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Parasites Elicited Cross-Reacting Antibodies to *Opisthorchis viverrini*

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SUMMARY **Two batches of crude antigens extracted from adult *Opisthorchis viverrini* worms were compared. One was derived from adult worms harvested from the livers of laboratory infected hamsters and another was obtained from worms sedimented from the faeces of opisthorchiasis patients following treatment with Praziquantel. SDS-PAGE and Coomassie brilliant blue staining revealed that the two preparations had similar protein components of which the predominant ones were the 17-18 kDa doublet. The antigens were used in an indirect ELISA for the detection of antibodies against *O.viverrini* in the sera of four groups of patients, *ie.* patients with opisthorchiasis (group 1), patients with mixed infections of *O.viverrini* and other parasites (group 2), patients with other parasitic infections (group 3), and normal-heathy, parasite-free individuals (group 4). The sensitivity of the test was high (91-92%), regardless of the batch of the antigen used. However, its specificity was relatively low (70-80%). Cross-reaction was observed with patients infected with *Paragonimus heterotremus*, *Schistosoma* spp.; *Taenia* spp.; *Trichinella spiralis*; *Strongyloides stercoralis;* hookworms; *Plasmodium* spp.; hookworms and *Plasmodium* spp.; *S. stercoralis, Blastocystis hominis* and yeast; and hookworms, *Ascaris lumbricoides, Trichuris trichiura* and *P.falciparum*. Western blot analysis revealed that sera of patients infected with these heterologous organisms contained antibodies reactive to *O.viverrini* antigenic components ranging from *Mr* 15.5 to 144.**

Opisthorchiasis viverrini, the liver fluke infection caused by *Opisthorchis viverrini* is a disease of public health concern of Thailand. The infection rarely produced acute clinical disease which is different from the infection caused *by O. felineus*, but rather be chronic in nature and may persist for many years.[1] The majority of infected individuals are symptomless.[2] Clinical manifestations of symptomatic cases vary from mild to severe form depending upon the number of worms present and the duration of the infection. Severe cases are more common in patients over 40 years old and males are more affected than females.[3,4] Some patients may harbour thousands of flukes at any one time, suggesting that the mechanism for parasite elimination is not effective and/or that re-infeciton is common in this disease. Chronic persistent infection with large number of worms can lead to chronic relapsing cholangitis and obstructive jaundice,[5] and may in some cases eventually lead to cholangiocarcinoma.[6,7]

Since *Opisthorchis viverrini* infections are not pathognomonic, diagnosis is based mainly on demonstration of the fluke eggs in stool, duodenal fluid or bile. Although examination of eggs in stool is reliable, it is useful only when intensity of infection is high. On the other hand, when the infection is light, examination of stool for eggs is uncertain. *O. viverrini* eggs are difficult to distinguish from eggs of small intestinal flukes, *eg. Prosto-*

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dendrium molenkampi, *Phanaeropsolus bonnei*, *Haplorchis taichui* and *H. pumillo*.[8] In severe infections, where there is a biliary obstruction, the eggs remain in the gall bladder and/or biliary tract and can not be found in the faeces. In these instances, canulation of the biliary canal is necessary for recovering the eggs. However, such procedure is not simple and may pose some danger to the patient. Alternative methods for diagnosis of opisthorchiasis included the use of DNA probe for detecting complimentary DNA of the fluke in stool,[9] the monoclonal antibody-based ELISA for antigen (E-S/somatic) detection[9,10] and various antibody detection assays.[11,12] However, none of them meets the ideal criteria on specificity and sensitivity as yet. The antibody detection assay, *eg.* an indirect ELISA may be as sensitive as the detection of eggs in stool[13] but cross-reaction with other parasitic infections are commonly and frequently found when crude parasite extract (which is the most convenient and available source of antigen) was used. The use of the parasite specific antigen, *eg.* the 89 kDa metabolic protein[14] might be an alternative in order to increase specificity of the antibody detection assay. However, sufficient amount of such antigen for a routine work is not available by the conventional method of preparation.

As mentioned above, the value of the antibody detection assay in opisthorchiasis is limited by the cross-reactive nature of the crude antigen used. In this communication, the parasitic infections which gives cross-reaction to the *O. viverrini* crude antigen are reported.

MATERIALS AND METHODS

Preparation of *O. viverrini* antigen

Collection of O. viverrini adult worms

Adult *O. viverrini* worms were from two different sources. Worms of the first batch were collected from infected individuals whereas the second batch was from infected hamsters.

First batch. Patients with opisthorchiasis viverrini admitted at the Bangkok Hospital for Tropical Diseases were treated with Praziquantel at 40 mg/kg body weight and given 45 ml of saturated magnesium sulphate *per orem* seven hours later. Stools were collected within twelve hours after the treatment with Praziquantel. Sedimentation was done on the stool specimens and adult *O. viverrini* were collected in normal saline solution (NSS).

Second batch. Young adult hamsters were individually infected with 100 *O. viverrini* metacercariae. The metacercariae were from muscles of laboratory-bred fresh water fishes belonging to the genus *Pontius* previously caught from the northeast of Thailand. Two months after the infection, the hamsters were sacrificed after ether euthanaesia and the livers were dissected out. The tissue was pressed in between two glass pads and examined for adult worms. The worms were carefully collected from the bile ducts using two needles. They were collected in sterile NSS.

Preparation of antigen

The adult *O. viverrini* worms were washed five times with NSS and once with distilled water. They were lyophilized. After the lyophilization they were ground homogeneously in sterile distilled water containing protease inhibitors. The preparation was subjected to an MSE ultrasonication at maximum input at 4°C for 10 minutes. The supernatant was collected and the pellet was discarded. Protein content of the supernatant was determined.[15] The preparation was kept in small aliquots at -20°C until used.

Serum samples

Serum samples were collected from four groups of individuals, *ie.* group 1 from 22 patients with *O. viverrini* infection only; group 2 from 31 patients infected with *O. viverrini* and other intestinal parasitic infections; group 3 were 141 patients with other parasitic infections; and group 4 from 24 normal, healthy, parasite-free individuals. The lists of serum specimens and the parasites found in patients of groups 2 and 3 are tabulated in Tables 1 and 2, respectively.

Indirect ELISA

Crude *O. viverrini* antigen at 2.5 μg protein per ml of carbonate bicarbonate buffer, pH 9.6 was used to coat all wells (100 μl per well) of microtitre plates (Greiner, Germany). The plates were incubated at 37°C for one hour and at 4°C overnight. After the unbound surface of each well was blocked with 200 μl of 1% bovine serum albumin (BSA) in PBS, 100 μl of serum samples at 1:400 dilution was added to appropriate wells except the blank wells, incubated for one hour, washed and 1:1,000

Table 1. Patients of group 2 whose faecal samples revealed *O. viverrini* and other parasites

Patient no.	Parasite(s) beside *O. viverrini* found in stools
8	hookworms, *Giardia lamblia*
12	hookworm, *Entamoeba coli*
24	*Blastocystis hominis*
32	*Echinostoma* spp.
34	*Echinostoma* spp., hookworms, *B. hominis*
45	*Taenia* spp.
47	Hookworms, *E. nana, B. hominis*
66	*Trichomonas hominis*
75	Hookworms, *Strongyloides stercoralis, Entamoeba coli, B. hominis*
109	Hookworms, *S. stercoralis, B. hominis*
110	Hookworms, *Echinostoma* spp.
118	Hookworms, *Echinostoma* spp., *G. lamblia*
124	Hookworms, *B. hominis*
130	Hookworms, *S. stercoralis, B. hominis*
116, 119	Hookworms, *S. stercoralis*
6, 16	Hookworms, *E. nana*
2, 100	*S. stercoralis*
7,9,14, 15, 20, 27, 28,30, 46, 49, 111	Hookworms

dilution of rabbit anti-human immunoglobulin-peroxidase conjugate was added (100 µl per well). After another incubation at 37°C for 30 minutes, the plates were washed and the substrate solution was added. Enzyme-substrate reaction was allowed to proceed for 20 minutes. The reaction was stopped by adding 50 µl of 1 N NaOH to each well. Optical densities of the contents of all wells were read against the blanks at 492 nm using an ELISA reader.

SDS-PAGE and Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out in vertical slab gel apparatus (Bio-Rad Laboratories, USA) according to the system of Laemnli. A 10% acrylamide separating gel and 4% acrylamide stacking gel were used in the process. The separated components of the crude *O. viverrini* antigen (40 µg per lane) was electroblotted onto a sheet of nitrocellulose (NC) paper. The blot was put in a blocking solution (3% BSA, 0.5% gelatin in PBS, pH 7.4) for one hour at room temperature with gentle rocking. The NC strip was washed three times with washing buffer (0.1 M PBS, pH 7.4 containing 0.05% Tween-20), then it was put into the serum specimen diluted at 1:400 for one hour at room temperature. The strip was then washed and the immune complexes were visualized using peroxidase conjugated rabbit anti-human immunoglobulins and its substrate.

RESULTS

Figure 1 illustrates protein components of crude extract of adult *O. viverrini* of the first batch. Similar pattern was also obtained when the second batch of antigen was tested. The components varied in molecular mass from *Mr* 17,000-71,000. A 17,000-18,000 kDa doublet were the most prominent components among all proteins.

When the first batch of antigen was used in the indirect ELISA to detect antibodies in serum samples of 24 normal controls, the mean ($\bar{X}$) of the optical densities was 0.030 and standard deviation (SD) was 0.012. If the $\bar{X}$ + 12 SD which was 0.174 was used as the cutoff limit between positive and negative opisthorchiasis, it was found that all of the 22 patients of group 1 and 27 from 31 patients of group 2 were positive (Figure 2). The number of ELISA positive among group 3 was 33 from 141 patients. Thus the diagnostic sensitivity, diagnostic specificity and positive and negative predictive values were 92%, 80%, 60% and 97%, respectively.

When the second batch of the antigen was used to capture the antibodies in the patients' sera, similar results of the ELISA were obtained. If the OD 0.174 was used as the cutoff optical density between positive and negative opisthorchiasis, 19 patients of group 1, 27 patients of group 2 and 50 patients of group 3 were positive (Figure 3). The diagnostic sensitivity, diagnostic specificity and predictive and negative values were 91%, 70%, 49% and 96%, respectively. The parasites found in patients whose serum samples were positive by ELISA using the second batch of antigen are given in Table 3.

Figure 4 illustrates representative Western blot patterns of

Table 2. List of organisms found in 118 patients of group 3

Patient no.	Parasite(s) beside *O. viverrini* found in stools
36	*T. trichiura*
37	*A. lumbricoides*, hookworm, *T. trichiura*, *P. falciparum*
38	*S. stercoralis*, *P. falciparum*
39	*T. hominis*, *A. lumbricoides*, *T. trichiura*, *P. falciparum*
40	Hookworms, *B. hominis*, *P. falciparum*
41	*T. trichiura*, hookworms, *S. stercoralis*
52	*S. stercoralis*
53	Hookworms, *P. falciparum*
55	S. stercoralis, *T. hominis*, yeasts, *P. falciparum*
56	Hookworms, *G. lamblia*, *P. falciparum*
60	Hookworms, *S. stercoralis*, *A. lumbricoides*, *P. falciparum*
64	*S. stercoralis*, *P. falciparum*
65	*T. saginata*
69	*S. stercoralis*, hookworms, *P. falciparum*
70	Hookworms, *P. falciparum*
73	Hookworms, *P. falciparum*
77	*A. lumbricoides*, *T. trichiura*, hookworms, *P. falciparum*
78	*A. lumbricoides*, *T. trichiura*
79	*S. stercoralis*, hookworms
80	*S. stercoralis*, hookworms, *P. falciparum*
2.1-2.21	*Paragonimus heterotremus*
3.1-3.10	*Schistosoma* spp.
4.1-4.21	*Trichinella spiralis*
5.1-5.13	*Taenia* spp.
6.1-6.20	*Strongyloides* spp.
7.1-7.12	Hookworms
8.1-8.3	*Entamoeba histolytica*
9.1-9.17	*Plasmodium* spp.
10.1-10.3	*Gnathostoma spinigerum*
11.1	Filaria

Table 3. Parasites found in patients whose serum samples gave false positive ELISA for the detection of antibodies to *O. viverrini* crude antigen (second batch)

Parasites	Number of cases which gave false positive reaction/total
Paragonimus heterotremus	14/21
Schistosoma spp.	2/10
Taenia spp.	4/13
Trichinella spiralis	16/21
Strongyloides stercoralis	8/21
Hookworms	1/12
Plasmodium spp.	1/17
Hookworms and *Plasmodium* spp.	2/3
S. stercoralis , *B. hominis* and yeast	1/1
Hookworms, *Ascaris lumbricoides*, *T. trichiura* and *P. falciparum*	1/1

SDS-PAGE separated *O. viverrini* antigen reacted with serum samples of patients which cross-reacted by ELISA to the antigen. Table 4 summarizes the serum reactivities of patients with opisthorchiasis, patients with other parasitic infections and healthy, parasite-free controls against the *O. viverrini* extract as revealed by SDS-PAGE and Western blot analysis.

DISCUSSION

The most predominant components of the crude extract of *O. viverrini* adult worms were the 17-18 kDa doublet. This finding is consistent with that reported by Ruppel *et al.*[16] and Wongratanacheewin *et al.*[14] who observed further that this doublet were glycoproteins and were most likely localized in the surface tegument of the parasite.

Sensitivities of the indirect ELISA were 92% and 91% using the first and second batches of crude extracts prepared from adult *O. viverrini* derived from two distinct sources, respectively. SDS-PAGE of the two antigens also revealed the same patterns of fractionated components. Thus one source of the *O. viverrini* adult worms can be an alternative of another. Four out of 53 patients of groups 1 and 2 gave false negative ELISA when the first batch of antigen was used. The same four cases and additional 1 case gave false negative result when the second batch of antigen was used. These cases had stool egg counts of less than 800 EPGF at the time the serum samples were collected thus classified as cases with light infection.[16] Egg output is known to correlate with the worm burden.[3] In the present study, the

Table 4. Serum samples of patients with parasitic infections and normal individuals of opisthorchiasis endemic (Thai) and non-endemic (Swedes) areas which contained antibodies reactive to antigenic components of *O. viverrini* somatic antigens

Serum sample which gave reaction	Antigenic component (mol.wt x 10^{-3})																			
	144	112	94	90	84	76	72	67	63	56	55	51	48	47	45	43	42	41	40	38
Opisthorchiasis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Paragonimiasis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Schistosomiasis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Gnathostomiasis	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-
Trichinellosis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hook worm infection	-	+	-	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Strongyloidiasis	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	-
Trichuriasis	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	-
Capillariasis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Filariasis (W.b.)	+	+	-	+	-	-	+	+	-	-	-	-	-	+	+	+	-	+	-	+
Taeniasis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malaria (P.f.)	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-	+	+
Malaria (P.v.)	+	+	+	-	-	+	+	+	+	-	-	-	-	+	+	+	-	+	-	+
Amoebiasis	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+
Normal Thai	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Normal Swedes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Serum sample which gave reaction	Antigenic component (mol.wt x 10^{-3})																			
	37	36	35	34	32	30	29	28	27	25.5	24.5	22	21.5	20.4	19.7	18.5	18	17	16.5	15.5
Opisthorchiasis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	+	+
Paragonimiasis	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	+	+	-	-
Schistosomiasis	+	-	-	-	+	+	+	+	-	-	-	+	-	+	-	-	-	-	-	-
Gnathostomiasis	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Trichinellosis	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-
Hook worm infection	+	-	+	+	+	+	-	+	-	+	-	-	-	+	-	-	+	+	-	+
Strongyloidiasis	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Trichuriasis	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Capillariasis	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Filariasis (W.b.)	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	+
Taeniasis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
Malaria (P.f.)	+	+	-	-	-	+	+	-	-	+	+	-	+	+	-	-	+	-	-	+
Malaria (P.v.)	-	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	+	+	-	-
Amoebiasis	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
Normal Thai	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Normal Swedes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

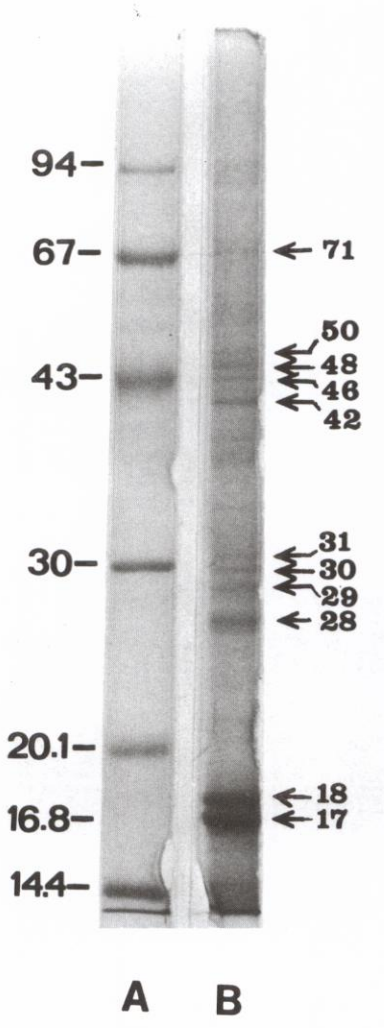

Fig. 1. Components of crude antigen extracted from adult *O.viverrini* (first batch) after SDS-PAGE and Coomassie brilliant blue staining (lane B). Lane A = molecular weight markers. Numbers at left and right are *Mr* x 10^{-3}.

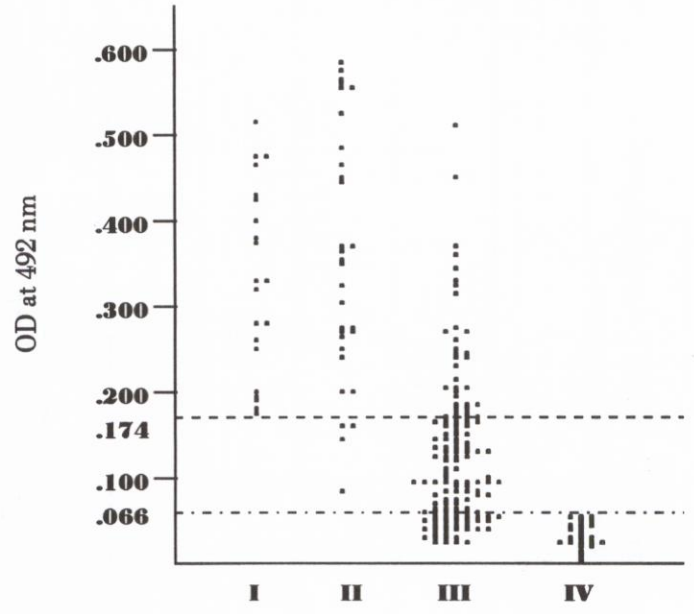

Fig. 2. Scattered diagram of the optical densities of indirect ELISA for the detection of antibodies to *O. viverrini* in serum samples of all groups when the first batch of antigen was used.

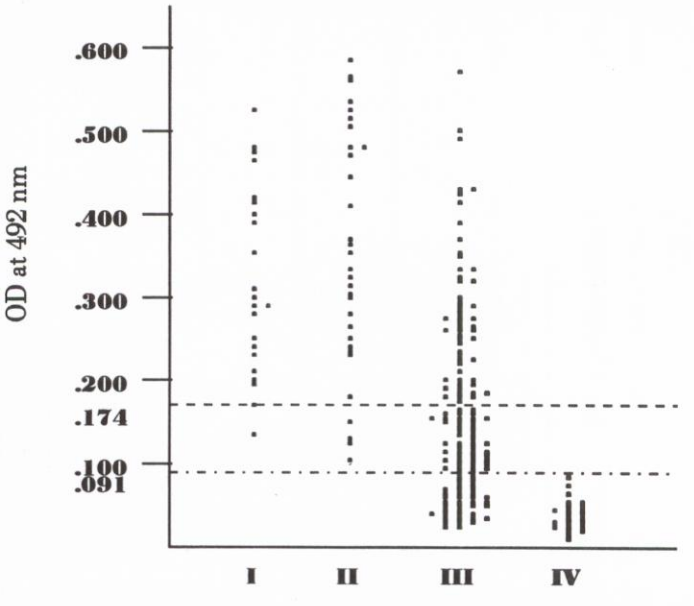

Fig. 3. Scattered diagram of the ELISA optical densities for the detection of specific antibodies to *O. viverrini* in all individuals when the second batch of antigen was used.

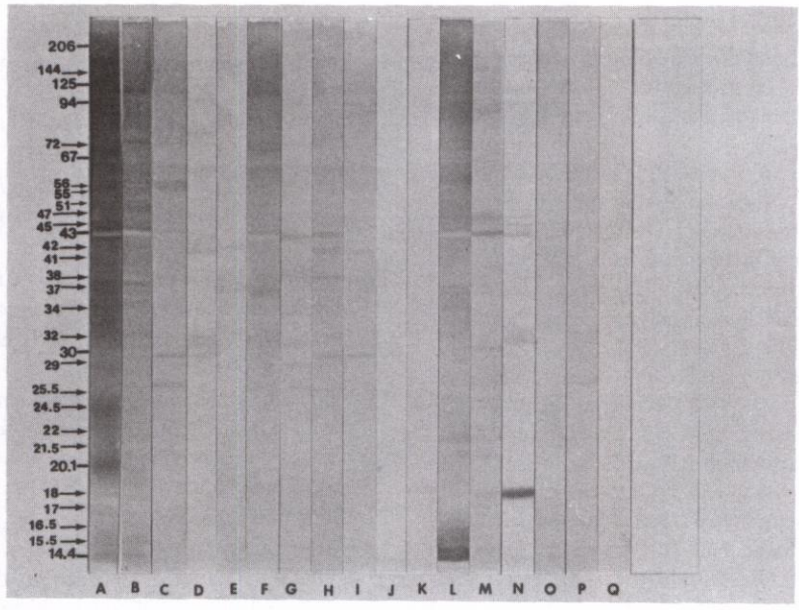

Fig. 4. Representative Western blot patterns of *O. viverrini* antigen and serum samples of patients which gave cross-reactive ELISA
B = Opisthorchiasis; C = Paragonimiasis; D = Schistosomiasis;
E = Gnathostomiasis; F = Trichinellosis; G = Hook worm infection;
H = Strongyloidiasis; I = Trichuriasis; J = Capillariasis;
K = Filariasis (W.b.); L= Taeniasis; M = Malaria (P.f.);
N = Malaria (P.v.); O = Amoebiasis; P = Normal Thai;
Q = Normal Swedes;
A = SDA-PAGE separated crude antigen stained with Con A.

stool egg counts and the indirect ELISA optical densities were significantly correlated ($p < 0.05$; Spearman rank sum test). These findings were consistent with those previously reported by Sriwatanakul *et al.*[13] Another possible explanation of this false negative reaction was that the patients might have been "misdiagnosed" cases of opisthorchiasis. This speculation has stemmed from the observation that it is difficult to distinguish *O. viverrini* eggs from the eggs of small intestinal flukes morphologically under the light microscope.[8] Possibly these patients could have been infected by the latter instead of the *O. viverrini*, but were diagnosed as opisthorchiasis cases.

The diagnostic specificity of the ELISA was relatively low compared with the sensitivity (80% and 70% using the first and second batches of antigen, respectively). Except the one patient with *Plasmodium* infection (Table 3), all of the sera that showed false positive reactions were from patients with other helminthic infections. These false positive reactions could probably be due to the complex natures of helminths, some components of which are shared by two or more parasites. This finding is, however, consistent with the data reported previously by several investigators who, likewise, used crude antigens.[17-19] Their studies demonstrated cross reactions between *O. viverrini* and other parasites, *ie. Paragonimus, Schistosoma, Fasciola* and *Gnathostoma*. From this study, besides the four previously reported cross-reactive parasites, cross-reactions were found in sera of patients infected with *Taenia, Trichinella, Strongyloides, Plasmodium, Blastocystis, Ascaris,* hookworms and *Trichuris*. However, among these, two patients with single infections, *ie.* one with hookworms and another one with *Plasmodium* need careful interpretation. The reason is that, there were altogether 12 cases with hookworms and 17 cases with *Plasmodium* tested; and yet only one of each gave positive results. Moreover, the ELISA optical densities were slightly higher than the cutoff optical density (0.174; Figures 2 and 3). The positive reaction might have been due to either a concomittant *O. viverrini* infection at an early stage when the worms were not reach the egg laying adult stage as yet or light infection which was not diagnosed by the stool examination. Previous studies on humoral immune responses in experimental opisthorchiasis had shown that low levels of antibodies could be detected as early as the second week of primary infection when the parasites were still in the juvenile stage.[20-22] Another possibility is that some of these false positive cases might have had previous exposure to *O. viverrini* and developed antibodies to the parasites and later they were cured by unknown mechanism(s). The antibodies against *O. viverrini* were found to last for several months

after the worms had been removed by Praziquantel treatment.[18,23]

The significant difference $p < 0.05$) in ELISA positive of those with *O. viverrini* infection (groups 1 and 2) and those without the infection (groups 3 and 4) suggests that the test could be used as a screening method for opisthorchiasis. However, the test could not be used alone for diagnosis as it could not differentiate between the present and past cure infections and could give also some false positive results. For definite diagnosis of the present infection it is required that either the parasite specific antigens and/or eggs are present in the patient's specimen.

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