

## RESEARCH ARTICLE

# Evaluation of the Recombinant *EgAgB8/2* Antigen for the Diagnosis of Cystic Echinococcosis in Tibetan Sheep on the Qinghai-Tibetan Plateau, China

Hong DUO <sup>1,a</sup> Yong FU <sup>1,b (\*)</sup> Xiuying SHEN <sup>1,c</sup> Zhihong GUO <sup>1,d</sup> Xueyong ZHANG <sup>1,e</sup> Yijuan MA <sup>1,f</sup>  
Mohammed YOSRI <sup>2,g</sup> Tiancong SUN <sup>1,h</sup> Youlu CHEN <sup>1,i</sup> Gengqiu JIUNAI <sup>3,j</sup>

<sup>1</sup> Qinghai Academy of Animal Science and Veterinary Medicine, Qinghai University, State Key Laboratory of Plateau Ecology and Agriculture, Xining 810016, CHINA

<sup>2</sup> The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo 11787, EGYPT

<sup>3</sup> Animal Husbandry and Veterinary Station, Zhenqin Township, Chindu County 815100, CHINA

ORCID: <sup>a</sup> 0000-0002-3013-9818; <sup>b</sup> 0000-0003-3354-9107; <sup>c</sup> 0000-0002-3804-2113; <sup>d</sup> 0000-0003-0049-607X; <sup>e</sup> 0000-0001-5076-9211

<sup>f</sup> 0000-0003-0423-1730; <sup>g</sup> 0000-0001-6197-0690; <sup>h</sup> 0000-0002-1009-4042; <sup>i</sup> 0000-0003-0029-4507; <sup>j</sup> 0000-0002-7926-1611

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## Abstract

The unique geographical and ecological conditions of the Qinghai-Tibetan Plateau (QTP) constitute the environmental conditions of the natural foci of cystic echinococcosis (CE). It is a rare and highly endemic area in the world. The main host of the pathogen, Tibetan sheep, is widely distributed, and the population density is on the rise in recent years, which increases the pressure on the transmission and risk of the natural epidemic source. Currently, the developments of serological diagnosis test for *Echinococcus granulosus* infection on the QTP Tibetan sheep are scanty, which seriously restricts the epidemiological investigation of CE in Tibetan sheep on the QTP. *EgAgB8/2* is one of the most important targets of host antibody response in CE and has better diagnostic performance in livestock. This study expressed the recombinant *EgAgB8/2* antigen of *E. granulosus* from Tibetan sheep in prokaryotic expression vector, and also preliminarily evaluated its potential value for diagnosing CE in Tibetan sheep using indirect ELISA. Our preliminary results shown that the recombinant *EgAgB8/2* antigen had good immunogenicity and exhibited high sensitivity (95%) and high specificity (100%) and no cross-reacted with both *Taenia multiceps* and *Taenia hydatigena*. Further studies are needed to collect more Tibetan sheep sera from *E. granulosus* and other parasitic infections, which may evaluate further sensitivity, specificity and cross-reactive of recombinant *EgAgB8/2* antigen in Tibetan sheep positive for CE of the QTP, China.

**Keywords:** Cystic echinococcosis, Tibetan sheep, *EgAgB8/2* antigen, Serological diagnosis

## Çin'in Qinghai-Tibet Platosu'ndaki Tibet Koyunlarında Kistik Ekinokokkozisin Tanısında Rekombinant *EgAgB8/2* Antijeninin Değerlendirilmesi

### Öz

Qinghai-Tibet Platosu'nun eşsiz coğrafyası ve ekolojisi, kistik ekinokokkozisin (KE) doğal odaklarının çevresel koşullarını oluşturur. Burası dünyada nadir bulunan ve oldukça endemik bir bölgedir. Patojenin ana konakçısı olan Tibet koyununun yaygınlığı ve popülasyon yoğunluğu son yıllarda artmaktadır ve bu durum doğal salgın kaynağı riskini ve bulaş üzerindeki baskıyı artırmaktadır. Günümüzde, Qinghai-Tibet Platosu'ndaki Tibet koyunlarında *Echinococcus granulosus* enfeksiyonunun serolojik tanı testlerindeki gelişmeler yetersizdir ve bu, bölgede Tibet koyunlarında KE'ya yönelik epidemiyolojik araştırmaları ciddi şekilde kısıtlamaktadır. *EgAgB8/2*, KE'da konakçı antikor yanıtının en önemli hedeflerinden birisidir ve çiftlik hayvanlarında enfeksiyonun teşhisinde daha iyi bir performansa sahiptir. Bu çalışmada, prokaryotik vektör kullanılarak Tibet koyunlarından elde edilen *E. granulosus*'un rekombinant *EgAgB8/2* antijeninin ekspresyonu sağlandı ve bu antijenin Tibet koyunlarında KE'un teşhisinde kullanım potansiyeline yönelik ön değerlendirilmesi indirekt ELISA ile gerçekleştirildi. Çalışmamıza ait ön bulgular, rekombinant *EgAgB8/2* antijeninin iyi bir immünojeniteye sahip olduğunu, yüksek duyarlılık (%95) ve özgüllük (%100) sergilediğini ve hem *Taenia multiceps* hem de *Taenia hydatigena* ile çapraz reaksiyon vermediğini gösterdi. *E. granulosus* ve diğer parazitik etkenlerle enfekte daha fazla Tibet koyunundan toplanacak serumlarla, rekombinant *EgAgB8/2* antijeninin duyarlılık, özgüllük ve çapraz reaksiyonlarının değerlendirilmesine yönelik daha fazla çalışma yapılmasına ihtiyaç vardır.

**Anahtar sözcükler:** Kistik ekinokokkozis, Tibet koyunu, *EgAgB8/2* antijeni, Serolojik tanı

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### (\*) Corresponding Author

Tel: +86 0971 5226221

E-mail: qhfuyong@163.com (Y. Fu)



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## INTRODUCTION

Echinococcosis is a zoonosis that can be caused by cestodes of the genus *Echinococcus*, especially cystic echinococcosis (CE) caused by the larvae of *Echinococcus granulosus*, occurs worldwide and is highly endemic on the Qinghai-Tibetan Plateau (QTP), China [1-3]. CE not only seriously endangers the health of both humans and livestock but also is a significant public health issue worldwide [4-7]. The diagnosis of CE is primarily based on imaging and ultrasound techniques which are sometimes inconclusive, but serological tests can be applied as a supplemented test [8]. Especially, the serological test is generally needed in livestock as imaging methods are not practical due to cost factors [9]. Tibetan sheep is the main livestock species on the QTP, which the infection rate of CE in Tibetan sheep is relatively high in this area [10,11]. Currently, epidemiological surveillance of CE in Tibetan sheep on the QTP is based mainly on necropsy procedures. In the absence of serological diagnostic test, infected Tibetan sheep remain in the morbid state, leading to substantial economic losses to the development of animal husbandry. Therefore, the development of an accurate serological diagnostic test of CE in Tibetan sheep is essential for devising effective strategies to control this disease on the QTP.

Over the past years, serological diagnostic test of CE in sheep mainly depends on hydatid cyst fluid (HCF), a crude protoscolex preparation and recombinant proteins as recombinant EG95 oncosphere protein and the AgB hydatid cyst fluid protein [12]. Particularly, *E. granulosus* hydatid cyst fluid has been extensively investigated in serological tests, and its protein components of EgAgB antigens has better diagnostic performance [13]. To date, five different but closely related genes of EgAgB have been identified, corresponding to genes EgAgB1, EgAgB2, EgAgB3, EgAgB4 and EgAgB5 [14]. Several studies have shown that EgAgB8/2 is the most promising subunit of EgAgB for the development of a reliable sensitive and specific test for the serodiagnosis of CE [14-16], which has been widely used in the serological test of CE in inter-mediate host including cattle, sheep, goats, buffaloes, camels, etc. [9,17-19]. Previous studies have shown that the recombinant EgAgB8/2 antigen is very immunogenic [20], and the recombinant EgAgB2 protein has been found to improve the performance of ELISA for serodiagnosis of CE [21], which developing the ELISA test has proved the sensitive and convenient for detecting CE.

Tibetan sheep are predominantly infected with cystic echinococcosis when compared with other animals on the Qinghai-Tibetan Plateau, China. However, there have been currently fewer reports on the development of serological tests for CE in Tibetan sheep. Therefore, in the present study, we expressed the recombinant antigen EgAgB8/2 from cystic echinococcosis in Tibetan sheep. In addition, we preliminarily investigated the recombinant EgAgB8/2

antigen potential value for diagnosing CE in Tibetan sheep using indirect ELISA. These findings are essential for large-scale epidemiological investigations of CE on the QTP endemic regions.

## MATERIAL AND METHODS

### Parasites and Sera Collection

Hydatid cysts were collected from Tibetan sheep at a local abattoir in Qinghai province, China. Hydatid cysts were identified as *E. granulosus* sensu stricto (G1) [22], and protoscolices were separated from hydatid cyst fluid as previously described [23]. Positive sera (n=20) and negative sera (n=20) for CE infection in Tibetan sheep were provided by the zoonosis laboratory, Qinghai University. Positive sera against *Taenia hydatigena* (n=20) and *Taenia multiceps* (n=20) were obtained from naturally infected Tibetan sheep. All sera were stored at -20°C.

### cDNA Synthesis of EgAgB8/2

Total RNA was isolated from the protoscolices using Trizol reagent (Sangon, Shanghai, China) according to the manufacturer's recommendations. The cDNA was synthesized with M-MLV kit (Sangon, Shanghai, China) following standard protocols of cDNA synthesis. The cDNA coding sequence of EgAgB8/2 target protein was PCR amplified using the primers 5'-GCGAATCCTCTGCGTGGA CATTGTGGAG-3' (including an *EcoR* I site, restriction sites underlined) and 5'-GCAAGCTTTGGCAAATCATGTGT CCGAC-3' (including a *Hind* III site, restriction sites underlined). The cDNAs coding of EgAgB8/2 protein was cloned in the pET32a (+) plasmid (Invitrogen, Carlsbad, California, USA). after their PCR amplification with primers designed with suitable restriction enzyme sites.

### Expression, Purification and Data Analyses of Recombinant Protein

The target fragment and the vector fragment were recovered individually and ligated using T4 DNA ligase (Invitrogen, Carlsbad, California, USA) at 4°C overnight, and subsequently transformed into *Escherichia coli* BL21 (DE3) (Solarbio, Beijing, China). The expression was induced using 0.5 mM/L IPTG at 37°C for 5 h. Then, the bacterial cells were harvested and lysed using ultrasonication. Briefly, the bacteria were resuspended in 25 mL of binding buffer and ultra-sonicated until the solution was clear. The solution was centrifuged, and the supernatant was collected and filtered through a 0.22 µm filter. The recombinant protein was purified to complete homogeneity using Ni-NTA agarose resin (Qiagen, Hilden, Germany). Briefly, the recombinant protein was allowed to bind to Ni-NTA resin at room temperature for 2 h with constant shaking. Affinity column was washed with wash buffer (pH 6.5) supplemented with 12 mM imidazole and recombinant protein eluted with elution buffer at pH 4.2. The expression of the EgAgB8/2

recombinant protein was analyzed by SDS-PAGE, and the protein concentration was determined with UV spectrophotometry.

### Western Blotting Analysis

The recombinant protein was run on 10% SDS-PAGE gel and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). Membranes were incubated using a 1:1000 dilution of a mouse anti-His monoclonal antibody (Sigma, St. Louis, USA), negative serum (1:200 dilution), and sheep anti-*E. granulosus* serum (1:200 dilution) at 37°C for 1 h. After washing for three times, a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, St. Louis, USA) was used as secondary antibody at 1:1500 dilution and was incubated at 37°C for 2 h. Finally, reactions were visualized using the TMB Chromogenic Reagent kit (Sangon, Shanghai, China) according to manufacturer's instructions.

### Establishment of Indirect ELISA

Optimization experiments were used to determine the optimum working conditions of the antigen *EgAgB8/2* and sera. Briefly, *EgAgB8/2* was diluted to concentrations from 20 µg/mL to 0.625 µg/mL in 1:2 dilution steps, and each dilution was added to the ELISA plate (100 µL/well) and incubated overnight at 4°C. The wells were blocked with 5% skim milk, washed, and incubated with serum samples in dilutions (1:10, 1:50, 1:100, 1:200 and 1:400) at 37°C for 1 h. The plates were then washed three times and incubated with HRP-labeled rabbit anti-goat IgG (Boster Bio-project Co, Wuhan, China) for 1 h. Color reactions were developed with *o*-phenylenediamine solution (OPD) for 15 min in the dark, and the reaction was terminated with 2 M H<sub>2</sub>SO<sub>4</sub>. The optical density at 450 nm was measured with a microplate reader (Thermo Scientific, Pittsburgh, PA, USA). The cut-off value, sensitivity and specificity were determined as previously described<sup>[24,25]</sup>. Briefly, the cut-off value was determined from the mean OD<sub>450</sub> of the 20 negative sheep serum samples plus three standard deviations. The

sensitivity of the recombinant *EgAgB8/2* antigen ELISA was evaluated using 20 positive sheep serum samples against *E. granulosus*. The specificity was evaluated using 20 negative sheep serum samples. The sensitivity and specificity were calculated as follows:

$$\text{sensitivity (\%)} = \text{ELISA positive/true positive} \times 100\%$$

$$\text{specificity (\%)} = \text{ELISA negative/true negative} \times 100\%$$

The true positive and true negative were determined by the necropsy of Tibetan sheep. The cross reactivity was evaluated using 20 positive serum samples against *T. hydatigena* and 20 positive serum samples against *T. multiceps*.

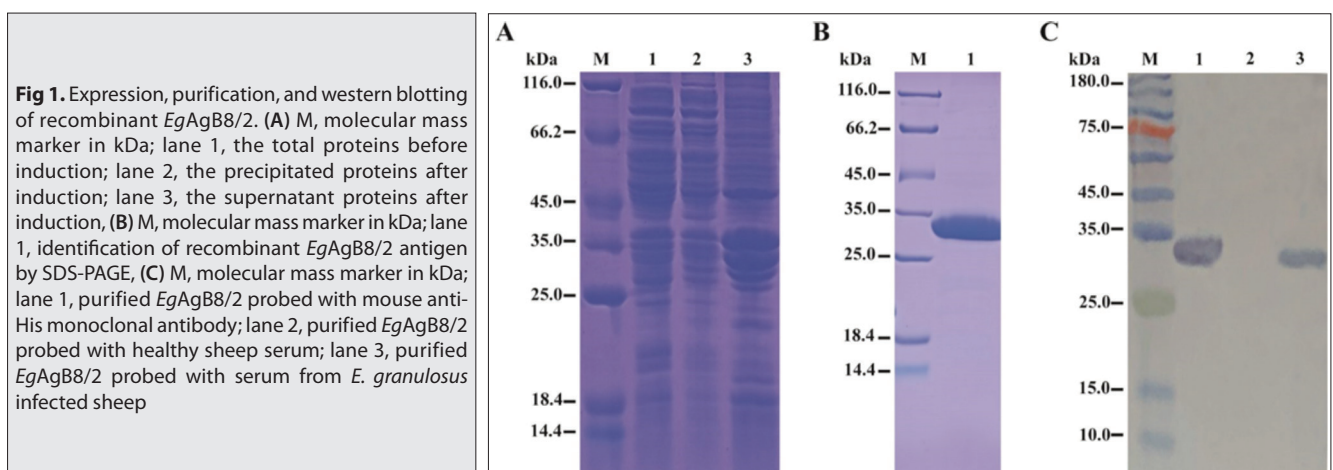
### Statistical Analysis

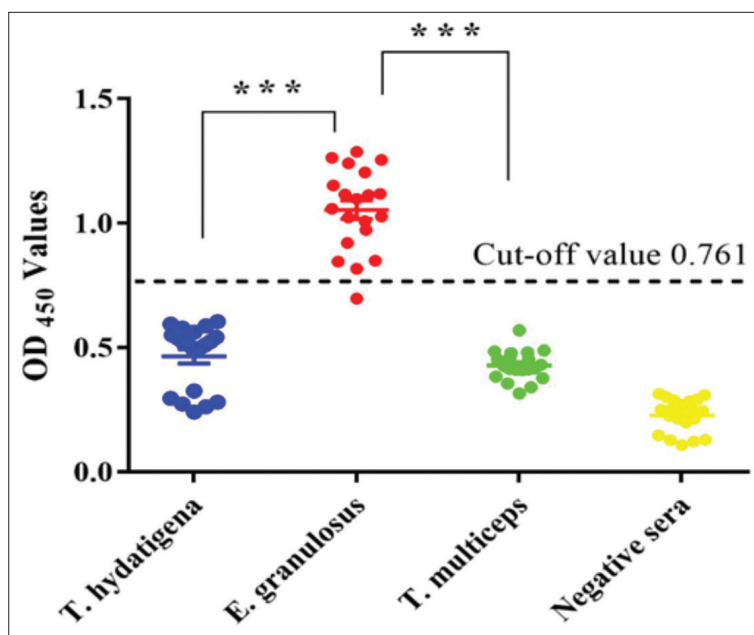
ELISA data are represented as the mean ± SEM. Statistical analyses were performed using Mann-Whitney test, and the cross-reactivity of the recombinant *EgAgB8/2* antigen with sera from Tibetan sheep infected with *T. hydatigena* and *T. multiceps* were plotted using GraphPad Prism software. Values of P < 0.05 are considered statistically significant.

## RESULTS

### Expression, Purification and Western Blotting

After IPTG induction, the expression products of *E. coli* BL21 (DE3) containing pET32a(+)-*EgAgB8/2* were analyzed by SDS-PAGE electrophoresis. Analysis of the protein solution analysis showed that *EgAgB8/2* was successfully expressed in *E. coli* and the recombinant protein was approximately 29 kDa (including a His-tag of 17 kDa), which was consistent with the expected size. The purified recombinant protein *EgAgB8/2* was a single band (Fig. 1). Western blotting analysis showed that the mouse anti-His monoclonal antibody could specifically bind to the recombinant protein, and the recombinant protein could react with serum from *E. granulosus*-infected sheep and it formed a single band. The results indicate that the protein is the predicted protein and could be specifically recognized by anti-*Eg* positive serum.





**Fig 2.** Indirect ELISA of Tibetan sheep sera showing specificity and sensitivity and cross-reactions of recombinant *EgAgB8/2* antigen for diagnosis of *E. granulosus*. Data are expressed as mean  $\pm$  SEM of 2 independent experiments. \*\*\*  $P < 0.001$  by Student's t test

### Evaluation of Recombinant Antigen by Indirect ELISA

The 96-well plate was coated with recombinant *EgAgB8/2* antigen (100  $\mu$ L/well) at the optimum concentration of 5.0  $\mu$ g/mL carbonate buffer (pH 9.6), and the optimal working concentration of serum was 1:100. The cut-off value was determined as 0.761 from the 20 samples of *E. granulosus*-negative serum. The sensitivity of *EgAgB8/2* was 95% (19/20) and the specificity was 100% (20/20). The recombinant *EgAgB8/2* antigen showed no cross-react with positive sera against *T. hydatigena* and positive sera against *T. multiceps* with OD<sub>450</sub> above cut-off value ( $P < 0.001$ ) (Fig. 2). There was a statistically significant difference in the mean OD values of *E. granulosus*-positive sera and *E. granulosus*-negative sera (Mann-Whitney  $U$ ,  $z = -5.410$ ,  $P < 0.001$ ).

## DISCUSSION

The serological tests for CE are mainly based on ELISA using hydatid cyst fluid (HCF) [26,27], but different sources of HCF to differing diagnostic effects when testing the sera. *Echinococcus* spp. harbor considerable variability of the strain from different geographical environments and different hosts [28-30], which cause variation in pathogenicity and antigen-antibody reactions between hosts [31]. The research showed that the sensitivity and specificity of CE serological diagnosis largely depends on HCF source, mainly due to the antigenic variability of the HF among different *E. granulosus* s. s. genotypes [32]. For a long time, crude preparations of HCF from *E. granulosus* cysts isolated from sheep was the only antigen available for serological diagnosis of CE in domestic animals [33]. However, HCF is scarce and heterogeneous for the detection of infected animals. Interestingly, *EgAgB* is the largest portion of the parasite-derived proteins and the most abundant ones inside the HCF [34], which became important reagents for

serological diagnosis in cystic echinococcosis. Currently, the recombinant *EgAgB* antigens from hydatid cyst fluid have been comprehensively investigated in serological diagnosis [9,35]. One of the the recombinant *EgAgB* antigens is *EgAgB2*, a secretory protein of the larval stage of *E. granulosus* [36], was a highly antigenic molecule in CE infections [37], and was used as an effective diagnostic antigen for sero-diagnosis of CE [15,38-39]. It has been demonstrated that the recombinant *EgAgB8/2* antigen has better sensitivity and specificity compared to the other recombinant antigens from hydatid cyst fluid in CE [9,40]. In this study, the recombinant *EgAgB8/2* antigen for CE in Tibetan sheep showed a sensitivity of 95% and specificity of 100%, which was consistent with the ranges of the different native antigens of HCF were from 64.8% to 100% for sensitivity, and from 40% to 100% for specificity. Therefore, it suggests the different diagnostic performance of CE in animals from different geographical areas.

Tibetan sheep is the main livestock species on the QTP, but relatively high infection rate of CE seriously affects the development of animal husbandry in this area [41-44]. Although the abattoir data of Tibetan sheep are important, particularly in the surveillance of CE, it can be very difficult to identify specifically small lesions in the liver and lungs of young animals without additional histological examination. In addition, DNA technology, particularly the advent of the polymerase chain reaction (PCR), provides an approach for the unambiguous diagnosis of *E. granulosus* but currently this necessitates using metacestode material excised from infected intermediate hosts [45,46]. Therefore, developing a serological diagnostic method would be useful for diagnosis in CE of Tibetan sheep. However, some studies found that there were cross-reactivity between *E. granulosus* and other parasitic infections such as *Echinococcus multilocularis*, *Taenia multiceps*, *Taenia*

*hydatigena*, *Taenia solium*, and *Fasciola hepatica*, etc, which result in reduced performance for the serological diagnosis of CE in animals [16,32,47]. Therefore, that is why recombinant antigen based diagnostic methods of CE in animals were sought. The recombinant EgAgB8/2 antigen in the hydatid cyst fluid was used to detect cystic echinococcosis, which was very immunogenic [20]. Currently, the serological test based on recombinant EgAgB8/2 antigen is well established in diagnosis of human CE, however little information is available in diagnostic or epidemiological assessment of *E. granulosus* infections in animal intermediate hosts.

In the current study, we expressed the recombinant EgAgB8/2 antigen in the HCF of *E.g* from Tibetan sheep, and preliminarily evaluated the extent of cross-reactivity of the recombinant EgAgB8/2 antigen for other cestode infection commonly found on the QTP such as *Taenia multiceps* and *Taenia hydatigena*. We found the recombinant EgAgB8/2 antigen had good immunogenicity and no cross-reacted with both *Taenia multiceps* and *Taenia hydatigena* which are common parasitic diseases on the QTP. However, more other cestode serums of Tibetan sheep need to be collected for cross-reaction verification.

In conclusion, this study may be a first step toward development of a reliable diagnostic test for CE infection in Tibetan sheep, and can also provide important experimental basis for the future study of CE epidemiological on the QTP. In the meanwhile, a larger panel of sera should be needed in future studies to evaluate the recombinant antigen with better sensitivity and specificity in the detection of CE infection in Tibetan sheep.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## ETHICAL STANDARDS

Not application.

## AUTHOR CONTRIBUTIONS

H.D., T.S., and Y.C. designed and performed experiments and analyzed results. X.S., Z.G., X.Z., Y.M. and G.J. collected samples. M.Y. provided advice. Y.F supervised the study and wrote the manuscript.

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