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Short communication

An ELISA using recombinant TmHSP70 for the diagnosis of *Taenia multiceps* infections in goats

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ABSTRACT

Infections with the tapeworm *Taenia multiceps* are problematic for ruminant farming worldwide. Here we develop a novel and rapid method for serodiagnosis of *T. multiceps* infections via an indirect ELISA (iELISA) that uses a heat shock protein, namely, TmHSP70. We extracted the total RNA of *T. multiceps* from the protoscoleces of cysts dissected from the brains of infected goats. Subsequently, we successfully amplified, cloned and expressed the TmHSP70 gene in *Escherichia coli* BL21 (DE3). Western blot analysis showed that the recombinant protein (~34 kDa molecular weight) was recognized by the coenurosis positive serum. Given these initial, robust immunogenic properties for recombinant TmHSP protein, we assessed the ELISA-based serodiagnostic potential of this gene. The indirect ELISA was then optimized to 2.70 μg/well dilution for antigen and 1:80 dilution for serum,while the cut-off value is 0.446. We report that our novel TmHSP ELISA detected *T. multiceps* sera with a sensitivity of 1:10240 and a specificity of 83.3% (5/6). In a preliminary application, this assay correctly confirmed *T. multiceps* infection in 30 infected goats, consistent with the clinical examination. This study has revealed that our novel iELISA, which uses the rTmHSP protein, provides a rapid test for diagnosing coenurosis.

1. Introduction

Taenia multiceps is a parasitic cestode, the larval stage of which is the causative agent of coenurus cerebralis, a zoonosis that affects both ruminant animals and people worldwide (Ghazaei, 2007). The impact of the disease on animal health is considerable and causes severe socio-economic consequences for ruminant farming (Nie et al., 2013). Often, the clinical symptoms of infection include: circling, ataxia, drowsiness, head pressing, blindness and coma (Wu et al., 2012).

Effective diagnosis is central in implementing control programs. Unlike imaging methods including radiology and ultrasonography, immunodiagnosis can be used for primary diagnosis and for follow-up examinations of infected animals after pharmacological treatment (Komnenou et al., 2000; Tirgari et al., 1987). For example, ELISA, a flexible immunodiagnostic method, can accommodate large number of samples and is becoming the predominant serologic assay for the field surveillance.To date, an ELISA has been developed by applying a purified recombinant protein coating that improved the specificity of diagnosis (An et al., 2011). Notably, the target antigens that have good immunogenic properties and potential of serologic diagnosis are still lacking.

Prior to numerous studies showed that the heat shock proteins (HSPs) within different parasites, including *Trypanosoma, Trichomonas* and *Haemonchus*, are ideal (Leeuwen, 1995; Bozner, 1997; Toaldo et al., 2001). For example, *HSP60*, Eg2HSP70 and HSP70, with their encoded proteins deemed immunodominant antigens, have been described in *Echinococcus granulosus*. (Martinez et al., 1999).

Given the lack of information on the characteristics of HSPs of *T. Multiceps* and the importance of this parasite in ruminant farming, it is crucial to develop a rapid, sensitive and specific serological method to improve diagnosis of coenurus cerebralis. In this study, therefore, we aimed to develop a novel, rapid tool to diagnose *T. multiceps* infections using a recombinant protein. Specifically, we developed an indirect ELISA based on purified TmHSP70 heat shock protein and tested its potential of serological diagnosis in the field.

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2. Materials and methods

2.1. Parasite and serum preparation

A total of 30 blood samples were collected from 30 moribund goats presenting with neurological symptoms of coenurosis from a goat farm in Sichuan province, China. Goats which were identified by surgical puncture to be affected by coenurosis were sacrificed by farmers. All the organs of the dead goats were inspected for cysts. *T. multiceps* protoscoleces were aspirated from cysts and then stored at 4 °C in sterile normal saline prior to RNA extraction. *Cysticercus tenuicollis* sera (6 samples) were isolated from naturally infected goats, while negative sera (30 samples) collected from healthy goats with no cysts by autopsy. All the goats were from goat farms in Sichuan Province.

2.2. Cloning of TmHSP from cDNA

Total RNA was extracted using Trizol reagent (Huashun, Shanghai, China) according to the manufacturer's instructions. The obtained RNA was reverse-transcribed (Fermentas, Shenzhen, China) and processed according to the manufacturer's instructions. The cDNA sequence of TmHSP used for designing primers was derived from unigene 18109 of the assembled *T. multiceps* transcriptome dataset (accession number JR934847 in Transcriptome Shotgun Assembly Sequence Database at NCBI). The open reading frame (ORF) of the nucleotide sequence was analyzed by Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The amino acid sequences predicted by oligo $(dT)_{18}$ primer (MBI Fermentas, Germany) were found with previously reported sequences in GenBank by the BLAST network server of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The forward primer, TmHSP-F 5′-CCG**GAATTCA**TGGGTCTCAAAGAAACAG-3′, contained a *BamH*I site, while the reverse primer, TmHSP-R 5′-CCG**CTCGAG**TCAGAATAGAGCCATTAACTC-3′, contained a *Xho*I site. The PCR products were digested with BamHI and XohI (TaKaRa, Dalian, China) and inserted into similarly digested pET32a(+) (Novagen, Madison, USA) to generate recombinant expression plasmid.The secondary structure of TmHSP was analyzed by use of DNAstar (Lasergene, USA).

2.3. Protein expression and purification

Recombinant expression plasmid pET32a(+) was transformed into *E. coli* BL21 (DE3) competent cells. For the expression of the fusion protein, transformed cells were grown at 37 °C in liquid LB medium containing 50 μg/mL ampicillin. When the OD600 reached approximately 0.6, cultures were induced with 1 mM isopropyl thiogalactoside (IPTG) at 37 °C for 3 h. The expressed recombinant proteins were purified using Ni^{2+} affinity chromatography (Bio-Rad, Hercules, CA, USA) according the manufacturer's instructions.

2.4. Western blot analyses

The recombinant protein was separated by SDS-PAGE (12%) and transferred to a nitrocellulose filter (0.2 μm, Bio-Rad). Non-specific binding sites were blocked with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.4) overnight at 4 °C. The serum of goats infected with *T. multiceps* was used at a dilution of 1:200 in TBS containing 0.1% (v/v) Tween (TBST) and 5% (w/v) BSA. The membrane was incubated with serum for 2 h at room temperature. Following a wash with TBST, the rabbit anti-goat IgG-HRP conjugate (Boster Bio-project Co, Wuhan, China) was used as the secondary antibody. After further washing, immunoreactive bands were visualized using Diaminobenzidine (DAB; TIANGEN, Beijing, China).

2.5. Development of the TmHSP ELISA

The 96-well ELISA plates were coated with 100 μL of two-fold diluted rTmHSP (ranging from 5.40 μg /well to 0.09 μg/well) in 0.1 M carbonate buffer (pH 9.6) for 12 h at 4 °C. All wells were washed by 300 μL of PBS containing 0.05% Tween-20 (PBST) and then each well was blocked with 5% skimmed milk (skimmed milk in PBS) for 1 h at 37 °C. The sera of infected and uninfected goats were diluted in PBS to dilutions of 1:20, 1:40, 1:80, 1:160, 1:320 or 1:640, then added to each well and incubated for 1 h at 37 °C. After three washes with PBST, the rabbit anti-goat IgG-HRP conjugate (100 μL; 1:3000) was added to the wells and the plates were incubated for a further 1 h at 37 °C (Boster Bio-project Co). Color reactions were developed by the addition of 100 μL of the substrate TMB (TIANGEN). This process was stopped with 100 μL of 2 M H_2SO_4. The optical density (OD) of each well was measured at 450 nm (OD450). We then determined the dilutions of rTmHSP antigen and goat serum that gave rise to the maximum difference at OD450 values between positive and negative sera (*P*/*N*), and the cutoff value. Negative and blank controls were included on each plate. The cutoff value was calculated as the mean +3 standard deviations from the OD450 value of the negative sera.

2.6. Specificity and sensitivity of the TmHSP ELISA

To determine the specificity of the TmHSP ELISA, we evaluated potential cross-reactivity with six *C. tenuicollis* sera (co-infections with these two tapeworms are common in goats). Three *T. multiceps*-positive served as positive controls. To evaluate sensitivity, the 96-well plates were coated with optimal concentrations of purified rTmHSP as described above. Three positive serum samples with twofold serial dilutions from 1:20 to 1:20480 were then added to each well and incubated with the antigen. The sensitivity of the TmHSP ELISA was defined as the highest dilution of the positive serum that produced an OD450 value higher than the cutoff value.

2.7. Repeatability and reproducibility of the TmHSP ELISA

To evaluate the repeatability and reproducibility of the TmHSP ELISA, six positive serum samples were used. Every sample was tested simultaneously to assess the intra-assay variability (repeatability), and then tested consecutively to assess the inter-assay variability (reproducibility). Repeatability and reproducibility tests were conducted three times and their corresponding mean OD450, standard deviation (SD), and coefficient of variation (CV) were calculated.

2.8. Clinical testing of the TmHSP ELISA

We assessed the clinical serodiagnostic potential of our TmHSP ELISA on 60 field-held goats, 30 of which were infected with *T. multiceps* and 30 of which were uninfected. The positive diagnostic rate was calculated by referring to the cutoff value. Each goat was tested twice.

3. Results

3.1. Characteristics of TmHSP70 and antibody reactivity

The entire TmHSP cDNA sequence comprised 408 bp (GenBank accession number GU205474) and showed an initiation and a stop codon. Analysis of the nucleotide sequence revealed 90% nucleotide