PRODUCTION AND EVALUATION OF RECOMBINANT *TRICHINELLA PAPUAE* TISSUE-TYPE PLASMINOGEN ACTIVATOR FOR IMMUNODIAGNOSIS OF HUMAN TRICHINELLOSIS PAPUAE

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Abstract. Trichinellosis is a serious worldwide disease arising from infection with zoonotic *Trichinella* nematodes. Immunodiagnosis is a crucial sensitive and relatively painless non-invasive method that combined with clinical symptoms and patient history allows species-specific diagnosis of trichinellosis. Currently, immunodiagnostic tests have been developed using different types of antigens, such as crude worm, excretory/secretory, tyvelose-bearing and, more recently, recombinant antigens, most of which are focused on trichinellosis spiralis (caused by *Trichinella* sp belonging to encapsulated clade). Here, we established for the first time a recombinant protein-based indirect IgG-ELISA for detection of human trichinellosis papuae (resulting from infection of *Trichinella* sp belonging to the non-encapsulated clade) using a 40 kDa recombinant tissue-type plasminogen activator (rTpaPLAT). Sensitivity, specificity, positive predictive value and negative predictive value of rTpaPLAT-based ELISA was 100, 86, 44, and 100%, respectively against a battery of 200 human sera from 32 different parasite infections and 26 from healthy controls. Noteworthy, there were no false positive results with trichinellosis spiralis sera. This technique should be of use in immunodiagnosis, seroprevalence and surveillance of human trichinellosis papuae.

Keywords: *Trichinella papuae,* human trichinellosis papuae, indirect IgG-ELISA, recombinant tissue-type plasminogen activator

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INTRODUCTION

Trichinellosis is a food-borne parasitic zoonosis caused by nematode worms of the genus *Trichinella* (Gottstein *et al*, 2009). The disease sporadically occurs in humans, domestic animals and wildlife worldwide. Genus *Trichinella* can be classified into two main clades, namely, encapsulated and non-encapsulated, the former consisting of *T. britovi, T. chanchalensis, T. murrelli, T. nativa, T. nelsoni, T. patagoniensis, T. spiralis,* and three unique genotypes, *Trichinella* T6, T8 and T9; and the latter consists of *T. papuae, T. pseudospiralis* and *T. zimbabwensis* (Gottstein *et al*, 2009; Sharma *et al*, 2020; Zarlenga *et al*, 2020).

Trichinellosis can cause significant morbidity and mortality in humans (Pozio, 2007). Domestic pig and wild boar served as main source of infection and human can acquire the disease via consumption of raw and/or improper cooked meat products. For life cycle, *Trichinella* muscle larvae were released into the stomach by digestion of gastric juice. Then, larvae penetrate the intestinal mucosa and reach to the adult stage. Female worm releases newborn larvae in the lymphatic vessels after mating and the newborn larvae reach the striated muscle and actively penetrate in the muscle cell (Gottstein *et al*, 2009). Although biopsy is the definitive diagnosis of trichinellosis, it is an invasive and often painful procedure (Dupouy-Camet *et al*, 2002). Immunodiagnosis provides a highly sensitivity and relatively less invasive technique (Bruschi and Murrell, 2002). Detection using an indirect ELISA with specific antibody against excretory/secretory products of *T. spiralis* muscle stage L1 larvae (Ts-ESL1) is recommended for human trichinellosis diagnosis by the International Commission on Trichinellosis (ICT) due to its high specificity (Gamble *et al*, 2004). Several studies have developed recombinant protein-based immunodiagnosis tests for human trichinellosis, which are undergoing validation trials (Nagano *et al*, 2008; Sun *et al*, 2018; Zhai *et al*, 2021).

Up to now, the majority of immunodiagnosis tests are focused on *Trichinella* encapsulated clade, in particular *T. spiralis* (Cui *et al*, 2015). Given that multiple outbreaks of trichinellosis papuae, due to infection with this non-encapsulated *Trichinella* sp, have occurred in Cambodia, Malaysia and Thailand, resulting in 33 infected, 8 died; 1 infected; and 28 infected, respectively (Caron *et al*, 2020), there is an urgent need to have an immunodiagnostic test specifically for human trichinellosis papuae. Recently, we have identified a 31 kDa protein from *T. papuae* muscle L1 larva crude worm antigen that is specific in immunodetection of human trichinellosis papuae, with no cross-reactivity with human trichinellosis spiralis (Sahaisook *et al*, 2022). Mass

spectrometry analysis indicates the identity of *T. papuae* 31 kDa antigen as putatively belonging to five *T. papuae* proteins, one of which is tissue-type plasminogen activator (PLAT) (40,364 Da).

PLAT is a serine protease involved in the dissolution of blood clots (Jilani and Siddiqui, 2022). In parasites, *Acantamoeba castellanii* PLAT degrades host fibrinogen and may contribute to the pathogenesis of acanthamoeba keratitis (Mitra *et al*, 1995). PLATs have been found in a variety of *Trichinella* sp, both encapsulated and non-encapsulated (Somboonpatarakul *et al*, 2018). However, there is no information on the application of *T. papuae* PLAT (or any other *T. papuae* antigens) for immunodiagnosis of human trichinellosis papuae.

Here, a recombinant tissue-type plasminogen activator (rTpaPLAT) was produced and applied in an indirect ELISA for diagnosis of human trichinellosis papuae. This study provides the first report of the use of this recombinant protein in indirect IgG-ELISA which can discriminate human trichinellosis papuae from trichinellosis spiralis.

MATERIALS AND METHODS

Parasites and experimental animal source

T. papuae, *Schistosoma mansoni* and *S. mekongi* were obtained from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Thailand and ICR mice (*Mus musculus*) were from National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand.

T. papuae muscle L1 larva crude worm antigen (Tpa-L1-CWA) preparation

Approximately 100 *T. papuae* larvae were orally infected into each mouse (n = 10), which were maintained in the Animal Science Unit Laboratory, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. At Day 45 post-infection (dpi), infected mice were euthanized by carbon dioxide inhalation, and Tpa-L1 larvae were isolated from carcasses by digestion with 0.7% (w/v) pepsin in 0.7% (v/v) HCl as previously described (Tattiyapong *et al*, 2011) and stored at -80°C until used.

Sera collection

Experimental mice sera were prepared from blood prior to and 45 dpi with *T. papuae* infection (n = 10), 45 dpi with *T. spiralis* (n = 10), 56 dpi with *Schistosoma mansoni* (n = 8), 56 dpi with *S. mekongi* (n = 9), and non-infected control (n = 10). Human sera were collected from Karen hill tribe patients (n = 22), Kaen-Makrood Subdistrict, Ban Rai District, Uthai Thani Province, Thailand presenting one or more symptoms compatible with trichinellosis or had a history of eating wild animal raw meat (Khumjui

et al, 2008; Kusolsuk *et al*, 2010). All serum samples were positive by immunodiagnosis against a 109-kDa protein of *T. spiralis* (Ts)L1-CWA and non-encapsulated *Trichinella* larvae were found in muscle biopsy from two patients, which were subsequently identified as *T. papuae* by molecular technique. All sera samples were stored at -80°C until used. In addition, stored human sera from 178 cases of 31 other parasitic diseases diagnosed through recovery of worms or worm products, clinical features and serological tests were used, designated as heterologous sera. All human sera were leftover specimens archived at the Immunodiagnostic Unit for Helminthic Infections, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University; permission to use these specimens were granted by Ethics Committee of Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (approval no. MUTM2017-059-01). Sera ($n = 26$) from subjects with negative stool examinations both simple smear examination and formalin-ether concentration technique (Ritchie, 1948) at the time of blood collection were used as negative control.

The study in animals was approved by the Faculty of Tropical Medicine Animal Care and Use Committee (no. FTM-ACUC 030/2017).

Heterologous expression and purification of recombinant (r) TpaPLAT

RNA was isolated from *T. papuae* L1 larvae using Trizol® reagent (Invitrogen, Carlsbad, CA) and used as template for 1st-strand cDNA employing a Universal Riboclone® cDNA synthesis system (Promega Corp, Madison, WI), which was then converted to dsDNA by PCR in a reaction mixture (25 µl) containing 1 µl of *T. papuae* 1st-strand cDNA, 12.5 µl of KAPA Taq ReadyMix PCR kit (KAPABIOSYSTEM, Wilmington, MA), 1 µM of TpPLAT-specific forward primer (5' ATAGGATCCATGAAAT-TTTTGTATAGTAAATCAGTCTG 3') and reverse primer (5' ATACTG-CAGTCAACGATTGCGGCTCATG 3') (*Bam*HI and *Pst*I restriction site respectively underlined), and nuclease-free water (to make up volume). Thermocycling was conducted in T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) as follows: 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds, 53°C for 45 seconds and 72°C for 3 minutes; with a final step at 72°C for 5 minutes. TpaPLAT cDNA was purified using Farvorprep™ Gel/PCR purification kit (Ping-Tung Agricultural Biotechnology, Ping-Tung, Taiwan) and was inserted into a TA cloning vector, pGEM®-T (Promega Corporation, Madison, WI) and transfected into *Escherichia coli* JM109 strain (Promega Corporation, Madison, WI). Blue-white screening, colony PCR and DNA sequencing were carried out (Bioneer Corporation, Daejeon, South Korea). Nucleotide

sequence of TpaPLAT was deposited with GenBank, accession no. KRZ67526.1.

Recombinant pGEM®-T containing TpaPLAT gene together with pQE30 (Qiagen GmbH, Hilden, Germany) were digested with *Bam*HI and *Pst*I (Thermo Fisher Scientific Inc, Vilnius, Lithuania) at 37°C for 2 hours, and linearized pQE30 and TpaPLAT were ligated using T4 DNA ligase (Thermo Fisher Scientific Inc, Vilnius, Lithuania) prior to transfection into *E. coli* JM109 strain (Promega Corporation, Madison, WI). After confirmation of transformants using colony PCR and restriction endonuclease, *Bam*HI and *Pst*I digestion, pQE30-TpaPLAT plasmid was used to transform *E. coli* M15 strain (Qiagen, Hilden, Germany) for recombinant protein expression. Heterologous expression and purification of rTpaPLAT were performed as previously described (Grams *et al*, 2006). In brief, *E. coli* M15 transformant carrying pQE30-TpaPLAT was cultured in DifcoTM LB broth (Becton, Dickinson and Company, Sparks, MD) containing 50 μg/ml ampicillin and 25 μg/ml kanamycin at 37°C with shaking until A_{600nm} = 0.4-0.6, then incubated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Thermo Fisher Scientific Inc, Vilnius, Lithuania) for a further 3 hours. Bacterial cells were harvested by centrifuging at 4,000 g for 20 minutes at 4°C and lysed in 10 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 6 M guanidine HCl; pH 8.0), which was centrifuged at 14,000 g and supernatant incubated with 1 ml of TALON® metal affinity resin (Takara Bio USA Inc, San Jose, CA) for 1 hour at 25°C prior to loading onto a 2-ml TALON® Disposable Gravity Column (Takara Bio USA Inc, San Jose, CA). Flow-through solution was collected, then column was washed twice with 5 ml of washing solution (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, and 8 M urea; pH 6.3), followed by sequential elution with 4 and 6 ml of 100 mM and 250 mM imidazole in eluting solution (50 mM NaH_2PO_4, 300 mM NaCl, and 8 M urea; pH 6.3), respectively. Each elution fraction was analyzed by 12% SDS-PAGE, stained with Coomassie brilliant blue G-250 dye (USB Corporation, Cleveland, OH), transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) using a semi-dry transfer cell (ATTO, Tokyo, Japan) and immunoreactive protein identified with a murine primary anti-His antibody (BioLegend, San Diego, CA) and goat secondary HRP-conjugated anti-murine IgG antibody (Southern Biotech, Birmingham, AL). Elution fractions containing rTpaPLAT were pooled, dialyzed against 0.5 M phosphate-buffered saline pH 7.4 (PBS), protein concentration was measured using Coomassie® Plus protein assay reagent (Pierce Chemical, Rockford, IL) and rTpaPLAT solution stored at -80°C until used.

Immunoblot assay of sera using rTpaPLAT

Twenty μg of rTpaPLAT solution was separated in a wide single well by 12% SDS-PAGE (Mini-PROTEAN® instrument; Bio-Rad Laboratories, Hercules, CA), transferred onto nitrocellulose membrane as described above, incubated with 5% skim milk in PBS containing 0.05 % (v/v) Tween 20 (PBS-T) for 1 hour on a rocking platform and then cut into small strips (3 mm wide). Each strip was incubated with individual murine serum (1:50 dilution in PBS-T containing 0.02 % (w/v) NaN3; diluent solution) at 4°C for 16-18 hours on a rocking platform, washed five times with PBS-T, then treated with horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) (1:2,000 dilution) and reactive band visualized by incubating with 2,6 dichloroindophenol substrate (Sigma-Aldrich Co, St Louis, MO) in PBS containing 30% H_2O_2 at 25°C for 3 minutes and reaction terminated by washing with distilled water.

Indirect enzyme-linked immunosorbent assay (ELISA)

In order to determine anti-TpaPLAT IgG level in experimental murine sera, 5 μg of rTpaPLAT were added to each well of 96-well microtiter plate (Nunc, Roskilde, Denmark), incubated overnight at 4°C, then 50-μl aliquot of individual murine serum (1:200 dilution in diluent solution) was added in duplicate and plate incubated at 37°C for 1.5 hours. After washing with PBS-T, 50-μl aliquot of HRP-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) (1:2,000 dilution) was added to each well and incubated at 37°C for a further 1.5 hours. After washing with PBS-T, reactions were visualized by addition of 50-μl of 0.3 g/l 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate (KPL ABTS® Peroxidase Substrate (1-component), Seracare, Milford, MA) and incubating for 30 minutes at 25°C. Reaction was terminated by adding 50 μl of 1% SDS and A_{405nm} measured using an ELISA micro-plate reader (Sunrise; TECAN, Mannedorf, Switzerland).

For assay of human sera, the above procedures were performed except human serum (1:800 dilution) and HRP-conjugated goat anti-human IgG (Southern Biotech, Birmingham, AL) (1:5,000 dilution) were used.

Data analysis

TpaPLAT nucleotide sequence was aligned with other orthologs using multiple alignment MUSCLE software (https://www.ebi.ac.uk/Tools/msa/muscle/) and a phylogenetic tree constructed based on deduced aa sequences using a maximum likelihood analysis and 1,000 bootstrap replications employing a MEGA version X program (Kumar *et al*,

2018). Antigenic B cell epitopes were predicted using a BCPred 1.0 program (Kolaskar and Tongaonkar, 1990; Jespersen *et al*, 2017).

Cut-off values of indirect ELISA were determined by a receiver operating characteristic (ROC) curve analysis [95% confidence interval (CI)] using a PASW Statistics for Windows, Version 18.0 (SPSS Inc, Chicago, IL). Sensitivity, specificity, and positive and negative predictive values were calculated as previously described (Florkowski, 2008).

RESULTS

Properties of TpaPLAT

TpaPLAT gene is composed of 1,068 bp encoding 355 amino acids (aa) residues (Fig 1), with a calculated molecular weight and isoelectric point (pI) of 40.4 kDa and 7.66, respectively [Pepstats software (Rice *et al*, 2000)]. TpaPLAT contains a signal peptide at aa 2-39 and does not have a transmembrane region [TMHMM server, v. 2.0 (Krogh *et al*, 2001)], indicating a secretory enzyme (Fig 1). Prediction of O-glycosylation

MKFLYSKSVCTFSKSQTMIPNFKRLLITWLLFQSLLVTAKRLHLRSKLKQKMKVASKHKE 60

LEGVGLESPF**NYLEVTTECGKYPFDLSNDA**LSPKDLKTYK**VKLPWVAYVVEKMSDGKFHP**120

ICLASIIPDANDEGSTVLMTLNQCIDKEKFENGMLYIYAGNTLPVTDKDDDDLYEIE**LYT**180

IDKNAPNPLLPNVAIVQIKDGIEYDDEKKAVCLPYSNAELQDNTLCAFSALKYSAAT**KTF**240

EEYLVEAVNDPNTCQATKVTLMVEKYTELCFMDFRKDVYL**AKGGPLICKINGLWTQMGIH**300

AKGQYSFDDYTLD**VSDKVAKNPQPATYVKVSRF**RSLMQNSQNLESIGRLHMSRNR 355

Fig 1 - *Trichinella papuae* tissue type plasminogen activator (TpaPLAT) amino acid sequence

Amino acid (aa) sequence was deduced from nucleotide sequence of TpaPLAT GenBank accession no. KRZ67526.1. Sequences in bold lettering are predicted B cell epitopes using BCPred server 1.0 (Jespersen *et al*, 2017; Kolaskar and Tongaonkar, 1990).

#Potential O-glycosylation site using NetOGlyc 4.0 Server (Steentoft *et al*, 2013)

*C predicted involved in forming disulfide bond: C79-C144 or C122-C144 and C226-C288 using DiANNA 1.1 program (Ferrè and Clote, 2006)

sites indicates potential sites at S46, S56 and T260 [NetOGlyc 4.0 Server (Steentoft *et al*, 2013)] but no potential N-glycosylation site [NetNGlyc 1.0 Server (Gupta and Brunak, 2002)]. Potential disulfide bonds were predicted between C79-C144 or C122-C144 and C226-C288 [DiANNA 1.1 program (Ferrè and Clote, 2006)]. In addition, TpaPLAT contained six potential antigenic linear B-cell epitopes (20 aa in length) [BCPred 1.0 program (Kolaskar and Tongaonkar, 1990; Jespersen *et al*, 2017)], with prediction score of sequence starting at aa 71, 101, 178, 238, 281, and 314 of 0.732, 0.875, 0.997, 0.793, 0.753, and 0.968, respectively.

A phylogenetic tree was constructed based on deduced aa sequences of TpaPLAT and homologs using a maximum likelihood analysis and 1,000 bootstrap replications carried out with a MEGA version X program (Kumar *et al*, 2018). The constructed tree revealed TpaPLAT is clustered in the same clade as *T. pseudospiralis* (non-encapsulated *Trichinella* group) and *T. zimbabwensis*, and apart from encapsulated *Trichinella* group (Fig 2).

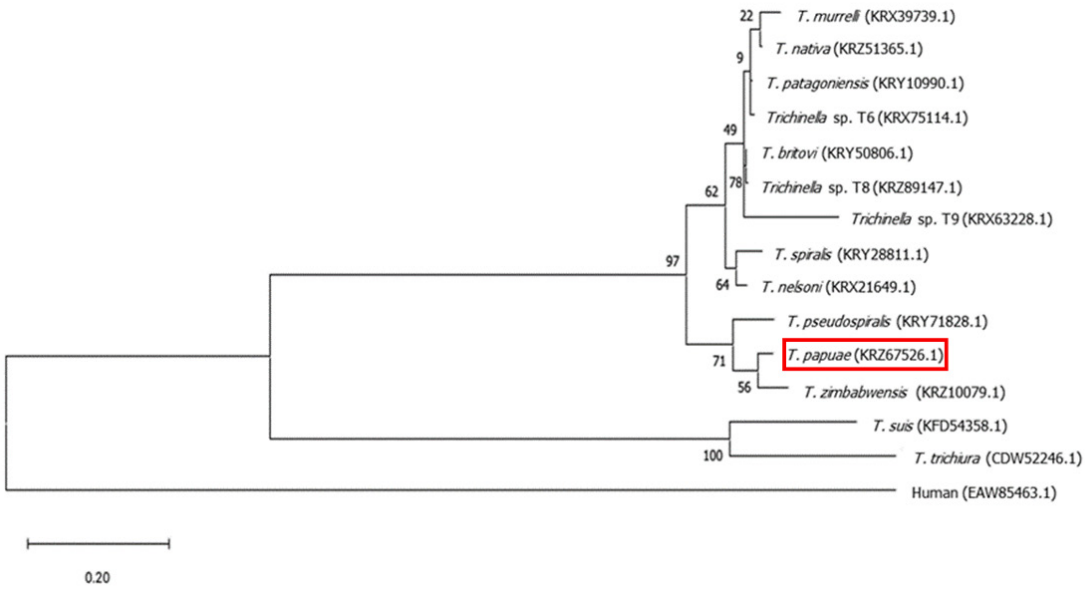

Fig 2 - Phylogenetic tree of *Trichinella* tissue type plasminogen activator orthologs

Phylogenetic tree was constructed using a maximum likelihood analysis with 1,000 bootstrap replications performed with a MEGA version X program (Kumar *et al*, 2018). The bootstrap values were indicated at node to demonstrate the relationship among homologs. *Trichinella papuae* from this study is shown in red box. GenBank accession number is indicated for each *Trichinella* sp. Scale below represents number of substitutions per site.

Heterologous expression and purification of rTpaPLAT

Heterologous expression of His-tag rTpaPLAT in *E. coli* M15 strain was induced by addition of IPTG resulting in presence of inclusion bodies, which upon solubilization in a buffer containing 6M GuHCl and 8M urea contained a protein of 40 kDa immunoreactive to anti-His antibody (Fig 3A). Purification of rTpaPLAT was achieved employing a TALON® metal affinity chromatography and elution in 100-250 mM imidazole (Fig 3B). The eluted fractions were pooled, dialyzed and used in subsequent investigations.

Application of rTpaPLAT in specific immunodetection of *T. papuae*-infected murine sera

Evaluation of the potential of rTpaPLAT to diagnose trichinellosis papuae in a mouse model was performed using sera from *S. mansoni*-infected mice on 56 dpi, *S. mekongi*-infected mice on 56 dpi, *T. papuae*-infected mice on 45 dpi, *T. spiralis*-infected mice on 45 dpi, and control uninfected mice, which were incubated with rTpaPLAT on a nitrocellulose membrane strip and IgG-rTpaPLAT complex visualized by treatment with HRP-conjugated goat anti-mouse IgG and 2,6 dichloroindophenol substrate. Bands were observed only with sera from *T. papuae*-infected mice (Fig 4). No cross-reactivity was observed with *T. spiralis*-infected mice.

Indirect ELISA detection of trichinellosis papuae using rTpaPLAT

After determination of IgG level in experimental murine sera, positive reaction was obtained only for the ten cases of *T. papuae*-infected mice (A_{405nm} >0.360 cut-off value, area under the ROC curve (AUC) = 1.000) (Fig 5). Sensitivity, specificity, and positive and negative predictive values were 100%.

For detection of human trichinellosis papuae, indirect ELISA was carried out as described above using sera from 22 cases of trichinellosis papuae, 13 of trichinellosis spiralis, 93 of other helminth infections, 27 of cestode infections, 27 of trematode infections, 18 of protozoon infections, and 26 of healthy controls. Positive results were obtained for all trichinellosis papuae (A_{405nm} >0.797 cut-off value, AUC = 1.000), none for trichinellosis spiralis, but false positives were obtained in 22% of taeniasis, 25% of enterobiasis, 25% of falciparum malaria, 25% of Malayan filariasis, 25% of paragonimiasis, 33% of amoebiasis, 33% of blastocystosis, 33% of echinococcosis, 33% of opisthorchiasis, 36% of Bancroftian filariasis, 43% of minute intestinal fluke infections, 62% of strongyloidiasis, 67% of fascioliasis, and 100% of creeping eruption cases (Table 1). The sensitivity, specificity, positive predictive value, and negative predictive value was 100, 86, 44, and 100%, respectively.

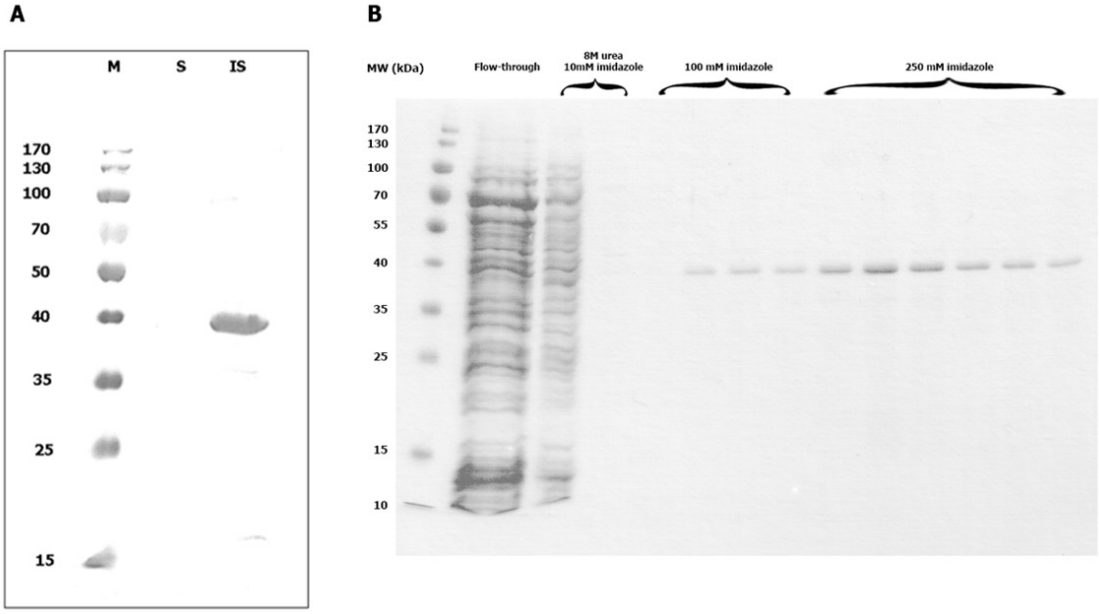

Fig 3 - Heterologous expression and purification of recombinant *Trichinella papuae* tissue type plasminogen activator (rTpaPLAT)

His-tag rTpaPLAT was heterologously expressed in *Escherichia coli* M15 strain under induction with isopropyl-β-D-thiogalactopyranoside. Cells were then lysed in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 6 M guanidine HCl; pH 8.0, centrifuged at 14,000 g and pellet dissolved in TALON® metal affinity resin solution (Takara Bio USA Inc, San Jose, CA) prior to separation on a TALON® column (Takara Bio USA Inc, San Jose, CA). Flow-through solution was collected, then column was washed twice with 10 mM imidazole in 50 mM NaH_2PO_4, 300 mM NaCl, and 8 M urea; pH 6.3, followed by sequential elution with 100 mM (100 mM imidazole) and 250 mM (250 mM imidazole) in 50 mM NaH_2PO_4, 300 mM NaCl, and 8 M urea; pH 6.3. Each elution fraction was analyzed by 12% SDS-PAGE, transferred onto nitrocellulose membrane and immunoreactive protein identified with a murine primary anti-His antibody (BioLegend, San Diego, CA) and goat secondary HRP-conjugated anti-murine IgG antibody (Southern Biotech, Birmingham, AL).

A: Immunoblot of *Escherichia coli* lysate; B: Immunoblot of eluent (50 mM NaH_2PO_4, 300 mM NaCl, and 8 M urea; pH 6.3) from TALON® column

His: histidine; HRP: horseradish peroxidase; IgG: immunoglobulin G; IS: pellet; kDa: kilodalton; M: Pageruler protein standard marker (Thermo Fisher Scientific Inc, Vilnius, Lithuania); mM: millimolar; S: *Escherichia coli* supernatant; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

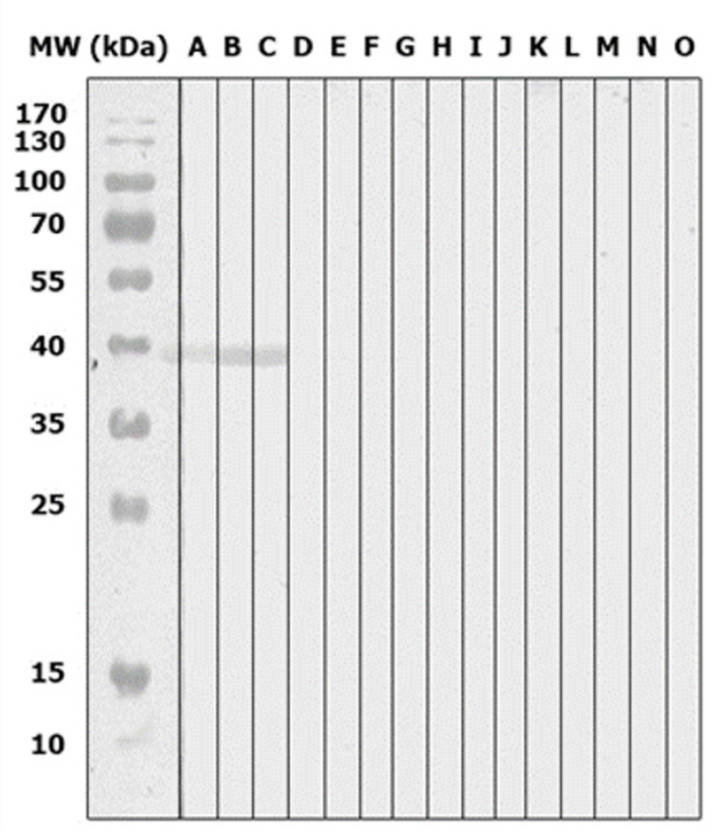

Fig 4 - IgG-immunoblots of parasite-infected murine sera using recombinant *Trichinella papuae* tissue type plasminogen activator (rTpaPLAT)

Twenty μg of rTpaPLAT solution was separated in a wide single well by 12% SDS-PAGE, transferred onto nitrocellulose membrane, incubated with 5% skim milk in phosphate-buffered saline containing 0.05 % (v/v) Tween 20 (PBS-T) and then cut into small strips. Each strip was incubated with individual murine serum (1:50 dilution in PBS-T containing 0.02 % (w/v) NaN_3) at 4°C for 16-18 hours, washed with PBS-T, then treated with horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) (1:2,000 dilution) and reactive band visualized by incubating with 2,6 dichloroindophenol substrate (Sigma-Aldrich Co, St Louis, MO).

Left lane: Pageruler protein standard marker (Thermo Fisher Scientific Inc, Vilnius, Lithuania); Lanes A-C: sera from *T. papuae* L1 larval infection on day 45 post infection (dpi); Lanes D-F: sera from uninfected mice; Lanes G-I: sera *T. spiralis* infection on 45 dpi; Lanes J-L: sera from *Schistosoma mekongi* infected on 56 dpi; Lanes M-O: sera from *S. mansoni* infection on 56 dpi

IgG: immunoglobulin G; kDa: kilodalton; MW: molecular weight; μg: microgram; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; v/v: volume by volume; w/v: weight by volume

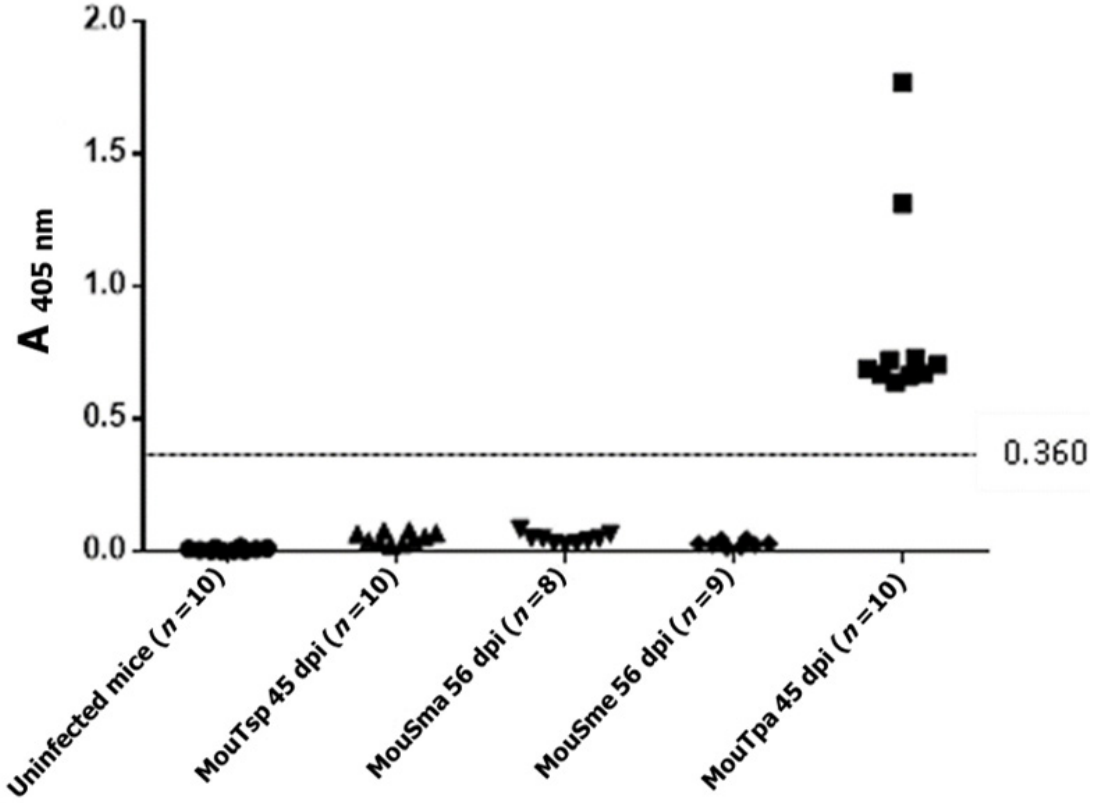

Fig 5 - Indirect IgG-ELISA detection of *Trichinella papuae*-infected murine sera

To each recombinant *Trichinella papuae* tissue type plasminogen activator-plated well of microtiter plate (Nunc, Roskilde, Denmark) murine serum was added and incubated at 37°C for 1.5 hours, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) and incubated at 37°C for a further 1.5 hours. Reaction was visualized by addition of 0.3 g/l 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) substrate (KPL ABTS® Peroxidase Substrate (1-component), Seracare, Milford, MA) and incubating for 30 minutes at 25°C. Reaction was terminated by 1% SDS and A_{405nm} measured using an ELISA micro-plate reader (Sunrise; TECAN, Mannedorf, Switzerland). Cut-off value A_{405nm} = 0.360. Sera were determined for each infected murine group.

MouSma 56 dpi: *Schistosoma mansoni*-infected mice on day 56 post-infection); MouSme 56 dpi: *S. mekongi*-infected mice on 56 dpi; MouTpa 45 dpi: *Trichinella papuae*-infected mice on day 45 post-infection; MouTsp 45 dpi: *T. spiralis*-infected mice on 45 dpi

A_{405nm}: Absorbance value at 405 nanometers; g/l: gram per liter; IgG: Immunoglobulin G; IgG-ELISA: determination of Immunoglobulin G level by enzyme-linked immunosorbent assay; SDS: sodium dodecyl sulphate

Table 1

IgG-ELISA detection of trichinellosis papuae using recombinant *Trichinella papuae* tissue type plasminogen activator

Parasitic disease	Number of cases	Diagnosis			IgG-ELISA	
		Parasite detection[a]	Immunological technique[b]	Molecular technique[c]	Number of positive cases	A_{405nm} range[d]
Nematode						
Trichinellosis papuae	22	+	+	+	22	0.802-1.644
Trichinellosis spiralis	13	+	+	ND	0	0.292-0.783
Gnathostomiasis	11	+	+	ND	0	0.336-0.654
Bancroftian filariasis	11	+	+	ND	4	0.379-1.041
Malayan filariasis	8	+	+	ND	2	0.203-0.914
Angiostrongyliasis	6	-	+	ND	0	0.439-0.658
Hookworm infections	8	+	ND	ND	0	0.272-0.714
Creeping eruption	1	+	ND	ND	1	0.972
Capillariasis	5	+	ND	ND	0	0.108-0.572
Toxocariasis	5	-	+	ND	0	0.294 - 0.608
Trichostrongyliasis	7	+	ND	ND	0	0.364 - 0.766
Ascariasis	9	+	ND	ND	0	0.162 - 0.685
Enterobiasis	4	+	ND	ND	1	0.394 - 1.016
Strongyloidiasis	8	+	+	ND	5	0.598 - 1.550
Dirofilariasis	3	-	+	ND	0	0.310 - 0.587
Trichuriasis	7	+	ND	ND	0	0.230 - 0.662

Table 1 (cont)

Parasitic disease	Number of cases	Diagnosis			IgG-ELISA	
		Parasite detection[a]	Immunological technique[b]	Molecular technique[c]	Number of positive cases	A_{405nm} range[d]
Cestode						
Neurocysticercosis	8	-	+	ND	0	0.182 - 0.634
Sparganosis	3	-	+	ND	0	0.240 - 0.619
Hymenolepiasis nana	3	+	ND	ND	0	0.475 - 0.645
Hymenolepiasis diminuta	1	+	ND	ND	0	0.487
Taeniasis	9	+	ND	ND	2	0.293 - 1.288
Echinococcosis	3	-	+	ND	1	0.707 - 1.134
Trematode						
Opisthorchiasis	6	+	+	ND	2	0.350 - 0.887
Minute intestinal fluke infections	7	+	ND	ND	3	0.508 - 1.037
Paragonimiasis	8	+	+	ND	2	0.350 - 0.883
Fascioliasis	3	-	+	ND	2	0.551 - 1.750
Schistosomiasis	3	+	+	ND	0	0.319 - 0.790

Table 1 (cont)

Parasitic disease	Number of cases	Diagnosis			IgG-ELISA	
		Parasite detection[a]	Immunological technique[b]	Molecular technique[c]	Number of positive cases	A_{405nm} range[d]
Protozoan						
Falciparum malaria	4	+	ND	ND	1	0.265 - 0.893
Vivax malaria	5	+	ND	ND	0	0.380 - 0.766
Amoebiasis	3	+	ND	ND	1	0.265 -1.140
Giardiasis	3	+	ND	ND	0	0.324 - 0.497
Blastocystosis	3	+	ND	ND	1	0.226 - 1.340
Total	200				50	
Healthy control	26	-	ND	ND	0	0.121 - 0.425

[a]egg, larva, cyst and/or worm detection; [b]positive for immunoblot and/or ELISA; [c]*T. papuae* ITS2-gene analysis; [d]Cut-off A_{405nm} value = 0.797

A_{405nm} range: minimum and maximum absorbance at 405 nm; IgG-ELISA: determination of Immunoglobulin G level by enzyme-linked immunosorbent assay; ND: not done

DISCUSSION

Until now, immunodiagnosis of trichinellosis, both from infections of encapsulate and non-encapsulated *Trichinella* clades, relies on indirect ELISA using excretory/secretory (ES) product of *T. spiralis* L1 larva (TsL1-ESP); however, false-negative results are obtained particularly in infection with *Trichinella* sp of non-encapsulated clade (Mahannop *et al*, 1992; Dvorožňáková and Hurnikova, 2012) Among *Trichinella* sp, genotypes, and encapsulated- and non-encapsulated clades, differences in immunogenicity of T and B cells, and in cytokine and antibody responses are observed (Dvorožňáková and Hurnikova, 2012). Moreover, differences in composition of TsL1-ESP preparations and low antigen yields constitute major problems. In order to overcome these obstacles, recombinant protein technology has been applied, such as recombinant 31 kDa trypsin-like serine protease present in TsL1-ESP and a target for protective antibodies is used for diagnosis of trichinellosis spiralis in humans and animals (Cui *et al*, 2015; Ren *et al*, 2018; Sun *et al*, 2018).

Hence, the serine protease tissue type plasminogen activator TpaPLAT, identified in the previous report (Sahaisook *et al*, 2022), was chosen for heterologous expression and application in immunodiagnosis of human trichinellosis papuae. Parasite PLAT is involved in breakdown of both mechanical and humoral barriers in the host during infection (Yang *et al*, 2015). The amino acid sequence of TpaPLAT has a high degree of conservation with *Trichinella* sp belonging to the non-encapsulated clade and little homology with other parasitic helminths, including those with close relationship, such as *Trichuris trichiura.*

We showed that IgG-ELISA employing rTpaPLAT produced 100% sensitivity, specificity and positive and negative predictive values in a murine trichinellosis papuae model both in immunoblotting and indirect ELISA procedures. ELISA employing recombinant *T. spiralis* serine protease inhibitor serpin (rTsSERP) has 100% sensitivity and specificity for detection of late infection of swine trichinellosis but is not appropriate for immunoblot method due to reduced specificity (Nuamtanong *et al*, 2012). Whether rTpaPLAT can be employed in immunoassay to detect swine trichinellosis remains to be determined.

Species-specific proteins play an important role in epidemiological study of *Trichinella* infections due to the variety of natural hosts (Pozio, 2007). Previously, Nagano *et al* (2008) reported that using an immunoblot assay of recombinant 53-kDa ES protein expressed from five *Trichinella* species (*T. spiralis*, *T. britovi, T. nativa, T. pseudospiralis*, and *T. papuae*) is able to differentiate among five *Trichinella* spp in an experimental murine model.

Sensitivity and negative predictive value of IgG-ELISA using rTpaPLAT for diagnosis of human trichinellosis were 100%, but percent specificity and positive predictive values were lower. The results showed that all 22 cases of trichinellosis papuae (100 %) were positive. Overall false-positivity with sera from other parasite infections was 14% (28/202 samples), but it was re-assuring that no false-positivity was obtained among trichinellosis spiralis cases. Previously, Somboonpatarakul *et al* (2018) reported that TsPLAT in immunodiagnosis is specie-specific with no cross-reactivity with trichinellosis pseudospiralis and trichinellosis papuae. One third of cases with trematode infections (n = 27) produced false-positive results, except for schistosomiasis (n = 3). *T. spiralis* larval antigen was used to diagnosis of human capillaria philippinensis (Intapan *et al*, 2006). On the other hand, IgG-ELISA using rTpaPLAT produced no false positives in capillariasis cases or in trichuriasis cases, where the infective agents belong to the superfamily Trichuroidea.

In conclusion, this is the first report of the use of recombinant *Trichinella papuae* serine protease tissue type plasminogen activator (rTpaPLAT) in indirect IgG-ELISA detection of human trichinellosis papuae with no cross-reactivity with trichinellosis spiralis. However, false positive results in cases with other parasitic infections need further study to obtain 100% sensitivity, specificity, positive predictive value, and negative predictive value. Furthermore, the applicability of rTpaPLAT immunodiagnosis for surveillance or seroprevalence of trichinellosis papuae in reservoir animals, such as rodents and reptiles, need to be determined.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

REFERENCES

Bruschi F, Murrell KD. New aspects of human trichinellosis: the impact of new *Trichinella* species. *Postgrad Med J* 2002; 78: 15-22.

Caron Y, Bory S, Pluot M, *et al*. Human outbreak of trichinellosis caused by *Trichinella papuae* nematodes, Central Kampong Thom Province, Cambodia. *Emerg Infect Dis* 2020; 26: 1759-66.

Cui J, Wang L, Sun GG, *et al*. Characterization of a *Trichinella spiralis* 31 kDa protein and its potential application for the serodiagnosis of trichinellosis. *Acta Trop* 2015; 142: 57-63.

Dupouy-Camet J, Kociecka W, Bruschi F, Bolas-Fernandez F, Pozio E. Opinion on the diagnosis and treatment of human trichinellosis. *Expert Opin Pharmacother* 2002; 3: 1117-30.

Dvorožňáková E, Hurnikova Z. Variation in immunogenicity and infectivity of encapsulating and non-encapsulating *Trichinella* species. New York: Nova Science Publishers Inc; 2012.

Ferrè F, Clote P. DiANNA 1.1: an extension of the DiANNA web server for ternary cysteine classification. *Nucleic Acids Res* 2006; 34: W182-5.

Florkowski CM. Sensitivity, specificity, receiver-operating characteristic (ROC) curves and likelihood ratios: communicating the performance of diagnostic tests. *Clin Biochem Rev* 2008; 29 (Suppl 1): S83-7.

Gamble HR, Pozio E, Bruschi F, Nöckler K, Kapel CMO, Gajadhar AA. International Commission on Trichinellosis: recommendations on the use of serological tests for the detection of *Trichinella* infection in animals and man. *Parasite* 2004; 11: 3-13.

Gottstein B, Pozio E, Nöckler K. Epidemiology, diagnosis, treatment, and control of trichinellosis. *Clin Microbiol Rev* 2009; 22: 127-45.

Grams R, Adisakwattana P, Ritthisunthorn N, Eursitthichai V, Vichasri-Grams S, Viyanant V. The saposin-like proteins 1, 2, and 3 of *Fasciola gigantica*. *Mol Biochem Parasitol* 2006; 148: 133-43.

Gupta R, Brunak S. Prediction of glycosylation across the human proteome and the correlation to protein function. *Pac Symp Biocomput* 2002; 7: 310-22.

Intapan PM, Maleewong W, Sukeepaisarnjaroen W, Morakote N. Potential use of *Trichinella spiralis* antigen for serodiagnosis of human capillariasis philippinensis by immunoblot analysis. *Parasitol Res* 2006; 98: 227-31.

Jespersen MC, Peters B, Nielsen M, Marcatili P. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res* 2017; 45: W24-9.

Jilani TN, Siddiqui AH. Tissue plasminogen activator, 2022 [cited 2022 Mar 14]. Available from: URL: https://www.ncbi.nlm.nih.gov/books/NBK507917/

Khumjui C, Choomkasien P, Dekumyoy P, *et al*. Outbreak of trichinellosis caused by *Trichinella papuae*, Thailand, 2006. *Emerg Infect Dis* 2008; 14: 1913-5.

Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett* 1990; 276: 172-4.

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 2001;

305: 567-80.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018; 35: 1547-9.

Kusolsuk T, Kamonrattanakun S, Wesanonthawech A, *et al*. The second outbreak of trichinellosis caused by *Trichinella papuae* in Thailand. *Trans R Soc Trop Med Hyg* 2010; 104: 433-7.

Mahannop P, Chaicumpa W, Setasuban P, Morakote N, Tapchaisri P. Immunodiagnosis of human trichinellosis using excretory-secretory (ES) antigen. *J Helminthol* 1992; 66: 297-304.

Mitra MM, Alizadeh H, Gerard RD, Niederkorn JY. Characterization of a plasminogen activator produced by *Acanthamoeba castellanii*. *Mol Biochem Parasitol* 1995; 73: 157-64.

Nagano I, Wu Z, Takahashi Y. Species-specific antibody responses to the recombinant 53-kilodalton excretory and secretory proteins in mice infected with *Trichinella* spp. *Clin Vaccine Immunol* 2008; 15: 468-73.

Nuamtanong S, Dekumyoy P, Adisakwattana P. Evaluation of recombinant serine protease inhibitor from *Trichinella spiralis* for immunodiagnosis of swine trichinosis. *Southeast Asian J Trop Med Public Health* 2012; 43: 1094-104.

Pozio E. World distribution of *Trichinella* spp. infections in animals and humans. *Vet Parasitol* 2007; 149: 3-21.

Ren HN, Guo KX, Zhang Y, *et al*. Molecular characterization of a 31 kDa protein from *Trichinella spiralis* and its induced immune protection in BALB/c mice. *Parasit Vectors* 2018; 11: 625.

Rice P, Longden I, Bleasby A. EMBOSS: the European molecular biology open software suite. *Trends Genet* 2000; 16: 276-7.

Ritchie LS. An ether sedimentation technique for routine stool examinations. *Bull US Army Med* Dept 1948; 8: 326.

Sahaisook P, Adisakwattana P, Reamtong O, Watthanakulpanich D, Dekumyoy P. Application of *Trichinella papuae* crude antigen in immunodiagnosis of human trichinellosis papuae and identification of immunodominant antigens. *Southeast Asian J Trop Med Public Health* 2022; 53: 232-53.

Sharma R, Thompson PC, Hoberg EP, *et al*. Hiding in plain sight: discovery and phylogeography of a cryptic species of *Trichinella* (Nematoda: Trichinellidae) in wolverine (*Gulo gulo*). *Int J Parasitol* 2020; 50: 277-87.

Somboonpatarakun C, Rodpai R, Intapan PM, *et al*. Immuno-proteomic analysis of *Trichinella spiralis, T. pseudospiralis*, and *T. papuae* extracts recognized by human *T. spiralis*-infected sera. *Parasitol Res* 2018; 117: 201-12.

Steentoft C, Vakhrushev SY, Joshi HJ, *et al*. Precision mapping of the human *O*-GalNAc glycoproteome through SimpleCell technology. *EMBO J* 2013; 32: 1478-88.

Sun GG, Song YY, Jiang P, *et al*. Characterization of a *Trichinella spiralis* putative serine protease. Study of its potential as sero-diagnostic tool. *PLoS Negl Trop Dis* 2018;12: e0006485.

Tattiyapong M, Chaisri U, Vongpakorn M, Anantaphruti MT, Dekumyoy P. Comparison of three antigen preparations to detect Trichinellosis in live swine using IgG-ELISA. *Southeast Asian J Trop Med Public Health* 2011; 42: 1339-50.

Yang Y, Wen Yj, Cai YN, *et al*. Serine proteases of parasitic helminths. *Korean J Parasitol* 2015; 53: 1-11.

Zarlenga D, Thompson P, Pozio E. *Trichinella* species and genotypes. *Res Vet Sci* 2020; 133: 289-96.

Zhai CC, Liu XL, Bai X, *et al*. Bioinformatic prediction and production of four recombinant proteins from different developmental stages of *Trichinella spiralis* and testing of their diagnostic sensitivity in mice. *Iran J Parasitol* 2021; 16: 122-35.