IDENTIFICATION OF EARLY DIAGNOSTIC ANTIGENS FROM *SPIROMETRA ERINACEIEUROPAEI* SPARGANUM SOLUBLE PROTEINS USING IMMUNOPROTEOMICS

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Abstract. In order to identify early specific diagnostic antigens of *Spirometra erinaceieuropaei* (syn. *S. erinacei* or *S. mansoni*) sparganum, soluble proteins were analyzed by two-dimensional electrophoresis (2-DE) and western blotting probed with immune sera from infected mice at 14 days post-infection. From a total of approximately 462 proteins spots mainly distributed in pI range of 5-6.6 and with molecular mass of 25-48 kDa, 6 immuno-reactive protein spots with molecular mass of 31.8-38.3 kDa were characterized by MALDI-TOF/TOF-MS. Three proteins were identified as *S. erinaceieuropaei* cysteine protease, *Toxoplasma gondii* hypothetical protein and *Pecten* spp actin, while the remaining were unidentified. The cysteine protease from *S. erinaceieuropaei* soluble proteins recognized by early infection sera might be developed as diagnostic reagent for early detection of sparganosis.

Keywords: *Spirometra erinaceieuropaei,* early diagnostic antigen, immunoproteomics, plerocercoid (sparganum), soluble proteins

INTRODUCTION

Sparganosis is a serious parasitic zoonosis caused mainly by plerocercoid (sparganum) of *Spirometra erinaceieuropaei* (syn. *S. erinacei* or *S. mansoni*). Human infections are acquired by drinking water contaminated with cyclops harboring procercoids, ingesting raw flesh of frogs and snakes infected with plerocercoids, or placing frog or snake flesh on open wound for treatment of skin ulcers or eye inflammation (Fukushima and Yamane, 1999; Magnino *et al*, 2009; Roberts *et al*, 2009). Sparganosis poses a serious threat to human health. The plerocercoids usually lodge in the subcutaneous tissues and muscles, but sometimes invade the central nervous system causing seizures, headache, epilepsy, paralysis, and several neurological disorders with progressive mental deterioration (Shirakawa *et al*, 2010). The disease is sporadically distributed worldwide, but most cases occur in Southeast Asia, Japan, Korea and Thailand (Anantaphruti *et al*, 2011). In the People's Republic of China, sparganosis

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is an important food-borne parasitic zoonosis, with more than 1,000 human cases reported in 27 out of 34 provinces, autonomous regions, or municipal districts (Cui *et al*, 2011b).

Enzyme-linked immunosorbent assay (ELISA) is a rapid and most commonly used serological method for the detection of sparganum infection in humans (Yeo *et al*, 1994). Using soluble (crude) antigens of plerocercoids, ELISA has high sensitivity, but the main disadvantage is false negative results during the early stage of infection and cross-reaction with sera from patients with other parasitic diseases, *viz* cysticercosis, paragonimiosis and clonorchiosis (Nishiyama *et al*, 1994). Results of SDS-PAGE analysis demonstrate that the protein components of sparganum crude antigens contained 19 protein bands (Li *et al*, 2010). Detection of specific IgG4 antibodies against the 31 and 36 kDa proteins in sparganum crude antigens appears to be reliable, but these molecules cross-reacted with sera of patients with cysticercosis, and show blurred, irregular and broad banding patterns (Chung *et al*, 2000; Rahman *et al*, 2011).

Immunoproteomics is an approach to identify specific antigenic proteins with high resolution for a wide range of proteins expressed by different organisms (Krah and Jungblut, 2004). The combination of two-dimensional electrophoresis (2-DE) with western blotting help support discovery of numerous novel antigens and the screening of novel serological diagnostic markers and vaccine candidates. The technique has proven to be highly successful in characterizing proteins expressed by such parasitic organisms as *Toxoplasma gondii*, *Neospora caninum*, *Eimeria tenella*, *Schistosoma japonicum*, and *Trichinella spiralis* (Zhong *et al*, 2010; Wang *et al*, 2012; Wang *et al*, 2013a). In the analysis of *S. erinaceieuropaei*, previous studies have addressed the differential protein expression in different developmental stages (Kim *et al*, 2009). However, to the best of our knowledge, crude antigens of *S. erinaceieuropaei* plerocercoids have not been analyzed by immunoproteomics and identified by tandem mass spectrometry.

The aim of the present study was to identify antigens in soluble proteins of *S. erinaceieuropaei* sparganum recognized by early infection sera. Crude proteins from plerocercoids were analyzed by 2-DE and western blotting using sera from mice infected with *S. erinaceieuropaei* spargana at 14 days post-infection (dpi). Then the immuno-reactive protein spots were characterized by matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF)/TOF-MS analyses in combination with bioinformatics analysis.

MATERIALS AND METHODS

Parasite and experimental animals

Plerocercoids (spargana) of *S. erinaceieuropaei* were collected from subcutaneous tissues and muscles of the naturally infected wild frogs (*Rana limnocharis* and *R. nigromaculata*), which were captured from the suburbs of Zhengzhou City, Henan Province, China. The collected worms were washed five times in physiological saline before being orally infected into 50 6-weeks old female specific pathogen free (SPF) BALB/c mice (2 plerocercoids/mouse), which were obtained from the Experimental Animal Center of Henan Province, Zhengzhou, China. All infected mice were sacrificed at 28 dpi by deep ether anesthesia, carcasses skinned and spargana were recovered, washed thoroughly in sterile normal saline solution and deionized water, and preserved in liquid nitrogen. All procedures of animal

experiment were approved by the Life Science Ethics Committee of Zhengzhou University.

Collection of sera and detection of anti-sparganum IgG antibodies

Serum samples from the infected mice were collected as described previously (Cui *et al*, 2011a). About 100 µl of tail vein blood were, on alternate days, collected from each mouse before infection and during 6-28 dpi. Anti-sparganum IgG antibodies in sera of infected mice at 6-28 dpi were assayed by ELISA as previously described (Cui *et al*, 2011a). In brief, 96-well ELISA plates (Corning, Bloomington, MN) were coated with 2.5 µg of ES antigens in 100 µl of bicarbonate buffer (pH 9.6) overnight at 4ºC, washed with 0.1% Tween-20 in PBS (PBS-T), and incubated in 3% skimmed milk in PBS-T for 2 hours at 37ºC. After washing with PBS-T, the following reagents were sequentially added and incubated for 1 hour at 37ºC: (1) mouse sera diluted 1:100 in PBS-T, and (2) HRP-conjugated anti-mouse IgG (Sigma, St Louis, MO) diluted 1:5,000. Then color was developed by incubating with a 50-µl aliquot of ortho-phenylene diamine (5 mg/10 ml of citrate–phosphate buffer) and 5 µl of 30% H_2O_2 for 30 minutes. The reaction was stopped by adding 50 µl of 2 M H_2SO_4. Optical density (OD) at 492 nm was measured with a microplate reader (TECAN, Grödig, Austria). All samples were analyzed in duplicate. Test sera/negative sera OD values < 2.1 were regarded as negative and those ≥ 2.1 as positive. The cut-off value of ES antigen ELISA for detection of experimentally infected mice was 0.34.

2-DE

Soluble proteins were prepared from *S. erinaceieuropaei* spargana as previously described with some modifications (Park *et al*, 2005). In brief, worms in liquid nitrogen were ground using a mortar and pestle for 30 minutes and dissolved in the lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1- propane-sulfonate (CHAPS), 65 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.2% immobilized pH gradient (IPG) buffer. Then, samples were ground on ice for 1 hour and sonicated for 30 minutes (JY92-II sonifier cell disrupter, China). Supernatants were collected and pooled after centrifugation at 15,000*g* for 60 minutes at 4ºC, dialyzed and then lyophilized (Heto Mxi-Dry-Lyo, Allerod, Denmark). Protein concentration was 8.59 mg/ml of deionized water (Bradford, 1976).

Protein sample was precipitated using 10% trichloroacetic acid (TCA in acetone for 3 hours at -20ºC, centrifuged at 15,000*g* for 15 minutes at 4ºC, washed with cold acetone and air-dried (Ni *et al*, 2010). Then, 700 µg of the protein pellet was suspended in 200 µl of rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol (DTT), 0.2% IPG buffer (pH 4-7) and 0.001% bromophenol blue] and centrifuged at 15,000*g* for 10 minutes at room temperature to remove the insoluble materials (Nareaho *et al*, 2006). The soluble protein in the supernatant was spotted onto 11-cm pH 4-7 immobilized pH gradient strip (Bio-Rad, Hercules, CA), incubated overnight in the supernatant and separated by isoelectric focusing (IEF) using a Protean IEF Cell (Bio-Rad, Hercules, CA) at 20ºC as follows: S1: 50 V for 12 hours; S2: 250 V for 30 minutes; S3: 1,000 V for 30 minutes; S4: 8,000 V for 4 hours; and S5: 8,000 V for 40,000 vh (using a limit of 50 µA/strip). Then, the IPG strip was sequentially equilibrated, first in equilibration buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS and 20%

Table 1
Identification using MALDI-TOF/TOF-MS of *Spirometra erinaceieuropaei* sparganum soluble proteins recognized by murine infection sera at 14 days post-infection.

Spot no.	Protein	GenBank accession No.	Protein score	Theoretical Mr/pI	Observed Mr/pI	Matched peptides	Coverage (%)
1	Cysteine proteinase (*S. erinaceieuropaei*)	gi|15146346	116	20.4/4.66	31.8/4.875	3	25
2	Not identified	-	-	-	31.8/4.954	-	-
3	Hypothetical protein TGME49_013480 (*Toxoplasma gondii*)	gi|237843579	80	39.8/5.04	31.8/5.046	6	32
4	Not identified	-	-	-	5.167/31.8	-	-
5	Actin (*Pecten* spp)	gi|224305	105	41.5/5.3	/32.5/5.333	8	36
6	Not identified	-	-	-	38.3/4.565	-	-

Mr, molecular mass (kDa); pI, isoelectric value.

glycerol) containing 2% DTT, followed by equilibration buffer containing 2.5% iodoacetamide. The second dimension was performed in 12% SDS-PAGE (Mini Protean cell; Bio-Rad, Hercules, CA) at 16ºC for 30 minutes at 10 mA and then at 23 mA until the dye front reached the bottom of the gel. The gels was either stained with Coomassie blue R-250 for proteomic analysis or used for immuno-blotting. All experiments were conducted in triplicate.

Western blotting analysis

Proteins from 2-DE gel was transferred onto polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer cell (Bio-Rad, Hercules, CA) for 1 hour at 20 V (Wang *et al*, 2013b). The membrane was incubated with 5% skimmed milk in tris buffered saline with Tween-20 (TBST, pH8.0) at 37ºC for 1 hour, washed with TBST, and incubated with serum of infected mice (1:100 dilution) overnight at 4ºC. After washing with TBST, membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, St Louis, MO) (1: 5,000 dilution) at 37ºC for 1 hour, washed and treated with an enhanced chemiluminescence (ECL) kit (CWBIO, Beijing, China). Sera collected from mice before infection were used as negative controls. Images of immunoblots were recorded using ImageScanner (GE Healthcare, Fairfield, CT) and aligned with equivalent proteins stained 2-DE gels using Image Master 2D Platinum 6.0 (GE Healthcare, Fairfield, CT). All experiments were conducted in triplicate.

Protein identification by MALDI-TOF/TOF-MS

The 2-DE gel electrophoresis protein spots recognized by infection sera at 14 dpi were excised manually from the Coomassie blue-stained gels, destained for 20 minutes in 200 mM NH_4HCO_3 containing 30% acetonitrile (ACN), lyophilized and digested overnight at 37ºC with 12.5 ng/ml trypsin in 25 mM NH_4HCO_3 (Li *et al*, 1997). The peptides were extracted three times with 60% ANC containing 0.1% trifluoroacetic acid (TFA). The extracts were pooled and dried completely by centrifugal lyophilization (Heto Mxi-Dry-Lyo, Allerod, Denmark).

The resulting dried peptides were analyzed by MALDI-TOF/TOF-MS as described previously (Robinson *et al*, 2007). In short, samples were spotted onto stainless steel sample target plates and mixed (1:1 ratio) with a matrix consisting of a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50% ACN containing 1% TFA. Peptide mass spectra were obtained using an Applied Biosystem Sciex 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). Data were acquired using CalMix5 standard (ABI4700 Calibration Mixture) to calibrate the instrument. MS spectra were recorded in reflector mode in a mass range from 800 to 4000 with a focus mass of 2000. For MS/MS spectra, up to 10 of the most abundant precursor ions per sample were selected for MS/MS acquisition, excluding the trypsin autolysis peaks and the matrix ion signals. In MS/MS positive ion mode, for each main MS spectrum, 50 subspectra with 50 shots per subspectrum were accumulated using a random search pattern. Collision energy was 2 kV, collision gas was air, and default calibration was using Glu1-Fibrino-peptide B ([M+H] + 1 = 570.6696) spotted onto Cal 7 positions of the MALDI target. Combined peptide mass fingerprinting (PMF) and MS/MS queries were performed using MASCOT search engine 2.2 (Matrix Science, London, UK) and submitted to MASCOT Sequence Query server (http://www.matrixscience.com) for identification against nonredundant NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) with the following parameter settings: 100 ppm mass accuracy, trypsin cleavage (one missed cleavage allowed), carbamidomethylation set as fixed modification, oxidation of methionine allowed as variable modification, and MS/MS fragment tolerance set at 0.4 Da. Criterion for successful identification of a protein is a protein score ≥ 66.

RESULTS

2-DE analysis of sparganum soluble proteins

The soluble proteins of *S. erinacei-europaei* spargana were separated on a 2-DE gel covering a pH 4-7, and the protein spots were visualized following Coomassie R-250 staining. A total of approximately 462 spots were detected with molecular weight from 6 to 97 kDa and pI values varying from 4 to 7 (Fig 1A). The majority of protein spots were distributed in the range of molecular size of 25-48 kDa and pI values 5-6.6. The 2-DE protein patterns were highly reproducible (data not shown).

Western blotting analysis of sparganum soluble proteins separated by 2-DE

The specific anti-sparganum antibodies were first detected at 8 dpi and the antibody positive rate was 100% at 14 dpi and persisted until the end of this experiment (28 dpi) (data not shown). Infection sera at 14 dpi were used to detect sparganum antigenic proteins. There were 6 protein spots recognized by 14 dpi sera (Fig 1B). Alignment of these 6 immunopositive protein spots with those stained by Coomassie R-250 showed them to be of 31.8-38.3 kDa in size and were selected to be analyzed by MS. Control negative sera did not show detectable immunoreactivity with any of the 6 protein spots (Fig 1C).

Identification of immuno-reactive proteins by MALDI-TOF/TOF-MS

MALDI-TOF/TOF-MS revealed that protein spots 1, 3 and 5 was identified as being indicative of *S. erinaceieuropaei* cysteine proteinase, *Toxoplasma gondii* hypothetical protein and *Pecten* spp actin

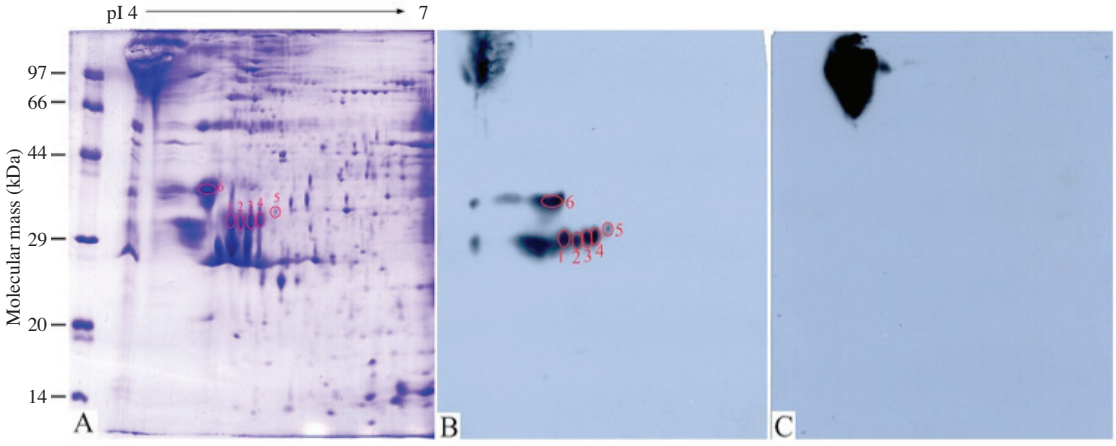

Fig 1–Two-dimensional electrophoresis (2-DE) and western blotting of *Spirometra erinaceieuropaei* sparganum soluble proteins. (A) 2-DE gel of sparganum soluble proteins separated in the first dimension by isoelectro-focusing electrophoresis in the pH range 4-7 and then in the second dimension by 12% polyacrylamide gel-electrophoresis. The gel was stained with Coomassie blue R-250. Molecular weight standard is on the left, and pI values are indicated on top. (B) Western blot of the soluble proteins immuno-reactive to murine infection sera at 14 days post-infection; immuno-reactive protein spots were detected using enhanced chemiluminescence. (C) Western blot using murine sera before infection. Protein spots selected for MALDI-TOF/TOF-MS analysis are numbered.

(Table 1). The other 3 protein spots did not match with any proteins in the NCBI database.

DISCUSSION

Plerocercoids can survive for decades in host tissues and stimulate host immune system to develop specific antibodies (Roberts *et al*, 2009). Therefore, anti-sparganum antibodies in sera have been considered useful biomarkers for the diagnosis of sparganosis. Hence, murine sera during the early stages of sparganum infection of *S. erinaceieuropaei* collected from infected frogs were used to identify soluble immunogenic proteins employing a combination of 2-DE, western blotting and MALDI-TOF/TOF-MS.

Out of six immuno-reactive *S. erinaceieuropaei* proteins spots, only one was identified *S. erinaceieuropaei* cysteine proteinase. Cysteine protease has previously been detected in *S. erinaceieuropaei* (Song *et al*, 1992; Chung and Yang, 2008). Cysteine protease produced extracellularly is the key factor in parasite pathogenicity, either by inducing tissue damage and facilitating invasion and/or by empowering parasites to generate metabolites from host proteins (Tort *et al*, 1999). Plerocercoids of *S. erinaceieuropaei* are also known to secrete a large amount of cysteine proteases, which hydrolyze collagen, hemoglobin IgG *in vitro*, and thus may be involved in digestion of host tissues (Song and Chappell, 1993; Kong *et al*, 1994; Liu *et al*, 2013). Plerocercoid cysteine proteases might come from the excretory and secretory products and cuticles (Hu *et al*, 2013), and thus are directly exposed to the host immune system.

There were 3 immuno-reactive proteins that were not identified from NCBI database. So far, there is very limited information on genome sequence or expressed sequences of *S. erinaceieuropaei* and genes coding for cysteine protease, superoxide dismutase, glyceraldehyde-3-phosphate dehydrogenase, and α-tubulin have been submitted to GenBank database (Kim *et al*, 2009). Determination of the amino acid sequences of the 3 putative *S. erinaceieuropaei* antigens in the future will be required to definitively identify them and to clone their genes.

In the present study, two spots of *S. erinaceieuropaei* proteins were recognized by immune sera from sparganum-infected mice, and identified as *T. gondii* hypothetical protein and *Pecten* spp actin, respectively, suggesting that *S. erinaceieuropaei* might have similar gene sequences and common antigens with *T. gondii* and *Pecten* spp. Thus, the immune sera from the sparganum-infected mice have cross-immune reaction with *T. gondii* hypothetical protein and *Pecten* spp actin.

In conclusion, the cysteine protease of *S. erinaceieuropaei* was recognized by early infection murine immune sera and has the potential to be developed as a diagnostic tool for early detection of sparganosis in humans.

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REFERENCES

Anantaphruti MT, Nawa Y, Vanvanitchai Y. Human sparganosis in Thailand: an overview. *Acta Trop* 2011; 118: 171-6.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.

Chung YB, Kong Y, Yang HJ, *et al.* IgG antibody responses in early experimental sparganosis and IgG subclass responses in human sparganosis. *Korean J Parasitol* 2000; 38: 145-50.

Chung YB, Yang HJ. Partial purification and characterization of a cysteine protease inhibitor from the plerocercoid of *Spirometra erinacei*. *Korean J Parasitol* 2008; 46: 183-6.

Cui J, Li N, Wang ZQ, *et al.* Serodiagnosis of experimental sparganum infections of mice and human sparganosis by ELISA using ES antigens of *Spirometra mansoni* spargana. *Parasitol Res* 2011a; 108: 1551-6.

Cui J, Lin XM, Zhang HW, *et al.* Sparganosis, Henan Province, central China. *Emerg Infect Dis* 2011b; 17:146-7.

Fukushima T, Yamane Y. How does the sparganosis occur? *Parasitol Today* 1999; 15: 124.

Hu DD, Cui J, Wang L, *et al.* Immunoproteomic analysis of the excretory-secretory proteins from *Spirometra mansoni* sparganum. *Iranian J Parasitol* 2013; 8: 408-16.

Kim JH, Kim YJ, Sohn WM, *et al.* Differential protein expression in *Spirometra erinacei* according to its development in its final host. *Parasitol Res* 2009; 105: 1549-56.

Kong Y, Chung YB, Cho SY, *et al.* Cleavage of immunoglobulin G by excretory-secretory cathepsin S-like protease of *Spirometra mansoni* plerocercoid. *Parasitology* 1994; 109: 611-21.

Krah A, Jungblut PR. Immunoproteomics. *Methods Mol Med* 2004; 94: 19-32.

Li G, Waltham M, Anderson NL, Unsworth E, TrestonA, Weinstein JN. Rapid mass spectrometric identification of proteins from two-dimensional polyacrylamide gels after in gel proteolytic digestion. *Electrophoresis* 1997; 18: 391-402.

Li N, Wang ZQ, Cui J, *et al.* [Specific diagnostic antigens in excretory-secretory products of sparganum mansoni]. *Chin J Pathog Biol* 2010; 5: 680-2.

Liu LN, Cui J, Zhang X, *et al.* Analysis of structures, functions, and epitopes of cysteine protease from *Spirometra erinaceieuropaei* spargana. *Biomed Res Int* 2013; 2013: 198250.

Magnino S, Colin P, Dei-Cas E, *et al.* Biological risks associated with consumption of reptile products. *Int J Food Microbiol* 2009; 134: 163-75.

Nareaho A, Ravanko K, Holtta E, *et al.* Comparative analysis of *Trichinella spiralis* and *Trichinella nativa* proteins by two-dimensional gel electrophoresis. *Parasitol Res* 2006; 98: 349-54.

Ni XD, Wang N, Liu YJ, *et al.* Immunoproteomics of extracellular proteins of the *Aeromonas hydrophila* China vaccine strain J-1 reveal a highly immunoreactive outer membrane protein. *FEMS Immunol Med Microbiol* 2010; 58: 363-73.

Nishiyama T, Ide T, Himes SR, Jr, *et al.* Immunodiagnosis of human sparganosis mansoni by micro-chemiluminescence enzyme-linked immunosorbent assay. *Trans R Soc Trop Med Hyg* 1994; 88: 663-5.

Park YK, Park JH, Guk SM, *et al.* A new method for concentration of proteins in the calcareous corpuscles separated from the spargana of *Spirometra erinacei*. *Korean J Parasitol* 2005; 43: 119-22.

Rahman M, Lee EG, Bae YA. Two-dimensional immunoblot analysis of antigenic proteins of *Spirometra* plerocercoid recognized by human patient sera. *Parasitol Int* 2011; 60: 139-43.

Roberts LS, Janovy J, Nadler S, Schmidt GD, Larry S. Foundations of Parasitology. 9th ed. Boston: McGraw-Hill, 2009: 670.

Robinson MW, Greig R, Beattie KA, *et al.* Comparative analysis of the excretory-secretory proteome of the muscle larva of *Trichinella pseudospiralis* and *Trichinella spiralis*. *Int J Parasitol* 2007; 37: 139-48.

Shirakawa K, Yamasaki H, Ito A, *et al.* Cerebral sparganosis: the wandering lesion. *Neurology* 2010; 74: 180.

Song CY, Chappell CL. Purification and partial characterization of cysteine proteinase from *Spirometra mansoni* plerocercoids. *J Parasitol* 1993; 79: 517-24.

Song CY, Choi DH, Kim TS, *et al.* [Isolation and partial characterization of cysteine proteinase from sparganum]. *Kisaengchunghak Chapchi* 1992; 30: 191-9.

Tort J, Brindley PJ, Knox D, *et al.* Proteinases and associated genes of parasitic helminths. *Adv Parasitol* 1999; 43: 161-266.

Wang L, Wang ZQ, Cui J. Proteomic analysis of the changed proteins of *Trichinella spiralis* infective larvae after co-culture in vitro with intestinal epithelial cells. *Vet Parasitol* 2013a; 194: 160-3.

Wang L, Wang ZQ, Hu DD, Cui J. Proteomic analysis of *Trichinella spiralis* muscle larval excretory-secretory proteins recognized by early infection sera. *BioMed Res Int* 2013b; 2013: 139745.

Wang SW, Wang ZQ, Cui J. Protein change of intestinal epithelial cells induced in vitro by *Trichinella spiralis* infective larvae. *Parasitol Res* 2011; 108: 593-9.

Wang ZQ, Wang L, Cui J. Proteomic analysis of *Trichinella spiralis* proteins in intestinal epithelial cells after culture with their larvae by shotgun LC-MS/MS approach. *J Proteomics* 2012; 75: 2375-83.

Yeo IS, Yong TS, Im K. Serodiagnosis of human sparganosis by a monoclonal antibody-based competition ELISA. *Yonsei Med J* 1994; 35: 43-8.

Zhong ZR, Zhou HB, Li XY, *et al.* Serological proteome-oriented screening and application of antigens for the diagnosis of *Schistosomiasis japonica*. *Acta Trop* 2010; 116: 1-8.