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# *Opisthorchis viverrini*: Evaluation of 28 kDa glutathione S-transferase as diagnostic tool in human opisthorchiasis

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ABSTRACT

The liver fluke *Opisthorchis viverrini* is the agent of human opisthorchiasis in Thailand with a high prevalence observed in the rural population of north and northeastern regions of the country. A focus of research has therefore been the development of diagnostic tools to indicate infection by this parasite. In the present study, a 28 kDa glutathione S-transferase of *O. viverrini* (OV28GST), which is found in the excretion/secretion product of the parasite, was evaluated for its application in diagnosis of human opisthorchiasis. Bacterially expressed and functionally active rOV28GST was used in immunoblots and indirect ELISA to detect anti-OV28GST antibody in sera of infected individuals. Crude whole worm extract, sera of uninfected individuals and a rabbit anti-rOV28GST antiserum were used as controls in the assays while positivity for parasite DNA by PCR and egg count in faeces were used as primary indicators of infection. The results showed weak or absent reactivity of the infected sera to immunoblotted rOV28GST and no significant difference in absorbance values when compared to uninfected sera in ELISA. In addition, a glutathione capture ELISA which was performed to test for circulating OV28GST in human and hamster sera showed negative results. In conclusion, OV28GST is not applicable as a diagnostic tool in established infections due to low specific antibody titre and abundance as circulating antigen.



## 1. Introduction

In 2003, Jongsuksuntigul and Imsomboon reported human opisthorchiasis in Thailand with an overall prevalence rate of 9.6% with 19.7 and 15.3% prevalence in North and Northeast Thailand. The infection may persist unnoticed for an extended period of time if the number of parasites is small and pathology limited. Treatment with Praziquantel is commonly used to terminate the infection but a high rate of reinfection has been reported (Upatham et al., 1988). Furthermore, in the past years a strong link has been established between long-time infection and the development of cholangiocarcinoma (for recent reviews see Kaewpitoon et al., 2008; Sripa and Pairojkul, 2008) and it is therefore recommendable to have a fast, robust routine diagnostic assay available which could be performed at low cost at local hospitals to prevent asymptomatic chronic infections. At present, microscopic examination of parasite eggs in the faeces of infected individuals is still a standard method for diagnosis of opisthorchiasis. Several attempts have been made to develop a more effective diagnostic assay including recent PCR approaches to detect the parasite's DNA in stool samples (Duenngai et al., 2008; Suksumek et al., 2008; Umesha et al., 2008). Native protein from *O. viverrini* has been used to detect specific antibody in serum (Srivatanakul et al., 1985; Tesana et al., 2007) but is not suitable for routine diagnosis due to the required amounts. In 2006, we evaluated a recombinant eggshell protein of the parasite as an diagnostic tool in human opisthorchiasis (Ruangsittichai et al., 2006). Expression in the bacterial host was low and specificity/sensitivity were not sufficient, moreover eggshell proteins carry secondary modifications (conversion of tyrosine to 3,4-dihydroxyphenylanaline [dopa]) which are not present in the recombinant protein. More recently, a recombinant asparaginyl endopeptidase of the parasite was analysed for its diagnostic potential (Laha et al., 2008). This protein needed *in vitro* refolding to become active and correct folding might also be necessary to obtain the highest sensitivity in a diagnostic application. In 2004, we described a 28 kDa glutathione S-transferase of the parasite (OV28GST, GenBank accession no. AAL23713) and demonstrated that it can be abundantly expressed as a functional protein in *Escherichia coli* (Eursitthichai et al., 2004). In this study, we have evaluated OV28GST as a diagnostic tool in human opisthorchiasis due to its presence in the excretion/secretion product of the parasite (Eursitthichai, V. Molecular cloning of antigen encoding

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**Table 1**
Case data of *O. viverrini* egg positive and negative individuals including mean and standard deviation for age, fecal egg count, and ELISA absorbance values against crude worm (CW) and recombinant OV28GST.

| No. | Sex | Age (years) | Fecal egg count | PCR | $A_{492}$ CW | $A_{492}$ GST |
|---|---|---|---|---|---|---|
| Egg-positive individuals | | | | | | |
| 1 | M | 45 | 90 | + | 3.162 | 1.766 |
| 2 | F | 64 | 513 | + | 2.929 | 1.572 |
| 3 | F | 63 | 15 | + | 1.741 | 2.476 |
| 4 | F | 35 | 2907 | + | 2.477 | 1.857 |
| 5 | M | 48 | 656 | + | 2.501 | 2.291 |
| 6 | F | 68 | 288 | + | 2.414 | 2.046 |
| 7 | F | 42 | 18.5 | + | >3.5[a] | 1.975 |
| 8 | F | 44 | 40 | + | 2.521 | 1.843 |
| 9 | M | 42 | 110 | + | 1.771 | 2.282 |
| 10 | F | 42 | 868 | + | 1.814 | 1.931 |
| 11 | M | 58 | 330 | + | 2.514 | 1.862 |
| 12 | M | 64 | 14.8 | + | 0.946 | 1.652 |
| 13 | F | 35 | 2899 | + | 1.864 | 2.303 |
| 14 | F | 32 | 74.3 | + | 1.855 | 2.584 |
| 15 | M | 46 | 210 | + | 2.159 | 1.763 |
| 16 | M | 58 | 37 | + | 1.929 | 1.855 |
| 17 | F | 51 | 30 | + | 1.264 | 1.829 |
| 18 | M | 62 | 2174.3 | + | 1.832 | 1.607 |
| 19 | M | 52 | 160 | + | 1.387 | 1.445 |
| Mean | | 50.05 ± 11.08 | 601.8 ± 955.7 | | 2.060 ± 0.575 | 1.944 ± 0.313 |
| Egg-negative individuals | | | | | | |
| 1 | M | 31 | 0 | – | 0.918 | 2.069 |
| 2 | M | 50 | 0 | – | 1.235 | 1.380 |
| 3 | F | 55 | 0 | – | 1.345 | 2.185 |
| 4 | M | 54 | 0 | – | 1.025 | 1.361 |
| 5 | F | 62 | 0 | – | 1.306 | 1.634 |
| Mean | | 50.40 ± 11.67 | 0 | | 1.166 ± 0.186 | 1.726 ± 0.384 |

[a] Excluded value (above upper limit of plate reader).

genes from *Opisthorchis viverrini* (liver fluke) and primary analysis of encoded protein. Ph.D. Thesis, Mahidol University, Bangkok, 2004).

## 2. Materials and methods

### 2.1. Human sera

The human sera used in this study were provided by the Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand. They were collected together with stool samples in a survey of the population from two villages in Khon Kaen Province, a highly *O. viverrini* endemic area in Northeast Thailand. The sera were classified as uninfected or *O. viverrini* infected sera based on presence/absence of parasite eggs in the stool samples as observed by microscopic examination and by PCR with *O. viverrini* specific primers (Umesha et al., 2008) (Table 1). The performed research was approved by the Human Ethics Committee of Thammasat University (22 October 2007, project no. 013/2007). The human sera were sampled in the project "Relationship between parasite-specific antibody levels, hepatobiliary diseases and prevalence of liver fluke infection in Khon Kaen Province" approved by the Khon Kaen University Ethics Committee (22 April 2005, project no. HE480315). Participants in the survey who tested positive received a single treatment with praziquantel to terminate the infection at local public health care centers.

### 2.2. Adult parasites and preparation of crude worm (CW) extract

Six- to eight-week-old hamsters (*Mesocricetus auratus*) were each orally infected using a stomach tube with 50 metacercariae obtained from naturally infected cyprinoid fishes from Khon Kaen Province, Thailand. Briefly, fishes were blended and incubated in pepsin solution (0.25% pepsin, 1.5% HCl in 0.85% NaCl) at 37 °C for 2 h with gentle shaking. After filtration to remove large particles the metacercariae were sedimented by gravity in normal saline solution. The isolated metacercariae were washed and kept in normal saline solution until used for infection. Parasites were isolated 8 weeks after infection from the livers of sacrificed hamsters, washed in normal saline solution and kept in liquid nitrogen. Serum samples were taken from the hamsters preinfection and at weeks 2, 4, 6 postinfection. For preparation of CW extract the parasites were suspended and homogenised in lysis buffer (10 mM Tris–HCl, pH 7.2, 150 mM NaCl, 0.5% Triton-X 100, 1 mM EDTA). The homogenate was rotated at 4 °C for 1 h and insoluble matter pelleted at 12,000 × *g* for 15 min. The supernatant was collected and used as CW extract. The protein concentration was determined using a Bradford protein assay (Bio-Rad Protein Assay Kit II, Bio-Rad Laboratories, Hercules, CA, USA).

### 2.3. Preparation of recombinant OV28GST (rOV28GST) and rabbit anti-rOVGST28 antiserum

The open reading frame-containing cDNA fragment of OV28GST (GenBank accession number AY057838) was inserted into the pET21a expression vector (Novagen, Madison, WI, USA) and, after transformation of *E. coli* strain BL21 with the recombinant plasmid, expression and purification of functional active rOV28GST was achieved as previously described (Eursitthichai et al., 2004). A New Zealand white rabbit was subcutaneously immunised three times with 50 μg rOV28GST in complete (first immunisation) and incomplete (second and third immunisation) Freund's adjuvant to obtain a polyclonal anti-rOV28GST antibody.

### 2.4. SDS-PAGE and immunoblots

Separation of CW extract (20 μg) and rOV28GST (400 ng) was performed by SDS-PAGE (12.5%) according to Laemmli (1970) using