



# Cloning, expression, characterization, and immunological properties of citrate synthase from *Echinococcus granulosus*

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## Abstract

The larval stages of the tapeworm *Echinococcus granulosus* (Cestoda: Taeniidae) are the causative agent of cystic echinococcosis, one of the most important parasitic zoonoses worldwide. *E. granulosus* has a complete pathway for the tricarboxylic acid cycle (TCA), in which citrate synthase (CS) is the key enzyme. Here, we cloned and expressed CS from *E. granulosus* (Eg-CS) and report its molecular characterization. The localization of this protein during different developmental stages and mRNA expression patterns during H<sub>2</sub>O<sub>2</sub> treatment were determined. We found that Eg-CS is a highly conserved protein, consisting of 466 amino acids. In western blotting assays, recombinant Eg-CS (rEg-CS) reacted with *E. granulosus*-positive sheep sera and anti-rEg-CS rabbit sera, indicating that Eg-CS has good antigenicity and immunoreactivity. Localization studies, performed using immunohistochemistry, showed that Eg-CS is ubiquitously expressed in the larva, germinal layer, and adult worm sections of *E. granulosus*. Eg-CS mRNA expression levels increased following H<sub>2</sub>O<sub>2</sub> exposure. In conclusion, citrate synthase might be involved in the metabolic process in *E. granulosus*. An assessment of the serodiagnostic potential of rEg-CS based on indirect ELISA showed that, although sensitivity (93.55%) and specificity (80.49%) are high, cross-reactivity with other parasites precludes its use as a diagnostic antigen.

**Keywords** *Echinococcus granulosus* · Citrate synthase · Tricarboxylic acid cycle · Immunohistochemical localization · mRNA expression

## Introduction

Cystic echinococcosis (CE), caused by infection with the larval stage of the tapeworm *Echinococcus granulosus*, is a chronic disease with parasitic helminths that form cysts in humans as well as in domestic and wild ungulates worldwide (Jenkins et al. 2005). The synanthropic life cycle of *E. granulosus*

involves two mammalian hosts, including an intermediate host, usually a wild or domestic herbivore (humans are accidental intermediate hosts) and a canine definitive host, such as the domestic dog (Guo et al. 2011; Thompson and McManus 2002). Larval stages (metacestodes) can persist asymptotically in the intermediate hosts for decades (Eckert et al. 2001), eventually causing a spectrum of debilitating pathologies and death (Garcia et al. 2007). It is estimated that at least 50 million humans are infected with *E. granulosus* globally (Garcia and Del Brutto 2005), with up to 10% of the population having detectable hydatid cysts in some areas (Li et al. 2011). The latest estimate for the global burden of CE is 1.84 million disability-adjusted life years (DALYs) per annum (Deplazes et al. 2017; Torgerson et al. 2015). Moreover, CE in livestock causes an annual loss of US\$ 3 billion (Torgerson and Macpherson 2011).

Citrate synthase (CS), located at a major metabolic branch point, is a key enzyme in the tricarboxylic acid (TCA) cycle. It catalyzes the reaction between acetyl-coenzyme A and oxaloacetate to give citrate and coenzyme A (CoA) and enables the cellular provision of reducing equivalents and building blocks via citric acid cycle activity or glyoxylic acid cycle activity

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Ning Wang and Hui Zhu contributed equally to this work.

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(Arndt et al. 2008). The CS gene was initially isolated and cloned from yeast (Suissa et al. 1984) and subsequently from mammals, including pigs and humans (Bloxham et al. 1982; Evans et al. 1988; Liu et al. 2000). To date, research into the characteristics and functions of the CS gene has mainly focused on plants, with some studies examining fungi or mammals (Zinsser et al. 2013; Monteiro et al. 2010). As it reported previously, *E. granulosus* has a complete pathway for the TCA cycle, indicating that CS might play a vital role in this tapeworm (Zheng et al. 2013). However, there was not any research about CS in *E. granulosus* has been performed.

In this present study, *E. granulosus* CS (Eg-CS) was cloned and characterized. The localization of this protein in the adult worm and larval stage, and its mRNA expression patterns in protoscoleces (PSCs) after oxidation treatment were determined. The antigenicity and immunoreactivity of recombinant Eg-CS (rEg-CS) were examined and the preliminary enzyme-linked immunosorbent assay (ELISA)-based serodiagnostic potential of rEg-CS was assessed.

## Materials and methods

### Animals and parasites

*E. granulosus* cysts were collected from the liver of naturally infected sheep in an official abattoir in Sichuan Province, China. Fertility of the cysts was determined by microscopy by the presence of PSCs in the hydatid fluid (Khan et al. 2001; Wang et al. 2017). PSCs, as well as cyst walls from fertile and infertile cysts, were isolated and treated aseptically as previously reported (Hu et al. 2015). Fresh PSCs were cultured in complete RPMI 1640 medium (Hyclone, Logan, UT) in an atmosphere containing 5% CO<sub>2</sub> at 37 °C for 1 day to observe their activity and then collected for further treatment. The adult worms of *E. granulosus* were obtained from the small intestine of artificially infected dogs (Wang et al. 2017). All cysts and PSCs used in this study were identified as G1 genotype.

### Sera

Serum samples from sheep naturally infested with *E. granulosus* (31 samples) and *Taenia multiceps* (7 samples), as well as serum samples from goats naturally infested with *Taenia hydatigena* (10 samples), were collected from a slaughterhouse in Sichuan Province. Cut-off values were established using sera from 24 sheep without echinococcosis. The control sera was collected from a non-echinococcosis epidemic area and the lack of echinococcosis was further confirmed by autopsy.

### Bioinformatic analysis

The complementary DNA (cDNA) sequence encoding the CS gene of *E. granulosus* (EgrG\_001028500) was obtained from the GeneDB database (<http://www.genedb.org/Homepage>) and translated into the amino acid sequence using DNASTar software. Basic physicochemical properties, signal peptides, transmembrane regions, subcellular localizations, B cell epitopes, and secondary structure of the CS protein were predicted as in our previous study (Guo et al. 2017; Wang et al. 2017). Furthermore, amino acid sequences of CS from different species were retrieved from NCBI and the GeneDB database. Sequences were aligned and an evolutionary tree was constructed using MEGA software 5.05 by the neighbor-joining (NJ) method.

### Cloning, expression, and purification of rEg-CS

Total parasite RNA from PSCs was extracted and cDNA synthesis performed (Wang et al. 2017). The sequence encoding CS was amplified by polymerase chain reaction (PCR) using the following specific primers: sense primer 5'-CCGGAATTCCTTTGTTCTTCCGATATGT-3' and antisense primer 5'-CCGCTCGAGTCAA GTGGCCTTGACGA-3', which introduce *EcoRI* and *XhoI* restriction enzyme sites (underlined), respectively. CS was amplified using the PCR cycling conditions as follows: 95 °C for 4 min, 35 cycles of amplification at 95 °C for 30 s, 50 °C for 60 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The amplified fragment was cloned into the pET-28a vector (Novagen, Madison, USA), which was transformed into *Escherichia coli* BL21 (DE3) cells (Cowin Biotech, Beijing, China) for protein expression, induced with 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) at 37 °C for 6 h. The rEg-CS was affinity purified and analyzed by 15% SDS-PAGE as previously described (Wang et al. 2017). The concentration of purified rEg-CS was determined using a BCA protein assay kit (Beyotime, Jiangsu, China).

### Preparation of antiserum

The antiserum for immunohistochemistry assays was obtained by immunizing rabbits once with 200  $\mu$ g purified rEg-CS with complete Freund's adjuvant and twice with incomplete Freund's adjuvant at 2-week intervals. The antiserum was collected 2 weeks after the last injection and IgG in antiserum was purified using HiTrap Protein A (GE healthcare, Germany), aliquoted, and stored at -80 °C. Serum was also collected before immunization to serve as a negative control and stored as described above.

## Western blotting

SDS-PAGE was performed to resolve rEg-CS protein which was subsequently transferred onto a nitrocellulose membrane (Boster, Wuhan, China) at a constant current (300 mA) for 1 h. The membrane was blocked with 5% skimmed milk in tris-buffered saline-Tween 20 (TBST) overnight at 4 °C and then incubated with *E. granulosus*-positive sheep sera or anti-rEg-CS rabbit sera at a dilution of 1:200 (*v/v*). After washing with TBST five times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody for 1 h at room temperature and visualized using a diaminobenzidine (Boster) substrate solution. Total PSC extract was also used to determine the specificity and sensitivity of rabbit anti-rEg-CS IgG. Non-infected sheep and pre-immunized rabbit sera were used as negative controls.

## Development of an indirect ELISA

One hundred microliters of rEg-CS antigen diluted in 0.1 M carbonate buffer (pH 9.6) and diluted twofold from 1:20–1:2560 was coated on 96-well microtiter plates at 4 °C overnight. After washing three times with phosphate-buffered saline-Tween 20 (PBST) to remove non-adsorbed antigen, the plates were blocked with 5% skimmed milk (*w/v*) in PBS at 37 °C for 1 h. Positive and negative sera isolated from sheep were twofold diluted in PBS ranging from 1:20 to 1:640 (3.2 µg/well to 0.1 µg/well) and incubated at 37 °C for 1 h. After washing, 100 µL of rabbit anti-sheep HRP-conjugated antibody (diluted to 1:3000 with PBS) was added to each well and incubation continued at 37 °C for 1 h. After a final wash, the enzyme reaction was visualized by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) at room temperature for 15 min and quenched with 0.5 M phosphoric acid. The optical density (OD) value was measured at 450 nm using a microplate reader.

## Evaluation and statistical analysis

The optimal dilutions of rEg-CS antigen and sera were determined, and sera from 62 sheep (31 *E. granulosus*-positive, 7 *T. multiceps*-positive and 24 negative controls) and sera from 10 goats (*T. hydatigena*-positive) underwent serodiagnosis using the indirect ELISA described above. *E. granulosus*-positive and negative sera were used in all plates, acting as the intra-plate controls. The sensitivity of the method was assessed by the percentage value of ELISA positive and true positives, while the specificity was evaluated by the percentage value of ELISA negative and true negatives.

The negative cut-off was defined as the mean value + 3× standard deviations (SD) from the OD values obtained from the 24 negative sera. The significance of comparisons between test sera groups was estimated by ANOVA (SPSS Inc., Chicago, IL, USA).

## Immunohistochemical localization of Eg-CS

Fertile and infertile cyst walls, PSCs, and adult worms were fixed for 36 h with 4% paraformaldehyde, embedded in paraffin, and sectioned. Pre-treatment of sections was implemented as described previously (Wang et al. 2017) followed by incubation with anti-CS rabbit IgG or native rabbit IgG (1:500 *v/v* dilutions in PBS) at 4 °C overnight. Sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG (1:200 *v/v* dilution in 0.1% Evans blue) in the dark at 37 °C for 1 h, glycerine was added following four washes with PBS, and images were collected under a fluorescence microscope (Nikon 55i).

## Eg-CS mRNA expression in PSCs after oxidation treatment

PSCs with more than 95% activity were cultured in 12-well microplates and incubated with 10 mM H<sub>2</sub>O<sub>2</sub> for 8 h at 37 °C to induce cell apoptosis and death. Every 2 h, PSCs were collected and stored at –80 °C for further study.

Total RNA was extracted and the corresponding cDNA was obtained as described above. To quantify the transcript levels of Eg-CS in PSCs after oxidation treatment, quantitative real-time reverse transcription PCR was conducted. All amplifications were performed as described above (Wang et al. 2017), except for the annealing temperatures: 56 °C for Eg-CS and ACTB. The primers for Eg-CS were 5'-GAT GTA TGG TGG TAT GCG AGG TG-3' and 5'-TTT GGA AGT AGC TCT TGG CAA TC-3'. The primers for the housekeeping gene *ACTB* (encoding β-actin) were 5'-ATG GTT GGT ATG GGA CAA AAG G-3' and 5'-TTC GTC ACA ATA CCG TGC TC-3'. Expression levels were calculated using the 2<sup>-ΔΔCT</sup> method.

## Results

### Bioinformatics analysis of Eg-CS

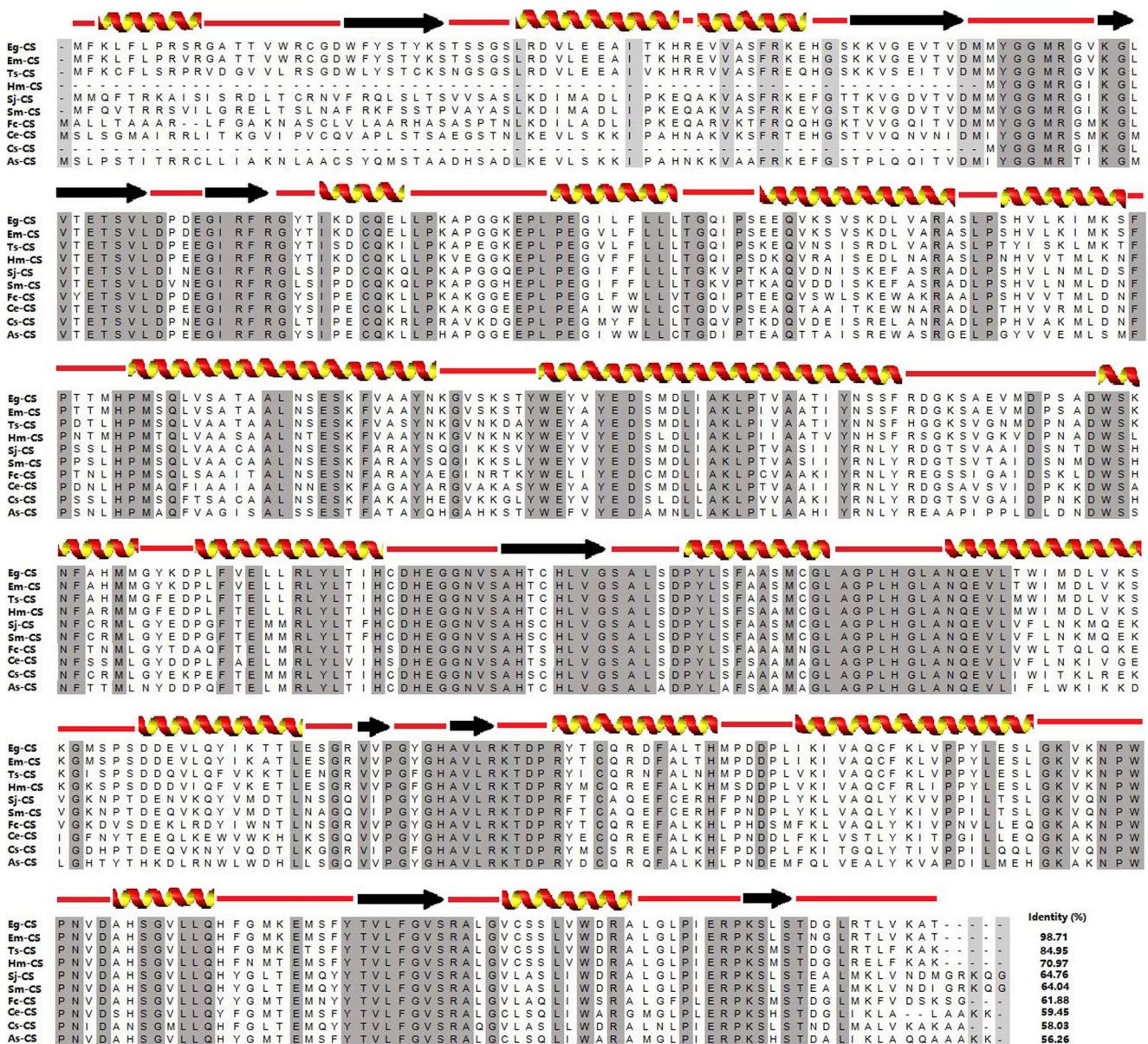
The sequence of Eg-CS was 100% similar to the reference sequence (EgrG\_001028500, <http://www.genedb.org/Homepage>). The full-length Eg-CS cDNA is comprised of 1398-bp and encodes a protein of 466 amino acids (aa), without any signal peptide. The predicted molecular weight (MW)



of Eg-CS is 51 kDa and the predicted pI is 8.26. There are no predicted transmembrane regions in Eg-CS. Subcellular localization analysis suggests that Eg-CS is located in the mitochondrion. Thirty-one B cell epitopes (aa 9–16, 23–35, 42–44, 53–64, 73–75, 78–81, 83–94, 99–100, 104–117, 128–138, 143, 147–148, 160–165, 169, 171, 174–183, 187–195, 197–201, 212–213, 215–217, 220–239, 265–273, 282–286, 288, 302–305, 319–330, 336–346, 348–361, 367–374, 389–405, 444–456) are found in Eg-CS. Secondary structure analysis predicted that Eg-CS contains 53.43, 8.15, 7.73, and 30.69% alpha helix,  $\beta$ -strand,  $\beta$ -turn, and coil, respectively.

### Sequence alignment and phylogenetic analysis

Amino acid sequences of CS from different species were retrieved from GenBank and GeneDB database. Multiple sequence alignment revealed that CS genes have high variability (Fig. 1). The Eg-CS sequence shows the highest sequence identity with CS from *Echinococcus multilocularis* (98.71%). CS from *Hymenolepis microstoma* shows 70.97% identity, *Taenia solium* shows 84.95% identity, and homology with other trematodes and nematodes ranges from 56.03 to 64.76%.



**Fig. 1** Sequence alignment of Eg-CS from different species. Accession numbers: *Echinococcus granulosus* (GeneDB: EgrG 001028500); *Echinococcus multilocularis* (GeneDB: EmuJ 001028500); *Taenia solium* (GeneDB: TsM 000710200); *Hymenolepis microstoma* (GeneDB: HmN 000685700); *Schistosoma japonicum* (GeneDB: Sjp

0037110); *Schistosoma mansoni* (GeneDB: Smp 204,770); *Felis catus* (NCBI: XP 003988937); *Caenorhabditis elegans* (NCBI:NP 499264); *Clonorchis sinensis* (NCBI: GAA48476); *Ascaris suum* (NCBI: ADY44536). Conserved residues are highlighted in dark gray while residues that are conserved in most species are highlighted in light gray

A phylogenetic NJ tree of CS genes was constructed based on the CS sequences obtained from NCBI and Gene DB databases (Fig. 2). Eg-CS is relatively homologous with CS of the congeneric cestode, trematode, and nematode, while it has a relatively distant relationships with the genes from protozoa and mammals.

### Expression and identification of rEg-CS

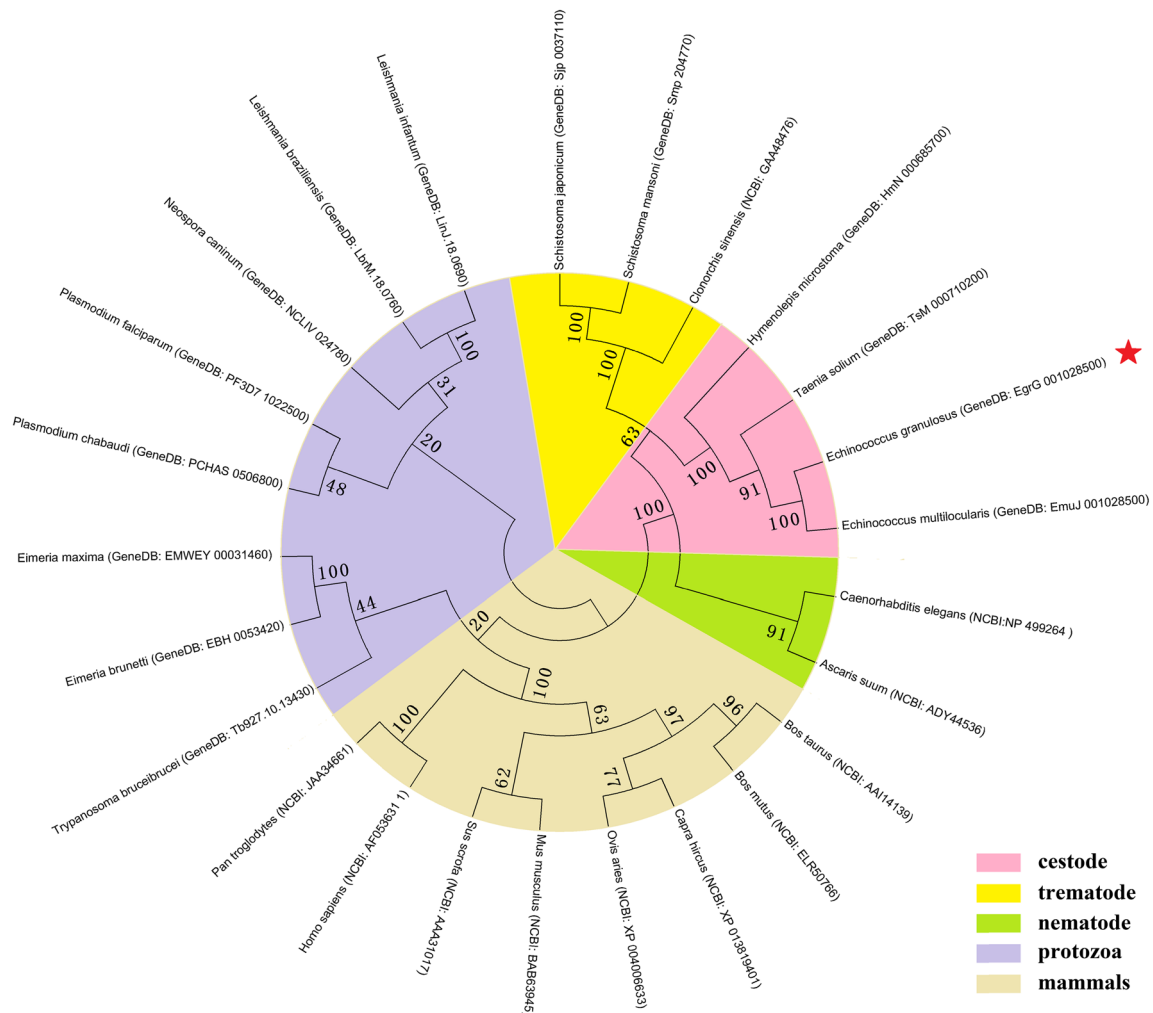
The Eg-CS gene was amplified from PSCs, and rEg-CS was successfully expressed. After purification, rEg-CS protein including the His-tag shows a single band near the predicted size of 54 kDa by 15% SDS-PAGE (Fig. 3).

In western blotting, rEg-CS reacted with CE-positive sheep sera and anti-Eg-CS rabbit sera. Moreover, the anti-Eg-CS rabbit sera IgG also recognizes the native proteins from total PSC extract at its predicted MW. The specific band corresponding to Eg-CS is not detected

following incubation with sera of non-infected sheep or pre-immunized rabbit serum (Fig. 3).

### Localization of Eg-CS in different life cycle stages of *E. granulosus*

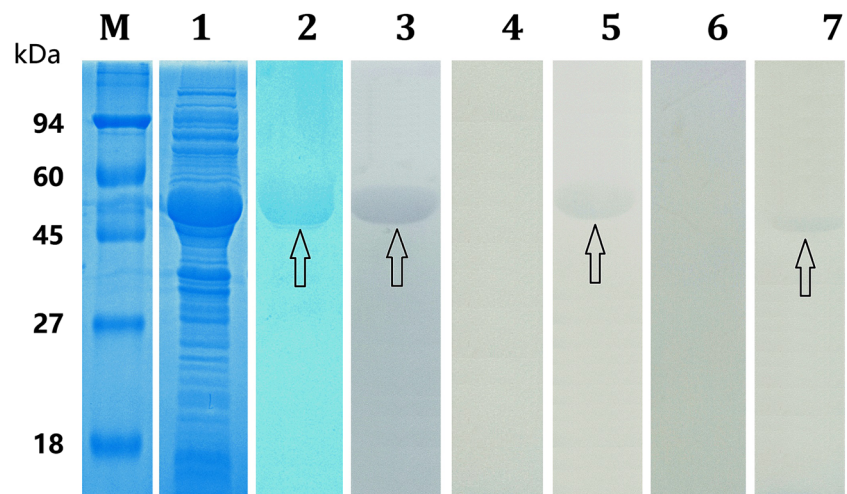
In immunofluorescence analysis, anti-Eg-CS rabbit IgG was applied for the detection of the native protein in adult worms, cyst walls (from fertile and infertile cysts), and PSCs. The results indicate that Eg-CS is distributed in almost all tissues of *E. granulosus* except for the laminated layer of the cyst wall, including the inner body and tegument of adult worms and germinal layer (GL) of cyst wall, as well as the parenchymal region and tegument of the PSCs (Fig. 4). It is interesting to note that the fluorescence intensity of Eg-CS in the GL of the fertile cyst wall is much higher than in the infertile cyst wall. Specific fluorescence is not observed with pre-immunized rabbit sera IgG.



**Fig. 2** The phylogenetic tree of Eg-CS, constructed using the neighbor-joining method. MEGA 5.05 software was used to construct an evolutionary tree with 1000 bootstrap replicates by the neighbor-joining (NJ) method with CS gene sequences from 25 species



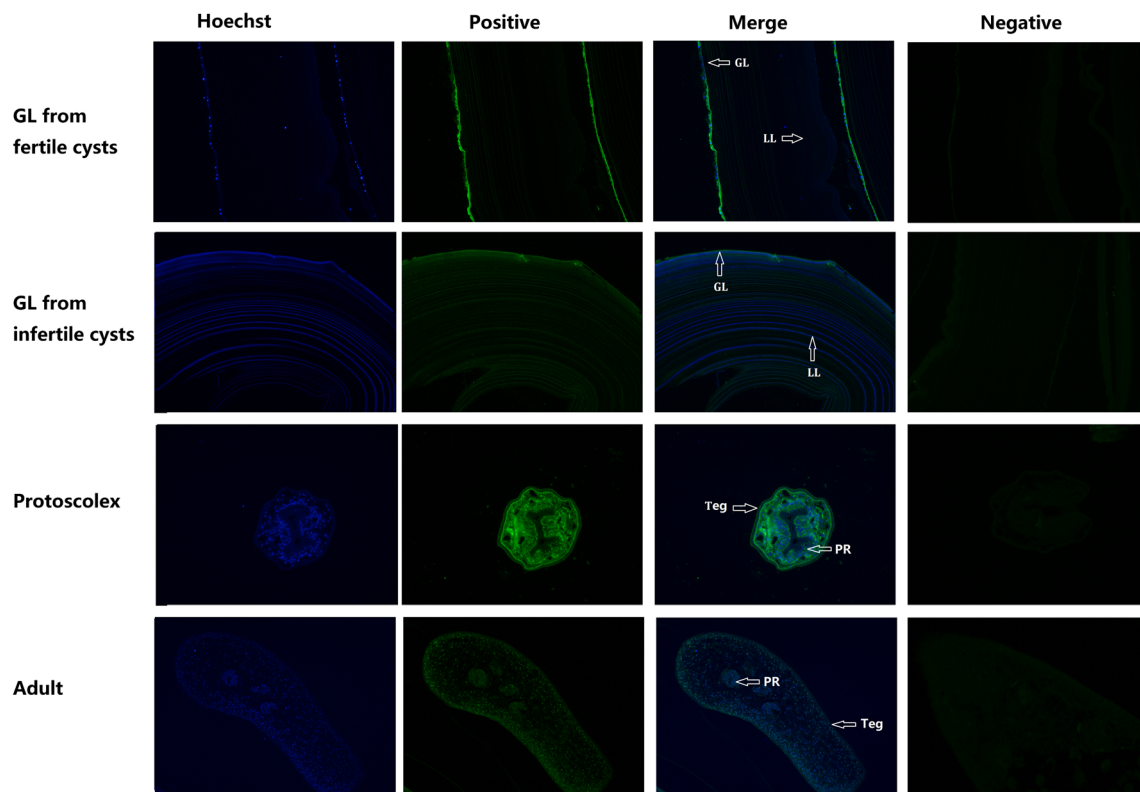
**Fig. 3** Purification of rEg-CS. M, molecular mass markers in kDa; lane 1, whole protein extract from *E. coli* BL21 (DE3) transformants containing pET28a(+)-Eg-CS induced by IPTG; lane 2, purified rEg-CS; lane 3, purified rEg-CS probed with anti-rEg-CS rabbit sera; lane 4, purified rEg-CS probed with pre-immunized rabbit sera; lane 5, purified rEg-CS probed with sera from CE-positive sheep; lane 6, purified rEg-CS probed with non-infected sheep sera; lane 7, total PSC extract probed with anti-Eg-CS rabbit sera IgG



### Indirect ELISA

Building on the good antigenicity and immunoreactivity of rEg-CS, the preliminary serodiagnostic potential of rEg-CS based on indirect ELISA was assessed. The optimal concentration of the rEg-CS antigen was 0.8  $\mu\text{g}/\text{well}$

and the best dilution of the sera was 1:320. The cut-off value of the rEg-CS-ELISA was 0.553 (mean = 0.443, SD = 0.037) which was inferred from the *E. granulosus*-negative sheep sera. Based on the cut-off value, a total of 62 sheep serum samples (31 *E. granulosus*-positive, 24 *E. granulosus*-negative and 7 *T. multiceps*-positive) and



**Fig. 4** Detection of Eg-CS localization at different stages of the *E. granulosus* life cycle by immunohistochemistry. Anti-rEg-BAG3/anti-rEg-EB1 rabbit IgG was used as the primary antibody and FITC-conjugated sheep anti-rabbit IgG was used as the secondary antibody to detect the native protein in PSCs, the cyst wall, and adult worms. Pre-

immunized rabbit sera was used as negative control. Images of PSCs are magnified  $\times 400$ ; cyst walls and adult worm are magnified  $\times 200$ . LL, laminated layer; GL, germinal layer; Teg, tegument; PR, parenchymal region

10 goat serum samples (*T. hydatigena*-positive) were tested. Twenty-nine serum samples from sheep infected with *E. granulosus* were detected as positive, indicating a sensitivity of 93.55% (29/31; Fig. 5). The OD values of 24 *E. granulosus*-negative sera and 4 *T. multiceps*-positive sera isolated from sheep and 5 *T. hydatigena*-positive sera isolated from goat were lower than the cut-off value. This indicated that the specificity of this assay is 80.49% (33/41), but its cross-reactivity is high (Fig. 6).

### Expression profile analysis of Eg-CS in response to oxidative stress

To assess Eg-CS mRNA expression in PSCs treated for different amounts of time, we performed qPCR using *ACTB* as a reference gene. As shown in Fig. 7, the mRNA expression level of Eg-CS increases with H<sub>2</sub>O<sub>2</sub> exposure, with upregulation observed at 2 h (Fig. 7). The expression gradually decreases by 8 h to 37% of initial expression levels due to the increased apoptosis of the PSCs.

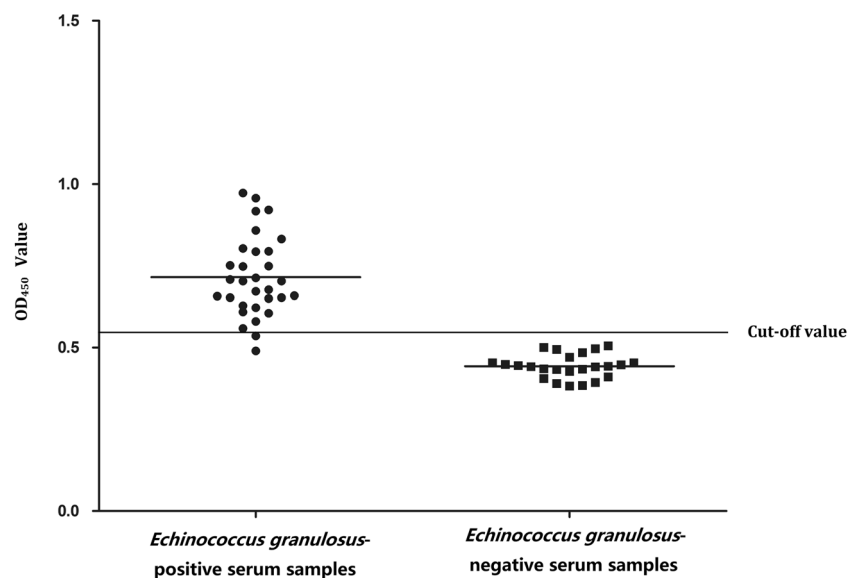
### Discussion

CS is a component of nearly all living cells and plays a critical role in the central metabolic pathway of organisms, the TCA cycle. The TCA cycle functions as a source of two reducing equivalents for the electron transport chain and as a source of intermediates required for the biosynthesis of amino acids for ketogenesis, lipogenesis, and gluconeogenesis (Wiegand and Remington 1986). The flux through the TCA is maintained by activated acetic acid originating as acetyl-coenzyme A from the degradation of carbohydrates, fatty acids, and amino acids.

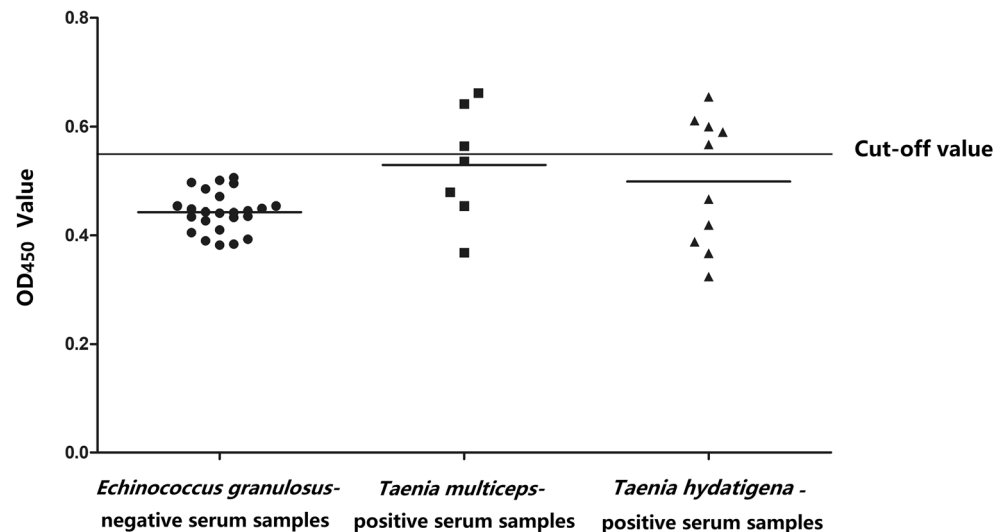
The activity of CS can regulate this cycle (Cheng et al. 2009). CS is widely distributed in most species. In recent years, it has attracted more and more attention due to its importance in agriculture and medicine. The CS gene was initially cloned from yeast and encodes 479 amino acids (Bloxham et al. 1981). It was subsequently cloned from pigs, chickens, humans, and other species (Liu et al. 2000; Linossier et al. 1997; Siu et al. 2003). However, there are few studies of this gene in parasites. Based on the analysis of the *E. granulosus* proteome, it was suggested that CS might be an antigen that could be used as a potential drug target for CE (Monteiro et al. 2010). *E. granulosus* has a complete pathway for the TCA cycle, indicating that CS might play a vital role in this tapeworm. CS was found to be widely distributed in adult worm, oncosphere, PSCs, and hydatid cyst, which suggests that it could be a candidate gene for serological diagnosis of CE (Zheng et al. 2013). In our study, CS from *E. granulosus* (Eg-CS) was cloned, expressed, and characterized. Furthermore, the location, immunological properties, and mRNA expression after oxidant stress of Eg-CS were explored.

Localization studies performed using immunohistochemistry revealed that Eg-CS is expressed in all stages of the *E. granulosus* life cycle. It is widely distributed in adult, PSCs, and the GL of both fertile and infertile cysts, which suggests that Eg-CS might play a crucial role in the growth and various physiological activities of *E. granulosus*. *E. granulosus* is structurally like a flipped gut, with nutrient absorption and waste excretion mainly carried out through the tegument (Smyth and Mcmanus 1989). The expression of Eg-CS in the GL of cyst walls and the tegument of adults and PSCs is particularly evident. These findings suggest that this protein might be involved in the metabolic process.

**Fig. 5** ELISA of serum samples from *E. granulosus*-infested sheep and negative control sheep



**Fig. 6** Cross-reactivity in the ELISA. The cut-off value of the Eg-CS-ELISA was 0.553 which was inferred from the *E. granulosus*-negative sheep sera. The OD values of 24 *E. granulosus*-negative sera and 4 *T. multiceps*-positive sera isolated from sheep and 5 *T. hydatigena*-positive sera isolated from goat were lower than the cut-off value



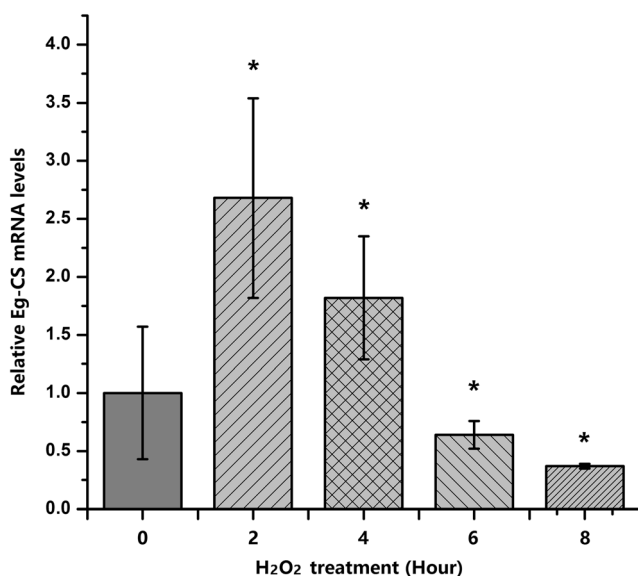
It is noteworthy that the fluorescence intensity of Eg-CS in the GL of fertile cysts is stronger than that in the GL of infertile cysts. The GL is the particular structure of *Echinococcus* tapeworms that provides an immunologic barrier for the survival and growth of PSCs. Cells in the GL are totipotent adult stem cells, which have the ability for self-renewal and differentiation. They are the basis for the continuous proliferation and development of the cysts.

It was reported that the glycogen and lipid content in fertile cysts is significantly lower than in infertile cysts, while the content of polypeptide is significantly higher. It was demonstrated that complete metabolic pathways for sugar, fat, and protein are present in the GL of *E. granulosus*, which are used in the formation and development of the daughter cysts and

PSCs (Leducq and Gabrion 1992; Turčeková et al. 2009; Irshadullah and Rani 2011). Due to the higher metabolic requirements in fertile cysts, the content and activity of Eg-CS is relatively higher.

Evidence suggests that apoptosis negatively regulates PSC generation and causes infertility of hydatid cysts, but the genes involved in regulating the process still unknown (Cabrera et al. 2008; Paredes et al. 2011; Spotin et al. 2012). Previous studies found that decreases in CS activity might lead to oxidative stress and excessive production of reactive oxygen species (ROS) (Orrenius 2007). Apoptosis would subsequently be induced by initiation of the caspase cascade or through regulation of the expression of apoptosis related proteins such as p53 and Bcl-2 (Turrens 2003; Hsieh et al. 2007; Rana 2008). CS was also shown to increase the sensitivity of cells to TNF and trigger apoptosis (Giordano et al. 2005). Therefore, we speculated that Eg-CS might involve in the formation of infertile cysts in *E. granulosus*, but further study should be taken to verify this assumption.

The mRNA expression profile of Eg-CS after  $H_2O_2$  exposure was analyzed to establish whether the protein responds to oxidative stress and to further explore its function in apoptosis. The results of qPCR showed that Eg-CS mRNA expression is upregulated after 2 h of  $H_2O_2$  treatment but becomes significantly downregulated after a longer treatment. After 8 h, the expression decreases to 37% of untreated levels. The increase in Eg-CS expression at beginning of apoptosis contradicts the previous studies discussed above which showed that decreases in CS induced apoptosis and triggered the production of ROS and release of cytochrome C (Chinta et al. 2009; Maríngarcía et al. 2009). The reason might be that apoptosis is a proactive and energy-consuming process (Zischka et al. 2008; Lee et al. 2009). In this process, sufficient adenosine triphosphate is needed to stimulate the activity and increase the expression of Eg-CS to ensure the progression of apoptosis. Apoptosis is a complex process and many



**Fig. 7** Expression profile of Eg-CS in response to  $H_2O_2$  treatment. Data represent the mean  $\pm$  SD of triplicate experiments. Statistically significant differences between the 0 h group (as the control) and the other groups were determined using Student's *t* test (\* $P < 0.05$ )



important factors such as toxins, cytokines, and hormones are involved in the process. However, with increased treatment time, the volume and transmittance of PSCs were reduced, and calcium particles became smaller and nebulous. At the end of treatment, a large number of PSCs had undergone cell death or even ruptured into pieces, which gradually reduced the required energy and metabolism. Therefore, as the key enzyme in the TCA cycle, the mRNA expression of Eg-CS decreased, and the decrease further promoted the apoptosis of PSCs. In summary, CS plays an important role in *E. granulosus* as a crucial factor in the TCA pathway and might maintain the fertility of hydatid cysts. Decreased levels of Eg-CS could cause the formation of infertile cysts.

In addition, bioinformatics analysis showed that the Eg-CS gene encodes 31 B cell antigen epitopes, indicating that this protein has potential as a drug target (Monteiro et al. 2010). In our study, immunoblotting analysis showed that rEg-CS is recognized by *E. granulosus*-positive sheep sera and anti-rEg-CS rabbit sera demonstrating that the protein has good reactivity. The native proteins from total PSC extract were recognized by the anti-Eg-CS rabbit sera IgG, showing high specificity. To determine whether a recombinant protein is suitable as a diagnostic antigen, it is necessary to consider the influence of host species at the same time. The aim of our study was to explore whether rEg-CS was suitable for diagnosing echinococcosis in sheep. In China, especially in Sichuan Province, sheep are highly infected with *E. granulosus*, but an effective and specific diagnostic antigen still needs to be explored. Like our previous studies (Song et al. 2016; Song et al. 2017; Wang et al. 2017; Wang et al. 2018; Wu et al. 2018), this research also lays a theoretical foundation for screening suitable antigens. Indirect ELISA was used to evaluate the diagnostic potential of rEg-CS. The results showed that rEg-CS has high sensitivity (93.55%) and specificity (80.49%), but high cross-reactivity to *T. multiceps* in sheep and *T. hydatigena* in goat. These results indicate that rEg-CS protein is not suitable for diagnosis of CE in sheep, which might be due to the high level of conservation of the gene among tapeworm species.

## Conclusions

In this study, *E. granulosus* gene encoding CS was successfully cloned, expressed, and characterized. The results of immunohistochemistry showed that Eg-CS is widely distributed in adult worms, PSCs, and the GL of both fertile and infertile cysts. The relative mRNA expression of Eg-CS was upregulated at the beginning of H<sub>2</sub>O<sub>2</sub> treatment, but significantly downregulated after a longer treatment. In conclusion, our study indicates that Eg-CS might play a crucial role in the growth and various physiological activities of *E. granulosus* as a key enzyme in a metabolic pathway. It could maintain the

fertility of hydatid cysts, with decreases in Eg-CS potentially causing the formation of infertile cysts. Although rEg-CS has good antigenicity and immunoreactivity, it is not suitable as a diagnostic antigen.

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**Authors' contributions** NW and GYY conceived and designed the study and critically revised the manuscript. JFZ, CG, NXS, XBG, and WML performed the experiments, analyzed the data, and drafted the manuscript. XRP and YX conducted the data analysis. HZ and GYY revised the manuscript. All authors have approved the final manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethics statement** All animals included in this study were purchased from the Laboratory Animal Center of Sichuan Agricultural University. All procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals and with approval from the Animal Ethics Committee of Sichuan Agricultural University (Ya'an, China; Approval No. 2013–028).

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