Genetics and Molecular Research

Cloning, identification, and bioinformatics analysis of a putative aquaporin TsAQP from *Trichinella spiralis*

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ABSTRACT. Vaccination as a preventative strategy against *Trichinella spiralis* infection is an ongoing effort, although no ideal vaccine candidates have been identified until now. Identification of more effective antigens that have a role in essential life stages of the parasite and that may be effective vaccine candidates is therefore of importance. In the present study, we identified a novel aquaporin gene (*TsAQP*) from *T. spiralis*, and the potential antigenicity of TsAQP was evaluated by epitope prediction. A total of 11 post-translational modification sites were predicted in the protein and fell into 4 categories: *N*-glycosylation; casein kinase II phosphorylation; protein kinase C phosphorylation; and *N*-myristoylation sites. TsAQP is a membrane intrinsic protein with high hydrophobicity; the main hydrophobic domains comprised up to 38.5% of the protein and were distributed at amino acid positions 21-43, 54-71,

83-91, 107-121, 163-174, 187-200, and 242-261. The protein consisted mainly of helices (39.58%) and loops (50%). The advanced structure of TsAQP was predicted using homology modeling, which showed that the protein was formed from 6 membrane-spanning domains connected by 5 loops. Based on these analyses, 6 potential B-cell epitopes and 4 potential T-cell epitopes were further predicted. These results suggest that TsAQP could be a promising antigen candidate for vaccination against *T. spiralis*.

Key words: *Trichinella spiralis*; Aquaporin; Bioinformatics analysis; Cloning

INTRODUCTION

Trichinella spiralis is an intracellular parasite and is recognized as one of the crucial pathogens of the zoonotic trichinellosis (Teunis et al., 2012). The parasite has a worldwide distribution and is capable of causing infection in almost all mammals, including humans (Feidas et al., 2014). Human infection is attributed mainly to the consumption of raw or undercooked meat, particularly pork, contaminated with muscle larvae (Dupouy-Camet, 2000). Pathologic lesions in the host are strictly related to the intestinal and muscular parasitic stages, and cause especial harm to the heart and brain (Dupouy-Camet, 2000). In recent years, the re-emergence of trichinellosis in many countries and areas has had an impact on the international commerce of food-producing animals, rekindling public health concerns over the parasite (Djordjevic et al., 2003; Cuperlovic et al., 2005; Blaga et al., 2007).

Albendazole and mebendazole are currently the principal drugs for treating trichinellosis (Gottstein et al., 2009). However, encapsulated larvae in muscle are refractory to treatment with these benzimidazole derivative drugs (Pozio et al., 2003). Investigation of effective vaccination strategies is a potential method to protect against *T. spiralis* infection. Several *T. spiralis* antigens have been developed and evaluated in mice (Gu et al., 2013; Fang et al., 2014), but none have been found to provide complete protection. Identification of novel functional proteins of *T. spiralis* is therefore greatly desired, for the purposes of developing new specific drugs or vaccines.

The biological membrane system of parasites may interact with receptors in hosts, and is often looked upon as a potential target for the development of drug or vaccine candidates (Kirk, 2004; Landfear, 2010). According to previous studies of *Leishmania major* and *Schistosoma mansoni*, Aquaporins, also named water channel proteins (Benga, 2009), are components of this parasite membrane system and are promising candidates for this purpose (Gourbal et al., 2004; Faghiri and Skelly, 2009). Aquaporins (AQPs) are the most abundant proteins in the biological membrane and belong to the major intrinsic protein (MIP) family. The protein group can be divided into water-specific channels (orthodox AQPs), and channels that additionally facilitate transport of glycerol, urea, ammonia, and other small, uncharged molecules (aquaglyceroporins), according to specific functions of each protein. AQPs have been identified in most parasites as having roles in many physiological functions, including osmotaxis; volume regulation; and uptake of nutrients and release of toxic metabolites (Beitz, 2006; Uzcategui et al., 2008; Faghiri et al., 2010). However, little is known about AQPs in *T.*

spiralis (*TsAQP*).

In the present study, a putative *TsAQP* was amplified from total RNA of *T. spiralis* yunnan isolate. Signal peptides, epitopes, topological structures, and other physical and chemical characteristics of the *TsAQP*-encoded protein were identified using multiple bioinformatic methods. These findings provide the foundation for our understanding of the biological function of TsAQP, and its potential application in development of epitope-based vaccine or drug.

MATERIAL AND METHODS

Mice and parasites

Specific-pathogen-free grade, female Kunming mice (8 weeks old) were purchased from the Center of Laboratory Animals at the Lanzhou Institute of Biological Products (Lanzhou, China). Mice were maintained on sterilized food and water and were used for attaining muscle larvae. All procedures involving mice were carried out in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China (2010, GB 14925-2010).

Muscle larvae of *T. spiralis* yunnan isolate from infected mice were artifically digested with pepsin-HCl at 35 days p.i. (Liu et al., 2012), and were purified using the method described by Gamble and Bessonov (Gamble et al., 2000). Muscle larvae were used for total RNA extraction.

Preparation of *T. spiralis* RNA and cloning of *TsAQP*

Total RNA was extracted from *T. spiralis* using Trizol (Invitrogen, USA), according to the manufacturer protocol. First strand cDNA was reverse transcribed using oligo (dT) primers at 42°C for 30 min. Then, *TsAQP* was amplified using a pair of specific primers AQPPF (5'-GTGAGCAGAGCATATTCACA-3') and AQPPR (5'-GGGGCTTTAGAAATGTGAGA -3') by PCR. Individual reactions for PCR amplification contained 1.0 µL cDNA; 2.0 µL 2.5 mM dNTP mix; 2.5 µL 10X PCR buffer; 1.0 µL 10 µM of each primer; 0.4 µL 2.5 U/mL Taq DNA polymerase; and 17.1 µL ddH_2O. Cycling conditions for PCR were: 5 min at 94°C; 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min 30 s; and a final extension at 72°C for 10 min.

Products of PCR (5 µL) were analyzed by electrophoresis on 1.0% agarose (w/v) gel stained with ethidium bromide. The gel was visualized using a gel ImageScanner system (PeiQing, Science & Technology, Shanghai, China).

Cloning, sequencing, and identification of *TsAQP*

The target gene fragment was purified using a gel extraction kit (TIANGEN, China) according to the manufacturer protocol. The fragment was ligated into a pMD18-T vector (TaKaRa, China), and transformed into *Escherichia coli* DH5α competent cells, which were then coated onto plates of Luria-Bertani (LB) supplemented with ampicillin (AMP, 100 µg/mL), and cultured at 37°C. Twelve hours later, 10 single colonies were selected for culture in LB liquid medium containing 100 µg/mL AMP, and shaken overnight at 37°C. Colonies were then screened by PCR amplification, and positive colonies were sent for sequencing to GENEWIZ™ (China). Sequencing results were aligned on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Positive recombinant plasmid was termed pMD-TsAQP.

Analysis of TsAQP amino acid sequence

General features of TsAQP including molecular weight, isoelectric point, amino acid (aa) composition, molecular extinction coefficient, and half-life were predicted using the ProtParam software (http://web.expasy.org/protparam/). Motifs were identified using the MotifScan software (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and hydrophilicity/hydrophobicity was analyzed using ProtScale.

The secondary structure of TsAQP was predicted using PredictProtein (https://www.predictprotein.org/). Then, determination of the topologic structural characteristics of TsAQP was performed using the online service SMART (Letunic et al., 2012), and the 3-dimensional (3D) structure estimated by homology modeling using the online service SWISS-MODEL (Kiefer et al., 2009). TsAQP was constructed via the automated modeling program using the software Swiss PDB-Viewer and the corresponding protein from *Plasmodium falciparum* as a template (code: 3c02). A 3D model of TsAQP was constructed using the program Swiss-PDB Viewer (Guex et al., 2009), and assessed using Verify_3D (http://services.mbi.ucla.edu/Verify_3D/).

The flexibility and accessibility of the structural proteins were analyzed by Karplus-Schulz and Plot-Emini methods, respectively, using the DNAStar 7.1 software (DNAStar, USA). Finally, the potential linear B-cell epitopes of TsAQP were predicted with the Jameson-Wolf index using the DNAStar software. The potential T cell epitopes were calculated using the online server SYFPEITHI (http:www.syfpE-thi.de/Scripts/MHCServer.dll/EpitopePrediction.htm) and Propred I (http:www.imtech.res.in/raghava/propred1/).

RESULTS

Identification of recombinant vector pMD-TsAQP

The recombinant plasmid pMD-TsAQP was identified by PCR. Agarose gel electrophoresis revealed the amplified fragment was approximately 1100 bp; this was consistent with the expected length (Figure 1). The aa sequence translated from the *TsAQP* open reading frame (ORF) demonstrated 45% identity to human AQP9. This indicated that insertion into the pMD18-T vector was successful.

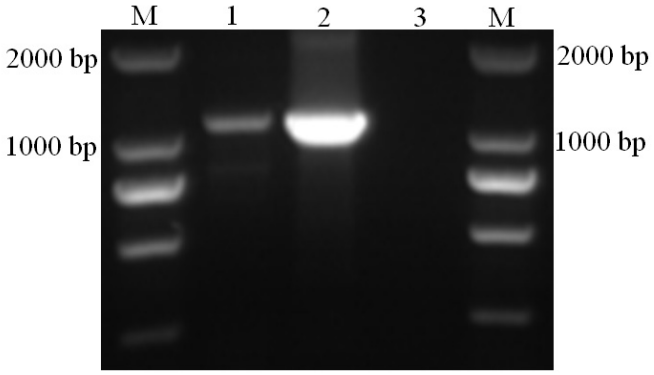

Figure 1. Identification of the recombinant vector pMD-TsAQP by polymerase chain reaction amplification. *Lane M* = DL2000 DNA markers; *lane 1* = PCR products of cDNA TsAQP; *lane 2* = identification of pMD-TsAQP by PCR; *lane 3* = control.

Physiological and biochemical characters of TsAQP

The ORF of *TsAQP* was found to be 867 bp in length, and encoded a 288-aa protein (Figure 2). The molecular weight of the TsAQP protein was 31 kDa, and its theoretical isoelectric point was 8.47. At 280 nm in water, the molar extinction coefficient of TsAQP was 52,035 mol/cm. The half-life of the protein in yeast and *Escherichia coli* was estimated as >20 and >10 h, respectively. TsAQP protein had an instability index of 32.48, and could therefore be classified as stable.

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gtgagcagagcatattcacagagcagcatttcagtgtacggagacggtcggttgatcgacttgtttgcttgtttgcatc
agtacactcactgctttgtttgtcggctttgatttattcagttagaagaaataattaatacaaaacaattaatataatc
catataataagaataa
ATG AAG AGA TTG CAG CAT ACG ATG GCA AAA CGA CTA CGT ACT GAT
  M   K   R   L   Q   H   T   M   A   K   R   L   R   T   D
CGA ACT TGG ATT CGA ATT GTT TTG GCC GAA TTT TTT GGC ACC CTG
  R   T   W   I   R   I   V   L   A   E   F   F   G   T   L
GTA CTT GTG ATG CTT GGT GAT GGA GCA ATT GCA CAG GCG GTT CTC
  V   L   V   M   L   G   D   G   A   I   A   Q   A   V   L
AGT CGA CAA CAA GCT GGA AAT TTT GTT ACC GTC AAC GTT GCC TAC
  S   R   Q   Q   A   G   N   F   V   T   V   N   V   A   Y
GCT CTC GCA TTG ACA TTT GCC ATT TAC GTC AGC GGT GGA GTT TCA
  A   L   A   L   T   F   A   I   Y   V   S   G   G   V   S
GGT GGT CAC GTC AAT CCT GCC GTT ACG CTT GCG CTG TGC AGT ATC
  G   G   H   V   N   P   A   V   T   L   A   L   C   S   I
GGT GCT TGT AGT TGG CAT ATG TTG CCT GCT TAT TGG TTT GGA CAA
  G   A   C   S   W   H   M   L   P   A   Y   W   F   G   Q
TAT TTA GGG GCT TTT ATT GGT TCG TCA ATT GTA CTT GTA ATT TAC
  Y   L   G   A   F   I   G   S   S   I   V   L   V   I   Y
ACG GAT GCA ATA AAT ACG TTT GAC ATG GGA CAT CGG TTG GTA ACA
  T   D   A   I   N   T   F   D   M   G   H   R   L   V   T
GGA CCT AAC GCA ACA GCC GGC ATT TTT GCA ACA TAT CCA AGT GAT
  G   P   N   A   T   A   G   I   F   A   T   Y   P   S   D
CAT ATA AGC ACT GCT GGA TCC TTT TTT GAT CAA GTT CTG GGC ACA
  H   I   S   T   A   G   S   F   F   D   Q   V   L   G   T
GCA ATT TTG TTG ATG GTT ATT GTC GCT GTA ACC GAT GAA CGG AAT
  A   I   L   L   M   V   I   V   A   V   T   D   E   R   N
ATG GAA GTG CCT AAA GCA ATT GCT CCA GTT TTG ATT GGA TTG GCA
  M   E   V   P   K   A   I   A   P   V   L   I   G   L   A
ATT TTT TCC GTC GCA TCA GGA TTT GGT TAC AAT TGT GGT GGA GCG
  I   F   S   V   A   S   G   F   G   Y   N   C   G   G   A
ATT AAT CCA GCT CGT GAT TTC AGT CCT CGC TTA CTG ACA TCT TTG
  I   N   P   A   R   D   F   S   P   R   L   L   T   S   L
ACT GGC TGG GGT TCT GAA ACG TTC AGC GTT CGA AAT TAT TGG TTC
  T   G   W   G   S   E   T   F   S   V   R   N   Y   W   F
TGG GTA CCA ATT ATT GGT CCT CAT ATT GGC GGT TTG ATC GGG GCA
  W   V   P   I   I   G   P   H   I   G   G   L   I   G   A
GCG TTG TAC AAA CTG TTT GTC GGT ATC CAT TGG CCA CCT GTA CAC
  A   L   Y   K   L   F   V   G   I   H   W   P   P   V   H
GAG ATT ACG ATG ATC AGT TCC CAG ATT TCT CGT AAT GAA CAT ATT
  E   I   T   M   I   S   S   Q   I   S   R   N   E   H   I
GGA TTG TCT TAG tttgattcttcgatcatgttttcttattcaattctttttaatcaatttaacttaaa
  G   L   S   *
gtatattttaatttctttctctcacatttctaaagccc
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Figure 2. Nucleotide and amino acid sequences of TsAQP.

Prediction of functional motifs

After alignment of TsAQP with other identified AQPs, the MIP family signature was predicted at position 78-86, and a cysteine residue at position 207. Some other post-translational modification sites, such as one *N*-glycosylation, two casein kinase II phosphorylation, three protein kinase C phosphorylation, and five *N*-myristoylation sites, were also predicted in TsAQP (Table 1).

Table 1. Post-translational modification (PTM) sites of the putative TsAQP protein.

PTM sites	Position	Numbers
N-glycosylation	138-141	1
Casein kinase II phosphorylation	157-160, 280-283	2
N-myristoylation	51-56, 72-77, 104-109, 142-147, 250-255	5
Protein kinase C phosphorylation	14-16, 218-220, 234-236	3

Prediction of hydrophilicity/hydrophobicity and secondary structure of TsAQP

Hydrophobic effect is a dominant force for protein folding and architectural stability (Malleshappa Gowder et al., 2014). The TsAQP protein was predicted to be characteristically hydrophobic, with an aliphatic index of 107.36, and grand average of hydropathicity of 0.558. Further analysis of the hydrophilic/hydrophobic regions of TsAQP, by the Kyte & Doolittle selection of Hphob within the ProtScale software program, indicated that the protein contained multiple hydrophobic regions (38.5%). High-scoring hydrophobic domains were predicted at aa positions 21-43, 54-71, 83-91, 107-121, 163-174, 187-200, and 242-261 (Figure 3).

PredictProtein results calculated the proportion of α-helixes, strands, and random coils in TsAQP as 39.58, 10.42, and 50%, respectively.

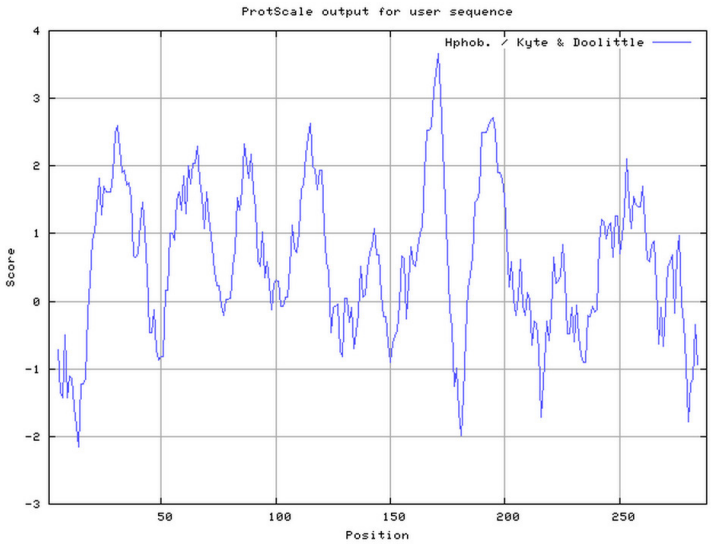

Figure 3. Prediction of hydrophobic domains of TsAQP.

Homology modeling of TsAQP

The tertiary structure of a protein always reveals several specific biological functions. Prediction of 3D protein structure, based on homologous models using a protein structure modeling strategy, is currently a popular method and has the benefits of being easier, faster, and more efficient than traditional physical and biochemical methods, such as X-ray crystallography and nuclear magnetic resonance (Biasini et al., 2014).

In the current study, TsAQP was found to consist of 6 membrane-spanning domains connected by 5 loops (Figure 4), which corresponded to the typical structure of aquaporin (AQP1, PfAQP). Analysis of the quality of the TsAQP model using Verify 3D online software showed that 87.92% of the residues had an averaged 3D/1D score ≥ 0.2, indicating the favored 3D structure of the protein was constructed.

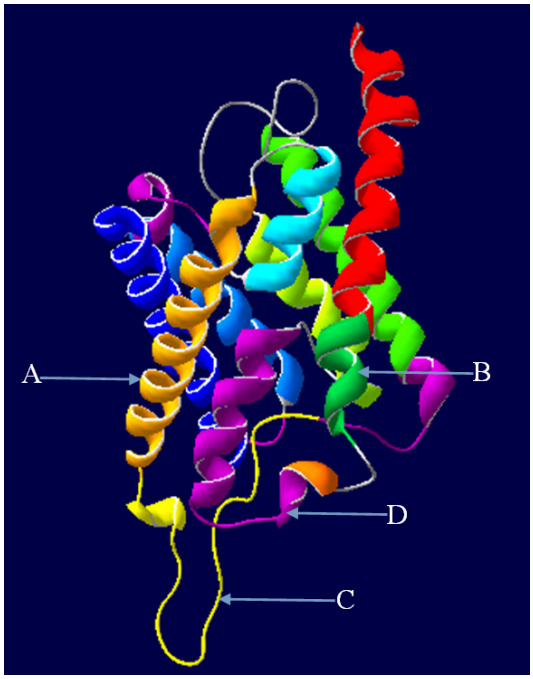

Figure 4. Tertiary structure prediction of TsAQP protein. Ribbon diagram of the α-helix (**A**), β-fold (**B**), random coil (**C**), partial potential epitopes (**D**; purple).

Potential B or T cell epitopes

B or T cell epitopes are usually discerned by the host immune system, and have the potential to induce protective immunity. Integrating consideration of the characters of variability, fragment mobility, hydrophilicity, surface accessibility and secondary structures, a total of 6 potential B cell epitopes of TsAQP were predicted (Table 2).

Based on integration of results from SYFPEITHI and Propred I software, 4 dominant T cell epitopes for alleles HLA-A*0201 were predicted; these were located at aa positions 22-30, 110-118, 167-175, and 191-199 (Figure 5).

Table 2. Potential B cell epitopes position and sequences.

Numbers	Position	Potential B cell epitopes sequences
1	11-16	RLRTDR
2	46-51	SRQQAG
3	71-78	SGGVSGGH
4	147-157	YPSDHISTAGS
5	213-232	PARDFSPRLLTSLTGWGSET
6	276-283	SSQISRNE

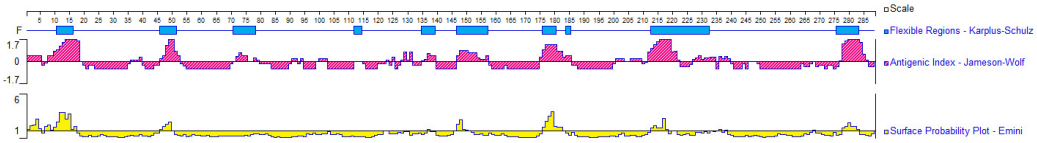

Figure 5. Analysis of TsAQP. Antigenic index (red), flexible regions (F; blue), and surface probability (yellow).

DISCUSSION

AQPs form homotetramers embedded in the lipid bilayer, and each monomer has an independent functional pore facilitating passive transport of water and/or other small neutral molecules (Gomes et al., 2009). These proteins are found in virtually all organisms, from vertebrates to invertebrates and even viruses, and are involved in various life processes (Hachez and Chaumont, 2010). However, due to a lack of genomic sequencing data, it was not previously known whether the conservative protein exists in *T. spiralis*. In the present study, the potential AQP was cloned in *T. spiralis*, and its physiological and biochemical characters and epitopes were predicted.

Four short single-read transcript sequences of the AQP gene were detected in *T. spiralis* EST database, but *TsAQP* was not annotated in the genomic sequencing data. We speculated that TsAQP lacked a classical signal sequence and was chimeric with putative kinesin motor protein, which lead to an erring annotation. Here, the cloned cDNA fragment of *TsAQP* was 1134 bp, and encoded a 288 aa protein with 45% identity to the AQP9 protein in humans. This suggests that TsAQP is an aquaglyceroporin, and may function to transport other molecules in addition to water.

There are two constriction points in AQPs. The narrowest pore mouth is termed the aromatic arginine (ar/R) constriction and is nearly the size of a single water or glycerol molecule (Campbell et al., 2008). In *Fasciola gigantica* AQPs, the Cys204 at the pore mouth is replaced by Tyr, which leads to a loss of water permeability (Geadkaew et al., 2011). The second constriction is located in the center of the channel formed by the NPA motifs, and has the ability to transport water molecules, but prevent leaking of proteins (Campbell et al., 2008). In *P. falciparum,* the AQP protein is comprised of an NLA/NPS motif, and mutation of this motif to NPS/NLA resulted in complete ablation of protein function (Hedfalk et al., 2008). Phosphorylation, pH and Ca^{2+} levels can also directly influence the permeability of many AQP proteins (Campbell et al., 2008).

Bioinformatics is becoming increasingly significant in the prediction of protein structure, function, and some other biological characteristics, and has the benefits of being effective and

low-cost compared to traditional methods (Yang and Yu, 2009; Zhang et al., 2014). In the current study, prediction of functional domains revealed that TsAQP contained a MIP family signature at position 78-86 and a cysteine residue at position 207. Studies of AQPs from humans and *S. mansoni* have demonstrated that MIP and cysteine residue are responsible for binding to inhibitors (for example, mercuric chloride), which may be potential drug targets (Kuwahara et al., 1997; Faghiri and Skelly, 2009). Phosphorylation of AQPs by protein kinase (PK), which can greatly affect their transport properties and subcellular localization, has been detected in both plants and animals (Hachez and Chaumont, 2010). Here, 3 PK C phosphorylation sites were identified in TsAQP at the position of 14-16, 218-220, and 234-236.

In analysis of protein secondary structures, α-helixes and β-sheets are considered to play roles in cell stability, mechanical signaling, and tissue constitution, whereas random coils are exposed to the protein surface (Perticaroli et al., 2014). In the present study, TsAQP protein was shown to consist mainly of α-helixes and random coils (approximately 90%), and to have good stability. To visualize secondary structures for analysis, the 3D model of TsAQP was also constructed by homologous modeling. This showed that the protein was consisted by 6 main transmembrane helices. The two NPA motifs located in the central channel as pseu-half transmembrane domains. The putative ar/R constriction at the narrowest pore mouth, and NPA motifs, were also predicted in TsAQP.

Linear T and B cell epitopes play important roles in inducing an acquired immune response, which holds undoubted potential for vaccine design (Yang and Yu, 2009; Flower, 2013). The ability to predict immunogenicity of unknown proteins, and identify antigen epitopes suitable for vaccine development, using bioinformatic techniques is both extremely useful and economical (Yang and Yu, 2009; Song and He, 2012). Combined with hydrophilicity/hydrophobicity, antigen index and surface accessibility analyses, 6 potential B cell epitopes, and 4 potential T cell epitopes were predicted in TsAQP.

In conclusion, a putative *TsAQP* was cloned in *T. spiralis*, and the encoded protein was predicted to be a multi-functional water channel protein, indicating an important role for the protein in the life cycle of this parasite. Further investigations of the potential for use of this protein as an antigenic target in vaccine and drug development, will extend our understanding of trichinellosis control. To this end, studies in suitable experimental animal models of *T. spiralis* infection are warranted.

Conflicts of interest

The authors declare no conflict of interests.

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