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Immunodiagnosis of opisthorchiasis using parasite cathepsin F

Salma Teimoori^{1,2}, Yuji Arimatsu¹, Thewarach Laha³, Sasithorn Kaewkes³, Piya Sereerak⁴, Sirikachorn Tangkawattana⁴, Paul J Brindley⁵, and Banchob Sripa¹

¹WHO Collaborating Centre for Research and Control of Opisthorchiasis (Southeast Asian Liver Fluke Disease), Tropical Disease Research Laboratory, Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand ²Centre of Research Excellence for Therapeutic Proteins and Antibody Engineering, Department of Parasitology, Faculty of Medicine, Siriraj hospital, Bangkok 10700, Thailand ³Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand ⁴Department of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand ⁵Department of Microbiology, Immunology and Tropical Medicine, and Research Center for Neglected Diseases of Poverty, School of Medicine & Health Sciences, George Washington University, Washington, D.C., 20037, USA

Abstract

Opisthorchis viverrini, a food-borne trematode parasite endemic in the lower Mekong countries, is conventionally diagnosed by stool examination. However, parasitological stool-based diagnosis can be unreliable in light infections. The goal of this study was to develop the immunodiagnosis of opisthorchiasis using cathepsin F cysteine protease of *O. viverrini* in both indirect and sandwich ELISA assays. A recombinant *O. viverrini* cathepsin F (*rOv-CF*) of 40 kDa was expressed in *E. coli* strain BL21 (DE3), affinity purified, and deployed in ELISA assays. Human sera from 272 cases were investigated by indirect *rOv-CF* based-ELISA. Positive antibody response to *rOv-CF* was found in 137 out of 272 cases (50.37%) using a cut off OD (0.400) determined by ROC analysis. In comparison to parasitological stool examined for fluke eggs, the gold standard, the *rOv-CF* indirect ELISA showed a sensitivity and specificity of 62.1% and 84.05%, respectively. Serum antibody levels correlated well with egg counts per gram feces (EPG) ($P < 0.001$). In addition chicken IgY antibody was raised against *rOv-CF* was tested in a sandwich ELISA for detection of coproantigen in the feces of experimentally infected hamsters. The sandwich ELISA using this chicken IgY in combination with rabbit antibody to *O. viverrini* somatic antigens showed sensitivity and specificity of 93.3% and 78.57%, respectively. Together these findings indicated the potential of *rOv-CF* for diagnosis of opisthorchiasis, including for uses with chicken IgY for detection of coproantigens of *O. viverrini*.

Keywords

Opisthorchis viverrini; cathepsin F; immunodiagnosis; coproantigen detection; opisthorchiasis; food-borne trematodiasis

Introduction

Infection with *Opisthorchis viverrini* is a major public health problem in Southeast Asia, particularly in Thailand and Lao People Democratic Republic, and is a key risk factor for development of cholangiocarcinoma, bile duct cancer (Sripa et al. 2011). The prevalence of the infection remains high even though control programs have been implemented (Sripa et al., 2011; Sithithaworn et al., 2012). Diagnosis of opisthorchiasis is conventionally accomplished by stool examination and, indeed, this assay has remained as the gold standard test for decades. However, not only is the method laborious, the performance is dependent upon the expertise of the technician/microscopist. Therefore, alternative sensitive and specific diagnostic approaches such as immunologic or molecular diagnostics are considered desirable (McCarthy et al. 2012). Earlier reports indicate that lysates of *O. viverrini* or excretory secretory products from these flukes are useful as antigens for serodiagnosis (e.g., Wongratanacheewin et al. 1988; Akai et al. 1995). However, defined or purified *Opisthorchis* antigens may provide more sensitivity and specificity (Wongratanacheewin et al. 2003) and several purified parasite proteins have been tested with variable results including in our own laboratory (Laha et al. 2008; Sripa et al. 2012).

Cysteine proteases are secreted from many parasitic helminths where they participate in excystation, invasion, nutrition and other aspects of the host-parasite relationship (Robinson et al., 2008; Robinson et al., 2013). The enzymes including in recombinant form are well established in immunodiagnosis in related infections, with the Sm31 antigen (a cathepsin B) well established in diagnostic assays for schistosomiasis mansoni (Noya et al. 2001) and similarly with cathepsin L for serodiagnosis of fascioliasis (Robinson et al. 2013). Cathepsin B from *Clonorchis sinensis* also has been extensively used for serodiagnosis (Chen et al., 2011; Cornelissen et al., 2001). Notably, cysteine proteases have been reported and are secreted by *O. viverrini* adult worms (Kaewpitoon et al. 2008; Pinlaor et al. 2009). This study therefore aimed to develop a novel immunodiagnostic test based on a recombinant cathepsin F cysteine protease from *O. viverrini*.

Materials and methods

O. viverrini crude somatic antigen preparation and fecal processing

Hamsters were infected with 50 *O. viverrini* metacercariae. Four months later, the hamsters were euthanized after which adult *O. viverrini* worms recovered from the biliary system. These worms were used for preparation of a somatic antigen as described (Sripa and Kaewkes 2000). Briefly, the worms washed several times with saline, homogenized in PBS, pH 7.4 and the homogenate clarified at 10,000 x g at 4 °C for 15 min. The supernatant and the pellet, crude somatic extracts, were stored at -20 °C. Feces from uninfected (control) and *O. viverrini* infected hamsters were processed for coproantigen detection. Single pellets of feces were mixed with one ml of 20 mM Tris-HCl, 1% SDS, 0.5 M NaCl and 8 M urea (pH 7.5) (lysis buffer) and the mixture rotated overnight. The fecal slurry was centrifuged at 8,000 x g at 4°C, and the supernatant stored at -20 °C.

Human and animal sera

Sera from blood of 272 people were collected in Northeast Thailand who enrolled in opisthorchiasis project of the Tropical Disease Research Laboratory, Khon Kaen University. These included 203 cases of parasitologically confirmed opisthorchiasis, 43 with other helminthic infections and 26 parasite-negative sera as controls. Sera were stored at 20 °C until used. The latter controls were from individuals resident in non-endemic areas and absence of liver fluke eggs in the feces of the controls was confirmed by microscopic examination. In addition, 60 hamster fecal samples (46 *O. viverrini* positive and 14 *O. viverrini* negative) were individually subjected to coproantigen detection by sandwich ELISA. The Human Ethic Committee of Khon Kaen University (HE451132) approved the collection and investigation of the human and hamster samples.

Production and purification of recombinant *O. viverrini* cathepsin F

Recombinant *O. viverrini* cathepsin F (rOv-CF) was produced in BL21 (DE3) *Escherichia coli*. The region without signal peptide encoding the 308 amino acids of the secreted cathepsin F, Arginine 19 to Lysine 326 (Pinlaor et al. 2009) from the full length cathepsin F-like cysteine protease *O. viverrini* with the following accession number ACN68966 was amplified from a cDNA library of adult *O. viverrini* worms (Laha et al. 2007) using the following pair of primers: forward primer (5'-GCA TAT GAG AAC TAC CCC ATT CGAGCC TG) and reverse primer (5'-GCA TAT GCT ATT TGA CAA CGG CTG TAG TAA CTG C) with the *Nde*I restriction enzyme sites (underlined) to the 5'-ends. Thermo-cycling conditions for the PCR were: 30 sec denaturation at 98°C, 30 sec, annealing at 60°C and 30 sec extension at 72°C for 35 cycles, using a high fidelity polymerase (Phusion High-Fidelity DNA polymerase, New England Biolabs, UK). Following electrophoresis through agarose gel, the amplicons were purified and subsequently ligated into the vector pCR®-Blunt using a kit (Zero Blunt® PCR Cloning Kit, Invitrogen, USA). Recombinant plasmid was produced in *E. coli* transformed with the ligation products, and isolated using a plasmid extraction kit (GeneJET, Plasmid Miniprep Kit, Thermo Scientific, USA). The insert was released by digestion with *Nde*I, ligated into the expression vector pET15-b (Novagen, USA), and the sequence of the insert confirmed by nucleotide sequencing. Recombinant cathepsin F of *O. viverrini* was expressed in BL21 (DE3) (Novagen®, USA) in Luria Bertani medium containing 50 µg/ml ampicillin, and 2% glucose. When the bacterial cell density reached A₆₀₀ 0.5, 1 mM IPTG (isopropyl- β-D-thiogalactoside) was added and the culture incubated for 3 h. Bacterial cells were recovered by centrifugation at 8,000xg for 20 min. The recombinant cysteine protease was located in inclusion bodies, which were solubilized in binding buffer containing 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole and 8 M urea followed by 10-minute sonication at 20% amplitude, 0.5 cycle. The supernatant was filtered by Whatman™ Periodic 25mm filter (GE healthcare, UK), applied to a one ml volume His-Trap pre-packed column (GE healthcare, UK) and refolded gradually over 18 hours to remove the urea. Bound protein eluted from the column matrix in 0.5 M imidazole in 20 mM Tris-HCl, 0.5 M NaCl.

SDS-PAGE and immunoblotting

SDS-PAGE was carried out using a Mini Protein® II cell (Bio-Rad) under reducing conditions. The *rOv*-CF was diluted 1:1 in 0.5 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, 0.02% 2-mercaptoethanol (sample buffer) (1 µg/strip) and was sized-separated through a 10% gel in Tris-glycine electrode buffer (250 mM Tris, 192 mM glycine, 1% SDS, pH 8.3). Separated proteins were transferred to nitrocellulose membrane using a semidry blotter (Bio-Rad, Hercules, CA, USA). The membrane was cut into strips and blocked with 3% non-fat milk powder in PBS, pH 7.4 containing 0.05% Tween 20 (PBST) at 2 h at room temperature. The membrane strips were incubated with human serum diluted 1:100 for 2 hours and then washed with PBST. The strips were incubated for 2 h in goat anti-human IgG conjugated with horseradish peroxidase (Thermo Scientific, USA) at a dilution of 1:1000. Subsequently, the strips were washed with PBST three times and a final wash with PBS after which immunoreactivity was visualized with the substrate *o*-diaminobenzidine (DAB) (Thermo Scientific, USA) (Sripa et al. 2012).

Enzyme-linked immunosorbent assay (ELISA)

Purified *rOv*-CF diluted in 15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6 (coating buffer) to a final concentration of 1 µg was applied to the 96-well ELISA plate (Nunc Maxi-Sorp Immuno Plate, Denmark), 100 µl/well, overnight at 4°C. Excess antigen was washed with 400 µl of 154 mM NaCl, 0.05% Tween-20 (wash buffer). Non-specific binding was blocked with 5% non-fat dry milk in coating buffer for 2 hours in 37°C. After washing, the wells were filled with 100-µl of pre-diluted (1:500) human sera in 137 mM NaCl, 9 mM Na₂HPO₄.2H₂O, 0.8 mM NaH₂PO₄.2H₂O, 0.05 % Tween-20 (incubation buffer) and incubated at 37 °C for 1 h. Horse radish peroxidase conjugated rabbit anti-human IgG (Thermo Scientific, USA) diluted 1:10,000 in incubation buffer was applied for 1 h at 37 °C, washed and developed using 1-step Ultra TMB-ELISA substrate (Thermo Scientific, USA). The reaction was stopped with 0.5 M H₂SO₄ and optical density was determined at A_{450nm} using Versa Max micro-plate reader.

Preparation and purification of anti-*rOv*-CF chicken IgY

Two 18-week egg laying chickens (*Gallus domesticus*) were immunized with 0.5 mg of purified *rOv*-CF mixed with equal amounts of complete adjuvant followed by four boosts at three weeks intervals. Eggs were collected and IgY was purified using polyethylene precipitation (Pauly et al. 2011). Briefly, egg-shells were cracked delicately and yolks were separated using yolk separator in order to remove as much egg white as possible. Twice the egg yolk volume of PBS was mixed with the yolk, after which polyethylene glycol 6000 (PEG) (Sigma Aldrich, Germany) (3.5 % of the total volume) was added and vortexed, followed by 10 min rolling on a rolling mixer. Following centrifugation at 13,000 x g, 4 °C, 20 min, the supernatant was transferred to 8.5 % PEG 6000. The mixture was vortexed and rolled on a rolling mixer and centrifuged as above. The pellet was dissolved in 1 ml of PBS to a final volume of 10 ml. The solution was then mixed with 12% PEG 6000 (w/v, 1.2 gram) and treated as mentioned above. Finally, the entire pellet was dissolved in 800 µl of PBS. Protein concentration was determined spectrometrically at 280 nm, and the yield visualized in Coomassie-stained SDS-PAGE (10%) gels. Confirmation of the specificity of

the protein was accomplished by immunoblotting (as above). After washing and blocking of non-specific binding, the membrane was sequentially incubated in anti *rOv*-CF chicken IgY (1:1000) and rabbit anti-chicken IgY (1:1000) for 1 hour at room temperature each. After the final wash with PBS, immunoreactivity was visualized with the substrate α -diaminobenzidine (DAB) (Thermo Scientific, USA).

Development of IgY-based sandwich ELISA

Anti CF-IgY with the final concentration of 5 μ g/well in coating buffer, was coated onto the 96-well Maxisorp ELISA plate. The plate was incubated overnight at 4°C and washed three times with washing buffer. Skimmed milk (5%) in coating buffer was used to block non-specific reactive sites and the plates incubated at 37°C for 2 hours. Plates were washed subsequent to 1 hour incubation with 100 μ l of lysed positive and negative hamster feces. *O. viverrini* crude somatic antigen was used as positive control at a final concentration of 1 μ g/well. Excess antigens were washed and rabbit anti *O. viverrini*-somatic antibody (1:500) (Sripa and Kaewkes 2000) was incubated at 37 °C for 1 h. After washing, the plates were incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (1:5,000) (Thermo Scientific, USA), colour developed using 50 μ l of TMB substrate and optical density was determined at 450 nm with a spectrophotometer.

Statistical analysis

The optimal cut-off value for ELISA was evaluated based on receiver operating characteristic (ROC) curve analysis that correlated with true and false positive rates [sensitivity and (1-specificity)] (Bon et al. 2010). ROC curve and area under the curve (AUC) were carried out using STATA 12 (<http://www.stata.com/stata12/>) for indirect ELISA and R-3.0.3 software (<http://www.r-project.org/>) for sandwich ELISA. Specificity and sensitivity were calculated as follows: specificity = $TN/(TN + FP) \times 100\%$ and sensitivity = $TP/(TP + FN) \times 100\%$ where TN stands for true negative, TP for true positive, FN for false negative and FP for false positive. The samples were recorded as positive or negative based on the cut-off value, which was calculated as the mean Abs_{450 nm} value of the samples plus two standard deviations (SD). The sensitivity, specificity and positive and negative predictive values analyzed using the formalin-ethyl acetate concentration technique (WHO, 1995) as the gold standard method. The statistical significance between the different groups was performed with one-way ANOVA and two sample *t*-test (Sripa et al. 2012). Correlation between OD₄₅₀ and the ordinal infection level variable was calculated using Spearman's correlation coefficient. The data were analyzed with the aid of R-3.0.3 for Windows. *P* values ≤ 0.05 were considered statistically significant.

Results

Production, purification and immunoreaction of *O. viverrini* recombinant cathepsin F protease

The genomic regions encoding the active site of cathepsin F was amplified from a cDNA library, as a band at 927 bp following agarose gel electrophoresis (Fig.1A). Analysis of the deduced amino acid sequences (GenBank accession number KC661264) revealed 99% and 89% homology compared to cathepsin F-like cysteine protease of *O. viverrini* (Pinlaor et al.

2009) and *Clonorchis sinensis* (Kang et al. 2004), respectively. SDS-PAGE analysis of bacterial lysates before and after purification showed a polypeptide of ~40-kDa. Protein purification was carried out by 18-hour gradient refolding and more than 95% pure preparation when viewed in Coomassie-stained SDS-PAGE gel (Fig. 1B). After purification, the protein concentration was found to be ~1 mg/ml. Immunoblot analysis showed that rOv-CF was recognized by *O. viverrini* positive human sera, revealing a major band of recognition at 40 kDa, whereas control, non-*O. viverrini* infected sera did not show reactivity (Fig. 1C).

Sensitivity and specificity of rOv-CF as an immunodiagnostic tool

Following the affinity column purification and protein elution, an indirect ELISA was developed to test 272 human sera from an opisthorchiasis endemic region of Northeast, Thailand. Checkerboard titration was used to optimize the detection. The cut-off OD₄₅₀ level for positive infection status in the ELISA as determined from the ROC curve was 0.4003 and the area under the curve (AUC) was 0.7579 (95% confidence interval (0.692 to 0.823)). The curve was plotted between true positivity rate (sensitivity) and false-positivity rate (1-specificity) for different cut-off points of OD (Fig. 2A). Sera from human cases with high egg counts per gram (EPG) produced high OD values against recombinant *O. viverrini* cathepsin F cysteine proteinase with 62.06% sensitivity. With analysis against sera from non-infected human individuals and other helminthic infection, the system showed a specificity of 84.05 % (Table 1). Cross-reaction using serum was not evident in sera from people diagnosed with mono-infections of hookworm, *Taenia* spp, *Strongyloides stercoralis*, *Trichuris trichiura* and *Echinostoma* spp. However, five out of nine (55.55%) sera from human cases diagnosed with infection with minute intestinal flukes cross-reacted by rOv-CF indirect ELISA. Levels of specific antibody to rOv-CF showed significant correlation with intensity of *O. viverrini* infection as determined by EPG; EPG 1–500 = 33, EPG 501–1000 = 153, EPG 1001–2000 = 14, EPG 2001–10000 = 10 and EPG > 10000 = 9 (as shown in Fig. 2B) (Spearman $\rho = 0.308$, $P = 0.001$).

Chicken anti rOv-CF antibody production, purification and antigen recognition

The chicken antibody raised against rOv-CF was purified by removal of lipids followed by precipitation with PEG to obtain IgY. SDS-PAGE under reducing condition separated the heavy chain and light chain IgY; the heavy chain showed a major band of 70 kDa (Fig. 3A). Immunoblot analysis revealed a single band at 40 kDa with antibody to rOv-CF antigen (Fig. 3B).

Coproantigen detection using IgY-based sandwich ELISA

An anti-rOv-CF IgY-based coproantigen detection using Sandwich ELISA was established. The limit of detection was evaluated by spiking various concentrations of rOv-CF into diluted fecal samples from uninfected hamsters. The system detected *O. viverrini* crude antigens with a minimal detection limit of 70 ng/ml (data not shown). Experimentally infected hamster cases were screened by anti rOv-CF IgY-based Sandwich ELISA with 93.3% sensitivity and specificity of 78.57 % (Table 2). The ROC curve was plotted between true positivity rate (sensitivity) and false-positivity rate (100-specificity) for different OD cut-off points. The curve represents the suitable cut-off point at OD₄₅₀ of 0.5601. This cut-

off OD gave an area under the curve (AUC) of 0.958 (95% confidence interval (0.872 to 0.992) and revealed a significant difference between *O. viverrini* positive and negative groups ($P = 0.001$) (Fig. 4).

Discussion

Liver fluke infection with the fish-borne trematode *Opisthorchis viverrini* remains a major public health problem in the countries of Mekong region (Sithithaworn et al. 2012). Humans acquire the infection by eating raw, undercooked or salted fish (Prasongwatana et al. 2013). Disease of the hepatobiliary tract follows infection with *O. viverrini* infection both in people and in experimentally infected model species including hamsters (Sripa et al., 2011; Wonkchalee et al. 2011; Wu et al. 2011). Prevention and control of opisthorchiasis is warranted to reduce the disease morbidity and mortality of this neglected tropical disease. Due to several rounds of control programs have been implemented in Thailand and adjacent endemic countries, the prevalence of infection has declined gradually over recent decades. Nowadays the majority of the cases in many regions possess light infection (Sithithaworn et al. 2012; Miyamoto et al. 2014; Sayasone et al. 2015). This has presented investigators with the challenge of more sensitive and specific diagnosis for the infection (Müller et al., 2007; McCarthy et al. 2012; Duenngai et al. 2013). We recently reported coproantigen detection using monoclonal antibody against *O. viverrini* (Watwiengkam et al. 2013). Here we established an approach to immunodiagnosis of opisthorchiasis using both antibody and coproantigen detection, based on recombinant cathepsin F of *O. viverrini*.

Cathepsins have long been used for immunodiagnosis of infections with helminths including trematodes (Dixit et al. 2008). For diagnosis of human infection with a number of trematode pathogens, various families of cathepsin proteases have been shown to provide tractable antigens. These include cathepsin B of *Clonorchis sinensis* (Chen et al. 2011) and the cathepsin B termed Sm31 from the blood fluke *Schistosoma mansoni* (Klinkert et al. 1991). Our study demonstrated that *rOv-CF*, produced in *E. coli*, was a cost effective, informative reagent for use as an antigen for immunodiagnosis of opisthorchiasis. With human sera, the indirect ELISA showed 62 % sensitivity and 84 % specificity, indicating the potential value of *rOv-CF* in immunodiagnosis of the infection. The diagnostic sensitivity and specificity of *rOv-CF* indirect ELISA were comparable to the performance of the *rCsCB* protease as antigen in serodiagnosis of clonorchiasis (Chen et al. 2011), Sm31 for *S. mansoni* (Klinkert et al. 1991) or indeed recombinant cathepsin B of *O. viverrini* (termed *Ov-CB-I*) for serodiagnosis of opisthorchiasis (Sripa et al. 2012).

Currently, as the consequence of mass chemotherapy for opisthorchiasis in selected provinces and towns in the Isaan region of Thailand (Yeoh et al. 2015), there are frequent cases that are ELISA positive but stool parasite negative, which likely indicates low infection intensity where stool examination for parasite ova may reveal false negatives (Sithithaworn et al. 1991; Duenngai et al. 2013). However, antibody detection has limited application in diagnosis of current infection since past infection may sustain antibody titer for years after treatment (Wongratanacheewin et al. 1988). Coproantigen detection for current infection has been described for infection with other trematodes, including *F. hepatica* and *C. sinensis* (Mazidur Rahman et al., 2012). For opisthorchiasis, coproantigen

detection using monoclonal antibody to *O. viverrini* provides comparable sensitivity with the formalin ethyl-acetate concentration technique (FECT) (Sirisinha et al. 1995; Watwiengkam et al. 2013). However, the availability of monoclonal antibodies is limited. The potential of chicken IgY antibody and its advantages over IgG in antigen detection using sandwich ELISA for helminths have been described (Lei et al. 2009; Lei et al. 2011). Here we showed the utility of chicken IgY raised against *rOv*-CF in a sandwich ELISA for coproantigen detection of opisthorchiasis. We observed high sensitivity and specificity using rabbit anti-*O. viverrini* somatic antigens as the detection probe and the chicken IgY- *rOv*-CF antibody as capture antibody mounted with native cathepsin F in infected hamster feces.

To conclude, this report describes methods for the immunodiagnosis of opisthorchiasis based on a recombinant *O. viverrini* cathepsin F both by antibody (indirect ELISA) and coproantigen detection. Both approaches yielded worthwhile sensitivity and specificity for diagnosis of opisthorchiasis. We are now undertaking mass screening for opisthorchiasis in regions of Northeastern Thailand using the *rOv*-CF IgY-based sandwich ELISA coproantigen detection.

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Fig. 1. Expression, purification and immunoreaction of recombinant cathepsin F from *Opisthorchis viverrini*

(A) Gel image of the gene encoding *Ov-CF* amplified from a cDNA library prepared from adult stage *O. viverrini*. M: 50 bp ladder. Lane 1: *O. viverrini* cathepsin F at 927 bp is shown by the white arrow. (B) SDS-PAGE analysis of the recombinant *Ov-CF* protease. M: molecular mass standards (masses shown on the left side); lane 1: lysate of *E. coli* with *rOv-CF* before induction; lane 2–4: lysate of *E. coli* with *rOv-CF* after induction for 1h, 2h and 3h respectively; lane 5: flow throw, Lane 6–9 *rOv-CF* fractions 1–4 after affinity purification and refolding. (C) Western blot analysis of a pool of positive human sera from individuals with parasitologically proven infection with *O. viverrini*. Pooled negative human sera was used as the control, (N and P) negative and positive human opisthorchiasis sera, respectively.

Fig. 2. Efficacy of rOv-CF as antigen in an immunodiagnostic test

(A) Receiver operating characteristic (ROC) curve of recombinant cathepsin F from *Opisthorchis viverrini* (rOv-CF) employed as the antigen in an indirect ELISA for parasitologically negative and positive opisthorchiasis individuals. The curve represents the suitable cut-off point at OD₄₅₀ 0.4003 and showed the area under the curve (AUC) was 0.7579 (95% confidence interval (0.692 to 0.823)). (B) Serodiagnosis of *O. viverrini* infection among different infection intensity groups using the rOv-CF indirect ELISA. Data represent means $\pm$ 2 SD and are representative of 1 and 4 parasitologically negative and positive group respectively; these data show the positive correlation between EPG and rOv-CF indirect ELISA OD₄₅₀.

Fig. 3. Production, purification and specific immunoreaction of chicken IgY antibody raised against *rOv-CF*

(A) Extraction and purification of anti *rOv-CF* chicken IgY. M: Protein standards ladder. Lane 1: Position of heavy chain of purified anti *rOv-CF* IgY indicated with an arrow. (B) Immunoblot analysis of anti *rOv-CF* IgY. M: Protein standards. Lane 1: anti *rOv-CF* IgY recognized the purified *rOv-CF* antigen by revealing a single band of 40 kDa, indicated with an arrow.

Fig. 4. Efficacy of rOv-CF as an antigen detection immunodiagnostic test

Receiver operating characteristic curve of recombinant cathepsin F (rOv-CF) in IgY-based sandwich ELISA, calculated from OD at 450 nm values for feces from parasitologically negative and positive hamsters for opisthorchiasis. The curve represents the suitable cut-off point at OD 0.5601 and showed that the area under the curve (AUC) was 0.958 (95% confidence interval (0.872 to 0.992)).

Table 1

Comparison of the rOv-CF ELISA and fecal examination using the formalin-ethyl acetate concentration technique (FECT) (the latter being the gold standard method for) diagnosis of opisthorchiasis in sera from each of 272 people.

rOv-CF ELISA	<u>Detection by stool examination (FECT)</u>		
	Positive	Negative	Total
Positive	126	11	137
Negative	77	58	135
Total	203 (62.06%)	69 (84.05%)	272

Table 2

Comparison of the anti r*Op*-CF IgY-based Sandwich ELISA and fecal examination using formalin-ethyl acetate concentration technique (FECT) in samples of feces from each of 60 hamsters.

<i>rOp</i> -CF ELISA result	<u>Detection by stool examination (FECT)</u>		
	Positive	Negative	Total
Positive	43	3	46
Negative	3	11	14
Total	46 (93.47%)	14 (78.57%)	60