APPLICATION OF *TRICHINELLA PAPUAE* CRUDE ANTIGEN IN IMMUNODIAGNOSIS OF HUMAN TRICHINELLOSIS PAPUAE AND IDENTIFICATION OF IMMUNODOMINANT ANTIGENS

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Abstract. Immunodiagnosis of human trichinellosis papuae by indirect ELISA and immunoblot was developed using *Trichinella papuae* muscle L1 larva crude worm antigens (TpaL1-CWA) and tested with human sera of trichinellosis papuae (n = 18), healthy control (n = 26) and other parasitic diseases (n = 160). IgG_2- and IgG_3-indirect ELISAs failed to elicit positive results, total IgG-, IgG_1- and IgG_4-ELISAs demonstrated sensitivity of 100, 100 and 66.7%, respectively and specificity of 90.3, 89.8 and 66.7%, respectively. Western blotting of TpaL1-CWA revealed predominant immunoreactive antigens of 31, 45 and 66.2 kDa against total IgG and IgG_1 of trichinellosis papuae sera, which, except for 31 kDa antigen, showed cross-reactivity with sera of several other parasitic diseases. Specificity, sensitivity, positive predictive value, and negative predictive value of *T. papuae* 31 kDa in IgG_1-immunoblot were 100%. Mass spectrometry analysis of the 31 kDa antigen revealed the presence (in decreasing order of identity score) of 3-hydroxyacyl-CoA dehydrogenase, GLIPR1-like protein 1, tissue-type plasminogen activator, hypothetical protein T10_8058, and hypothetical protein T10_12289. Future studies should focus on evaluating the most appropriate candidate 31 kDa antigen to prepare recombinant protein for application in trichinellosis papuae immunodiagnosis.

Keywords: *Trichinella papuae*, ELISA, immunoblot, liquid chromatography-mass spectrometry, serodiagnosis

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INTRODUCTION

Trichinellosis is a zoonotic disease caused by nematode worms belonging in genus *Trichinella* (Gottstein *et al*, 2009). There are two main clades with nine species and three genotypes. Encapsulated clade consists of *T. britovi*, *T. nativa*, *T. murrelli*, *T. nelsoni*, *T. patagoniensis*, *T. spiralis*, *Trichinella* T6, *Trichinella* T8, and *Trichinella* T9; and non-capsulated clade consists of *T. papuae*, *T. pseudospiralis* and *T. zimbabwensis* (Gottstein *et al*, 2009; Krivokapich *et al*, 2012). Human trichinellosis is documented in 55 countries and *Trichinella* spp infections occur in domestic animals and wildlife in 43 and 66 countries, respectively (Pozio, 2007).

Humans acquire infection via consumption of raw and/or improper cooked meat products containing infective larvae from domestic pig and wild boar, which serve as main source of the infection, and from a variety of other animals (Murrell and Pozio, 2011). *Trichinella* sp cycle in the host body, the enteral phase, *Trichinella* infected muscle tissues are digested in the stomach. Larvae are released and penetrate the intestinal mucosa of the small intestine and reach the adult stage within 48 hours post infection. After mating, female worm releases newborn larvae in the lymphatic vessels. In the parenteral phase, the newborn larvae reach the striated muscle and actively penetrate in the muscle cell (Gottstein *et al*, 2009). Clinical symptoms of human trichinellosis are associated with the parasitic cycle, intestinal phase and muscular phase. The patients may present with fever, myalgia, eosinophilia and prolong diarrhea. After one or two weeks of the infection, the symptoms may be due to migration of the larvae into different organs including facial edema, myalgia, headache, fever and macro-papular rash (Dupouy-Camet *et al*, 2002). Diagnosis of human trichinellosis is based on a combination of clinical diagnosis, laboratory investigation and epidemiological study (Gottstein *et al*, 2009). The definitive diagnosis of human *Trichinella* infection is detection of the parasite via tissue biopsy; however, this test is not often performed due to its low sensitivity and (often painful) invasive procedure (Sun *et al*, 2015). Serodiagnostic methods provide an alternative approach for diagnosis of both human and animal trichinellosis (Ilić *et al*, 2004).

In Thailand, human trichinellosis is due to three species, namely, *T. papuae*, *T. pseudospiralis* and *T. spiralis* (Jongwutiwes *et al*, 1998; Kaewpitoon *et al*, 2006; Khumjui *et al*, 2008; Kusolsuk *et al*, 2010). Immunodiagnostic tests have been developed for *T. spiralis* infection while information on *T. papuae* infection is limited, being based on tests using *T. spiralis* antigens, which may have low immunogenicity for *T. papuae* detection (Khumjui *et al*, 2008; Kusolsuk *et al*, 2010; Caron *et al*, 2020).

Here, crude antigen from murine muscle-infected *T. papuae* L1 larvae (TpaL1-CWA) were used to evaluate IgG and IgG subclasses indirect ELISA of human trichinellosis papuae. In addition, western blotting was performed to detect immunoreactive antigens and mass spectrometry to identify the most promising antigen for future development of recombinant protein-based immunodiagnostics of human trichinellosis papuae.

MATERIALS AND METHODS

Trichinella papuae muscle larvae collection

Trichinella papuae muscle L1 larva (TpaL1) were harvested from ICR mice (*Mus musculus*) (National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand) inoculated using stomach tube with 100 infective larvae (Department of Helminthology, Faculty of Tropical Medicine, Mahidol University). On day 45 post-infection, mice (*n* = 10) were euthanized using carbon dioxide and carcasses were minced and digested with artificial gastric solution (0.7% (w/v) pepsin in 0.7% hydrochloric acid) as previously described (Tattiyapong *et al*, 2011). Larvae were collected using Baermann's method (Garcia and Bruckner,1997), washed three times with normal saline solution followed by a final wash with distilled water and stored at -80°C until used.

This part of the study was approved by Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (approval no. FTM-ACUC 030/2017).

Human sera collection

Stored human sera were obtained from the Immunodiagnostic Unit for Helminthic Infections, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University with the permission of the Institutional Review Board (approval no. MUTM2017-059-01), Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Sera were divided into three groups: Group 1 sera (*n* = 18) were obtained, prior treatment, from Thai hill tribe patients with symptoms compatible with trichinellosis following consumption of raw wild boar meat. Patients (*n* = 2) were hospitalized because of severe symptoms and underwent surgical biopsy of gastrocnemius muscle, revealing the presence of non-encapsulated *Trichinella* larvae, which were subsequently confirmed as *T. papuae* by molecular techniques (Khumjui *et al*, 2008; Kusolsuk *et al*, 2010). Group 2 sera (*n* = 160) were from patients infected with other parasites as diagnosed through recovery of worms or worm products, clinical features and serological tests (Table 1). Group 3 sera (*n* = 26) were from subjects with negative stool examinations by both simple smear and formalin-ether concentration technique (Ritchie, 1948) at time of blood collection.

TpaL1-CWA preparation

Fresh frozen TpaL1 sample was macerated with alumina in distilled water, sonicated at 30 seconds interval for 10 minutes (Sonics® vibra cell VCX750 equipped with tapered microtip no. 630-0418, diameter 3 mm; Sonics® and Materials Inc, Newtown, CT), centrifuged at 20,000 g for 60 minutes at 4°C, and supernatant protein concentration determined using Coomassie® Plus Protein Assay (Pierce Chemical, Rockford, IL) before being stored at -80°C until used.

Indirect ELISA determination

Indirect ELISA was carried as previously described (Dekumyoy *et al*, 1998). Prior to evaluation of IgG and IgG subclasses (IgG_{1-4}), checkerboard titrations using six serum samples of trichinellosis papuae and healthy controls respectively were performed to determine optimal conditions, results of which demonstrated that only total IgG, IgG_1 and IgG_4 could detect TpaL1-CWA. Subsequently, 50-μl aliquots of 2.5 μg/ml TpaL1-CWA (for total IgG-ELISA) or 5 μg/ml (for IgG_1-and IgG_4-ELISAs) in carbonate-bicarbonate buffer pH 9.6 were placed in each well of a 96-well Maxisorp Nunc-Immuno plate (Thermo Fisher Scientific, Roskilde, Denmark), and incubated at 37°C for 60 minutes, followed by an overnight incubation at 4°C. After treating with 1% skim milk in 0.5M phosphate-buffered saline pH 7.4 (PBS), serum (diluted 1:800 for determination of total IgG or 1:200 for determination of IgG_1 and IgG_4) was added to well in duplicate, followed by affinity purified horse radish peroxidase (HRP)-conjugated goat anti-human IgG (γ) antibody (KPL, Gaithersburg, MD) (1:5,000 dilution), hinge-HRP-conjugated mouse anti-human IgG_1 or pFc′-HRP-conjugated mouse anti-human IgG_4 antibody (Southern Biotech, Birmingham, AL) (1:2,500 dilution). To each well was added 50-μl aliquot of 0.3 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma-Aldrich Co, St Louis, MO), then the plate was incubated at 25°C for 30 minutes before the reaction was terminated with 1% SDS and $A_{405\ nm}$ measured using an ELISA spectrophotometer (Sunrise; TECAN, Mannedorf, Switzerland).

Western blotting analysis

TpaL1-CWA (625 μg) in a broad single well, together with an aliquot of a broad range protein molecular weight marker solution (Bio-Rad Laboratories, Hercules, CA) in a reference well, were separated by a 10-20% gradient SDS-PAGE at a constant current of 20 mA (Laemmli, 1970). Proteins were then electro-transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) using a semi-dry transfer cell (ATTO, Tokyo, Japan) at a constant current of 450 mA for 4 hours (Anantaphruti *et al*, 2005). Membrane was stained with Ponceau S-200 (Sigma-Aldrich Co, St Louis, MO), washed with distilled water, and the reference lane containing protein molecular markers was excised from the rest of the membrane, the latter then was washed with PBS containing 0.05% Tween 20, incubated with 3% skim milk containing 0.02% (w/v) NaN_3 in PBS for 1 hour at 25°C and washed with PBS. A strip of membrane was incubated with 1:50 diluted serum overnight at 25°C, followed by incubation separately with 1:1000 dilution of each of the three HRP-conjugated antibodies at 25°C for 2 hours. After a wash with PBS, membrane was incubated with freshly prepared 2 mg/ml 2,6-dichlorophenol indophenol substrate (Sigma-Aldrich, St Louis, MO) in PBS containing 30% H_2O_2 at 25°C for 2 minutes, then washed 3 times with distilled water. Immunoreactive proteins on membrane appeared as brown colored bands.

Protein identification by mass spectrometry (MS) technique

MS protein identification was conducted as previously described (Reamtong *et al*, 2020). In brief, 30 µg of TpaL1-CWA was separated by SDS-PAGE as described above and stained with Coomassie Blue G dye (Bio-Rad Laboratories, Hercules, CA). Based on immunoblot result, gel section containing protein band of interest (31 kDa) was excised and destained with 25 mM ammonium bicarbonate buffer containing 50% acetonitrile (Thermo Scientific, Rockford, IL). In-gel protein was sequentially treated with 4 mM dithiothreitol (Sigma-Aldrich, St Louis, MO) in 50 mM ammonium bicarbonate buffer at 60°C for 15 minutes, 250 mM iodoacetamide (Sigma-Aldrich, St Louis, MO),100% acetonitrile (Thermo Scientific, Rockford, IL) at 25°C for 15 minutes, and 10 ng trypsin (Sigma-Aldrich, St Louis, MO) in 200 µl of 50 mM ammonium bicarbonate buffer containing 5% acetonitrile (Thermo Scientific, Rockford, IL) overnight at 37°C. Peptides were extracted with 200 µl of acetonitrile (Thermo Scientific, Rockford, IL) and dried completely using centrifugal concentrator (CC-105, Tomy Kogyo Co Ltd, Tokyo, Japan), and dissolved in 0.1% formic acid for analysis in an UltiMate 3000 nano-liquid chromatography system (Thermo Scientific, Rockford, IL) coupled with a micrOTOF-Q electrospray ionization quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA), with tolerance of 0.6 m/z. Protein identification was performed by search of NCBI database using a MASCOT software (Matrix Science, London, UK), with allowance for one missed tryptic peptide.

Data analysis

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

RESULTS

Indirect ELISA

Three groups of human sera, namely, group 1 (individuals diagnosed with trichinellosis papuae) (*n* = 18), group 2 (individuals diagnosed with other infectious diseases) (*n* = 160) and normal control (not infected with any parasite) (*n* = 26) (Table 1), were analyzed for immunoreactivity against TpaL1-CWA using total IgG, IgG_1 and IgG_4 indirect ELISA (detection employing IgG_2 and IgG_3 were negative). Cut-off $A_{405\ nm}$ value for IgG, IgG_1 and IgG_4 was 0.525 (Area under the curve (AUC) = 0.980, 95 % CI: 0.962-0.997), 0.250 (AUC = 0.971, 95 % CI: 0.948-0.993) and 0.113 (AUC = 0.753, 95 % CI: 0.654-0.853), respectively.

Based on total IgG, 7/27 of other parasitic diseases produced false positive results, namely, capillariasis (100%), fascioliasis (100%), Malayan filariasis (20%), minute intestinal fluke infections (14%), strongyloidiasis (11%), trichinellosis spiralis (80%), and trichuriasis (11%) (Table 2). $A_{405\ nm}$ of false positives were close to cut-off value in cases of capillariasis, fascioliasis (1/3 cases), Malayan filariasis, minute intestinal fluke infections,

Table 1

Serum samples from individuals with parasitic infections

Group	Infection	Number of serum samples	Diagnostic method
1[ab]	Trichinellosis papuae	18	Presence of *Trichinella papuae* non-encapsulated larvae in muscle biopsy (*n* =2), ITS2-gene analysis and positive immunoblotting test against 109 kDa antigen of crude *T. spiralis* larvae from murine muscle. All cases were from two outbreaks[a].
2[b]	Trichinellosis spiralis	10	Larva and immunoblot detection
	Gnathostomiasis	10	Larva and immunoblot detection
	Bancroftian filariasis	10	Microfilariae detection
	Malayan filariasis	10	Microfilariae detection and ELISA using recombinant antigen
	Angiostrongyliasis	10	Larva and immunoblot detection
	Hookworm infections	10	Egg detection
	Creeping eruption	2	Symptoms, negative for strongyloidiasis and gnathostomiasis

Table 1 (cont)

Group	Infection	Number of serum samples	Diagnostic method
2[b]	Capillariasis	2	Egg, larva and adult worm detection
	Toxocariasis	10	Immunoblot detection
	Ascariasis	6	Egg and worm detection
	Enterobiasis	2	Egg detection
	Strongyloidiasis	9	Larva detection
	Dirofilariasis	1	Worm detection
	Trichuriasis	9	Egg detection
	Neurocysticercosis	8	Cyst and immunoblot detection
	Sparganosis	1	Sparganum detection
	Hymenolepiasis nana	3	Egg detection
	Taeniasis	9	Egg or segments (*Taenia solium* or *T. saginata*)
	Echinococcosis	3	Protoscolices detection
	Opisthorchiasis	6	Worm detection
	Minute intestinal fluke infections	7	Worm detection
	Paragonimiasis	8	Worm and egg detection
	Fascioliasis	3	Egg and immunoblot detection
	Malaria	2	Blood stage detection
	Amoebiasis (*Entamoeba histolytica*)	3	Cyst detection
	Giardiasis	3	Cyst detection
	Blastocystosis	3	Cyst detection
3[b]	Healthy control	26	Negative results of stool examinations with both simple smear and formalin-ether concentration technique at time of serum collection

[a]Khumjui *et al* (2008); Kusolsuk *et al* (2010); [b]Immunodiagnostic Unit for Helminthic Infections, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University

ELISA: enzyme-linked immunosorbent assay; ITS2: internal transcribed spacer 2

Table 2

Positivity of human sera to *Trichinella papaue* muscle L1 larva crude somatic antigen by indirect enzyme-linked immunosorbent assay (ELISA)

Infection	Number of samples	Total IgG		IgG_1		IgG_4	
		Number of samples	$A_{405\ nm}$ range	Number of samples	$A_{405\ nm}$ range	Number of samples	$A_{405\ nm}$ range
Trichinellosis papuae (Group 1)	18	18	0.526-0.809	18	0.254-0.687	12	0.056-0.449
Other parasitic infection (Group 2)							
Trichinellosis spiralis	10	8	0.402-0.721	8	0.166-0.864	1	0.021-0.177
Gnathostomiasis	10	0	0.236-0.373	0	0.080-0.193	1	0.019-0.130
Bancroftian filariasis	10	0	0.315-0.518	1	0.075-0.255	8	0.048-0.478
Malayan filariasis	10	2	0.363-0.609	0	0.051-0.197	6	0.075-0.331
Angiostrongyliasis	10	0	0.251-0.402	0	0.036-0.187	5	0.034-0.302
Hookworm infections	10	0	0.213-0.432	0	0.048-0.217	2	0.010-0.225
Creeping eruption	2	0	0.291-0.339	0	0.076-0.104	1	0.094-0.124
Capillariasis	2	2	0.540-0.550	0	0.240-0.244	1	0.006-0.437
Toxocariasis	10	0	0.165-0.481	0	0.047-0.174	4	0.013-0.554
Ascariasis	6	0	0.302-0.377	0	0.100-0.196	0	0.001-0.064
Enterobiasis	2	0	0.464-0.501	2	0.330-0.331	1	0.086-0.124
Strongyloidiasis	9	1	0.357-0.557	2	0.046-0.353	5	0.045-0.187
Dirofilariasis	1	0	0.517	0	0.224	1	0.114
Trichuriasis	9	1	0.284-0.600	1	0.039-0.269	5	0.030-0.569

Table 2 (cont)

Infection	Number of samples	Total IgG		IgG_1		IgG_4	
		Number of samples	$A_{405\ nm}$ range	Number of samples	$A_{405\ nm}$ range	Number of samples	$A_{405\ nm}$ range
Neurocysticercosis	8	0	0.214-0.428	1	0.061-0.273	0	0.022-0.107
Sparganosis	1	0	0.302	0	0.123	0	0.077
Hymenolepiasis nana	3	0	0.264-0.386	0	0.101-0.162	1	0.046-0.119
Taeniasis	9	0	0.249-0.476	0	0.034-0.171	1	0.003-0.124
Echinococcosis	3	0	0.281-0.378	0	0.060-0.177	2	0.077-0.335
Opisthorchiasis	6	0	0.307-0.508	0	0.105-0.200	1	0.003-0.379
Minute intestinal fluke infections	7	1	0.246-0.532	0	0.092-0.227	2	0.023-0.138
Paragonimiasis	8	0	0.369-0.462	0	0.036-0.246	7	0.107-0.507
Fascioliasis	3	3	0.565-0.701	3	0.255-0.373	1	0.027-0.168
Malaria	2	0	0.417-0.521	1	0.167-0.384	0	0.013-0.057
Amoebiasis	3	0	0.292-0.484	0	0.162-0.217	1	0.057-0.147
Giardiasis	3	0	0.372-0.399	0	0.104-0.145	0	0.029-0.081
Blastocystosis	3	0	0.372-0.452	0	0.155-0.191	1	0.050-0.180
Total	160	18		19		58	
Healthy control (Group 3)	26	0	0.179-0.387	0	0.039-0.236	4	0.002-0.163

$A_{405\ nm}$ range: minimum and maximum absorbance at 405 nm; IgG: Immunoglobulin G; IgG_1: Immunoglobulin G subclass 1; IgG_4: Immunoglobulin G subclass 4 strongyloidiasis, and trichuriasis (data not shown). All cases of cestodiasis did not cross-react with TpaL1-CWA. Based on IgG_1, 8/27 of other parasitic diseases produced false positive results, namely, Bancroftian filariasis (10%), enterobiasis (100%), fascioliasis (100%), malaria (50%) neurocysticercosis (12%), strongyloidiasis (22%), trichinellosis spiralis (80%), and trichuriasis (11%). Eight of 10 false-positive trichinellosis spiralis cases showed $A_{405\ nm}$ values equivalent to those of trichinellosis papuae (data not shown). Based on IgG_4, all other parasitic diseases except ascariasis, giardiasis, malaria, neurocysticercosis and sparganosis produced false positive results (22/27) and 4 healthy sera were false positive. It is worth noting that fascioliasis, strongyloidiasis, trichinellosis spiralis, and trichuriasis resulted in false-positive results using total IgG and IgG_1 indirect ELISA but all capillariasis cases produced false positives with only total IgG indirect ELISA. Interestingly, as the highest false positivity, IgG_4 indirect ELISA of ascariasis, sparganosis and giardiasis demonstrated no false positive as total IgG-and IgG_1-ELISAs. Among total IgG-, IgG_1-and IgG_4-ELISAs, IgG_4 of trichinellosis spiralis resulted only one false positive case but individual total IgG and IgG_1 demonstrated 8 false positive cases of this disease. Also, between IgG_1-and IgG_4-ELISAs, neurocysticercosis and malaria were not false positive but IgG_1 resulted in false positive.

Sensitivity, specificity, positive predictive value and negative predictive value of total IgG, IgG_1 and IgG_4 indirect ELISAs for human trichinellosis papuae detection using TpaL1-CWA were 100%, 90.3%, 50%, 100%; 100%, 89.8%, 48.7%, 100% and 66.7%, 66.7%, 16.2% and 95.4 %, respectively (Table 3). Total IgG and IgG_1 indirect ELISAs were promising for specific diagnosis for human trichinellosis papuae as sensitivity was 100% and specificity 90%. Although total IgG- and IgG_1-ELISAs could not differentiate between human trichinellosis papuae and trichinellosis spiralis, the ELISA tests still could be performed for screening of human trichinellosis cases from other parasitic diseases.

Detection and identification of *T. papuae*-specific antigen

As indirect ELISAs using TpaL1-CWA could not produce 100% specificity in discriminating trichinellosis papuae from other infectious diseases, western blot analysis was carried out to identify *T. papuae*-specific antigen(s) detected by total IgG and IgG_1 indirect ELISAs. Using sera samples from group 1 (trichinellosis papuae cases), group 2 (cases of other parasitic diseases) producing false-positive ELISA results and group 3 (healthy controls), western blotting of group 1 sera revealed that 31, 45, and 66.2 kDa antigens were consistently immune-reactive to both total IgG and IgG_1 (66.2 kDa antigen to a lesser degree), while among all other

Table 3

Sensitivity, specificity, positive predictive value and negative predictive value of total IgG, IgG_1 and IgG_4 indirect enzyme-linked immunosorbent assay in detection of human trichinellosis papuae using *Trichinella papaue* muscle L1 larva crude somatic antigen

Test	Indirect ELISA		
	Total IgG	IgG_1	IgG_4
Cut off value ($A_{405\ nm}$)	0.525	0.250	0.113
Sensitivity (%)	100	100	66.7
Specificity (%)	90.3	89.8	66.7
Positive predictive value (%)	50	48.7	16.2
Negative predictive value (%)	100	100	95.4

ELISA: enzyme-linked immunosorbent assay; IgG: Immunoglobulin G; IgG_1: Immunoglobulin G subclass 1; IgG_4: Immunoglobulin G subclass 4; $A_{405\ nm}$: Absorbance value at 405 nanometers

serum samples (including 7 group 3 cases to total IgG), only 31 kDa antigen was immune-reactive to IgG_1 in all 18 trichinellosis papuae cases (Table 4). Cross-reactivity of total IgG indirect ELISA between trichinellosis papuae and trichinellosis spiralis was due to immune-reactive 45 and 66.2 kDa antigens, including other antigenic bands. Representative immunoblots are shown in Fig 1. Determination of sensitivity, specificity, positive predictive value and negative predictive value of trichinellosis papuae detection showed confirmed the superiority of *T. papuae* 31 kDa antigen in total IgG and IgG_1 immunoblot assays (Table 5).

MS was employed to identify *T. papuae* 31 kDa antigen by comparing the profile of tryptic peptides (n = 36) against reference proteins stored in NCBI database. Among the ~31 kDa *Trichinella* proteins (n = 28), *T. papuae* 3-hydroxyacyl-CoA dehydrogenase (34,665 Da) had the highest protein identity score, but other *T. papuae* proteins identified included GLIPR1-like protein 1, hypothetical protein T10_8058, hypothetical protein T10_12289, and tissue-type plasminogen activator (Table 6).

DISCUSSION

In addition to clinical manifestations and epidemiological studies, tissue biopsy and laboratory assays provide confirmatory evidences to discriminate human trichinellosis from other helminth infections (Gottstein *et al*, 2009). Due to the invasive nature of

Table 4

Serum samples immunoreactive to *Trichinella papuae* muscle L1 larva proteins

Infection	Total IgG				IgG_1			
	Number of ELISA positive samples	Number of immuno-positive samples			Number of ELISA positive samples	Number of immune-positive samples		
		31 kDa	45 kDa	66.2 kDa		31 kDa	45 kDa	66.2 kDa
Trichinellosis papuae (Group 1)	18	18	18	18	18	18	18	18
Other parasitic infections (Group 2)								
Trichinellosis spiralis	5	0	5	5	5	0	0	0
Gnathostomiasis	3	0	3	0	3	0	0	0
Malayan filariasis	8	3	3	1	3	0	0	0
Bancroftian filariasis	3	0	3	0	3	0	0	0
Angiostrongyliasis	3	0	0	0	3	0	0	0
Hookworm infections	3	0	3	1	3	0	0	0
Creeping eruption	1	0	1	0	1	0	0	0
Capillariasis	2	0	2	2	2	0	0	0
Toxocariasis	3	0	3	1	3	0	0	0
Ascariasis	3	0	1	0	3	0	0	0
Enterobiasis	2	0	2	1	2	0	0	0
Strongyloidiasis	3	0	3	1	3	0	0	0
Dirofilariasis	1	0	0	0	1	0	0	0
Trichuriasis	3	0	3	0	3	0	0	0

Table 4 (cont)

Infection	Total IgG				IgG_1			
	Number of ELISA positive samples	Number of immuno-positive samples			Number of ELISA positive samples	Number of immune-positive samples		
		31 kDa	45 kDa	66.2 kDa		31 kDa	45 kDa	66.2 kDa
Neurocysticercosis	3	0	1	0	3	0	0	0
Sparganosis	1	0	1	0	1	0	0	0
Hymenolepiasis nana	3	1	3	1	3	0	0	0
Taeniasis	3	0	2	0	3	0	0	0
Echinococcosis	2	0	1	0	2	0	0	0
Opisthorchiasis	3	1	3	0	3	0	0	0
Minute intestinal fluke infections	7	1	6	1	3	0	0	0
Paragonimiasis	3	0	3	0	3	0	0	0
Fascioliasis	3	0	3	0	3	0	1	0
Malaria	2	0	2	0	2	0	0	0
Amoebiasis	3	0	2	1	2	0	0	1
Giardiasis	3	0	3	1	2	0	0	0
Blastocystosis	3	1	3	1	2	0	0	0
Total	87	7	65	18	75	0	1	1
Healthy control (Group 3)	5	0	0	1	5	0	0	0

IgG: Immunoglobulin G; IgG_1: Immunoglobulin G subclass 1; kDa: kilodalton

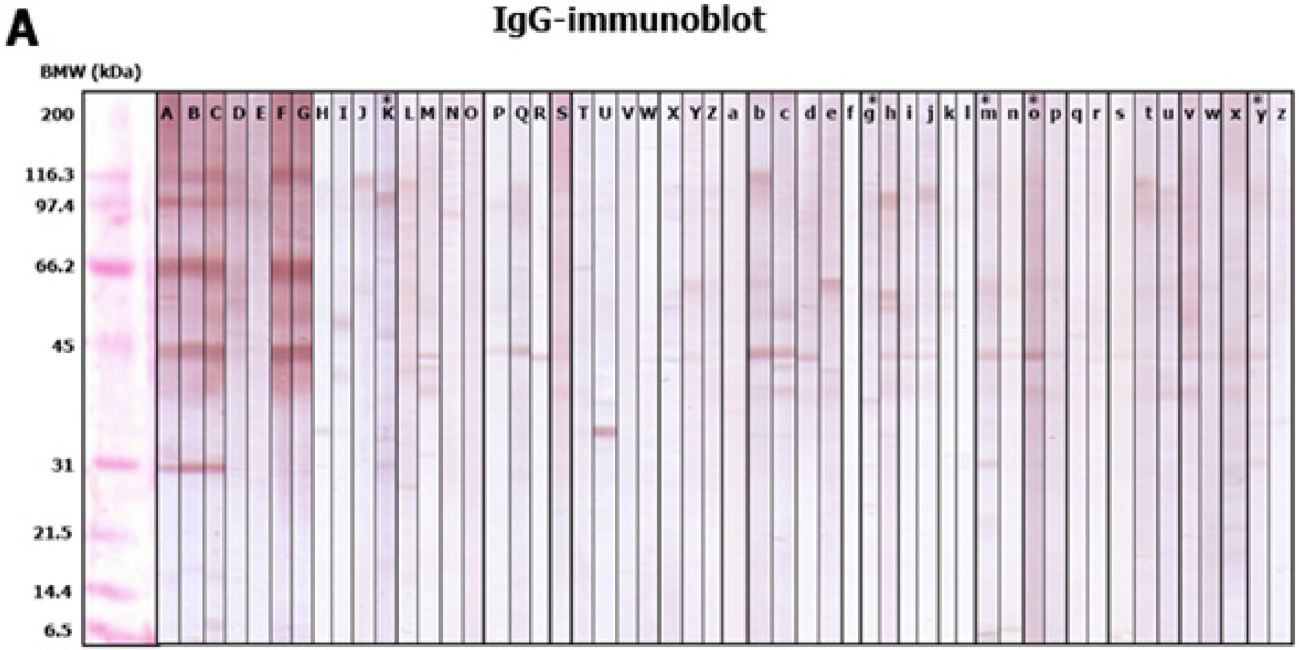

Fig 1 - Representative human serum immunoblots of *Trichinella papuae* muscle L1 larva proteins

T. papuae muscle L1 larva proteins (625 μg) were separated using a 10-20 % gradient SDS-PAGE, then electro-transferred onto nitrocellulose membrane, washed with phosphate-buffered saline (PBS) containing 0.05 % (v/v) Tween 20, incubated with 3% (w/v) skim milk containing 0.02% (w/v) NaN_3 in PBS, washed with PBS, and incubated with 1:50 dilution of human serum overnight at 25°C, followed by incubation with 1:1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (A) and HRP-conjugated mouse anti-human IgG_1 (B), and then treated with 2,6-dichlorophenol indophenol substrate in PBS containing 30% H_2O_2.

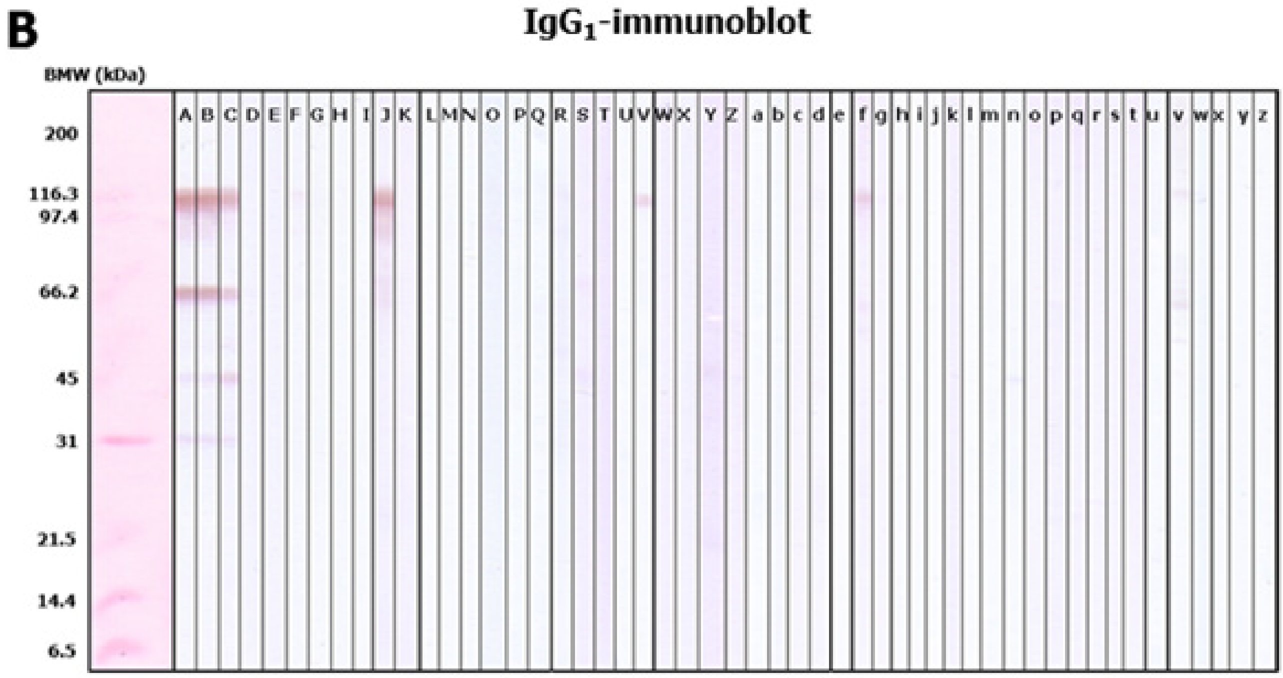

Fig 1 - (cont)

BMW Lane: Broad range protein molecular weight markers comprising those of 6.5, 14.4, 21.5, 31, 45, 66.2, 97.4, 116.2, and 200 kDa (Bio-Rad Laboratories, Hercules, CA); Lanes A-C: trichinellosis papuae sera; Lanes D-E: healthy human control sera; Lanes F-G: trichinellosis spiralis sera; Lanes H-I: gnathostomiasis sera; Lanes J-K: Malayan filariasis sera; Lanes L-M: bancroftian filariasis sera; Lanes N-O: angiostrongyliasis sera; Lanes P-Q: hookworm infection sera; Lane R: creeping eruption serum; Lane S: capillariasis serum; Lanes T-U: toxocariasis sera; Lanes V-W: ascariasis sera; Lane X: enterobiasis serum; Lanes Y-Z: strongyloidiasis sera; Lane a: dirofilariasis serum; Lanes b-c: trichuriasis sera; Lanes d-e: neurocysticercosis sera; Lane f: sparganosis serum; Lanes g-h: hymenolepiasis nana sera; Lanes i-j: taeniasis sera; Lanes k-l: echinococcosis sera; Lanes m-n: opisthorchiasis sera; Lanes o-p: minute intestinal fluke infection sera; Lanes q-r: paragonimiasis sera; Lanes s-t: fascioliasis sera; Lane u: malaria serum; Lanes v-w: amoebiasis sera; Lane x: giardiasis serum; Lanes y-z: blastocystosis sera

*Cross reaction with 31 kDa *Trichinella papuae* muscle L1 larva proteins

IgG: Immunoglobulin G; IgG_1: Immunoglobulin G subclass 1; kDa: kilodalton

Table 5

Sensitivity, specificity, positive predictive value and negative predictive value of trichinellosis papuae detection by immunoblot analysis of *Trichinella papuae* muscle L1 larva proteins

Test	*T. papuae* muscle larva protein					
	31 kDa		45 kDa		66.2 kDa	
	Total IgG	IgG_1	Total IgG	IgG_1	Total IgG	IgG_1
Sensitivity (%)	100	100	100	100	100	100
Specificity (%)	92	100	25	99	79	99
Positive predictive value (%)	72	100	23	95	50	95
Negative predictive value (%)	100	100	100	100	100	100

IgG: Immunoglobulin G; IgG_1: Immunoglobulin G subclass 1; kDa: kilodalton

parasite detection by tissue biopsy, immunodetection of antibodies specific to each *Trichinella* sp is a rapid, simple, sensitive and relatively non-painful invasive technique for diagnosis of human trichinellosis. The primary role of immunodiagnosis of human trichinellosis is to identify acute infection to allow early anthelminthic treatment and allows retrospective diagnosis and provides information for epidemiological studies (Ljungström, 1983). The majority of immunodiagnostic studies of human trichinellosis were conducted on *T. spiralis* using excretory/secretory (ES) products and somatic extracts of muscle larvae and adult worm as antigens for detection of *Trichinella* sp.-specific antibodies (Gomez-Morales *et al*, 2008; Mahannop *et al*, 1992; Sun *et al*, 2015) or purified larval antigens (Escalante *et al*, 2004). However, when using *T. spiralis* antigen for diagnosis, infections with other *Trichinella* spp, especially non-encapsulated clade may cause false-positive results. Variations in immunogenicity of T and B cells, cytokines and antibody responses were also observed in studies among different species, genotypes and encapsulated- and non-encapsulated clades (Dvorožňáková and Hurnikova, 2012). As a result, use of antigen prepared from non-encapsulated *Trichinella* spp such as *T. papuae* in this study should be appropriate for diagnosing trichinellosis papuae and trichinellosis due to other non-encapsulated clades.

Although excretory/secretory antigen (ESAg) has been shown to have a higher specificity than CWA

Table 6

Identification of *Trichinella papuae* 31 kDa antigen protein by mass spectrometry

Protein	GenBank accession number	Score*	Molecular size (Da)
3-hydroxyacyl-CoA dehydrogenase [*Trichinella papuae*]	KRZ71753.1	483	34,665
Deoxyribonuclease-2-alpha [*Trichinella zimbabwensis*]	KRZ17524.1	243	37,780
32 kDa beta-galactoside-binding lectin lec-3 [*Trichinella zimbabwensis*]	KRZ13803.1	231	35,223
32 kDa beta-galactoside-binding lectin [*Trichinella pseudospiralis*]	KRZ09574.1	220	31,736
Guanine nucleotide-binding protein subunit beta-2-like 1 [*Trichinella zimbabwensis*]	KRZ08615.1	163	35,209
GLIPR1-like protein 1 [*Trichinella papuae*]	KRZ67622.1	152	42,528
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial [*Trichinella pseudospiralis*]	KRZ33696.1	138	39,969
Inositol monophosphatase ttx-7 [*Trichinella zimbabwensis*]	KRZ15242.1	116	30,284
hypothetical protein T4E_10685 [*Trichinella pseudospiralis*]	KRY00910.1	87	24,549
Malate dehydrogenase, cytoplasmic [*Trichinella pseudospiralis*]	KRZ30718.1	83	36,155
Tissue-type plasminogen activator [*Trichinella papuae*]	KRZ67526.1	81	40,364
hypothetical protein T10_8058 [*Trichinella papuae*]	KRZ65410.1	78	50,659
Fructose-bisphosphate aldolase 1 [*Trichinella zimbabwensis*]	KRZ06039.1	71	39,429
hypothetical protein T4E_1920 [*Trichinella pseudospiralis*]	KRY00643.1	70	31,362
60S ribosomal protein L8 [*Trichinella zimbabwensis*]	KRZ14652.1	68	28,572
hypothetical protein T11_12895 [*Trichinella zimbabwensis*]	KRZ00193.1	68	27,511

Table 6 (cont)

Protein	GenBank accession number	Score*	Molecular size (Da)
Heat shock protein 83, partial [*Trichinella zimbabwensis*]	KRZ05730.1	67	87,383
Palmitoyl-protein thioesterase 1 [*Trichinella murrelli*]	KRX40271.1	66	33,455
Phosphoenolpyruvate carboxykinase [GTP] [*Trichinella zimbabwensis*]	KRZ09090.1	66	73,305
40S ribosomal protein S3a, partial [*Trichinella patagoniensis*]	KRY17636.1	63	34,159
Prohibitin [*Trichinella zimbabwensis*]	KRZ06125.1	59	30,392
Disorganized muscle protein 1 [*Trichinella zimbabwensis*]	KRZ08200.1	57	35,822
hypothetical protein T10_12289 [*Trichinella papuae*]	KRZ79058.1	52	27,965
hypothetical protein T4B_13703 [*Trichinella pseudospiralis*]	KRZ28753.1	48	36,783
Elongation factor G, mitochondrial [*Trichinella zimbabwensis*]	KRZ07995.1	46	118,336
Intermediate filament protein ifa-1, partial [*Trichinella zimbabwensis*]	KRZ06996.1	46	74,673
60S ribosomal protein L6 [*Trichinella patagoniensis*]	KRY07193.1	44	30,933
hypothetical protein T11_9147 [*Trichinella zimbabwensis*]	KRZ18717.1	44	33,935

*Protein score

Da: dalton; kDa: kilodalton

in immunodiagnosis of trichinellosis, particularly trichinellosis spiralis, its preparation requires experience and a sterile culture facility, and, furthermore, low yield makes scaling-up difficult (Elefant *et al*, 2016). In addition, cross-reactivity with other parasite diseases has been observed using ESAg of *T. spiralis* L1 larvae (Yera *et al*, 2003; Gamble *et al*, 2004; Gomez-Morales *et al*, 2008). In this regard, optimization of ELISA by validation of IgG-subclasses and presence of a distinct antigenic band by immunoblot of CWA, particularly for *Trichinella* spp other than *T. spiralis*, should be the first step towards developing an immunodiagnostic test and, in the future, recombinant protein-based immunodiagnosis.

There has been little information on trichinellosis papaue immunodiagnosis, with only ESAg-ELISA being used to identify anti-*T. papaue* IgM and IgG (Caron *et al*, 2020). In our study, TpL1-CWA was employed for evaluation of its immunodiagnostic potential. Sensitivity and specificity of both total IgG- and IgG_1-ELISAs was 100% and 90%, respectively; cross-reactivity with nematodiasis was higher compared to trematodiasis, but cross-reactivity of IgG_1-ELISA was found in one disease of cestodiasis and protozoa infections, not with cestodiasis and protozoa infections to IgG. Unfortunately, using TpL1-CWA both total IgG- and IgG_1-ELISAs could not distinguish trichinellosis spiralis from trichinellosis papuae.

Western blotting employing IgG_1 allowed identification of a 31 kDa *T. papaue*-specific antigen with no cross-reactivity with trichinellosis spiralis and all 27 other test parasitic infection cases. MS analysis identified 3-hydroxyacyl-CoA dehydrogenase (34,665 Da) as having the highest identity score among 28 proteins present in the 31 kD antigen, as well as four other *T. papaue* proteins. Previously, a 109 kDa *T. spiralis* muscle larvae crude worm antigen (TsL1-CWA) was employed for immunodiagnosis of trichinellosis spiralis (Mahannop *et al*, 1995), as well as of suspected trichinellosis papuae cases (Khumjui *et al*, 2008; Kusolsuk *et al*, 2010).

Although species-specific recombinant 53 kDa ES protein of *T. britovi, T. nativa, T. papuae, T. pseudospiralis,* and *T. spiralis* have demonstrated the ability to detect their cognate species in a trichinellosis murine model (Nagano *et al*, 2008), this is the first report of an immunodiagnosis specific to human trichinellosis papuae. A trypsin-like serine protease 31 kDa *T. spiralis* antigen from TsL1-ESP has been identified as a target of protective antibodies and is being explored as a potential diagnostic antigen for trichinellosis spiralis in humans and animals (Cui *et al*, 2015; Ren *et al*, 2018; Sun *et al*, 2018). It would be of interest to employ MS to identify

T. spiralis 109 kDa and *T. papaue* 66.2 kDa, which was responsible for cross-reactivity with trichinellosis spiralis sera.

In conclusion, the study identifies for the first time a 31 kDa *T. papuae* from a crude murine muscle-infected *T. papuae* L1 larval preparation that was specific in immunodiagnosis of human trichinellosis papuae, with no cross-reactivity with trichinellosis spiralis, and mass spectrometry analysis demonstrated 3-hydroxyacyl-CoA dehydrogenase (34,665 Da) as the protein with highest protein identity score present in the 31 kDa antigen, but four other *T. papuae* proteins were also identified. Future studies should focus on choosing the most appropriate *T. papuae* protein candidate for production of recombinant antigen in the development of immunodiagnosis method specific for human trichinellosis papuae.

ACKNOWLEDGEMENTS

The authors thank the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University for providing the human sera. The study was supported by a Joint Royal Golden Jubilee PhD Programme of the Thailand Research Fund (TRF) and Huachiew Chalermprakiet University (PHD/0201/2556), and by the Faculty of Tropical Medicine, Mahidol University.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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