*Ace*-CRT to express the target protein. The antigenicity of *Ace*-CRT was investigated by western blotting with canine serum. Mouse splenocytes and canine peripheral blood lymphocytes were stimulated with the recombinant protein *in vitro* to explore its proliferation activity and effect on the transcription level of cytokines. The recombinant protein was co-incubated with sensitized sheep erythrocytes to analyze the inhibitory effect on the complement-mediated hemolysis. Results showed that the r*Ace*-CRT was abundantly expressed in *Escherichia coli*, with good antigenicity. The recombinant protein stimulated the proliferation of mouse splenocytes and canine peripheral blood mononuclear cells (PBMCs). Quantitative reverse-transcription polymerase chain reaction showed that r*Ace*-CRT mainly promoted the expression of Th2 cytokines, especially IL-13, in canine peripheral blood lymphocytes. *In vitro*, r*Ace*-CRT inhibited complement-mediated sheep erythrocytes hemolysis. These findings indicate that *Ace*-CRT has an immunomodulatory role and might be a promising candidate molecule for the hookworm vaccine.

Introduction

Hookworms are soil-derived nematodes that parasitize the intestines of humans and animals. The buccal capsule contains hook-shaped teeth that attach to and lacerate the host's intestinal mucosa to suck blood (Loukas et al. 2001), leading to host malnutrition and iron-deficiency anemia. Hookworm disease is a serious but easily overlooked intestinal parasitic disease. Globally, there are up to 500 million human hookworm infection cases (Bartsch et al. 2016). This disease is estimated to cause about 65,000 global deaths annually (WHO 2002). However, the long-term sequelae of hookworm-associated anemia and malnutrition may account for losing up to 22 million disability-adjusted life-years annually (Diemert et al. 2008). *Necator americanus* and *Ancylostoma duodenale* can only infect humans, while *Ancylostoma ceylanicum* and *Ancylostoma caninum* are zoonotic species that can infect dogs, cats as well as humans (Aula et al. 2020). Studies have shown that *Ancylostoma ceylanicum* has become the second-largest hookworm infecting people in Southeast Asian areas (Traub et al. 2013), causing abdominal pain, diarrhea, malnutrition, and iron-deficiency anemia (Sungkar et al. 2019). Therefore, it is urgent to develop new methods to control this disease.

Calreticulin (CRT) is a multifunctional protein that mainly regulates calcium ions homeostasis, intracellular calcium concentration, and calcium storage in the endoplasmic reticulum (Krause and Michalak 1997). Calreticulin also functions as an intracellular chaperone for glycoproteins, engaging carbohydrate side chains via well-defined lectin domains (Coppolino et al. 1997). In addition, CRT can also participate in signal transduction and cell adhesion, regulating gene expression and other functions

(Tang et al. 2005; Johnson et al. 2001). The detection of CRT protein homologs in various parasites, including *Trypanosoma carassii, Trichinella spiralis*, *Necator americanus*, and *Brugia malayi*, suggests that this protein may play a variety of conserved roles (Pritchard et al. 1999; Ferreira et al. 2004, Oladiran and Belosevic 2010; Yadav et al. 2014; Zhao et al. 2017). When parasites invade the host, they may secrete CRT as a defense mechanism to divert host defensive responses (Ferreira et al. 2004). This protein became a target for the host's immune response (possibly generating antibodies cross-reactive with host CRT). Parasites may take advantage of CRT's anti-thrombotic and complement-inhibitory properties to suppress host defense (Ferreira et al. 2004), facilitating the establishment of parasitic infection. CRT binds to the host complement C1q and mannose-binding lectin (MBL) (Ramírez-Toloza et al. 2020), inhibiting the classic complement pathway and the lectin pathway's activation, counteracting the host immune system. *Trichinella spiralis*-CRT binding to C1q inhibited C1q involved pathogen-clearance functions, reducing the recruitment of immune cells (neutrophils, eosinophils, and macrophages) to the parasitic infection site and decreasing the release of reactive nitrogen intermediates and reactive oxygen intermediates (Zhao et al. 2017). *B. malayi*-CRT inhibited C1q-dependent lysis of immunoglobulin-sensitized red blood cells (Yadav et al. 2017). Presently, the function of *Necator americanus* calreticulin has been preliminarily explored. *Na*-CRT stimulated human basophils and mast cells to release type II cytokines (Pritchard et al. 1999), but there is no report on the study of *A. ceylanicum* CRT.

This study aims to reveal the biological function of *A. ceylanicum* calreticulin (*Ace*-CRT). We cloned and expressed *Ace*-CRT and then analyzed the antigenicity, immunogenicity of the recombinant protein and its effect on sensitized red blood cells. This study may lay a foundation to screen hookworm vaccine candidate molecules.

Materials And Methods

PCR amplification of Ace-CRT gene

Ancylostoma ceylanicum cDNA used to amplify cDNA encoding *Ace*-CRT was provided by our laboratory. A pair of specific primers, *Ace*-CRT-F (5´-ACGGCCAGTGAATTCATGGCTGT-3´) and *Ace*-CRT-R (5´-CTATAAAAGCTT GGCG-3´), were designed according to *A. ceylanicum* CRT gene (EYB83200.1) in GenBank and synthesized by Sangon Bioengineering (Shanghai) Co., LTD. *Ace*-CRT-F and *Ace*-CRT-R primers contained the restriction sites for *Xcm*Ⅰ (CCAGTGAATT CATGG) and *Hin*dⅢ (AAGCTT) endonuclease, respectively. The PCR reaction (25 μL) contained cDNA (0.5 μL), 1 μL of each forward and reverse primers, 2× PrimeStar HS PCR Master Mix (12.5 μL, TaKaRa, Dalian, China), and ddH_2O (10 μL). The cycling conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 54.8°C for 40 s and 72°C for 90 s; and final extension at 72°C for 8 min. PCR products were visualized by 1% agarose gel electrophoresis and harvested by the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA).

Cloning and sequence analysis of Ace-CRT gene

The PCR product and pUC-57 vector (Tsingke, Beijing, China) were double-enzyme digested with *Xcm*I and *Hind*Ⅲ (New England Biolabs, Ipswich, UK) and ligated with T4 ligase (TaKaRa, Dalian, China). Then, the mixture was transformed into *Escherichia coli* (*E. coli*) DH5α competent cells (TaKaRa, Dalian, China), following the manufacturer's protocol. Single white colonies were selected and inoculated into a 2 mL centrifugal tube containing 1 mL Luria-Bertani (LB) broth with 1 μL ampicillin, followed by shaking at 180 rpm, 37°C for 8 h. The positive plasmids were identified by double-enzyme digestion and bacterial liquid PCR identification and sent to Sangon Biotech Company for sequencing. The amino acid sequences of *Ace*-CRT were predicted using the DNAMAN software. The size and position of signal peptide in the CDS region of *Ace*-CRT amino acid sequence were analyzed by the online software SignalP5.0 (http://www.cbs.dtu.dk/services/SignalP). The trans-membrane domain of *Ace*-CRT amino acid sequence was examined by the online tool TMPred (https://bio.tools/TMPred). The software ProtScale (https://web.expasy.org/ protscale/) was used to determine *Ace*-CRT hydrophobicity/hydrophilicity. The protein tertiary structure was predicted using the online tool Swiss-model (http://swissmodel. expasy.org/).

Expression and purification of Ace-CRT recombinant protein

According to the *Ace*-CRT sequence (without the signal peptide), a pair of primers, F: 5´-GATAAGCTAGCATTCATGGC-3´ and R: 5´-CCGTCCCAAGCTTTTATAG T-3´, were designed to amplify the mature peptide sequence of *Ace*-CRT. The underlined parts represent *Nhe*I and *Hind*III's restriction sites. The PCR reaction mixture and cycling program were the same as mentioned above in the PCR amplification of *Ace*-CRT. The PCR products were examined on 1% agarose gel and harvested by the E.Z.N.A.® Gel Extraction Kit. The purified PCR products and the expression vector pET28a were digested by *Nhe*I and *Hin*dIII enzymes (New England Biolabs, Ipswich, UK), analyzed by 1% agarose gel electrophoresis, and then purified from the gel. The recovered products were ligated, transformed into *E. coli* BL21 competent cells (Sangon Biotech, Shanghai, China) and spread on LB plates supplemented with kanamycin. A single transformed colony was mixed into LB broth/ 0.1% kanamycin and incubated (200 rpm, 37℃) until 0.6 OD value. Then, the isopropyl *β*-D-1-thiogalactopyranoside (IPTG) with the final concentration of 0.5 mM was added to induce expression for 16 h. The bacterial solution was centrifuged (10,000 × g, 4℃, 5 min), and the supernatant was discarded. The bacteria pellets were resuspended in phosphate buffer saline (PBS). The mixtures were crushed in an ice bath by an ultrasonic cell disruptor (Toshiba, Tokyo, Japan) at 250W for 40 min with an interruption for 3 s every 2 s of sonication. After centrifuging at 12,000 × g for 5 min at 4℃, supernatant and precipitate were separated. The supernatant was transferred to the Ni-NTA Affinity Chromatography Column (Beyotime, Shanghai, China), let stand overnight at 4℃ and eluted with a 0–50 mM gradient imidazole solution. The supernatants, precipitates, and eluted proteins were mixed with SDS-PAGE loading buffer, heated at 95°C for 5 min and analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, followed by staining with Coomassie Brilliant Blue (Solarbio, Beijing, China). The Bicinchoninic Acid (BCA) Protein Assay Kit (Sangon Biotech, Shanghai, China) was utilized to measure the purified recombinant protein concentration.

Analysis of the specificity and antigenicity of recombinant Ace-CRT

Blood samples (2 mL) were collected from the cephalic vein of *A. ceylanicum* infected and healthy dogs in collection tubes and refrigerated at 4°C overnight. The positive serum of infected dogs and the negative serum of healthy dogs were collected by centrifuging blood samples at 4000 × g for 15 min. After SDS-PAGE analysis, purified *Ace*-CRT protein was electrotransferred to nitrocellulose (NC) membrane (Sangon Biotech, Shanghai, China). Western blot was performed with mouse anti-His tag antibody (1:3000, Abbkine, California, USA), positive dog serum, and negative dog serum as primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-mouse and rabbit anti-dog antibodies (1:3000, Abbkine, California, USA) were used as the corresponding secondary antibodies. The specificity and antigenicity of the recombinant protein were tested by the 3,3´-diaminobenzidine substrate (DAB) Western blot detection kit (Solarbio, Beijing, China).

The effect of r Ace -CRT on the splenocyte proliferation in mouse

The primary splenocytes were isolated from BALB/c mice (6–8 weeks old). The mice were sacrificed and soaked in alcohol for disinfection. The spleen was removed from the abdominal cavity, crushed into pieces, and filtered through a 200-mesh sieve to collect the primary splenocytes. Then, RPMI-1640 medium containing 10% fetal bovine serum was added to isolated splenocytes and centrifuged at 2400 × g for 5 min, decanting the supernatant. Washing and centrifugation of splenocytes were repeated. The cell pellets were harvested and resuspened in RPMI-1640 medium, and viable cells were counted after staining with trypan blue (Sangon Biotech, Shanghai, China). Samples with viability over 95% were selected for subsequent analysis. The mouse splenocytes (2×10^3) were added to each well in a 96-well plate and cultured in a 5% CO_2 cell incubator at 37℃. After stabilization, 100 μL of recombinant *Ace*-CRT at 15 μg/mL, 30 μg/mL, 60 μg/mL, and 90 μg/mL and BSA (negative control) were added at each well and placed in a 37°C, 5% CO_2 cell incubator for 24 h. Then, the absorbance values at 570 nm were measured in each group by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (van Meerloo et al. 2011).

The effect of r Ace -CRT on PBMC cytokine expression

An anticoagulated blood sample (2 mL) was collected from a healthy dog and used to isolate canine peripheral blood mononuclear cells (PBMCs) using Lymphocytes Isolation Kit (TBD, Tianjin, China). The precise isolation method was mentioned by He et al. (2021). Isolated lymphocytes were resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The cell density was adjusted to 3×10^6 cells/mL and inoculated to a 24-well culture plate. The cells were cultured in a 5% CO_2 incubator at 37℃. After the lymphocyte growth was stabilized, the experimental groups (with purified protein), blank control and negative control (BSA protein) were set up. Eight working concentrations of purified protein (5, 10, 15, 30, 45, 60, 75 and 90 μg /mL) were used in experimental groups, with three repetitions for each group. The prepared plate was incubated in a cell culture incubator (37□C, 24 h, 5% CO2). Following centrifugation of the cell suspensions, the precipitates were harvested

and used to extract PBMC total RNAs according to the instructions of the E.Z.N.A.® Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA). A microplate reader was used to assess the quality of the RNA (OD260/OD280 ≈ 2.0). Agarose gel electrophoresis was performed to determine RNA integrity.

The recovered PBMC total RNA was used to synthesize the first-strand cDNA by reverse transcription using the HiScript III RT SuperMix for qPCR (+ gDNA wiper) (Vazyme, Nanjing, China) following the manufacturer's protocol. Briefly, genomic DNA contamination was removed by mixing 1 μg template RNA with 4 μL of 4× gDNA wiper Mix and RNase free ddH_2O up to 16 μL, followed by incubation at 42℃ for 2 min. Then, 4 μL of 5× Hiscript III qRT SuperMix was gently mixed with the previous mixture and incubated at 37℃ for 15 min and 85℃ for 5 s. The resulting cDNA was stored at -20°C.

The fluorescence quantitative reverse-transcription PCR (qRT-PCR) was performed to detect the cytokines expression in PBMCs stimulated by *Ace*-CRT different concentrations using a LightCycler® 96 Real-time PCR. The sequence and lengths of qPCR primers used for amplifying canine 18S rRNA, and cytokine IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-23, IFN-γ genes are shown in Table 1. The reaction systems (10 μL) were as follows: 5 μL of ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), 0.4 μL of each forward and reverse primers, cDNA (1 μL), and double-distilled water (3.2 μL). qPCR reaction conditions comprised 50 cycles of 95℃ for 1 min, 95℃ for 10 s, and 60℃ for 30 s. The 18S rRNA was used as the internal reference gene, and each sample was examined in triplicate. Relative expression levels of cytokines were calculated by the 2-ΔΔCT method (Livak and Schmittgen 2001).

Table 1
Primers used for the quantitative real-time PCR in this study

Target gene	Primer sequence (5′- 3′)	Fragment size	References
18S rRNA	F: CTCAACACGGGAAACCTCAC R: CGCTCCACCAACTAAGAACG	110	Espíndola et al.(2012)
IL-4	F: CACCTCCCAACTGATTCCAA R: CTCGCTGTGAGGATGTTCAA	123	Li et al. (2021)
IL-5	F: ACCTGCAAGTATTTCTTGGTGTA R: AAGCCGGTTTGTTCTCAACTT	67	Fairfax et al.(2018)
IL-6	F: TCCACAAGCGCCTTTCTCCCTGG R: TCACGCACCTCATCCTGCGACTG	80	Varga et al.(2021)
IL-10	F: CAGAGCACCCTACCTGAGGA R: AAGTCTTCACCCTCCCGAAG	97	Huang et al.(2021)
IL-12	F: AAGCCACCTGGACCACCTTA R: AATATTCCTGGGCTCGGTGA	66	Kurata et al.(2004)
IL-13	F: CCTCCTCAGAGCAAAGTG R: CCCAGCACAAACAAAGAC	148	Chenery et al. (2021)
IFN-γ	F: GCGCAAGGCGATAAATGAAC R: CTGACTCCTTTTCCGCTTCCT	82	Abe et al. (2019)

Ace -CRT inhibitory effect on complement-mediated hemolysis of sheep erythrocytes

A hemolysis test was slightly improved from the method of Rose et al. (1997). The whole blood of a healthy dog was collected into a clean collection tube, left stand for 1 h at room temperature, kept at 4℃ for 2 h and centrifuged at 6000 × g for 10 min at 4℃ to collect serum. The canine serum sample was serially diluted at 1:2, 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160 with barbitone buffer solution (BBS-GM) to determine the optimal dilution of serum. Each serum dilution was mixed with sensitized sheep erythrocytes and BBS-GM (200 µL each) and used for the hemolysis test, with three replicates for each group. A mixture of sheep erythrocytes (2%, 100 µL) and ultrapure water (500 µL) was considered the complete hemolysis group. The absorbance of this group was used as the standard to calculate the hemolysis rate. Next, the optimal dilution of canine serum (200 µL) was mixed with 200 µL of r*Ace*-CRT of different concentrations (5, 10, 15, and 25 µg/mL). Then serum/ r*Ace*-CRT suspensions were gently mixed with 200 µL of sensitized sheep erythrocytes, incubated at 37°C for 30 min in a water bath and

centrifuged at 2400 × g for 10 min. The supernatant (150 μL) was collected from each group to measure the absorbance value at 405 nm (A1). BBS-GM, r*Ace*-CRT, and sensitized sheep red blood cells were used as the control group to measure the absorbance at 405 nm (A2). BBS-GM, canine serum, and sensitized sheep erythrocytes (200 μL each) were set as the complement group for measuring the absorbance value at 405 nm (A0). Hemolysis inhibition rate =[1-(A1-A2)/A0] ×100%.

Data analysis

The data were expressed as the mean ± SD values. All results were analyzed with SPSS 22.0 (SPSS Inc., USA) and GraphPad Prism 6.2 (GraphPad Inc., USA). A significant difference was considered when p-value < 0.05.

Results

Amplification and cloning of Ace-CRT gene

The cDNA encoding *Ace*-CRT was amplified from *A. ceylanicum* cDNA with a PCR product size of approximately 1200 bp (Fig. 1A). Following cloning, the bacterial liquid PCR demonstrated that the size of amplified bands corresponded to the expected fragment size (Fig. 1B). After double-enzyme digestion of the recombinant plasmid pET28a-*Ace*-CRT, a fragment of nearly 1200 bp was resulted, consistent with the expected target fragment size (Fig. 1C).

Bioinformatics analysis of Ace-CRT

Sequencing results showed that the *Ace*-CRT gene was 1224 bp in total length (OM280324) and encoded an open reading frame of 407 amino acids. The amino acid sequence of *Ace*-CRT was analyzed by bioinformatics software. The results showed that the average hydrophilicity coefficient of the protein was less than zero, indicating that *Ace*-CRT is a hydrophilic protein (Fig. 2A). There was a transmembrane domain (Fig. 2B), and the first 20 amino acids of the protein represented a signal peptide sequence (Fig. 2C). The quality assessment value of the protein's 3D structure model was 0.66, indicating good modeling quality (Fig. 2D).

Expression, purification and antigenicity analysis of r Ace -CRT

We used SDS-PAGE to analyze the bacterial culture (before and after expression induction), precipitate, supernatant, and purified protein. Results showed that the protein of interest was mainly expressed in the supernatant, and the purification result was optimal with 50 mM imidazole. The size of the recombinant protein was about 50 kDa (Fig. 3), which was consistent with the expected size of the target protein.

After Western blot analysis, this recombinant protein could be recognized by anti-His-tag antibodies, with a specific band at 50 kDa (Fig. 4A). The recombinant protein was also identified by positive canine serum with a clear band at 50 kDa. No reaction bands were formed with negative canine serum (Fig. 4B), indicating good antigenicity of the recombinant protein.

Effect of r Ace -CRT on the proliferation of mouse primary splenocytes

To assess r*Ace*-CRT effect on splenocyte proliferation, we measured the OD570 nm value of mouse primary splenocytes after co-culturing with different r*Ace*-CRT concentrations. As shown in Fig. 4, r*Ace*-CRT significantly stimulated the mouse primary splenocyte proliferation. When r*Ace*-CRT concentration was 15 and 30 μg/mL, the proliferation activity of splenocytes was the highest, significantly different from the control group. Additionally, the splenocyte proliferation simulation at the concentrations 60 and 90 μg/mL was significantly higher than in the control group (Fig. 5).

Effect of r Ace -CRT on PBMC cytokine expression

After stimulation of canine PBMCs with different concentrations of r*Ace*-CRT, the expression levels of IL-4, IL-10, IL-12, and IL-13 increased compared with the control group, of which IL-13 was significantly different from the control group. However, the expression levels of IL-5, IL-6, and IFN-γ decreased compared with the control group (Fig. 6).

Inhibitory effect of r Ace -CRT on sensitized erythrocyte hemolysis

Increasing the canine serum dilution ratio gradually reduced the hemolysis of sensitized sheep erythrocytes. When the serum dilution ratio was 1:50, the hemolysis rate was 90.7% (Fig. 7A). Therefore, 1:50 was selected as the optimal dilution ratio. Compared to the control group, r*Ace*-CRT at different concentrations inhibited the hemolysis of sensitized sheep erythrocytes. The hemolysis inhibition rate increased with increasing protein concentration (Fig. 7B).

Discussion

Currently, benzimidazoles are mainly used for deworming and controlling hookworm disease (Sungkar et al. 2019). Still, this measure cannot prevent re-infection of hookworm and is prone to drug resistance (Soukhathammavong et al. 2012). Therefore, there is an urgent need to find a new vaccine candidate molecule for further prevention and control measures. Calreticulin (CRT) is a multifunctional protein with anti-inflammatory, hemostatic, and immune potential. Pritchard et al. (1999) reported that CRT of *Necator americanus* (*Na*-CRT) can directly induce human basophils and mast cells to produce type II cytokines, causing allergic reactions. When researchers combined the purified recombinant protein with human complement protein C1q, the hemolytic function of C1q was significantly inhibited (Kasper et al. 2001; Pritchard et al. 2003; Logan et al. 2020). This finding indicates that *Na*-CRT has an immunomodulatory role. In addition, upon *Necator americanus* larval challenge, mice vaccinated with *Na*-CRT showed a 43–49% reduction rate in worms recovered from lung compared with the control group, indicating the potential of *Na*-CRT as a vaccine (Winter et al. 2005). So far, no studies on CRT of *Ancylostoma ceylanicum* have been reported.

In this study, the *Ace*-CRT gene was cloned and expressed for the first time. The cDNA encoding *Ace*-CRT length was 1224 bp, and the recombinant protein size was about 50 kDa. SDS-PAGE analysis of the pellet

and supernatant showed that the recombinant protein was expressed in the supernatant in large quantities, which provided great convenience for subsequent purification, consistent with the results of bioinformatics analysis (hydrophilicity). This recombinant protein could bind to His-tag antibodies and be recognized by positive canine serum, indicating good antigenicity. Co-incubation of r*Ace*-CRT with mouse primary splenocytes at different concentrations showed that r*Ace*-CRT significantly stimulated lymphocyte proliferation. This result suggests that *Ace*-CRT could promote the proliferation of immune cells and have an immunomodulatory role.

Cytokines are polypeptide molecules synthesized and secreted by immune cells that regulate cell differentiation and immune function (Ramakrishnan et al. 2012). This study showed that the expression levels of cytokines IL-4, IL-10, IL-12, and especially IL-13 were significantly increased in canine PBMCs stimulated by *Ace*-CRT recombinant protein. IL-4 is a product of T cells and can induce helper T cells (naive $CD4^+$ T cells) differentiation into the Th2 subset of effector cells. This subset is characterized by the stereotypic production of a suite of cytokines by committed Th2 cells, including IL-4, IL-5, IL-10 and IL-13 (Balic et al. 2006). Studies have shown that IL-4 plays a crucial role in parasites' primary and secondary infection, contributing to the host resistance to parasite invasion (Rzepecka et al. 2009). Mosmann et al. (1990) initially classified IL-10 as a Th2 cytokine. However, subsequent research has revealed that IL-10 is a key anti-inflammatory regulatory cytokine produced by various cell types, including both T and non-T cell classes. Recently, IL-10 production has been linked to a new $CD4^+$ T cell subpopulation with regulatory rather than effector properties (Treg cells) (Grous et al. 1998; Balic et al. 2006). As an anti-inflammatory cytokine, IL-10 can down-regulate the immunopathological response of the host, inhibit the differentiation of Th1 cells, causing T cell response to tilt toward the Th2 type and remains a critical component of resistance and survival during helminth infection (Balic et al. 2006). IL-13 is a typical type Th2 cytokine with an anti-inflammatory effect (Anthony et al. 2007), which can inhibit the activation of macrophages and stimulate the proliferation of B cells. IL-12 secreted by antigen-presenting cells promotes the differentiation of Th0 to Th1 cells, which primarily resist intracellular pathogen infection but cannot effectively counteract hookworms. In addition, the results of this study showed decreased expression levels of IL-5, IL-6 and IFN-γ. IL-5 cytokine stimulates the maturation of eosinophil and induces Th2 inflammatory response (Liu et al. 2007). The primary function of IL-6 is to enhance the proliferation of T cells (Rzepecka et al. 2009), and INF-γ antagonizes IL-4, activates macrophages and inhibits the proliferation of Th2 cells. Therefore, we concluded that calreticulin of *A. ceylanicum* might mainly contribute to the dominance of Th2 immune response in the host to resist the parasite infection, similar to the conclusion of the research on calreticulin of *Taenia solium* (Mendlovic et al. 2017).

C1q is the recognition protein of the classical complement pathway and the main connecting protein between innate immunity driven by the classical complement pathway and acquired immunity mediated by IgG or IgM (Gasque 2004; Yadav et al. 2014). In this study, canine serum was used as a complement, and then the complement activity (CH50) method (Kageyama et al. 2021) was used to detect the reaction between r*Ace*-CRT and complement. When the dilution ratio of dog serum was 1:50, the hemolysis rate was 90.7%, and r*Ace*-CRT could effectively inhibit the hemolysis of sensitized sheep erythrocytes.

Similarly, *Necator americanus* calreticulin could bind to human C1q and inhibit hemolysis mediated by C1q (Logan et al. 2020). This finding could contribute to bloodsucking by hookworm. Calreticulin of *Haemonchus contortus* could bind to C1q and inhibit the cleavage of sensitized erythrocytes mediated by C1q (Suchitra et al. 2005). These data were conducive to the host's survival, consistent with the results of this study. Due to the limitation of experimental conditions, whether r*Ace*-CRT can affect other pathways of complement activation cannot be directly explored, and further research is needed.

In summary, cDNA encoding *Ancylostoma ceylanicum* calreticulin was cloned for the first time in this study. The recombinant plasmid was constructed, and the expression, purification and function of recombinant proteins were explored. Recombinant *Ace*-CRT had good antigenicity and could stimulate the proliferation of mouse spleen cells and canine peripheral blood mononuclear cells. *Ace*-CRT also stimulated the secretion of Th2 cytokines and inhibited complement-mediated hemolysis. These findings lay the foundation to screen candidate molecules for *A. ceylanicum* vaccine.

Declarations

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Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval

Animal experiments involved in this study were performed following the guidelines of the Animal Welfare Law and Regulations of the Department of Health and Human Services, China and were reviewed and approved by the South China Agricultural University Animal Care and Use Committee (approval number SYXK(Yue)2019-0136).

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Figures

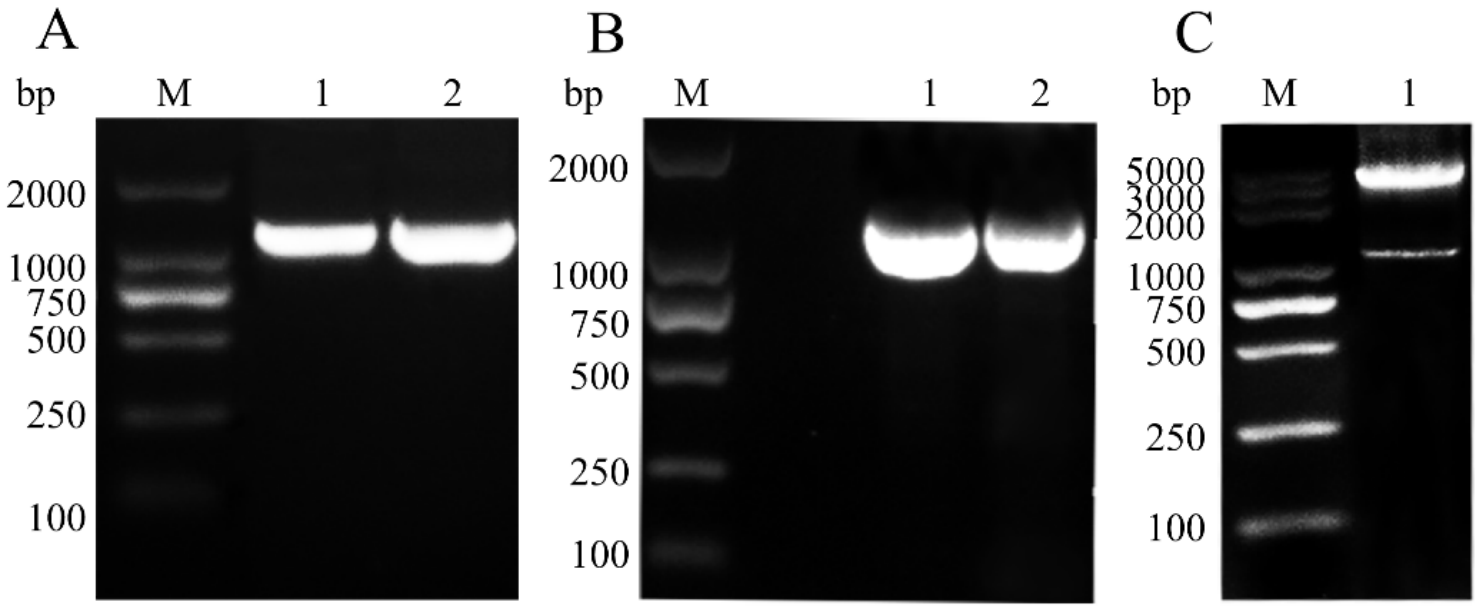

Figure 1

Amplification of *Ace*-CRT gene (A), bacterial fluid PCR (B) and recombinant plasmid enzyme-digested (C) identification. A. M: DL2000; lane 1: Full-length gene fragment; lane 2: Gene fragment except signal peptide B. M: DL2000; lane 1: Monoclonal colonies with full-length gene; lane 2: Monoclonal colonies with gene fragment except signal peptide; C. M: DL5000; lane 1: Enzyme-digested product of pET28a-*Ace*-CRT recombinant plasmid.

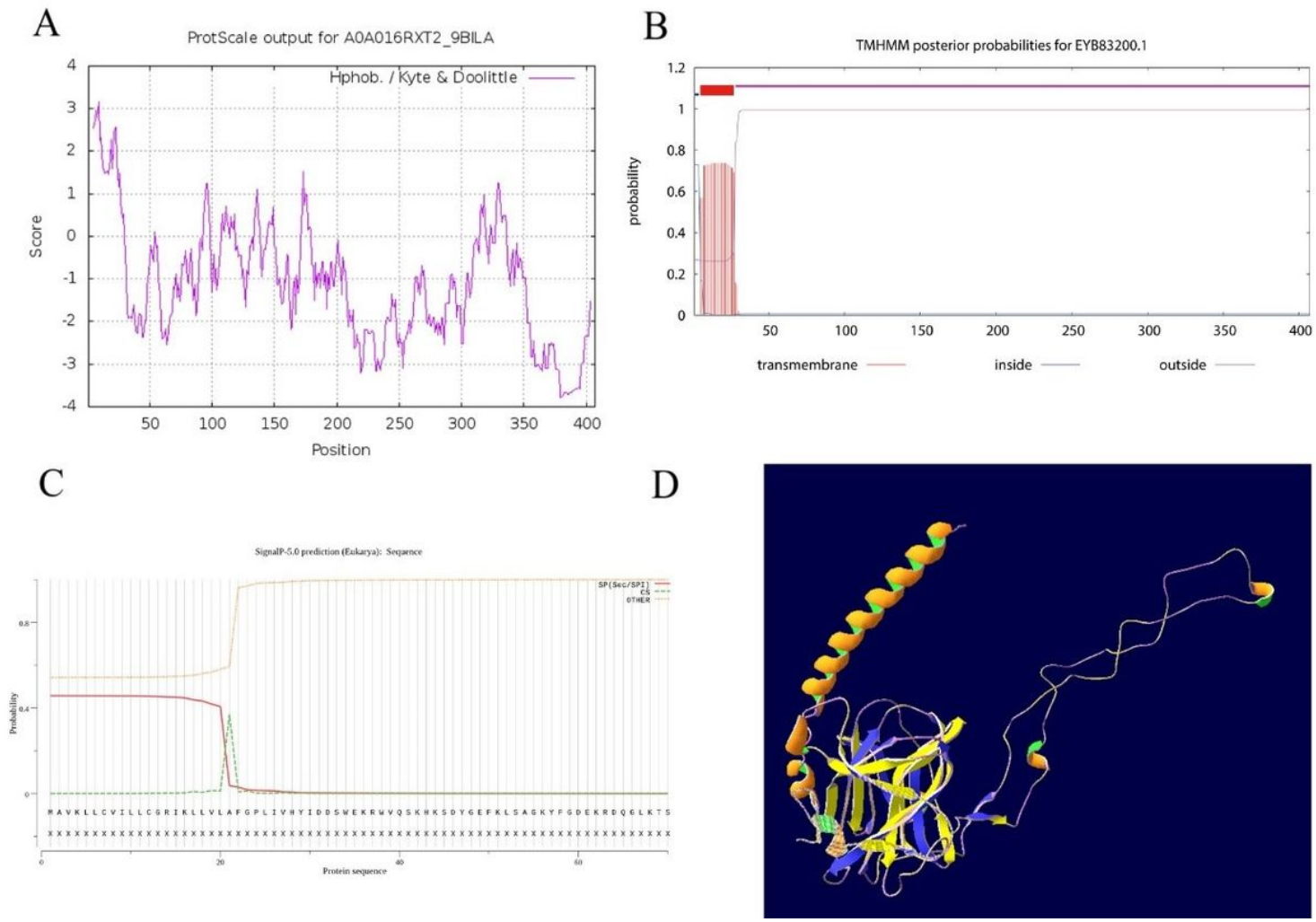

Figure 2

Bioinformatics analysis of *Ace*-CRT. A: Hydrophilic and hydrophobic analysis; B: Prediction of transmembrane domain; C: Signal peptide analysis; D: Prediction of tertiary structure.

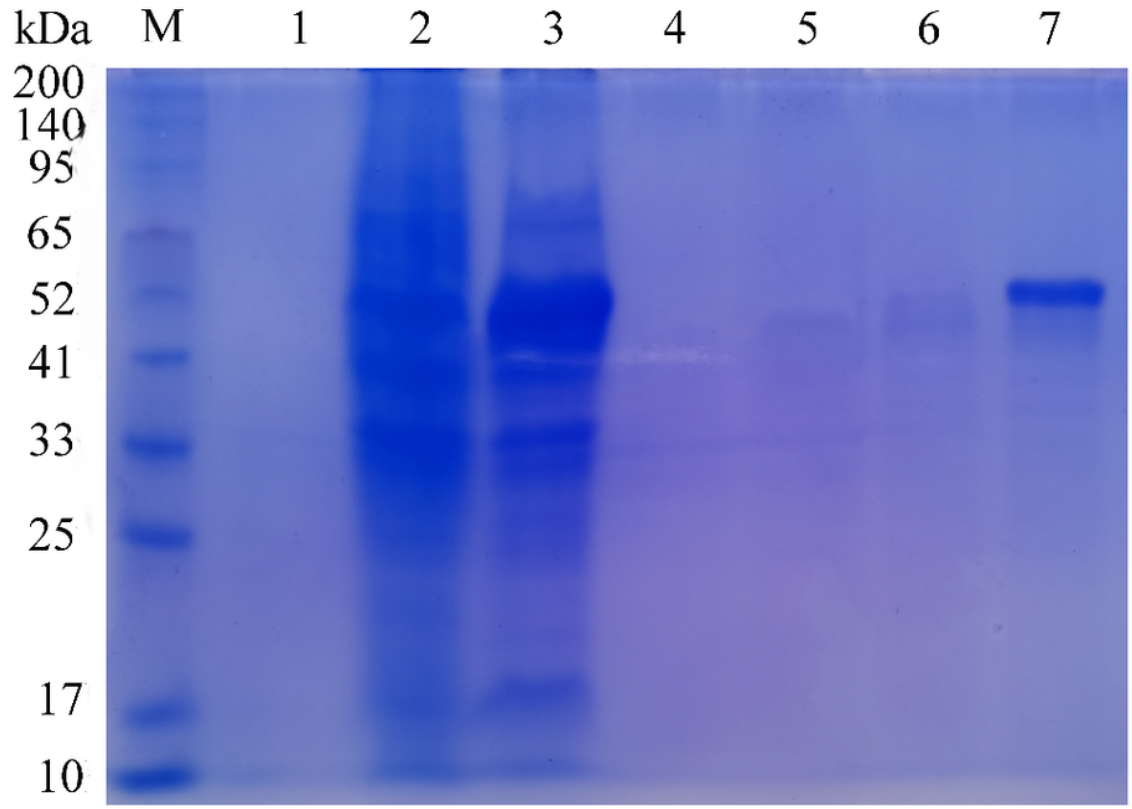

Figure 3

SDS-PAGE analysis of purified *Ace*-CRT recombinant protein. M: Protein molecular weight marker; lane 1: Uninduced recombinant bacteria; lane 2: Precipitation of induced recombinant bacteria; lane 3: Supernatant of induced recombinant bacteria; lane 4: 0 mM imidazole eluate; lane 5: 2 mM imidazole eluate; lane 6: 10 mM imidazole eluent; lane 7: 50 mM imidazole eluent.

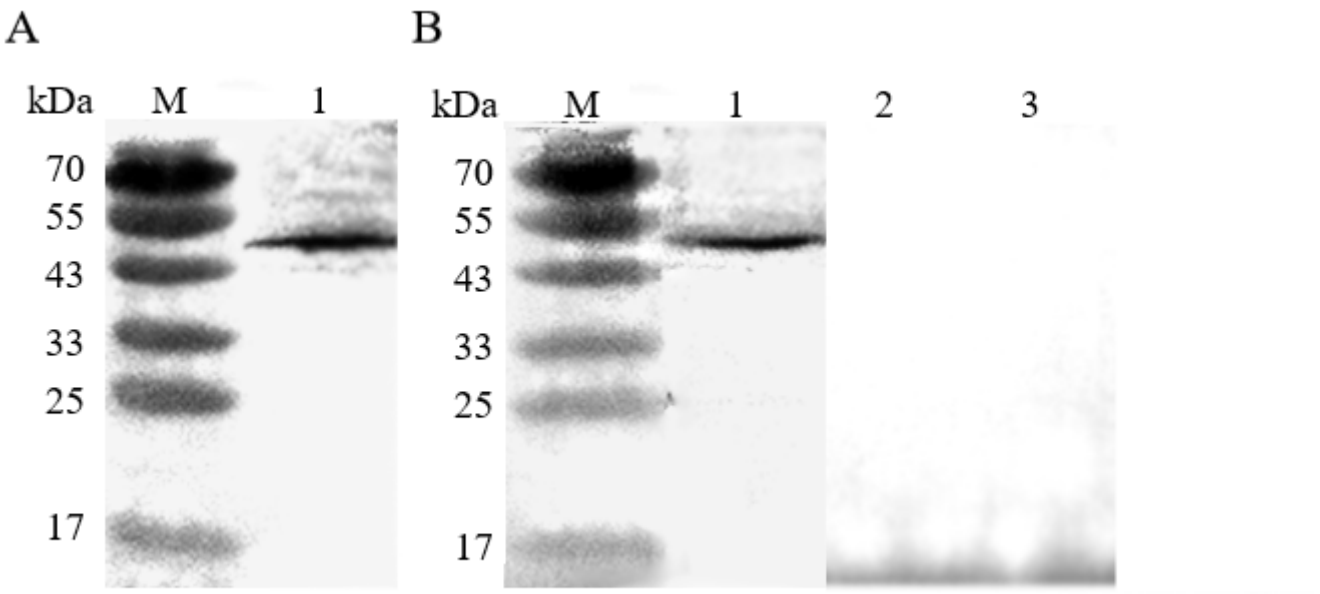

Figure 4

Western blot analysis of *Ace*-CRT recombinant protein. A. M: Protein molecular weight marker; lane 1: The r*Ace*-CRT reacted with anti-His tag antibody; B. M: Protein molecular weight marker; lane 1 The r*Ace*-CRT reacted with positive serum of infected dogs; lane 2 The r*Ace*-CRT reacted with negative serum of healthy dogs; lane 3: Negative control.

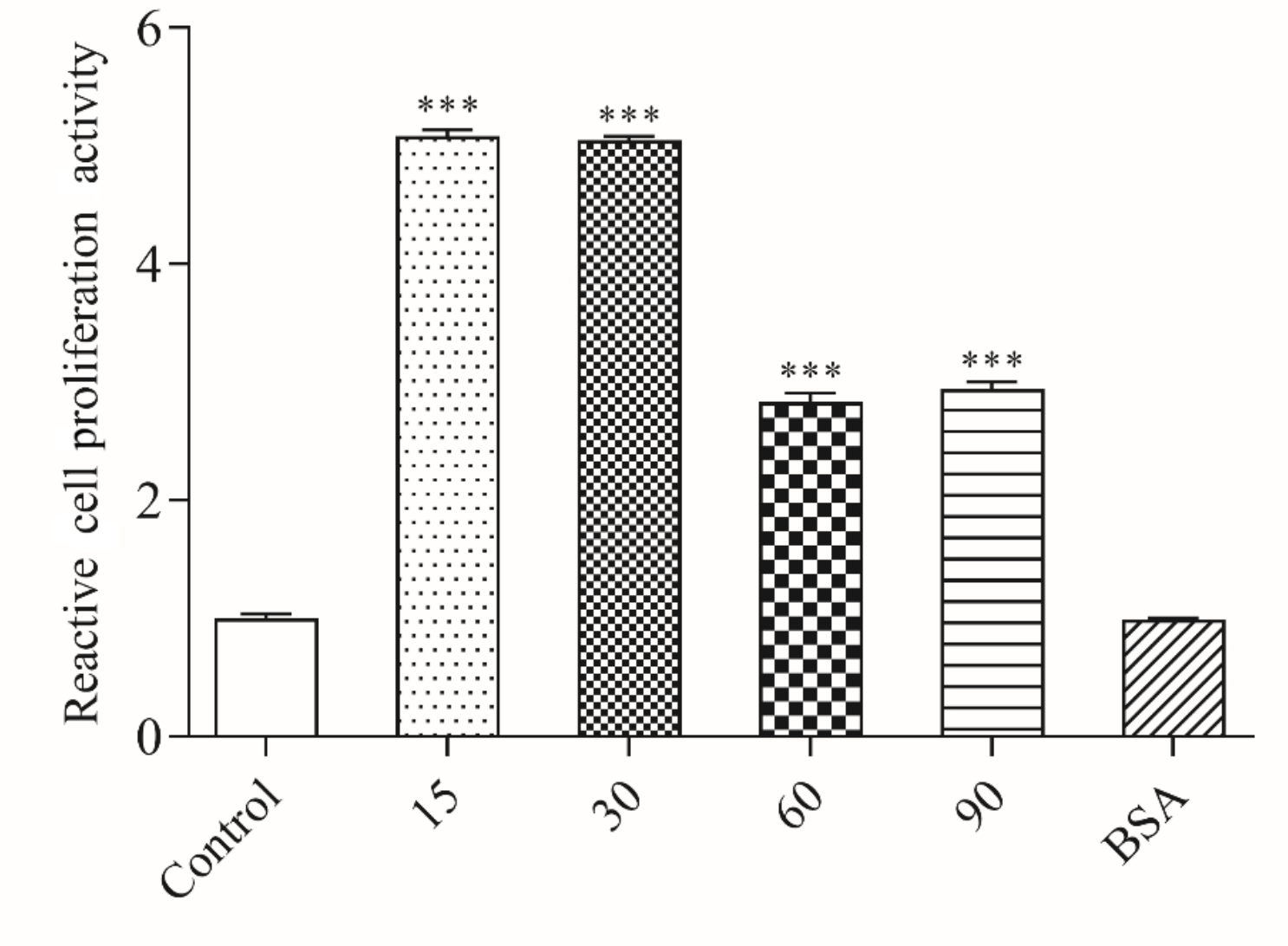

Figure 5

Stimulating activity of r*Ace*-CRT on mouse splenocyte proliferation. *** on the column diagram means p 0.001.

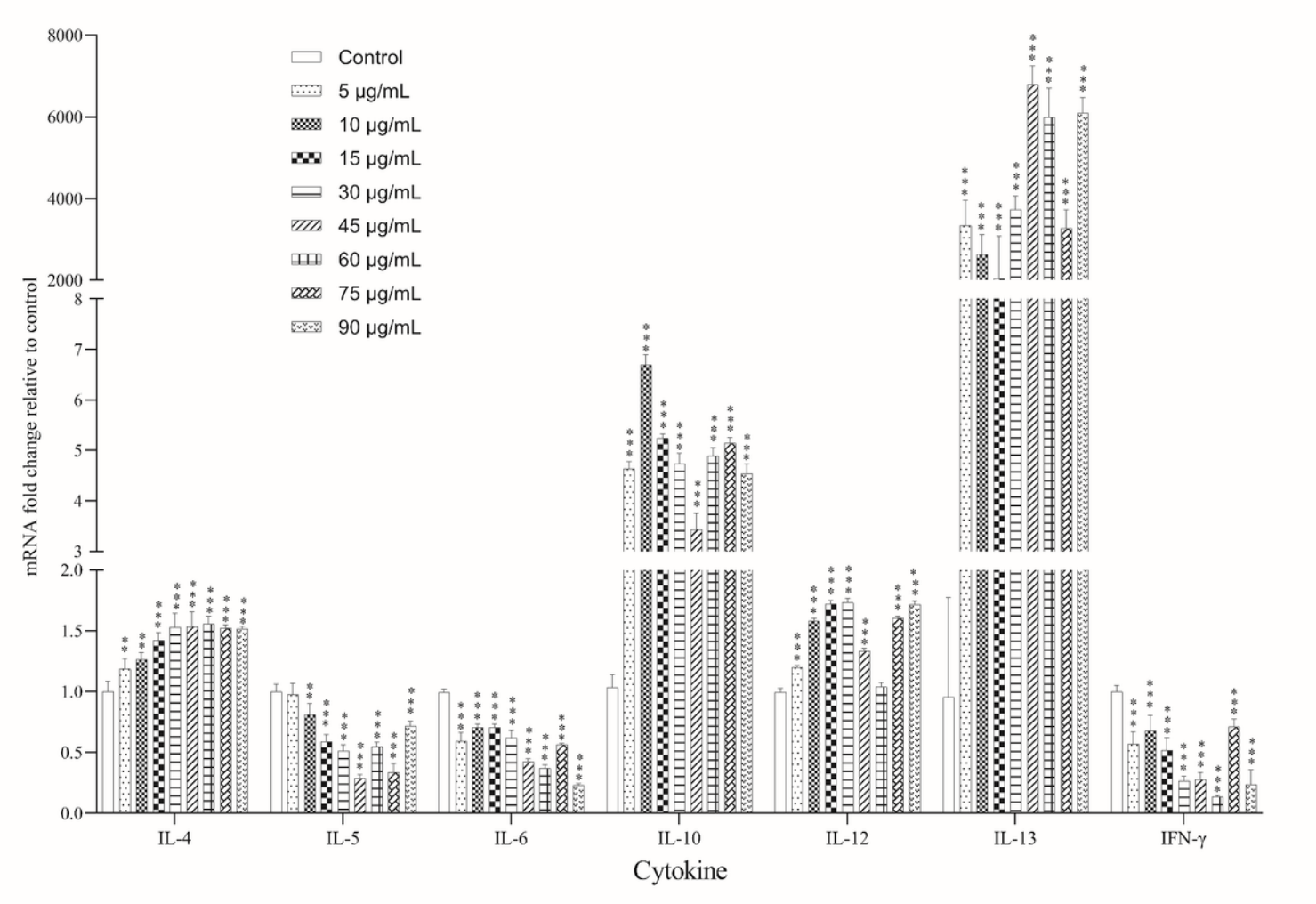

Figure 6

Expression levels of multiple cytokines stimulated by the recombinant *Ace*-CRT. PBMCs were incubated with the recombinant *Ace*-CRT, the mRNAs encoding IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, and IFN-γ were quantified by real time PCR. ** on the column diagram means $p<0.01$ and *** means $p<0.001$.

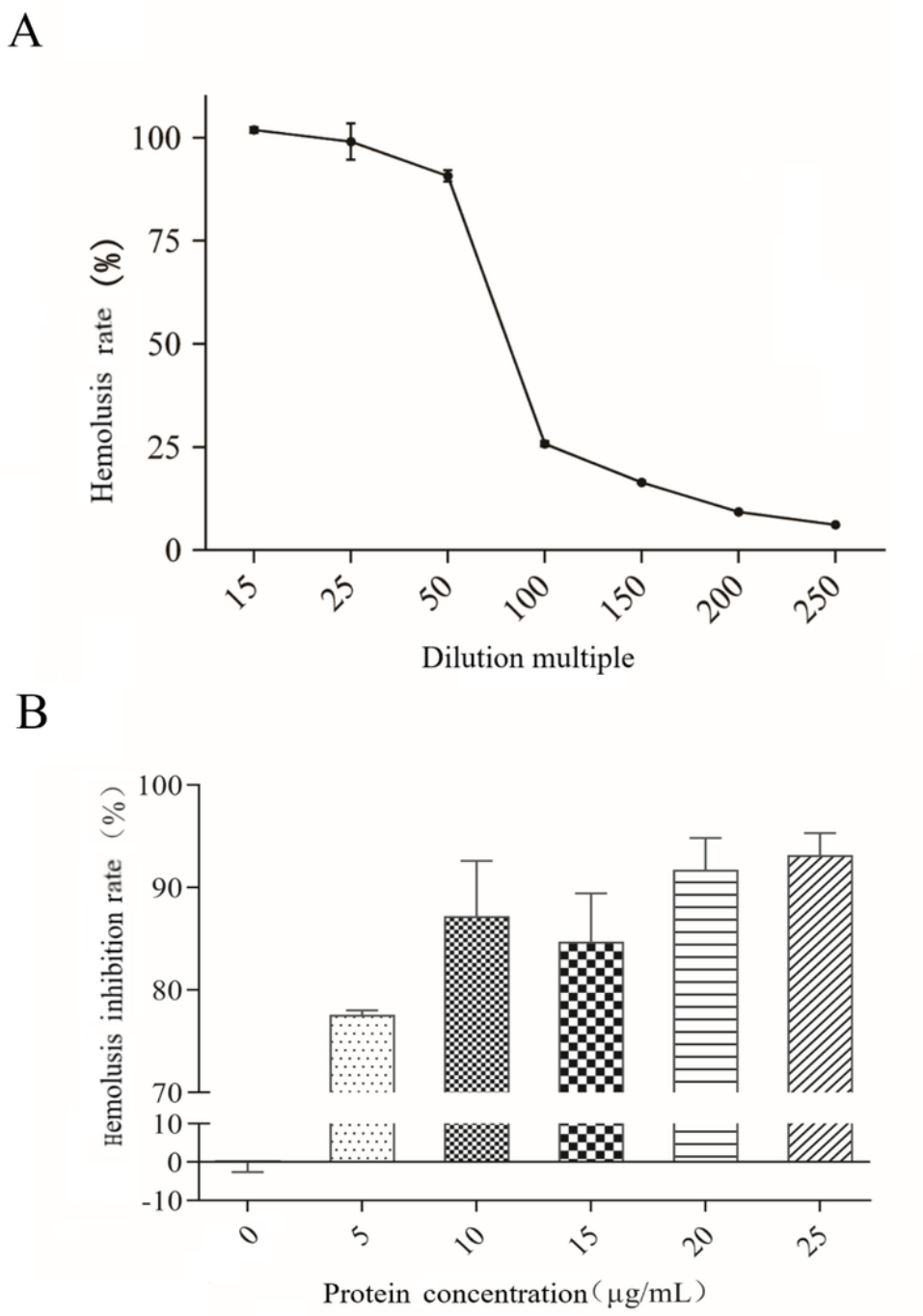

Figure 7

Selection of canine serum optimal dilution (A) and inhibition activity of r*Ace*-CRT on hemolysis (B).