Evaluation of liver fluke recombinant cathepsin B-1 protease as a serodiagnostic antigen for human opisthorchiasis

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**A R T I C L E   I N F O**

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**A B S T R A C T**

A cathepsin B-like cysteine protease belonging to family C1 is abundantly expressed in the transcriptome and proteome of the caragicenic liver fluke of humans, *Opisthorchis viverrini*. This enzyme is present in excretory/secretory (ES) products released by parasites cultured in vitro. This study evaluated the performance of recombinant O. viverrini cathepsin B1 (rOv-CB-1) as an antigen for immunodiagnosis of opisthorchiasis. The full length Ov-CB-1 cDNA was cloned and recombinant protein was produced in catalytically active form in *Pichia pastoris*. The recombinant Ov-CB-1 (rOv-CB-1) was affinity purified via nickel-NTA chromatography and tested in enzyme-linked immunosorbent assays (ELISA) with human sera from an opisthorchiasis endemic area. Sera from egg-positive *O. viverrini* infections produced a strong IgG antibody response to rOv-CB-1 both in ELISA and immunoblot analysis. The sensitivity and specificity of the ELISA test was 67% and 81%, respectively. These findings support the feasibility of using recombinant Ov-CB-1 in ELISA for the serodiagnosis of human opisthorchiasis.

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**1. Introduction**

Opisthorchiasis remains a major public health problem in Southeast Asia, particularly in Thailand where the highest prevalence occurs in northeastern provinces including Khon Kaen, Maha Sarakham, Nong Khai and Sisaket [1]. Although control programs have been implemented at the community level for more than three decades, prevalence remains high [1–3]. Appropriate methods to ascertain prevalence and infection intensity are critical for designing suitable interventions and control. To date, several techniques have been developed to diagnose opisthorchiasis, in addition to parasitological methods focused on microscopical identification of *Opisthorchis viverrini* eggs in stool samples. These latter techniques exhibit only low sensitivity in light infections [2,4,5]. Although fecal examination for the characteristically shaped eggs provides definitive diagnosis of infection with *O. viverrini*, serodiagnosis for the detection of specific antibody can be expected to have advantages as an alternative diagnostic approach for opisthorchiasis when investigating large populations in endemic regions such as the provinces of the Khorat Plateau of Thailand and the Lao provinces bordering the Mekong River, and especially for epidemiological surveys.

Detecting antibody in serum is a suitable method to estimate infection rates [6]. In past epidemiological surveys, crude extracts of *O. viverrini* worms – either from the tissues (somatic antigen preparations) and/or the excretory/secretory products (ES) released by cultured flakes in vitro – have been employed as antigens for serodiagnosis [7,8]. However, preparation of fluke-derived antigens is tedious with maintenance of the *O. viverrini* parasite in the laboratory and samples of metacercariae to infect laboratory hamsters reliant on natural sources of infected, wild fishes, which are unpredictable and variable depending on the season [9]. In addition, potential batch-to-batch variability in serodiagnostic performance compund the unsatisfactory attributes of reliance on somatic and ES antigens of *O. viverrini* for routine serodiagnosis.

By contrast, the use of a recombinant antigen for antibody detection is advantageous since recombinant proteins can be produced on preparative scale to develop more convenient and inexpensive serological assays. Recently, the performance of recombinant asparaginyl endopeptidase of *O. viverrini* was described. However, wide scale deployment of this antigen for serodiagnosis has been hampered by difficulties in refolding the recombinant protease to a natural, soluble conformation [10]. By contrast, the Sm31
antigen or cathepsin B (SmCB-1) of the human blood fluke, *Schistosoma mansoni* has been widely used for serodiagnosis of schistosomiasis mansoni [11,12]. In the present study, we produced a recombinant form of the cysteine protease cathepsin B1 of *O. viverrini* and investigated its performance and potential in an enzyme linked immunosorbent assay for serodiagnosis of human opisthorchiasis.

2. Materials and methods

2.1. Source of serum samples

A total of 145 human serum samples used for the establishment and testing of ELISA and immunoblotting assays were collected from the villages in opisthorchiasis endemic areas in Khon Kaen province, Thailand, supplied by the Tropical Disease Research Laboratory, Khon Kaen University. The samples included 87 sera from subjects with egg-positive *O. viverrini* infection and 58 sera from subjects who were negative by fecal microscopy for *O. viverrini* infection but positive for *Strongyloides stercoralis* (8), minute intestinal flukes (14), echinostomes (11), hookworms (15), *Taenia* species (3) and *Giardia lambila* (7). Twenty sera from subjects who were negative by fecal examination for *O. viverrini* and lived in a non-endemic area were used as negative control samples. In each of these 145 cases, the infection status for several species of gastro-intestinal parasites was established by a single microscopic examination of stool samples prepared using the formalin ethyl acetate concentration technique [13]. Corresponding sera were aliquoted and stored at −20 °C until used. Collection of these samples was approved by the Ethic Committee of Khon Kaen University, approval number HE451132.

2.2. Production of recombinant *O. viverrini* cathepsin B1

Recombinant *O. viverrini* cathepsin B1 (rOv-CB-1) (GenBank accession number ACT99885) was produced in *Pichia pastoris* as described [14]. Recombinant proteins were purified using Ni-NTA affinity columns (Novagen) and dialyzed against phosphate buffered saline (PBS) at pH 7.2–7.4 through dialysis membrane (SnakeSkin™ Pleated Dialysis Tubing, Pierce) for 4 h at 4 °C and stored at −20 °C until used. Protein concentration was determined by the method of Bradford [15]. The recombinant enzyme was catalytically active as described by us previously [14].

2.3. SDS-PAGE and immunoblotting

SDS-PAGE [16] was carried out using a Mini Protein® III cell (Bio-Rad) under reducing conditions. The rOv-CB-1 was diluted 1:1 in a sample buffer (0.5 M Tris–HCl pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, 0.02% 2-mercaptoethanol) (100 μl, 0.5 mg/ml) and was sized-separated through a 15% gel in Tris–glycine electrode buffer (25 mM Tris, 192 mM glycine, 1% SDS, pH 8.3). Following SDS-PAGE, separated proteins were transferred to nitrocellulose membrane (Bio-Rad) using a semidry blotter (Bio-Rad). The membrane was cut into strips and blocked with 5% non-fat milk powder in PBS, pH 7.4 containing 0.05% Tween 20 (PBST), overnight at 4 °C. The membrane strips were incubated with human serum at a dilution of 1:100 for 2 h and then washed with PBST, five times, each wash for 5 min. The strips were incubated for 2 h with goat anti-human IgG conjugated with horseradish peroxidase (HRP, Zymed) at a dilution of 1:1000. Subsequently, strips were washed with PBST four times, 5 min each and a final wash with PBS after which immunoreactivity was visualized with the substrate o-diaminobenzidine (DAB) (Zymed).

2.4. Enzyme linked immunosorbent assay (ELISA)

rOv-CB-1 in PBS was diluted to 0.5 μg/ml in 15 mM Na2CO3, 35 mM NaHCO3, pH 9.6 (coating buffer) and applied to a 96-well plate (Nunc Maxi-Sorp Immuno Plate, Denmark), 100 μl/well, overnight at 4 °C. Each well was washed with 200 μl of 154 mM NaCl, 0.05% Tween-20 (wash buffer) for 3 min, three times. Antigen was blocked with 200 μl/well of 3% BSA in coating buffer at 37 °C for 2 h. Plates were washed with 200 μl/well of wash buffer for 3 min, three times. One hundred microliters of serum diluted 1:1,000 in 3% BSA in 137 mM NaCl, 9 mM Na2HPO4, 2H2O, 0.8 mM NaH2PO4, 2H2O, 0.05% Tween-20 (incubation buffer) was added and incubated at 4 °C overnight. Plates were washed with wash buffer, as above. The secondary antibody, anti-human IgG (Fc)-HRP (Zymed), was diluted 1:3,000 and 100 μl was added and incubated for 1 h at 37 °C. Plates were washed with wash buffer as above followed by an additional wash with PBS for 3 min. Tablets of substrate o-phenylenediamine (OPD) (Zymed) dissolved in citrate buffer pH 5.0 (one tablet/12 ml) were used to develop colorimetric changes by adding 100 μl/well OPD solution and incubating for 30 min at 37 °C. Reactions were terminated by addition of 0.5 M sulfuric acid (H2SO4), 100 μl/well. Optical density at 492 nm (OD492) was determined using a plate reader (TECAN). All samples were assayed in duplicate. Each plate was standardized with blank (non-coated antigen), positive and negative control sera. The positive control was a pool of sera from persons stool positive for *O. viverrini* egg in feces. The positive pool of sera exhibited high titers of serum IgG against ES. The negative control was a pool sera from persons who were negative by fecal examination and lived in non-endemic area of opisthorchiasis.

2.5. 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)] [17]. ROC curve and area under the curve (AUC) were carried out using MedCalc software (http://www.medcalc.be/) (Mariakerke). The sensitivity, specificity and positive and negative predictive values were calculated using the formalin-ethyl acetate concentration technique (FECT) as the gold standard method [13]. The quantitative variables were individual test for normality with one-sample Kolmogorov–Smirnov test. The
statistical significance between the different groups was performed with one-way ANOVA. Analysis of the relationship between OD492 and O. viverrini eggs per gram of human feces (EPG) was performed with the Kruskal–Wallis H test (nonparametric analysis of variance). The data were analyzed using SPSS 16.0 for Windows. P values of ≤0.05 were considered to be statistically significant.

3. Results

3.1. Sera from opisthorchiasis subjects recognized recombinant Ov-CB-1

rOv-CB-1 was expressed and secreted in soluble form into culture medium by P. pastoris. Affinity purified rOv-CB-1 migrated in SDS-PAGE as a single species at ~44 kDa, which is the active form of the enzyme [14] (Fig. 1, lane 3). Immunoblot analysis showed that rOv-CB-1 was recognized by O. viverrini positive human sera, revealing a major band of recognition at 44 kDa, whereas control, non-O. viverrini infected sera showed no reactivity (Fig. 1).

3.2. Efficacy of rOv-CB-1 as an immunodiagnostic tool

An indirect ELISA was developed to test 145 human sera collected from an endemic area for opisthorchiasis in Khon Kaen province, Thailand. There were 87 positive O. viverrini infection cases, 44 male and 43 female individuals. The O. viverrini EPG range for these 87 cases was 8 to 6668 EPG, determined using a modified FECT. Persons positive for fecal O. viverrini egg were classified into 4 groups based on range of EPG. The number of subjects in each group were EPG 1–500 = 63; EPG 501–1000 = 11; EPG 1001–1500 = 4; EPG >1500 = 9 (as shown in Fig. 2). The cut-off OD492 level for positive infection status in the ELISA test as determined from the ROC curve was 0.76 (Fig. 2A). Human sera from persons diagnosed parasitologically with O. viverrini infection produced high OD values, with 67% sensitivity. Sera from humans infected with other gastrointestinal parasites showed a specificity of 82.76% (Table 1). The positive and negative predictive values of the test were 85.29% and 62.34%, respectively.

The correlation of IgG level and fecal egg counts among several infection intensity categories indicated that the IgG titers of the high intensity group were higher than the low intensity group (Fig. 2B). However, the mean level of IgG in the heavy infection group (EPG >1500) was lower than in persons with lighter infection levels. The significantly lower antibody responses in heavy infections compared to titers in persons with milder infection may be the result of O. viverrini infection induced immunosuppression [18]. Accordingly, rOv-CB-1 exhibits potential as a diagnostic antigen for ELISA-based...
serodiagnosis for opisthorchiasis in this endemic area of Thailand as indicated by the concordance of absorbance value with the intensity of *O. viverrini* infection.

### 4. Discussion

The findings from this present study indicate that *Ov*-CB-1 shows potential for development and deployment as an immunodiagnostic tool for opisthorchiasis. Human sera from parasitologically proven cases of *O. viverrini* infection showed strong reactivity with r*Ov*-CB-1. An indirect ELISA for detection of serum IgG antibodies specific for r*Ov*-CB-1 yielded levels of sensitivity (67%) and specificity (82%). The relative ease and modest expense of production of r*Ov*-CB-1 in *P. pastoris* and affinity purification of a soluble form of this enzyme are key attributes that make r*Ov*-CB-1 particularly attractive as a serodiagnostic antigen [14]. By way of comparison to similar antigens, the diagnostic efficiency of r*Ov*-CB-1 is similar to that exhibited by Sm31 for serodiagnosis of human schistosomiasis mansoni [11,19]. Nonetheless, cross reactivity with antigens from other helminthes and/or that past infection with *O. viverrini* may complicate the interpretation of positive ELISA outcomes. In addition, it was shown that FECT cannot detect light infection but the IgG level against *O. viverrini* antigen still can be detected in light infection group [4,13]. The cases that were FECT negative but ELISA positive might represent low intensity infections, where only few eggs are shed in the feces (and therefore missed by the single egg count) but an active infection is present and detectable via circulating antibodies to secreted proteins such as r*Ov*-CB-1.

An important approach will now be to determine whether *Ov*-CB-1 can differentiate between present and past infection. Fortunately, we have potential access to informative serum samples from individuals both before and following treatment with praziquantel for *O. viverrini* infection. Through a large longitudinal study we are undertaking on liver fluke-induced cholangiocarcinoma in Khon Kaen province [20]. Moreover, we plan to investigate whether synthetic peptides from *O. viverrini* cathepsin B might improve sensitivity and specificity, as has been seen with Sm31 in regions of low prevalence [21].

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### Table 1

<table>
<thead>
<tr>
<th>Parasites</th>
<th>No. of serum samples</th>
<th>No. positive of <em>Ov</em>-CB-1 ELISA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Echinostome</td>
<td>11</td>
<td>2 (3.45)</td>
</tr>
<tr>
<td><em>Giardia lamblia</em></td>
<td>7</td>
<td>2 (3.45)</td>
</tr>
<tr>
<td>Hookworm</td>
<td>15</td>
<td>2 (3.45)</td>
</tr>
<tr>
<td>Minute intestinal fluke</td>
<td>14</td>
<td>2 (3.45)</td>
</tr>
<tr>
<td>Strongyloides stercorealis</td>
<td>8</td>
<td>1 (1.72)</td>
</tr>
<tr>
<td><em>Taenia</em> sp</td>
<td>3</td>
<td>1 (1.72)</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>10 (17.24)</td>
</tr>
</tbody>
</table>

### References


