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Research paper

Expression and serodiagnostic potential of antigen B and thioredoxin peroxidase from *Taenia multiceps*

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ABSTRACT

Coenurosis is a serious parasitic disease of herbivorous animals caused by the metacestode of *Taenia multiceps* (*Coenurus cerebralis*). Accordingly, a significant amount of research is currently dedicated to the development of appropriate antigens for use in rapid and accurate coenurosis diagnosis kits. In the present study, antigen B (AgB) and thioredoxin peroxidase (TPx) from *T. multiceps* were cloned and expressed using a prokaryotic system, molecular characterization of *Tm*-AgB was determined by bioinformatical analyses. The serological diagnostic potentials of r*Tm*-AgB and r*Tm*-TPx were evaluated by indirect ELISA and compared with those of previously reported r*Tm*-AnxB2, r*Tm*-HSP70, and r*Tm*-GST. The results showed that *Tm*-AgB is a specific lipoprotein of cestodes with good thermal stability. The ELISA assay showed that r*Tm*-AgB exhibited a sensitivity of 95.8% and a specificity of 87.5%, indicating its strong potential for serological diagnosis of *T. multiceps*.

1. Introduction

Coenurosis is caused by the metacestode of *Taenia multiceps* (*Coenurus cerebralis*), which parasitizes the central nervous systems and subcutaneous and muscular tissues of herbivores such as goats, sheep, cattle, and yaks (Oryan et al., 2015). Furthermore, humans can act as intermediate hosts if food contaminated with *T. multiceps* eggs is eaten (Dehghani et al., 2016; Hermos et al., 1970; Li et al., 2018). Coenurosis is often fatal to the host, thus causing significant economic loss to animal husbandry industries in Europe, the USA, Africa, and Asia. Accordingly, the rapid and accurate diagnosis of coenurosis-infected animals is currently of interest in veterinary research (Huang et al., 2017; Wu et al., 2013, 2012).

Current diagnostic methods for coenurosis can be divided into clinical and laboratory methods (Pau et al., 1990, 1987; Wang et al., 2018). In the laboratory, immunological diagnosis methods are more convenient and appropriate. However, the antigens used in such methods are usually derived from the crude extracts of protoscolices or cystic fluid, which are often unstable, causing problems in terms of mass production. Consequently, ELISA assays based on recombinant antigens have become increasingly adopted to diagnose this disease.

The recombinant proteins *Tm*-P2, *Tm*-HSP70, *Tm*-GP50, *Tm*-GST, and *Tm*-HSP60 have been used as diagnostic antigens to establish reliable ELISA methods (Huang et al., 2015; Huang et al., 2016; Sun et al., 2017; Wang et al., 2015; Liu et al., 2019). These recombinant antigens could possibly allow the industrial production of reagent kits, but their thermal stabilities remain an issue in their mass production.

Consequently, further development of antigens is required to expand the choice of agents with serodiagnostic potential for *T. multiceps*. Accordingly, in the present study, antigen B (AgB) and thioredoxin peroxidase (TPx) of *T. multiceps* were cloned and their serodiagnostic potentials were evaluated. AgB, a lipoprotein specific to tapeworms, has been used as a diagnostic antigen for the larval stage of other cestodes. Furthermore, AgB exhibits good thermal stability owing to the high α-helix content of its secondary structure, and this presents several advantages in terms of its production and application to diagnostic kits (Monteiro et al., 2007). On the other hand, TPx is an important antioxidant in organisms and has been demonstrated to exhibit immunoreactivity, indicating that it can be recognized by the host during the invasion of *T. multiceps* (Li et al., 2009).

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In the present study, we expressed the recombinant proteins r*Tm*-AgB and r*Tm*-TPx and established indirect ELISA methods based on these proteins. To evaluate their potential utility for serological diagnosis, the results were compared with those achieved with r*Tm*-AnxB2, r*Tm*-HSP70, and r*Tm*-GST.

This study is the first to employ r*Tm*-AgB and r*Tm*-TPx in the detection of *T. multiceps*. Thus, it is hoped that this study will contribute to the reliable and rapid diagnosis of coenurosis.

2. Materials and methods

2.1. Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University (SYXK 2014-187). All animal procedures used in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Bethesda, MD, USA) and the ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines).

2.2. Sera

Positive sera against *T. multiceps* (24 samples) were isolated from experimentally infected goats; a total of 36 serum samples, including sera positive against *Taenia hydatigena* (12 samples), *Fasciola hepatica* (12 samples) and *Haemonchus contortus* (12 samples) were isolated from naturally infected goats in the Sichuan province; and a further 24 samples, including sera positive against *Echinococcus granulosus* (12 samples) and *Moniezia expansia* (12 samples) were isolated from naturally infected sheep. Serum samples from uninfected captive goats were also confirmed by autopsy and used as negative controls (24 samples). All sera were determined by autopsy.

2.3. Preparation of antigens

TRIzol reagent (Tiangen, Beijing, China) was used to extract total RNA from protoscolices, cDNA was reverse-transcribed using a RevertAid First Strand cDNA Synthesis Kit following the manufacturer's instructions (MBI Fermentas, Germany). Primers for *Tm*-AgB were designed based on transcriptome data, and primers for *Tm*-TPx were designed based on a previous study (Li et al., 2009). Target fragments were amplified, ligated into the pET32a(+) vector (TaKaRa, Dalian, China), and transformed into BL21 (DE3) competent cells. Expression of the recombinant proteins was induced by 1 mM isopropyl β-D-1-thio-galactopyranoside (IPTG), and purification was achieved using Ni^{2+} affinity chromatography (Bio-Rad, Hercules, CA, USA). r*Tm*-AnxB2, r*Tm*-HSP70, and r*Tm*-GST were provided by the Sichuan Agricultural University Department of Parasitology. The concentration of all purified antigens were determined by NanoDrop One (Thermo Fisher Scientific, Madison, USA).

2.4. Bioinformatics analyses of Tm-AgB

Bioinformatics analyses were performed using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to predict open reading frames (ORFs) and TMHMM Sever 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0) to identify transmembrane regions. Signal peptides were predicted using the SignalP server (http://www.cbs.dtu.dk/Services/SignalP/). Subcellular localization and B-cell epitopes were predicted using BaCelLo (http://gpcr.biocomp.unibo.it/bacello/pred.htm) and Antibody Epitope Prediction (http://tools.immuneepitope.org/bcell/), and the protein secondary structure was predicted using Jpred prediction (http://www.compbio.dundee.ac.uk/jpred/). Multiple sequence alignment was performed with Clustal X software version 1.83, and a phylogenetic tree was constructed by the maximum likelihood method using MEGA7.0 software.

2.5. Western blotting analysis

The recombinant proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using an electrophoretic transfer cell (Bio-Rad) for 30 min. After washing with TRIS-buffered saline containing Tween-20 (TBST), the membranes were incubated with 5% (*w/v*) skimmed milk at 37 °C for 2 h and then at 4 °C with serum from infected goats (1:200, *v/v* dilution) for 12 h. Finally, they were incubated with a 1:1000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Boster, Wuhan, China) for 2 h. Signals were measured using an Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen).

2.6. ELISA

To evaluate the serodiagnostic potential of the recombinant proteins, indirect ELISAs were performed following the standard checkerboard titration procedures (Crowther, 2000). Briefly, proteins were diluted to different concentrations using 0.1 M carbonate buffer (pH 9.6) and coated at 100 μL/well in 96-well polystyrene microtiter plates. After incubation at 4 °C for 12 h, plates were washed three times using phosphate buffered saline-Tween-20 (PBST) then blocked with 5% (*w/v*) skim milk at 37 °C for 1.5 h. After washing three times, the plates were incubated with 100 μL serum samples diluted to different concentrations with PBS at 37 °C for 1 h. Following washing, 100 μL of HRP-labeled rabbit anti-goat or sheep IgG (Boster) diluted 1:2000 with PBS was added and the plate was incubated at 37 °C for 1 h. Reaction with 3,3′,5,5′-tetramethylbenzidine (Tiangen) was used for staining and was stopped with 2 M H_2SO_4. The optical density at 450 nm (OD_{450}) was then measured using a microplate reader (Thermo Fisher Scientific). We selected the antigen concentration and serum dilution that gave the highest P/N value as the working condition, and the cut-off value was determined as the mean OD_{450} plus three standard deviations (SD) using 24 negative goat serum samples.

To further investigate the feasibility of the indirect ELISA method, 24 serum samples from goats infected with *T. multiceps* were evaluated and the sensitivity was calculated as (ELISA positive × 100)/true *T. multiceps*-positive. Furthermore, a total of 24 negative serum samples were evaluated to determine the specificity of the indirect ELISA, calculated by (ELISA negative × 100)/true *T. multiceps*-negative. An additional 60 serum samples (12 samples for each parasite) derived from sheep infected with *E. granulosus* and *M. expansia*, and goats infected with *T. hydatigena*, *F. hepatica*, and *H. contortus* were used to evaluate cross-reactivity.

The repeatability (intra-assay) and reproducibility (inter-assay) of the indirect ELISA method were tested using six serum samples positive against *T. multiceps*. With two groups set up, every sample in one plate was detected to assess the repeatability, and then three different plates were tested consecutively to assess the reproducibility. After three repeats, the coefficient of variation (CV) was calculated.

2.7. Statistical analyses

All data are presented as the mean ± SD. Statistical analyses were performed using the Mann-Whitney U test (for comparison between different serum groups). All statistical tests were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL). P values < 0.05 were considered to be significant.

3. Results

3.1. Bioinformatics analyses of Tm-AgB

The 261 nucleotide ORF in the cDNA sequence of *Tm*-AgB (GenBank: MK784567) encodes an 86 amino acid polypeptide. Signal peptides were predicted at amino acids 1–20 of the sequence, and it also

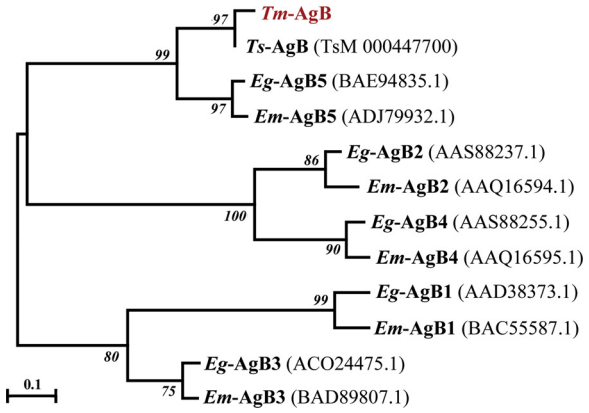

Fig. 1. The phylogenetic tree of *Tm*-AgB (maximum likelihood). Genbank access numbers are given in parenthesis.

appears to possess transmembrane regions. The predicted subcellular localization of *Tm*-AgB is secretory.

The phylogenetic tree (Fig. 1) shows that *Tm*-AgB is more closely related to AgB5 of *E. granulosus* and *Echinococcus multilocularis*. *Tm*-AgB shares 96.5%, 73.3%, and 72.1% sequence identity with orthologs in *T. solium* (geneDB ID: TsM 000447700), *E. granulosus* (GenBank: BAE94835.1), and *E. multilocularis* (GenBank: ADJ79932.1; Fig. 2), respectively. After the signal peptide was removed, the α-helix content of the secondary structure of *Tm*-AgB is 77.27%.

3.2. Preparation of antigens

Tm-AgB and *Tm*-TPx genes from protoscolices were amplified. After induction by 1 mM IPTG, the recombinant proteins were expressed in a prokaryotic expression system, and the resulting fusion proteins yielded single bands of ˜26 kDa and ˜28 kDa, (including the ˜18 kDa epitope tag fusion peptide from pET-32a) following separation by 12% SDS-PAGE (Fig. 2). After purification, the concentration of r*Tm*-AgB, r*Tm*-TPx, r*Tm*-AnxB2, r*Tm*-HSP70, and r*Tm*-GST were respectively 1 mg/mL, 2 mg/mL, 3 mg/mL, 5 mg/mL and 3 mg/mL.

3.3. Western blotting analysis

All recombinant antigens were recognized by sera from goats infected with *T. multiceps*, but not by the negative control serum, indicating the strong reactivity and good antigenicity of the recombinant proteins (Fig. 3).

3.4. Serodiagnostic potential of rTm-AgB, rTm-AnxB2, and rTm-TPx

The optimal antigen concentrations and serum dilutions were determined based on standard checkerboard titration procedures, and the cut-off values were determined by the average OD_{450} value from 24

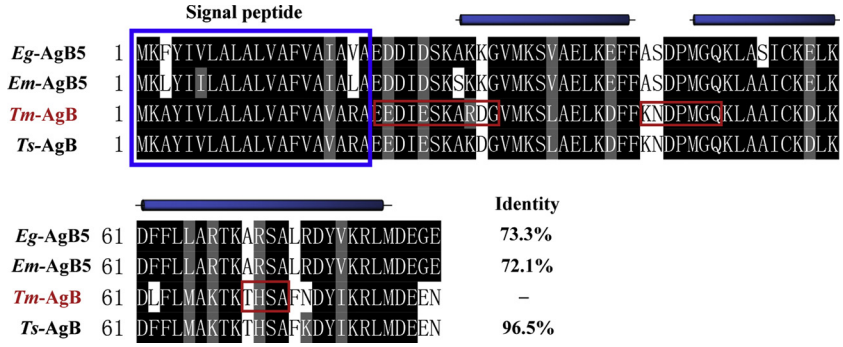

Fig. 2. Multiple sequence alignment of *Tm*-AgB. Multiple sequence alignment of the deduced amino acid sequence of *Tm*-AgB with homologous sequences of related proteins from other cestaodes: *Echinococcus granulosus* (GeneBank: BAE94835.1), *Echinococcus multilocularis* (GeneBank: ADJ79932.1) and *Taenia solium* (GeneDB ID: TsM_000447700). Conserved residues are highlighted by a black background. B-cell epitopes are marked with a red box. Signal peptides marked with blue boxes. α-Helices marked by blue cylinders (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

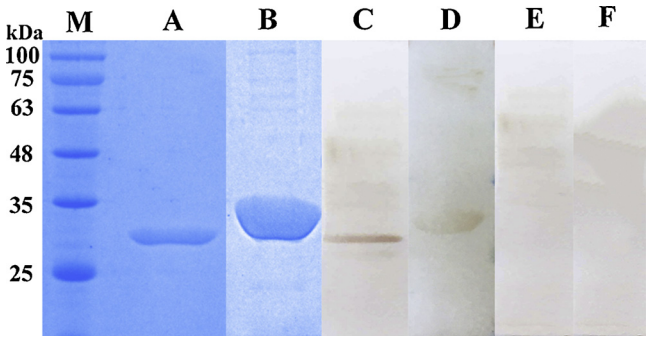

Fig. 3. SDS-PAGE and western blot analysis of *Tm*-AgB and *Tm*-TPx. Lane M: protein molecular weight markers; Lane A: purified r*Tm*-AgB; Lane B: purified r*Tm*-TPx; Lane C: Western blot of purified r*Tm*-AgB (10 μg) incubated with *Coenurus cerebralis*-infected goat serum; Lane D: Western blot of purified r*Tm*-TPx (10 μg) incubated with *Coenurus cerebralis*-infected goat serum; Lanes E and F: negative control goat serum.

negative serum samples plus three times the SD under optimal conditions. The cut-off values for r*Tm*-AgB, r*Tm*-AnxB2, and r*Tm*-TPx were 0.259, 0.43, and 0.442, respectively. All the intra- and inter-assay variabilities of the indirect ELISA method were < 10%.

The sensitivities for the indirect ELISA method identified by positive serum samples were 95.8% for r*Tm*-AgB (23/24), 79.2% for r*Tm*-AnxB2 (19/24), and 75.0% for r*Tm*-TPx (18/24), compared with 79.2% for r*Tm*-HSP70 (19/24) and 83.3% for r*Tm*-GST (20/24). Furthermore, the specificities as obtained using negative serum samples were 87.5% for r*Tm*-AgB (21/24), 95.8% for r*Tm*-AnxB2 (23/24), and 91.7% for r*Tm*-TPx (22/24), compared with 83.3% for r*Tm*-HSP70 (20/24) and 91.7% for r*Tm*-GST (22/24). Details of their cross-reactions with the sera of *T. hydatigena, F. hepatica, H. contortus, E. granulosus*, and *M. expansia* (60 samples in total) are presented in Fig. 4.

4. Discussion

Coenurosis causes serious, often fatal, health problems in herbivores and thus severe financial losses to areas and communities economically dependent upon animal husbandry (Guo et al., 2017). Especially when the intermediate host and the definitive host (carnivorous animals such as dogs and foxes) coexist, the intermediate host is more likely to be exposed to the eggs. Thus, coenurosis occurs frequently in areas where animal grazing is prevalent. However, timely diagnosis and treatment can reduce occurrence of the disease and the associated economic loss. Therefore, it is important to develop immunoreactive antigens for rapid and accurate diagnostic kits. Accordingly, in the present study, we evaluated the serological diagnostic potentials of r*Tm*-AgB and r*Tm*-TPx and compared with other antigens reported previously. In this study, the sensitivity with r*Tm*-TPx is 75.0%, and that with r*Tm*-AnxB2 is 79.2%.

AnxB2 is a phosphatide binding protein with calcium binding

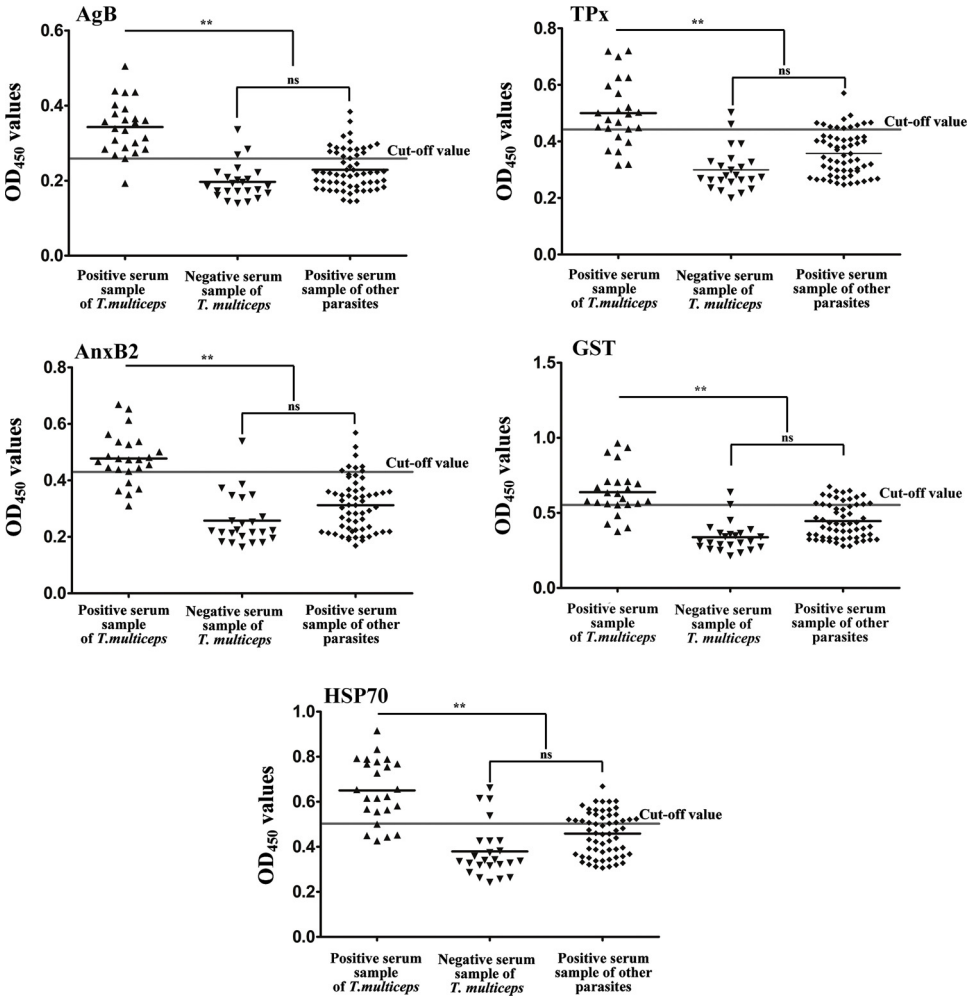

Fig. 4. Sensitivity, specificity, and cross-reactivity in indirect ELISA. The bold horizontal line represents the cut-off value. Statistical analyses were performed using Mann-Whitney U-tests for different serum groups. All statistical tests were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). *P* values < 0.05 were considered to be significant.

activity and belongs to the annexin family. Annexins are known to maintain biofilm structure and play important roles in various biological activities, such as cell membrane transport, signal transduction, and calcium channel formation (Chander et al., 2007; Mortimer et al., 2008; Ursula and Volker, 2004). Annexins have also been proved to be involved in immune regulation during invasion by *T. solium* and *E. granulosus* (Gao et al., 2007; Solano et al., 2006; Song et al., 2016). AnxB2 was used to establish indirect ELISA method for comparison in this study, since it has been proved to have immunoreactivity in previous studies (Guo et al., 2018). However, from our work, the sensitivity with r*Tm*-AnxB2 is only 79.2%, it seems that the effect of using AnxB2 as antigen to establish ELISA method is not well.

Tpx is an important sulfhydryl-specific antioxidant protein that belongs to the peroxiredoxin family. It has the ability to eliminate excess reactive oxygen species produced by normal cells (Henkle-Duhrsen and Kampkotter, 2001; Wood et al., 2003). TPx has been studied in parasites such as *Schistosoma mansoni*, *Brugia malayi*, and *Trichinella spiralis*, confirming its antioxidant role and immunogenicity (Eisinger et al., 1998; Kwatia et al., 2000; Williams et al., 2001; Zhang et al., 2013). TPx has also been used effectively in indirect ELISA methods for the detection of hydatid disease in both humans and animals (Li et al., 2004). In previous studies, TPx of *T. multiceps* was also found to exhibit antigenicity (Li et al., 2009). A situation similar to that of AnxB2 in *T. multiceps*, TPx also have the potential to serve as reagent for serological diagnosis from previous studies, but their sensitivities in our work are insufficient for detecting the antibodies.

However, r*Tm*-AgB has the much higher sensitivity of 95.8% and a specificity of 87.5%. AgB is a lipoprotein characteristic of cestoda that belongs to the hydrophobic ligand-binding protein (HLBP) family. HLBPs comprise both intracellular and extracellular members and are able to bind a variety of fatty acids, retinoids, and some sterols. Since cestoda have lost catabolic and biosynthetic pathways for fatty acids and cholesterols, metacestode are thought to use AgB to transport the lipids of hosts for their own use (Eung-Goo et al., 2010; Obal et al., 2012; Olson et al., 2012; Silva-Álvarez et al., 2015). This lipoprotein has excellent diagnostic potential for metacestodiasis, and it also presents advantages in terms of production and application since the protein part of AgB exhibits good thermal stability (Kamenetzky et al., 2005; Majid et al., 2013; Saghir et al., 2000; Sánchez et al., 2010). Interestingly, independent gene expansion in these families appear to have occurred in different cestode lineages, giving rise to species and gene-specific monophyletic clades. The *E. granulosus* and *E. multilocularis* antigen B families comprise five clades each, named *Eg*-AgB1 to *Eg*-AgB5 and *Em*-AgB1 to *Em*-AgB5, respectively (Arend et al., 2004; Wulamu et al., 2006; Zhang et al., 2010). According to the current data, the AgB of *T. multiceps* has closer relationships with *Eg*-AgB5 and *Em*-AgB5 than with the other members. This genotype has a higher α-helix content in its secondary structure, endowing it with superior thermal stability. r*Tm*-AgB is subjected to some cross-reactions, especially with eight of the 12 serum samples of *E. granulosus*, owing to the relative

conservatism of AgB. But its high sensitivity and thermal stability make it a potentially stronger serological diagnostic tool in comparison with the other agents considered in this study. And non-homologous short fragments containing more antigenic epitopes may improve the situation of cross-reaction in the future.

5. Conclusions

In this study, we expressed the recombinant proteins *Tm*-AgB and *Tm*-TPx, analyzed the molecular characteristics of *Tm*-AgB, and confirmed the relationship between *Tm*-AgB and antigen B from other cestodes. We also evaluated the serologic diagnostic potentials of r*Tm*-AgB, r*Tm*-TPx, and r*Tm*-AnxB2 as compared with other reported antigens. r*Tm*-AgB exhibited a sensitivity of 95.8% and 87.5% specificity, demonstrating its excellent promise for serological diagnosis. Thus, our study provides a reliable candidate antigen for the accurate and rapid clinical diagnosis of coenurosis.

Authors' contributions

YCL and GYY conceived and designed the study. YCL, YDY, and XWD performed the experiments. YCL and JX analyzed the data and drafted the manuscript. XBG and WML conceived the study and collected experimental materials. YX, BJ and XRP collected and analyzed the raw data. GYY assumed overall responsibility for this study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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