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Chicken IgY-based coproantigen capture ELISA for diagnosis of human opisthorchiasis

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Abstract

Diagnosis of *Opisthorchis viverrini* infection by conventional stool examination is increasingly difficult due to the low intensity of the infection after several rounds of control programmes in endemic regions as well as coinfections with intestinal flukes. Therefore sensitive and specific diagnostic test is needed. In this study, a coproantigen sandwich ELISA using recombinant *O. viverrini* cathepsin F (*rOv*-CF) was developed. This sandwich ELISA employing chicken IgY raised against *rOv*-CF in combination with rabbit IgG antibody to the somatic *O. viverrini* antigens showed a lower detection limit (LLD) of 70 ng native *O. viverrini* somatic antigens by spiking the parasite antigens into control feces. When applied to the diagnosis, the IgY-based sandwich ELISA exhibited sensitivity and specificity of 93.3% and 76.7%, respectively, in an investigation of 90 human cases positive or negative for opisthorchiasis. The positive predictive value (PPV) and negative predictive value (NPV) for this coproantigen detection were 66.7% and 95.2%, respectively. This IgY-based sandwich ELISA using parasite cathepsin F detection shows a promising immunodiagnostic alternative for human opisthorchiasis in endemic regions.

Graphical abstract

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Keywords

Opisthorchis viverrini; Cathepsin F; Immunodiagnosis; Coproantigen; Sandwich ELISA; Chicken IgY

1. Introduction

Human liver fluke infection caused by *Opisthorchis viverrini* and *Clonorchis sinensis* is a major public health concern in Southeast Asia, particularly Thailand, Laos People's Democratic Republic, Cambodia and Vietnam [1–3]. Generally the infection is acquired by ingesting raw or undercooked cyprinid fish products. Chronic and repeated infection with the liver flukes is associated with the fatal cholangiocarcinoma, an otherwise uncommon form of liver cancer [1]. Diagnosis is conventionally accomplished using the Kato–Katz method, although the eggs may not be detected due to biliary obstruction or intermittent egg excretion [4]. Multiple conventional stool examinations have shown to increase the sensitivity [5]; however, it is time consuming and difficult to do in practice. In addition, similarity in egg morphology to other trematodes such as *Haplorchis taichui* makes a difficulty in differential diagnosis [6]. Several serological and molecular tests have been introduced for substitution of conventional egg examination in the past decades [7–9]. Our group have also been working on serological and molecular diagnosis of opisthorchiasis for several years [10–12]. However, more sensitive and specific techniques are still needed. Recently, the WHO Disease Reference Group on Helminths (DRG4) prioritized research areas on coproantigen detection as for diagnosis of foodborne trematodiasis as it serves all the aforementioned problems [13].

Helminth cysteine proteases play important roles in many helminthic parasite activities that make the proteases potential targets for new immunotherapeutic and immunodiagnosics strategies [14]. Cathepsins have been extensively studied and their potential relevance as prognostic and diagnostic biomarkers has been discussed [15]. Due to the relatively high abundance in *O. viverrini*, focus on helminth cysteine proteases [16] for coproantigen detection offers a potential route forward in development of more sensitive diagnostic approaches. Given cathepsins B and F are abundant cysteine proteases in *O. viverrini* adult worm secretions, both cathepsins have potential for serodiagnosis of opisthorchiasis [17,18]. Unlike antibody detection, however, coproantigen assays can detect current infection even

more sensitive than egg examination particularly during the prepatent period, such as in *C. sinensis* infection [19]. *O. viverrini* cathepsin F may be of value in coproantigen detection.

Chicken IgY antibody, the major serum immunoglobulin in birds, has functions similar to mammalian IgG [20]. It is a stable immunoglobulin that can be simply obtained from egg yolk with high quantity with no necessity to bleed the laboratory animal. Interestingly, IgY does not activate the mammalian complement system, and does not bind to mammalian rheumatoid factors, protein A or G or cross-react with mammalian immunoglobulin which make it a better choice for both diagnostic and therapeutic applications [21]. With these advantages of chicken IgY and the abundance of *O. viverrini* cathepsin F, we have successfully developed IgY-based capture ELISA in a hamster model of opisthorchiasis [22]. In this study, we applied this coproantigen detection technique to the diagnosis of human opisthorchiasis from an endemic region of northeastern Thailand.

2. Materials and methods

2.1. *O. viverrini* crude somatic antigen

Syrian golden hamsters, *Mesocricetus auratus*, were infected with 50 *O. viverrini* metacercariae. Adult worms were collected four months later from the hamsters at necropsy. Somatic antigen was prepared from the liver flukes as described [23]. In brief, worms from hamster liver were carefully washed with normal saline and pulverized in PBS buffer. The homogenate was clarified by centrifugation at $10,000 \times g$ at 4°C for 20 minutes. The supernatant, the crude somatic antigen, was stored in aliquots at -20°C. The animal experiments and care complied with the national standard guideline for laboratory experimental animal and approved by Institutional Animal Ethics Committee (AEKKU Number 100/2555).

2.2. Recombinant cathepsin F of *O. viverrini*

The coding region of secretory *O. viverrini* cathepsin F was expressed in *Escherichia coli*, BL-21 strain (DE3) as previously [22]. Briefly, the cDNA encoding the protease was amplified from an library of *O. viverrini* cDNAs [24] using the forward primer 5'- GCA TAT GAG AAC TAC CCC ATT CGAGCC TG and the reverse primer 5'- GCA TAT GCT ATT TGA CAA CGG CTG TAG TAA CTG C; *Nde* I restriction enzyme sites are underlined. The amplicon was ligated into the expression vector pET15-b, after which BL21 (DE3) *E. coli* cells were transformed with the construct. Expression of recombinant cathepsin F was induced by 1 mM IPTG. Insoluble His tagged *O. viverrini* cathepsin F was solubilized in 20 mM Tris-HCl containing 0.5 M NaCl, 5 mM imidazole and 8 M urea followed by sonication. Recombinant cathepsin F was isolated on Ni²⁺ resin (1 ml pre-packed His Trap column, GE Healthcare, UK), subjected on the matrix to gradual refolding over 18 hours, and the recovered by elution from the column with 0.5 M imidazole in 20 mM Tris-HCl, 0.5 M NaCl.

2.3. Anti rOv-CF chicken IgY

Egg laying *Gallus domesticus* chickens were immunized as described [22]. Briefly 0.5 mg of purified rOv-CF was mixed with equal amount of complete adjuvant followed by 4 boosts

with 3 weeks intervals. Eggs were collected and the IgY was purified using polyethylene glycol precipitation [25]. The precipitated antibody was dissolved in 800 µl of PBS. Subsequently, the IgY was purified by gel filtration using HiPrep 16/60 Sephacryl S-200 HR (GE Healthcare, UK) and clarified by SDS-PAGE gel electrophoresis.

2.4. Cathepsin F immunoreaction

The rOv-CF (1 µg/strip), *O. viverrini* somatic antigen (1 µg/strip) and *O. viverrini* excretory secretory antigen (1 µg/strip) were subjected into a 10% SDS-PAGE and the separated proteins were transferred to nitrocellulose membranes at 15V for 30 minutes. Membranes were washed by washing buffer and blocked 3% non-fat milk. Each strip was individually treated with rabbit anti *O. viverrini* somatic antibody and chicken anti rOv-CF IgY. The strips were subsequently incubated with horseradish peroxidase (HRP) conjugated, goat anti- rabbit antibody (1:5,000) and rabbit anti-chicken IgY antibody (1:1,000), each for one hour, respectively. Signal detection by enzymatic reaction was developed using diaminobenzidine (DAB) as the substrate.

2.5. Human feces and preparation of coproantigen

Samples of fresh human feces were collected from liver fluke endemic areas of Khon Kaen province in Northeast Thailand during 2012–2014 and stored frozen in our sample banks (Ethics approval, HE480528). Thirty of each *O. viverrini* positive and negative individuals, and those with other helminthic infections were randomly selected for this study. One gram of each sample was individually mixed with lysis buffer containing Tris-HCl 20 mM, 1% SDS and 8 M urea. The mixture was rotated overnight at 4°C and centrifuged at $8,000 \times g$ for 10 minutes. The aqueous layer was collected and centrifuged again at $10,000 \times g$ for 5 minutes. The supernatant was collected and stored at 4°C.

2.6. Coproantigen capture ELISA

Coproantigen detection ELISA was applied to 30 *O. viverrini* positive and 30 negative individuals, and 30 samples with other helminthic infections. A coproantigen sandwich ELISA for detection of *O. viverrini* cathepsin F was carried out as described in experimentally hamsters [22]. Briefly wells of 96-well Maxisorp ELISA plates (Nunc, Denmark) were coated with 5 µg of CF-IgY in 100 µl of carbonate buffer, pH 9.6. The plate was incubated overnight at 4°C and the excess captured antibody removed by washes (3×) with washing buffer. The plate was incubated with 5% non-fat milk at 37°C to block nonspecific reactive sites and incubated thereafter with 100 µl of *O. viverrini* hamster positive and negative fecal samples for one hour. Excess fecal antigens were washed away and diluted rabbit antibody to *O. viverrini* somatic antigens (1:500) [10] applied for one hour at 37°C. After washing, the plate was incubated with goat anti-rabbit immunoglobulin -HRP conjugate (1:5,000) (Thermo Scientific, USA) and washed. Signals were developed using TMB (Thermo Scientific, USA) as the substrate, and measured at 450 nm using a spectrophotometer.

2.7. Limit of detection of capture ELISA for coproantigen

Sensitivity or detection limit of the established capture ELISA was determined by spiking the somatic antigen preparation of *O. viverrini* into samples of feces, negative for *O. viverrini* at decreasing antigen concentrations: 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, and 0.039 µg in 100 fecal µl of slurry. The Maxisorp ELISA plates were coated with CF-IgY antibody where sequentially incubated with fecal solution and rabbit anti *O. viverrini*-somatic antibody as above. After thoroughly washing steps, the plates were color developed in TMB substrate and optical density was determined at 450 nm. Lower Limit of Detection (LLD) was determined from the mean OD value of 20 replicates of the zero standard $\pm$ SD. Limit of detection was considered as mean OD + 2SD higher than mean OD of the blank.

2.8. Sensitivity and specificity of immunoassay

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)] [26]. ROC curve and area under the curve (AUC) were carried out using R 3.0.3 software (<http://www.r-project.org/>). Specificity and sensitivity were calculated respectively as 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 absorbance at 450 nm value of the samples plus two standard deviations (SD). The sensitivity, specificity, and positive and negative predictive values were analyzed using the formalin-ethyl acetate concentration technique as the gold standard method [27]. Groups were compared using one-way ANOVA and Student *t*-tests [17]. The data were analyzed with the aid of R-3.0.3 for Windows. A *P* value 0.05 was considered statistically significant. The coefficient of variation (CV) of the ELISA was less than 2 %.

3. Results

3.1. rOv-CF specific immunoreaction

Immunoblot analysis showed that native cathepsin F antigen was recognized by chicken anti rOv-CF IgY as a single band of 40 kDa. However, the rabbit anti-*Ov* somatic antibody detected several bands indicating the presence of different components of crude somatic antigens of *O. viverrini* (Fig. 1A). The two antibodies also recognized rOv-CF as a single band of 40 kDa (Fig 1.B).

3.2. Human opisthorchiasis coproantigen capture ELISA

An IgY-based coproantigen detection using sandwich ELISA was studied in feces from 90 human cases. The feces investigated included samples from 30 people who were positive for fecal eggs of *O. viverrini*, from 30 people who were negative for fecal eggs of *O. viverrini* and from 30 persons that were stool-positive for other helminth species (but negative for *O. viverrini*). The IgY based capture ELISA correlated well with the EPG data (infection intensity) ($R^2=0.43$, $P=0.05$). Receiver operating characteristic (ROC) curve constructed

from 60 individuals (each of 30 *Opisthorchis* positive- and negative cases) was used to determine the cut-off point: the optimal OD of 0.32 with an area under the curve (AUC) was 0.92 (95% confidence interval (0.82 to 0.96) was obtained (Fig. 2). With this cut-off point, the anti rOv-CF IgY-based sandwich ELISA gave 93.3% sensitivity and 76.7 % specificity against the fecal samples from 90 individual of *Opisthorchis* positive, negative and with other helminthic infections (Table 1). *Opisthorchis* positive group showed statistically significant higher levels of coproantigen (OD₄₅₀) than those without (Fig. 3) ($P = 0.001$). The positive predictive value (PPV) and negative predictive value (NPV) for the system were 66.7% and 95.2%, respectively. Cross-reaction from feces from cases with mono-infection of *Strongyloides stercoralis* was not seen, although weakly positive signals were evident from persons infected with *Taenia* spp, *Echinostoma* spp and/or minute intestinal flukes (MIF) (Table 2).

3.3. Detection limit of coproantigen capture ELISA

We characterized the limit of detection of the IgY-based sandwich ELISA by spiking serially diluted *Opisthorchis* somatic antigens in egg-negative, fecal slurries. From 20 replicate experiments; the mean $\pm 2SD$ of each concentration was plotted against the blank control without antigens. The SD bar did not show overlap at a concentration of 0.078 μg antigens, which was significantly different between the test and control (t -test, $P = 0.001$) (Fig 4). Accordingly, the lower limit of detection (LLD) of this IgY-based sandwich ELISA was 70 ng.

4. Discussion

Liver fluke infection caused by *Opisthorchis viverrini* remains a major health issue in Mekong countries where more than 10 million are infected [1]. Regardless of the efforts aiming to develop a more sensitive and specific detection, diagnosis largely remains based on conventional, stool examination – a method that both is time consuming and highly depended on the expertise of the technician. Moreover, the majority of cases nowadays harbour light infection, and can harbour coinfections with minute intestinal flukes (MIF) that possess similar egg morphology [6] which, together, make the diagnosis of opisthorchiasis challenging. Therefore, WHO prioritized the coproantigen detection as the approach to be used for diagnosis of foodborne trematodiasis [13]. Our IgY-based capture ELISA reported in this study showed a promising result for coproantigen detection and diagnosis of human opisthorchiasis.

Cysteine proteases, especially cathepsins, have long been used for immunodiagnosis of helminth parasitic infections [28]. These include the cysteine protease-like protein from the hookworm *Ancylostoma caninum* [29], plerocercoid cathepsin L-like protease of *Spirometra erinaceieuropaei* [30], and cathepsin B, an abundant enzyme of *O. viverrini* [17]. Coproantigen detection by capture ELISA has been popularly used for immunodiagnosis of many helminth infections because of its specificity [31]. Moreover, it indicates current (rather than past) infection unlike antibody detection where the antibody titre may not wane after treatment. There are reports describing the coproantigen detection in trematodes such as *F. hepatica* [32] and *C. sinensis* [33]. We have reported the establishment of coproantigen

detection method using recombinant *OV*-CF in hamsters infected with *O. viverrini* [22]. Here we applied the cathepsin F capture ELISA for coproantigen diagnosis of human opisthorchiasis. Making use of chicken IgY, which does not cross react with mammalian immunoglobulins [34,20] as a capture antibody for native cathepsin F in the feces and a rabbit anti-*Opisthorchis* somatic antigens as the second antibody, we observed sensitivity greater than 90%. Stability and proper binding of chicken IgY to ELISA plate are additional advantages of utilizing this antibody as a capture antibody [35].

This novel chicken IgY raised against immunogenic *O. viverrini* cathepsin F showed admirable recognition of both r*OV*-CF and native cathepsin F by western blot (Figure 1), providing assurance that it can react with native fecal cathepsin F. The optimized r*OV*-CF IgY capture ELISA for human opisthorchiasis coproantigen detection provided 93.3 % sensitivity and 76.7% specificity. The high sensitivity with LLD of > 70 ng of the test suggested that r*OV*-CF IgY-based capture ELISA is promising for application in immunodiagnosis of human opisthorchiasis. However, this capture ELISA showed a slight cross reactivity especially to individuals infected with MIF. However this may not be so problematic in opisthorchiasis endemic areas since the prevalence of infection with MIF nowadays is low, 0.1–2.9% [36]. Sharing similar antigens of over 70% of homology between *O. viverrini* cathepsin F and other flukes [18] likely explain the weak cross reactivity. Our overall results is in consistent with the studies of coproantigen detection for other helminthic infection including hookworm [37], *Fasciola hepatica* in cattle and sheep [38,39] as well as detection of circulating antigen of *F. gigantica* using sandwich ELISA [21,35,39,40].

Taken together, this report details an additional test targeting coproantigen to diagnose opisthorchiasis. The sandwich ELISA using r*OV*-CF IgY-based coproantigen detection is facile and indeed more convenient than fecal egg examination. The cost of the test may be apparently higher than stool examination but once developed; it can be scaled up for high throughput screening. We are now developing into a r*OV*-CF IgY-based immunochromatography that may be feasible for point of care (POC) diagnosis in the future. All in all, it holds clear promise as a new diagnostic for opisthorchiasis in endemic areas of northeastern Thailand and nearby Mekong countries.

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Highlights

- Recombinant *Opisthorchis viverrini* cathepsin F was expressed in *E. coli*.
- Coproantigen detection using *rOv*-CF based sandwich ELISA was established.
- Chicken IgY based sandwich ELISA is promising for diagnosis of human opisthorchiasis.

Figure 1. Immunoreaction of native and recombinant cathepsin F from *Opisthorchis viverrini*
 (A) Immunoblot analysis of *Opisthorchis viverrini* somatic antigen. Lane M Page Ruler Pre-stained Protein Ladder (Thermo Scientific), Lane 1: Somatic antigen treated with rabbit anti *Ov*-somatic antibody, Lane 2: Somatic antigen treated with chicken anti-r*Ov*-CF antibody. Two antibodies recognized the native cathepsin F by revealing a single band of ~40 kDa (indicated with arrow). (B) Immunoblot analysis of r*Ov*-CF. Lane M Page Ruler Pre-stained Protein Ladder (Thermo Scientific) Lane 1: r*Ov*-CF treated with rabbit anti *Ov*-somatic antibody, Lane 2: r*Ov*-CF treated with chicken anti r*Ov*-CF IgY. Both antibodies recognized the recombinant cathepsin F by revealing a single band of ~40 kDa (indicated with arrow).

Figure 2. Efficacy of rOv-CF as an antigen detection immunodiagnostic test

Receiver operating characteristic curve (ROC) for human opisthorchiasis in a total of 60 human positive and negative opisthorchiasis cases. The area under the curve was 0.921 with the 95% confidence interval.

Figure 3. Levels of *Opisthorchis viverrini* cathepsin F coproantigen rOv-CF-IgY-based capture ELISA in infected and non-infected individuals

Data represent means $\pm$ SD in parasitologically negative (0) and positive groups (1) ($P < 0.001$) analyzed by unpaired Student *t*-tests.

Figure 4. Detection limit of diagnostic assay

Quantitative coproantigen capture ELISA for examining the lower limit of detection; 2-fold varied concentration of *Ov*-somatic antigens were spiked into negative feces and subjected to r*Ov*-CF IgY based capture ELISA. Significant difference between high and low concentrations of *Ov*-somatic antigen was evident. The LLD considered as mean OD+ 2SD higher than the LOB (the mean OD of blank). Slight difference is seen between the sample without antigen (0) and the blank control (B). From this graph, the lower limit of detection (LLD) in IgY-based sandwich ELISA is equal to 70 ng (0.7µg). The dotted line represents the cut-off the ELISA.

Table 1

Comparison of IgY capture ELISA with fecal examination in humans: the formalinethyl acetate concentration technique (FECT) was used as the gold standard and ELISA method was compared to FECT for diagnosis of opisthorchiasis in a total of 90 human feces samples. In comparison to FECT, the r*Op*-CF sandwich ELISA showed 93.3% sensitivity and 76.7% specificity.

Ig Y-based sandwich ELISA	Detection by stool examination (FECT)		
	Positive	Negative	Total
Positive	28	14	42
Negative	2	46	48
Total	30 (93.33%)	60 (76.66%)	90

Table 2

Cross reactivity of coproantigen rOv-CF IgY-based capture ELISA with other-helminthic infections.

Helminth pathogen	Number Positive/Total (%)
Minute intestinal flukes (MIF)	3/9 (33%)
<i>Taenia</i> spp	1/6 (16.16%)
<i>S. stercoralis</i>	0/7 (0%)
<i>Echinostoma</i> spp	2/8 (25%)
Total	6/30 (20%)