Evaluation of a Commercial Enzyme-Linked Immunosorbent Assay Kit and In-House *Fasciola gigantica* Cysteine Proteinases-Based Enzyme-Linked Immunosorbent Assays for Diagnosis of Human Fascioliasis

Na T. D. Tran,^{1,2} Phuong Anh Ton Nu,² Kitti Intuyod,¹ Ly T. K. Dao,³ Pornpip Pinlaor,⁴ Yukifumi Nawa,⁵ Kiattawee Choowongkamon,⁶ Amornrat Geadkaew-Krenc,⁷ Nanthawat Kosa,⁷ Rudi Grams,⁷ and Somchai Pinlaor^{1*}

¹Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand; ²Department of Parasitology, Hue University of Medicine and Pharmacy, Hue, Vietnam; ³Institute of Malariology, Parasitology and Entomology Quy Nhon, Quy Nhon, Vietnam; ⁴Faculty of Associated Medical Sciences, Centre for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University, Khon Kaen, Thailand; ⁵Faculty of Medicine, Tropical Diseases Research Centre, Khon Kaen University, Khon Kaen, Thailand; ⁶Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand; ⁷Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand

Abstract. Fascioliasis, caused by *Fasciola hepatica* and *Fasciola gigantica* infection, is a major food-borne trematodiasis in many places of the world, with the central region of Vietnam being reported as a highly endemic area. Stool examination for *Fasciola* eggs is not a sensitive method, and immunodiagnostic methods are preferable. We investigated various enzyme-linked immunosorbent assays (ELISAs) to evaluate their efficacy for fascioliasis diagnosis. Test sera used are primarily screened using an ELISA kit produced in Vietnam (VN kit; Viet Sinh Chemical Producing & Trading Co. Ltd., Ho Chi Minh City, Vietnam): Seropositive individuals having symptoms compatible with fascioliasis were regarded as clinically diagnosed fascioliasis cases. A commercial *Fasciola* IgG ELISA kit from Diagnostic Automation/Cortez Diagnostics, Inc. (USA kit; Woodland Hills, CA), which has been commonly used in Vietnam, was assessed and compared with in-house ELISA systems, including a cystatin-capture (CC) ELISA using crude worm extract (CWE) and an indirect ELISA using a synthetic peptide Ac-TPTCHWECQVGYNKTYDEE-NHMe designed from the *F. gigantica* cathepsin B (FgCB5) molecule. The USA kit was suitable for routine diagnosis after recalibration of the manufacturer's suggested cutoff point. Cystatin-capture ELISA with CWE provided good sensitivity and specificity with perfect agreement to the results of the USA kit. In dot-blot ELISA, recombinant FgCB5 reacted more strongly with human antisera than did other *F. gigantica* antigens tested. Enzyme-linked immunosorbent assay using the synthetic peptide fragment of the FgCB5 exhibited nearly 80% sensitivity and specificity, but the test results showed low agreement with CC-ELISA or the USA kit. In conclusion, the commercially available *Fasciola* IgG ELISA kit from the United States and the in-house CC ELISA using CWE are suitable for practical diagnosis for fascioliasis.

INTRODUCTION

Fascioliasis is a disease caused by liver flukes of the genus *Fasciola*. It is acquired by eating watercress or other water plants contaminated with *Fasciola* metacercariae. At least 2.4 million people in more than 70 countries are infected, with several million more at risk.¹ The disease is most commonly caused by *Fasciola hepatica*, which has a worldwide distribution but primarily in temperate climates, particularly in regions of Asia and Africa.² Fascioliasis is highly endemic in the central region of Vietnam (more than 20,000 cases in 2011)³ where *Fasciola gigantica* is the main causative species.⁴

Fecal egg detection is a “gold standard” for the diagnosis of human fascioliasis. However, the egg detection rate is rather low because humans are not very suitable hosts for the parasite, and worms do not lay eggs at the acute stage. Eggs are also undetectable in cases of ectopic fascioliasis. Even during the chronic phase, eggs are only intermittently released. The World Health Organization has recommended confirming the diagnosis of fascioliasis based on the clinical signs supplemented with either parasitological (egg detection) or immunological techniques.⁵ Hence, immunodiagnosis based on antigen or antibody detection in the sera is preferable for diagnosis. Various diagnostic antibody-detection systems using excretory–secretory (ES) or crude worm extracts (CWE)

as the antigen have been introduced for the diagnosis of human fascioliasis, and some of them are now commercially available in convenient formats for routine use. To avoid or reduce false-positive results due to cross-reactions, use of specific antigens has been suggested and new techniques have been developed to improve the reliability of the assay system. For instance, because of the high immunogenicity, cysteine proteinases (CPs) of *Fasciola*, of which cathepsin L and cathepsin B are the major components,^{6,7} have been used as antigens for immunodiagnosis with high specificity.^{8–10} Related to this, recombinant proteins and synthetic peptides based on these molecules have also been used for immunodiagnosis.^{11–15}

Cathepsin Bs are expressed in the early stages of infection.^{16–18} For example, *F. gigantica* cathepsin B5 (FgCB5) has been found in juveniles and in mature adult worms, indicating its potential to detect fascioliasis at the early stage of infection.¹⁹ Besides, the cystatin-capture (CC) enzyme linked-immunosorbent assay (ELISA) can detect anti-CP antibodies without the need for purified/recombinant CPs.^{10,13}

In Vietnam, especially in the central part, the seroprevalence of human fascioliasis based on commercial ELISA kits is very high, ranging from 7% to 12.8%.^{20–23} However, commercial kits produced in the developed countries are not affordable for routine screening in the endemic areas and may not have been tested in, for example, Vietnam.

In the present study, CC ELISA with CWE and synthetic peptide-based ELISA using a peptide fragment of the FgCB5 molecule were evaluated for diagnostic application in parallel

* Address correspondence to Somchai Pinlaor, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. E-mail: psomec@kku.ac.th

with the imported commercial kit produced by Diagnostic Automation/Cortez Diagnostics, Inc. (USA kit).

MATERIALS AND METHODS

Ethical approval. This study was approved by the Ethical Review Committee of Khon Kaen University, Thailand (HE611175). The collection of human sera at the Hospital of the University of Medicine and Pharmacy and the Institute of Malariology, Parasitology and Entomology Quy Nhon, Vietnam, was approved by the Ethical Review Committee of Hue University of Medicine and Pharmacy, Vietnam (H2017/290). Sample collections were in strict accordance with the Human Ethics Procedures.

Parasites. *Fasciola* sp. adult worms were collected from the liver of cattle at the slaughterhouse in Quang Nam Province, Vietnam, and also at the slaughterhouse in Vientiane, Lao People's Democratic Republic. The worms were washed repeatedly to remove any traces of bile and blood, and frozen before being transferred to Thailand.

Identification of *Fasciola* worms by morphology and molecular techniques. The flukes were initially identified based on the morphological and morphometric parameters.²⁴ To avoid inclusion of female genitalia that might contain foreign sperms, genomic DNA was extracted from a small portion of the apical anterior end using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA fragments of nuclear ribosomal internal transcribed spacer-1 (ITS1) and ITS2 were amplified using the primer sets and polymerase chain reaction (PCR) cycling conditions as described by Itagaki et al.²⁵ PCR products were visualized on an agarose gel followed by DNA sequencing using the PCR primers as sequencing primers (1st BASE, Selangor, Malaysia). Sequences acquired were compared with other nucleotide sequences in the GenBank database using BLASTN, and phylogenetic analysis was performed using MEGA version 6.0 (<https://www.megasoftware.net/home>).

Human sera. Positive sera of human fascioliasis and two cases of human toxocariasis were obtained from the Parasitology Laboratory of Hue University of Medicine and Pharmacy and Institute of Malariology, Parasitology and Entomology, Quy Nhon province, Vietnam. Sixteen fascioliasis-positive sera tested using the ELISA kit produced by the Viet Sinh Chemical Producing & Trading Co. Ltd. (VN kit), and nine positive sera tested using the *Fasciola* IgG ELISA kit from Diagnostic Automation/Cortez Diagnostics, Inc. (Cat #8119-35) (USA kit) were selected. All of these patients had clinical symptoms consistent with fascioliasis, such as eosinophilia (> 10%), hepatomegaly, and a history of consuming raw vegetables. These sera are hereafter referred to as "sero-clinical positive" samples. Twenty-eight samples that were seropositive using the VN kit but were lacking clinical symptoms compatible with

fascioliasis were hereafter referred to as "seropositive" samples. In addition, sera were obtained from 45 healthy adults and from 64 otherwise healthy adults with other parasitic infections: ascariasis ($n = 2$), hookworm infection ($n = 2$), taeniasis ($n = 7$), giardiasis ($n = 1$), opisthorchiasis ($n = 16$), strongyloidiasis ($n = 29$), and mixed infections ($n = 7$). All of these samples were obtained from the Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand. Those sera were obtained from a fascioliasis non-endemic area in Thailand, and stool examinations were performed using the formalin–ether concentration method at the time of blood collection. Fifteen sera from sero-clinical positive cases and 17 sera from healthy individuals were used as positive and negative control stock sera, respectively, by pooling an equal volume of sera from each serum sample. These sera were selected after testing with the USA kit (details in the following).

Assessment of the *Fasciola* IgG ELISA kit produced in the United States and selection of positive control sera. To assess the USA kit, a total of 93 human serum samples in four groups (Table 1) were used: 1) sero-clinical positive according to the VN kit ($n = 16$), 2) seropositive according to the VN kit without clinical manifestations ($n = 28$), 3) healthy controls ($n = 17$), and 4) other parasitic diseases ($n = 32$). These sera were processed strictly according to the manufacturer's instructions. Sera of the sero-clinical positive group showing an optical density (OD) value higher than the determined cutoff point (see following paragraphs) were used as the positive control sera.

Crude worm extract. The CWE antigen of *Fasciola* was prepared using RIPA lysis and extraction buffer (Cell Signaling Technology, Danvers, MA) as described elsewhere with some modification.²⁶ Worms were washed extensively with ice-cold phosphate-buffered saline (PBS) until the solution became clear. Each worm was added to 250 μ L RIPA buffer (Cell Signaling Technology) with a protein inhibitor cocktail and homogenized on ice until completely broken down. After the addition of further RIPA buffer, the material was incubated on ice for 20 minutes. The homogenate was centrifuged at $20,000 \times g$ for 10 minutes at 4°C. The supernatant was transferred into a new tube as a pooled CWE, aliquoted, and kept at -20°C . Before freezing, the protein concentration was determined using the Pierce™ bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc., Waltham, MA).²⁷

Cystatin capture ELISA with CWE. The CC ELISA with CWE from *F. gigantica* adult worms was tested with the sera of 1) sero-clinical positive control sera ($n = 24$), 2) seropositive sera ($n = 28$), 3) healthy sera ($n = 17$), and 4) sera from cases with other parasitic diseases ($n = 32$). The protocol of the CC ELISA followed that described previously¹⁰ and was optimized using checkerboard titration. Wells of microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were sensitized with 1 μ g of chicken egg white cystatin (Sigma, St. Louis, MO) in 100 μ L of

TABLE 1
The results according to the USA kit with different serum groups using 0.63 as the cutoff point

Type of sera	Group	Range	Mean $\pm$ SD	No. positive
Sero-clinical positive* ($n = 16$)	1	0.621–3.468	2.404 $\pm$ 0.881	15/16
Seropositive† ($n = 28$)	2	0.268–3.484	2.572 $\pm$ 0.711	27/28
Healthy controls ($n = 17$)	3	0.077–0.411	0.178 $\pm$ 0.101	0/17
Other parasitic diseases‡ ($n = 32$)	4	0.051–0.630	0.141 $\pm$ 0.104	0/32

* Sera were those seropositive according to the VN kit. Fifteen sero-clinical positive sera were used as positive controls: All had OD values significantly higher than the cutoff point.

† Sera were those seropositive according to the VN kit.

‡ Sera from cases with other parasitic infections.

0.1 M NaHCO₃ buffer (pH 9.6) overnight at 4°C. The wells were incubated with 1.5 µg of CWE/100 µL diluted with 1% bovine serum albumin (BSA)/PBS/tween (T) at 4°C for 4 hours, followed by blocking with 2% BSA in PBS/T for 1 hour at room temperature (RT). Then the wells were washed and incubated with 100 µL human serum diluted 1:8,000 at 37°C for 1 hour. After washing, 100 µL of peroxidase-conjugated goat antihuman IgG (Thermo Fisher Scientific, Inc.) diluted 1:4,000 was added to each well. The reaction was stopped after 10 minutes by adding TMB substrate solution (Invitrogen, Carlsbad, CA), and the absorbance was measured at 450 nm using a microplate ELISA reader (TECAN Group Ltd., Männedorf, Switzerland).

Dot-blot ELISA using recombinant *F. gigantica* proteins.

Recombinant FgCatL-A (UniProt: Q9XYL8), FgCB5 (UniProt: A0A0U4HGR1), FgStefin-1 (UniProt: C6GC97), FgStefin-2 (UniProt: K4P3W9), FgMDC-d10 (UniProt: A0A097GX18), FgCaBP-1 (UniProt: Q45TR6), and FgDM9-2 (UniProt: A0A142BYJ5) were expressed as previously described.^{19,28–32} An equal amount of each recombinant protein (500 ng) and 3 µg of *F. gigantica* CWE (positive control) were dotted onto a nitrocellulose membrane and air-dried. Pooled human fascioliasis antisera (1:100) were used to probe the membrane-bound antigens at 4°C overnight. Polyclonal rabbit antihuman IgG/AP (DAKO; Agilent Technologies, Santa Clara, CA) was used as the secondary antibody (1:500) at RT for 1 hour. The immune complex was enzymatically detected using the chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate in conjunction with nitro blue tetrazolium (BCIP/NBT).

Synthetic peptides design based on FgCB5 sequence.

First, the tertiary structure of FgCB5 was built using homology modeling with SWISS-MODEL.³³ The resolved structure of cathepsin B1 from *Schistosoma mansoni* (PDBID: 3QSD; UniProtKB:Q8MNY2; DOI: <https://www.rcsb.org/structure/3qsd>) was selected from the protein data bank as a template model based on query coverage, identity, score A⁰, Ramachandran plot, and best similarity with FgCB5. Previously, the peptide sequence located between amino acid residues 133–138 of human cathepsin B was identified to be the epitope for a particular monoclonal antibody.³⁴ We decided to select the same region of the FgCB5 molecule because the tertiary structure of this region is highly conserved despite the amino acid sequence variation. Accordingly, we selected the peptide TPTCHWECQVGYNKTYDEE (amino acids 126–144 in mature FgCB5), which includes the putative epitope region with some additional residues at each end. A second peptide, KCAHHGSSGGYKPCPEQYY (amino acids 106–124) was also selected based on the B-cell epitope prediction tool, BepiPred linear epitope prediction,³⁵ with the span of almost the full length of the occluding loop of the proteinase (amino acids 103–127). The two selected peptide sequences derived from FgCB5 were then synthesized by Cellmano Biotech Ltd. (Anhui, China) using the solid-phase approach with modifications of acetylation at the N-terminus and NHMe at the C-terminus. To analyze epitope specificity, BLASTP searches, using the epitope sequence and FgCB5 protein sequence as queries, were performed against all available sequences in GenBank.

Indirect ELISA with synthetic peptide antigen based on FgCB5. Diagnostic value of the synthetic peptide-based ELISA was evaluated using 1) positive control sera ($n = 24$), 2) seropositive sera ($n = 27$), 3) healthy sera ($n = 45$), and 4) sera from other parasitoses ($n = 66$). The format of the indirect ELISA was optimized by checkerboard titration. Each well of

the ELISA plates (Maxisorp; Nunc) was sensitized with 1 µg of peptide antigen for 2 hours at 37°C in 100 µL of 0.1 M NaHCO₃ (pH 9.6). The wells were washed five times with 10 mmol/L PBS (pH 7.4) containing 0.05% Tween 20 (PBS/T) and blocked with 2% BSA/PBS/T for 1 hour at RT. After washing with PBS/T, the wells were incubated at 37°C for 1 hour with 0.1 mL of human sera diluted 1:100 in 1% BSA in PBS/T. After washing with PBS/T, peroxidase-conjugated goat antihuman IgG (Thermo Fisher Scientific, Inc.) diluted 1:2,000 was added. The wells were washed and incubated with 0.1 mL TMB substrate (Invitrogen). The reaction was stopped, and the absorbance measured at 450 nm using a microplate ELISA reader (TECAN). The OD value of the blank well on each plate was subtracted.

Statistical analysis. All statistical analyses were performed using GraphPad Prism release 7.0. The cutoff values for CC ELISA with crude antigen and for the synthetic peptide ELISA were established using a receiver–operator curve (ROC) analysis. The test agreement and cutoff point were analyzed using MedCalc version 18.2.1 (Ostend, Belgium). The cutoff value of the USA ELISA kit was obtained according to the manufacturer's recommendation and ROC analysis. Each test was evaluated for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), area under the curve (AUC), and accuracy (AC). The degree of agreement between the ELISA assays using crude antigen or the USA kit and sero-clinical positive samples was determined using kappa index (k) values with 95% confidence intervals and classified according to the Fleiss scale: 0.00–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; 0.81–0.99, very good; and 1.00, perfect. The normal distribution of data was evaluated by the Kolmogorov–Smirnov test. For the USA kit, significant differences (taken as $P < 0.05$) were detected using a Kruskal–Wallis test.

RESULTS AND DISCUSSION

Identification of *Fasciola* species. All worms were identified as *F. gigantica* based on morphometrics and molecular phylogenetic analysis. Phylogenetic trees based on the ITS1 region (Supplemental Data 1) and the ITS2 region (Supplemental Data 2) place our worms together with sequences of *F. gigantica* in a separate clade from sequences of *F. hepatica*. Neither ITS1 nor ITS2 contained the ambiguous sites, which are typical of hybrid *Fasciola* specimens.³⁶ Morphological and morphometric parameters, such as body length to width ratio, and distance between the ventral sucker and the posterior end of the body, also support the identification of our specimen as *F. gigantica* (S2).

Assessment of the *Fasciola* IgG ELISA kit from the United States and selection of positive control sera for subsequent experiments. Ninety-three samples were tested with the USA-made ELISA kit, and the results are shown in Figure 1 and Table 1. The manufacturer claims 100% sensitivity and specificity when an OD of 0.1 is accepted as the cutoff point. However, in our study in an endemic country, ROC analysis showed that the OD values of positive and negative sera were clearly distinguished after changing the cutoff point to 0.63 ($P < 0.0001$). Accordingly, we determined 0.63 to be a more appropriate cutoff point for the USA kit. Even using this more stringent criterion, the kit was very effective at distinguishing between positive and negative groups (the positive group being seropositive according to a different test

FIGURE 1. *Fasciola* IgG enzyme-linked immunosorbent assay (ELISA) kit from Diagnostic Automation/Cortez Diagnostics, Inc. (USA kit) against sera collected using the VN kit: (1) sero-clinical positive sera ($n = 16$), (2) seropositive patients ($n = 28$), and (3) negative sera (from healthy sera and sera from cases with other parasitic diseases [$n = 49$]). Experiments were performed on samples from different groups of individuals. Significant differences between the negative control and seropositive and sero-clinical positive groups were analyzed using the Kruskal–Wallis test ($P < 0.0001$). *Cut off value obtained by a receiver–operator curve analysis (0.63). #Cut off suggested by the manufacturer of the commercial kit (0.10).

kit and having clinical signs and symptoms compatible with fascioliasis). Thus, in this study, selection of positive control sera principally relied on clinical symptoms and a seropositive result using the USA kit. This approach is consistent with other studies of diagnosis of acute fascioliasis, in that similar clinical signs to those we observed were used and the diagnosis was confirmed using serological methods.^{37,38} The 16 sero-clinical positive sera according to the Vietnam kit were investigated again with the USA kit, and 15 of them had OD values significantly higher than the newly set cutoff point of 0.63 and were selected as positive controls in this study. Additional nine samples from patients with symptoms compatible with fascioliasis and ELISA OD values > 0.63 in the USA kit were also selected as positive controls for subsequent experiments. The total number of positive control sera selected based on clinical symptoms and serological tests was therefore 24. Twenty-seven samples of the seropositive group determined using the Vietnam kit were also positive by the USA kit. Our research is the first evaluation of the *Fasciola* IgG ELISA kit from the Diagnostic Automation/Cortez Diagnostics, Inc. (USA kit) using the new cutoff points (0.63) to differentiate sera of sero-clinical positive and seropositive groups from the sero-negative group ($P < 0.0001$) (Table 1). This finding will help clinical laboratories and clinicians to refine the diagnosis of fascioliasis.

Diagnostic efficacy of CC ELISA using crude worm extract. The results are plotted in Figure 2 with the cutoff point (0.141) obtained by ROC analysis. This ELISA system had 100% sensitivity (95% CI: 85.8–100.0) and specificity (95% CI: 92.7–100.0) (Table 2). Previous studies reported that the CC ELISA platform with ES antigen can discriminate fascioliasis from paragonimiasis and schistosomiasis.^{10,13} In our study,

FIGURE 2. The performance of cystatin capture enzyme-linked immunosorbent assay with positive control sera ($n = 24$); negative controls were sera from healthy individuals and from cases with other parasitic diseases ($n = 49$). The significant difference between groups was detected using a Mann–Whitney test ($P < 0.0001$). Data are shown as a scatter plot (A) and a box plot (B). *Cut off obtained by receiver–operator curve analysis (0.141).

CWE was used for the first time using this platform: It proved excellent for the diagnosis of fascioliasis, with a low cutoff point (at 0.141, it was lower than the 0.63 of the USA kit) and without cross-reactions with two cases of toxocariasis. Previously, CWE, used in indirect ELISA, was found to exhibit some cross-reactions with toxocariasis sera.⁹ Crude worm extract can be produced in large quantities without maintenance of living organisms. In this respect, it has an advantage over ES antigen, although both CWE and ES antigens are similarly effective antigens in serodiagnosis of human fascioliasis caused by *F. gigantica* infection.³⁹ Cystatin capture ELISA with CWE, without the requirement of cysteine proteinase purification, provides a good sensitivity and markedly high specificity, and yields a more conclusive diagnosis as possessing a low cutoff value.

Selection of recombinant proteins using dot-blot analysis. We assessed various recombinant proteins using dot-blot ELISA (Figure 3). Dot blots showed that all antigens except MDC-d10 and DM9-2 reacted with pooled positive sera. Dot-blot ELISA also indicated stronger immunogenicity of CPs (cathepsin B5 and cathepsin L) than other *F. gigantica* antigens. Invading host tissue trematodes such as *Paragonimus*,

TABLE 2
Comparisons of ELISAs using (A) cystatin capture with crude worm extract and (B) synthetic peptide for antibody detection

Test	Cut off	Sample size	Se%	CI 95%	Sp%	CI 95%	PPV	NPV	AC	AUC
Cystatin capture	0.141	75	100% (24/24)	85.8–100.0	100% (49/49)	92.7–100.0	100%	100%	100%	1.000
Peptide ELISA	0.706	135	79.2% (19/24)	57.8–92.9	79.3% (88/111)	70.5–86.4	45.2%	94.6%	79.3%	0.845

AC = accuracy; AUC = area under ROC curve; ELISA = enzyme-linked immunosorbent assay; NPV = negative prediction value; PPV = positive predictive value; ROC = receiver-operator curve; Se = sensitivity; Sp = specificity.

Fasciola, and *Schistosoma* are assumed to induce high antibody responses of the host to fluke CPs because these antigens are regularly released from the parasites. Among recombinant proteins tested, recombinant FgCB5 yielded the strongest immune reaction in the dot blot, especially in comparison with *F. gigantica* cathepsin L. Cathepsin B is a major secreted protein in early infection, whereas cathepsin L is the main CP released from the adult.^{6,7} Notably, our positive control sera were collected from acute patients exhibiting high eosinophilia and other clinical symptoms compatible with early fascioliasis. Of the proteins tested against human antiserum using dot-blot ELISA, FgCB5 demonstrated the strongest immune response, indicating a possible early detection of the disease based on this molecule.

Peptide selection and efficacy of synthetic peptide-based ELISA in comparison with other ELISA systems. The tertiary structure model of FgCB5 and the regions of the selected peptides are shown in Figure 4. Peptides are comparatively cheap and easy to produce in large quantities with consistent quality. They can be designed to contain highly specific and reactive epitopes of pathogen proteins. Compared with the putative orthologous *F. hepatica* cathepsin B (UniProtKB: A0A2H1C281, 94.4% sequence identity), the two peptides differ at one (KCAHHGSSGGYKPCPEeYY) and two positions (TPTCHWkCQVGYNKTYDEd), respectively. Peptide KCAHHGSSGGYKPCPEQYY, representing the occluding loop, a well-characterized surface-located feature of cathepsin B,⁴⁰ and strongly supported by epitope prediction (Supplemental Data 3), failed to distinguish positive from negative control sera (data not shown). The data from ELISA using the TPTCHWECQVGYNKTYDEE peptide were analyzed using ROC (Figure 5A). The peptide-based ELISA exhibited nearly 80% sensitivity and specificity (79.2% sensitivity and 79.3% specificity). Analysis of AUC using ROCs (Figure 5B)

FIGURE 3. Immunodetection of *Fasciola gigantica* antigens such as (1) CatL-A, (2) CB5, (3) Stefin-1, (4) Stefin-2, (5) MDC-d10, (6) CaBP-1, (7) DM9-2, and (8) crude worm extract (CWE). Dot-blot enzyme-linked immunosorbent assay of *F. gigantica* antigens with pooled human fascioliasis antisera. All of the proteins except MDC-d10 and DM9-2, a strictly intracellular protein, indicated an immune response. *Fasciola gigantica* cathepsin B5 was the strongest detected protein; CWE was used as a positive control.

confirmed that positive and negative sera groups could be differentiated (AUC = 0.845 and 79.3% AC). However, the PPV was lower (PPV = 45.6%) than the NPV (94.6%) (Table 2). The NPV (94.6%) gives high confidence to identify true negative patients. Higher NPVs than PPVs were also observed previously in peptides derived from cathepsin L1, which exhibited 100% sensitivity for the diagnosis of animal fascioliasis,¹¹ and 100% sensitivity and 97.3% specificity for the diagnosis of human fascioliasis.¹⁴ By contrast, another putative epitope of CL1⁴¹ demonstrated a similarly low sensitivity (77%) as our peptide, implying that this peptide is not capable of detecting human fascioliasis. We noted cross-reactions of the synthetic peptide antigen with giardiasis (1/1), taeniasis (2/7), strongyloidiasis (8/29), opisthorchiasis (3/16), other parasitoses (2/7), and healthy controls (7/45). It can be argued that short synthetic peptides, although generated as exact copies of likely epitopic regions, might not fold in the same way as the full-length protein, reducing their affinity to the specific antibody. The stability of a peptide is highly dependent on amino acid composition and sequence. Although our peptides were

FIGURE 4. Homology model of mature *Fasciola gigantica* cathepsin B (FgCB5) based on *Schistosoma mansoni* cathepsin B1 (PDBID: 3QSD). The locations of the peptides TPTCHWECQVGYNKTYDEE (T126-E144) and KCAHHGSSGGYKPCPEQYY (K106-T124) used in this analysis are indicated by their respective N- and C-terminal residues. Discovery Studio Visualizer (Discovery Studio, version 4.5: Accelrys, Inc., San Diego, CA) was used to create the graphical representation of FgCB5. This figure appears in color at www.ajtmh.org.

FIGURE 5. The performance of the peptide based on enzyme-linked immunosorbent assay with positive control sera ($n = 24$), negative control sera ($n = 11$), sera from healthy control ($n = 45$), and sera from cases with other parasitic diseases ($n = 66$). (A) Scatter dot plot with the cutoff line by receiver-operator curve (ROC) analysis. (B) Area under the ROC was analyzed by MedCalc (area under the curve [AUC] = 0.845, $P < 0.001$). *Cutoff point was obtained by ROC analysis (0.706).

modified at both ends to improve stability, they were quite sensitive after being dissolved.

Cystatin capture ELISA provided better AC (100%) than peptide-based ELISA (AC 79.3%). The USA kit, CC, and peptide-based ELISAs were tested using the same positive control sera ($n = 24$), seropositive sera ($n = 28$), healthy sera ($n = 17$), and sera from other parasitoses ($n = 32$). We found excellent agreement (Kappa = 1.000) between CC ELISA and the USA kit that is similar to other reported evaluations between DRG *Fasciola* commercial kits and an in-house assay.⁴² Crude worm extract is assumed to be the antigen used in the USA kit, hence produced the same result as our CC ELISA. However, these two systems showed only fair agreement with the peptide-based ELISA (Kappa = 0.387; CI: 0.209–0.565), an agreement based mainly on the positive control (79%, 19/24) and negative control (78%, 38/49) groups: Results for the seropositive group were only 50% accurate (14/28). The lower diagnostic sensitivity of the synthetic peptide than that of crude antigen⁴³ could explain the relatively low agreement between CC and peptide-based ELISA systems for this group. The use of a combination of more than two synthetic peptides

might yield improved results, as was previously demonstrated in visceral leishmaniasis.⁴³ Although finding of eggs in stool is the “gold standard” for the diagnosis for helminthiases, it is not always an option open to clinicians for reasons mentioned in the introduction. There is also a possibility of mistaking eggs of other trematodes for those of *Fasciola*.⁴⁴ Clinicians, therefore, rely heavily on immunological tests, especially for the diagnosis of early infection. However, serological tests also have limitations.⁴⁴ These include possibility of cross-reactions with antibodies produced against other species of parasites (multi-parasitism is common in rural Vietnam), long persistence of antibodies after treatment rendering serology a poor indicator of cure, and low sensitivity in late chronic infections when circulating antibodies drop to low levels.⁴⁴ Despite such limitations, we should assess the quality of diagnostic techniques being used in the developing countries (e.g., Vietnam) to determine the best ELISA platforms and specific antigens to use because of their practical applicability in rural hospitals.

In conclusion, the *Fasciola* IgG ELISA from the Diagnostic Automation/Cortez Diagnostics, Inc. (USA kit) is suitable for routine diagnosis when the newly determined cutoff point is applied and is applicable for patients with or without supportive clinical findings. Any commercial kit produced in non-endemic areas of fascioliasis should be validated in this way before being applied to endemic areas. The CC ELISA platform with CWE is promising as an effective tool of diagnosis for fascioliasis because the results of this platform showed perfect agreement with the results obtained by the USA kit with a lower cutoff point. The peptide TPTCHWECQVGYNKTYDEE derived from FgCB5 was not as capable of detecting antisera of human fascioliasis as were the other ELISA systems in this study. However, the peptide sequence appears to contain an immunogenic epitope region; therefore, this peptide or a similar one has a potential for early diagnosis of fascioliasis.

Received October 18, 2018. Accepted for publication November 26, 2018.

Published online January 21, 2019.

Note: Supplemental Data appear at www.ajtmh.org.

Acknowledgments: We would like to acknowledge David Blair from Publication Clinic KKU, Thailand, for his comments and editing the manuscript.

Financial support: This research was supported by Invitation Research, Faculty of Medicine (IN61241), and the Postgraduate Scholarship for International Students, Faculty of Medicine, Khon Kaen University.

Authors' addresses: Na T. D. Tran, Kittit Intuyod, and Somchai Pinlaor, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand, E-mails: t.tran@kku.ac.th, kitti.i@kku.ac.th, and psomec@kku.ac.th. Phueng Anh Ton Nu, Department of Parasitology, Hue University of Medicine and Pharmacy, Hue, Vietnam, E-mail: tnpnh@huemed-univ.edu.vn. Ly T. K. Dao, Institute of Malaria, Parasitology and Entomology Quy Nhon, Quy Nhon, Vietnam, E-mail: khangly75@yahoo.com. Porntip Pinlaor, Faculty of Associated Medical Sciences, Centre for Research and Development of Medical Diagnostic Laboratories, Khon Kaen University, Khon Kaen, Thailand, E-mail: porawa@kku.ac.th. Yukifumi Nawa, Faculty of Medicine, Nanthawat Kosa, and Rudi Grams, Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand, E-mails: amomrut_goad@hotmail.com, nantha.ko@hotmail.com, and rgrams@tu.ac.th.

REFERENCES

- World Health Organization (WHO) Ntd, 2018. *Human Fascioliasis: Review Provides Fresh Perspectives on Infection and Control*. Available at: http://www.who.int/neglected_diseases/news/fascioliasis-review-provides-new-perspectives-infection-control/en/. Accessed September 10, 2018.
- Mas-Coma S, Valero MA, Bargues MD, 2009. Chapter 2. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv Parasitol* 69: 41–146.
- Bui TD, Doanh PN, Saegerman C, Losson B, 2016. Current status of fasciolosis in Vietnam: an update and perspectives. *J Helminthol* 90: 511–522.
- Mas-Coma S, Bargues MD, Valero MA, 2018. Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. *Parasitology* 145: 1665–1699.
- World Health Organization (WHO), 2018. *Foodborne Trematode Infections. Fascioliasis. Fascioliasis Diagnosis, Treatment and Control Strategy*. Available at: http://www.who.int/foodborne_trematode_infections/fascioliasis/fascioliasis_diagnosis/en/. Accessed November 19, 2018.
- Smooker PM, Jayaraj R, Pike RN, Spithill TW, 2010. Cathepsin B proteases of flukes: the key to facilitating parasite control? *Trends Parasitol* 26: 506–514.
- Robinson MW, Menon R, Donnelly SM, Dalton JP, Ranganathan S, 2009. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. *Mol Cell Proteomics* 8: 1891–1907.
- Rojas-Caraballo J, López-Abán J, Del Villar LP, Vizcaino C, Vicente B, Fernández-Soto P, Del Olmo E, Patarroyo MA, Muro A, 2014. In vitro and in vivo studies for assessing the immune response and protection-inducing ability conferred by *Fasciola hepatica*-derived synthetic peptides containing B- and T-cell epitopes. *PLoS One* 9: e105323.
- Rokni MB, Massoud J, Haniilo A, 2003. Comparison of adult somatic and cysteine proteinase antigens of *Fasciola gigantica* in enzyme linked immunosorbent assay for serodiagnosis of human fasciolosis. *Acta Trop* 88: 69–75.
- Ikedo T, 1998. Cystatin capture enzyme-linked immunosorbent assay for immunodiagnosis of human paragonimiasis and fascioliasis. *Am J Trop Med Hyg* 59: 286–290.
- Meshgi B, Jalousian F, Fathi S, Jahani Z, 2018. Design and synthesis of a new peptide derived from *Fasciola gigantica* cathepsin L1 with potential application in serodiagnosis of fascioliasis. *Exp Parasitol* 189: 76–86.
- Santana BG, Dalton JP, Camargo FV, Parkinson M, Ndao M, 2013. The diagnosis of human fascioliasis by enzyme-linked immunosorbent assay (ELISA) using recombinant cathepsin L protease. *PLoS Negl Trop Dis* 7: e2414.
- Tantrawatpan C, Maleewong W, Wongkham C, Wongkham S, Intapan PM, Nakashima K, 2005. Serodiagnosis of human fascioliasis by a cystatin capture enzyme-linked immunosorbent assay with recombinant *Fasciola gigantica* cathepsin L antigen. *Am J Trop Med Hyg* 72: 82–86.
- Intapan PM, Tantrawatpan C, Maleewong W, Wongkham S, Wongkham C, Nakashima K, 2005. Potent epitopes derived from *Fasciola gigantica* cathepsin L1 in peptide-based immunoassay for the serodiagnosis of human fascioliasis. *Diagn Microbiol Infect Dis* 53: 125–129.
- O'Neill SM, Parkinson M, Dowd AJ, Strauss W, Angles R, Dalton JP, 1999. Immunodiagnosis of human fascioliasis using recombinant *Fasciola hepatica* cathepsin L1 cysteine proteinase. *Am J Trop Med Hyg* 60: 749–751.
- Creaney J, Wilson L, Dosen M, Sandeman RM, Spithill TW, Parsons JC, 1996. *Fasciola hepatica*: irradiation-induced alterations in carbohydrate and cathepsin-B protease expression in newly excysted juvenile liver fluke. *Exp Parasitol* 83: 202–215.
- Law RH, Smooker PM, Irving JA, Piedrafito D, Ponting R, Kennedy NJ, Whisstock JC, Pike RN, Spithill TW, 2003. Cloning and expression of the major secreted cathepsin B-like protein from juvenile *Fasciola hepatica* and analysis of immunogenicity following liver fluke infection. *Infect Immun* 71: 6921–6932.
- Meemon K, Grams R, Vichasri-Grams S, Hofmann A, Korge G, Viyanant V, Upatham ES, Habe S, Sobhon P, 2004. Molecular cloning and analysis of stage and tissue-specific expression of cathepsin B encoding genes from *Fasciola gigantica*. *Mol Biochem Parasitol* 136: 1–10.
- Siricoon S, Vichasri Grams S, Lertwongvisarn K, Abdullohfaakeyeh M, Smooker PM, Grams R, 2015. *Fasciola gigantica* cathepsin B5 is an acidic endo- and exopeptidase of the immature and mature parasite. *Biochimie* 119: 6–15.
- Quy TM, Yeatman H, Flood V, Chuong NC, Tuan BV, 2015. Prevalence and risks of fascioliasis among adult cohorts in Binh Dinh and Quang Ngai provinces-central Viet Nam. *Vietnam J Public Health* 3: 46–61.
- Huong NT, Duong TD, Tinh TT, 2013. Human *Fasciola* sp infection in community Nghe an province and comparison of an antibody ELISA detection for serodiagnosis of fascioliasis (in Vietnamese, English abstract). *J Vietnam Pract Med* 11: 156–160.
- Duong TD, Huong NT, Tinh TT, 2013. Research applied the technical standards in diagnostic human fascioliasis in hospital of the national institute of malariology, parasitology and entomology (2011–2013) (in Vietnamese, English abstract). *J Vietnam Pract Med* 11: 67–71.
- Van De N, Thi Xuan L, Van Chau L, 2003. Prevalence of first survey of human fascioliasis in Khanh Hoa province, Vietnam (in Vietnamese). *J Vietnam Pract Med* 3: 77–80.
- Periago MV, Valero MA, El Sayed M, Ashrafi K, El Wakeel A, Mohamed MY, Desquesnes M, Curtale F, Mas-Coma S, 2008. First phenotypic description of *Fasciola hepatica*/*Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta, Egypt. *Infect Genet Evol* 8: 51–58.
- Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T, Fukuda K, 2005. Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology* 131: 679–685.
- McVeigh P et al., 2014. RNAi dynamics in juvenile *Fasciola* spp. Liver flukes reveals the persistence of gene silencing in vitro. *PLoS Negl Trop Dis* 8: e3185.
- Walker JM, 1994. The bicinchoninic acid (BCA) assay for protein quantitation. Walker JM, ed. *Basic Protein and Peptide Protocols*. Totowa, NJ: Humana Press, 5–8.
- Phadungsil W, Smooker PM, Vichasri-Grams S, Grams R, 2016. Characterization of a *Fasciola gigantica* protein carrying two DM9 domains reveals cellular relocalization property. *Mol Biochem Parasitol* 205: 6–15.
- Geadkaew A, Kosa N, Siricoon S, Grams SV, Grams R, 2014. A 170 kDa multi-domain cystatin of *Fasciola gigantica* is active in the male reproductive system. *Mol Biochem Parasitol* 196: 100–107.
- Siricoon S, Grams SV, Grams R, 2012. Efficient inhibition of cathepsin B by a secreted type 1 cystatin of *Fasciola gigantica*. *Mol Biochem Parasitol* 186: 126–133.
- Tarasuk M, Grams SV, Viyanant V, Grams R, 2009. Type I cystatin (stefin) is a major component of *Fasciola gigantica* excretion/secretion product. *Mol Biochem Parasitol* 167: 60–71.
- Vichasri-Grams S, Subpipattana P, Sobhon P, Viyanant V, Grams R, 2006. An analysis of the calcium-binding protein 1 of *Fasciola gigantica* with a comparison to its homologs in the phylum Platyhelminthes. *Mol Biochem Parasitol* 146: 10–23.
- Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, Bordoli L, 2018. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res* 46: W296–W303.
- Mirkovic B, Premzl A, Hodnik V, Doljak B, Jevnikar Z, Anderluh G, Kos J, 2009. Regulation of cathepsin B activity by 2A2 monoclonal antibody. *FEBS J* 276: 4739–4751.
- Jespersen MC, Peters B, Nielsen M, Marcotili P, 2017. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res* 45: W24–W29.
- Nguyen S, Amer S, Ichikawa M, Itagaki T, Fukuda Y, Nakai Y, 2012. Molecular identification of *Fasciola* spp. (digenea: plathyelminthes) in cattle from Vietnam. *Parasite* 19: 85–89.
- Fica A, Dabanch J, Farias C, Castro M, Jercic MI, Weitzel T, 2012. Acute fascioliasis—clinical and epidemiological features of four patients in Chile. *Clin Microbiol Infect* 18: 91–96.

38. Weisenberg SA, Perlada DE, 2013. Domestically acquired fascioliasis in northern California. *Am J Trop Med Hyg* 89: 588–591.
39. Maleewong W, Intapan PM, Wongkham C, Tomanakan K, Daenseekaew W, Sukeepaisamjaroen W, 1996. Comparison of adult somatic and excretory-secretory antigens in enzyme-linked immunosorbent assay for serodiagnosis of human infection with *Fasciola gigantica*. *Southeast Asian J Trop Med Public Health* 27: 566–569.
40. Musil D et al., 1991. The refined 2.15 Å X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. *EMBO J* 10: 2321–2330.
41. Rokni M, Gharavi M, 2002. Evaluation of a pre-selected epitope of *Fasciola hepatica* cathepsin-L1 for the diagnosis of human fasciolosis by IgG-ELISA test. *Iran J Public Health* 31: 80–82.
42. Adela Valero M, Victoria Periago M, Pérez-Crespo I, Rodríguez E, Jesús Perteguer M, Gárate T, González-Barberá EM, Mas-Coma S, 2012. Assessing the validity of an ELISA test for the serological diagnosis of human fascioliasis in different epidemiological situations. *Trop Med Int Health* 17: 630–636.
43. Fargeas C, Hommel M, Maingon R, Dourado C, Monsigny M, Mayer R, 1996. Synthetic peptide-based enzyme-linked immunosorbent assay for serodiagnosis of visceral leishmaniasis. *J Clin Microbiol* 34: 241–248.
44. Mas-Coma S, Bargues MD, Valero MA, 2014. Diagnosis of human fascioliasis by stool and blood techniques: update for the present global scenario. *Parasitology* 141: 1918–1946.