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Diagnosis of Opisthorchiasis by Enzyme-Linked Immunosorbent Assay Using Partially Purified Antigens.

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SUMMARY *Opisthorchis viverrini* **antigens were partially purified from adult worms collected from liver and extrahepatic biliary system of infected hamsters. Tegument fraction was obtained by chemical extraction, whereas other fractions were purified by Sephadex G-200 gel filtration chromatography. Five fractions of** *O. viverrini* **antigens were obtained, namely tegument extract, somatic extract, fraction 1 (P_1), fraction 2 (P_2) and fraction 3 (P_3), respectively. The enzyme-linked immunosorbent assay technique was used to compare the reactivity of the five partially purified antigens. The sensitivity and specificity of all five antigens were compared by testing against the sera of 78** *O.viverrini* **-infected individuals from** *O. viverrini* **endemic areas and 70 individuals from non-endemic areas infected with hookworm,** *Trichuris* **and** *Ascaris* **including 49 individuals with negative stool examination. The assays performed with tegument extract, somatic extract and P_1 fraction were found to have 100% sensitivity, whereas the sensitivities of those with P_2 and P_3 were 96.1% and 83.3%, respectively. The tegument extract had the highest specificity as demonstrated by the lowest cross-reactivity with other parasites. Our results indicated that surface tegument is the most suitable antigen for use in immunological diagnosis of opisthorchiasis.**

Opisthorchiasis is one of the diseases of public health and economic importance in Thailand. The prevalence of *Opisthorchis viverrini* infection among the population in the northeastern part of the country has risen from 3.5 million cases in 1965 to 5.4 million cases in 1981.[1] Recently, it has been estimated that at least 7 million people are infected with this parasite.[2] Current methods for the diagnosis of *O. viverrini* infection are based on the demonstration of *Opisthorchis* eggs in either stool, duodenal fluid or bile. However, the techniques are tedious and unreliable in some cases, especially in cases of light infection and in cases of bile duct obstruction. Several attempts have been made to develop immunological methods for the diagnosis of the liver fluke infection.[3-7] The results are still not satisfactory due to the low sensitivity and cross-reactivity of the techniques.

It has been known that a more refined antigen would provide a more specific serological test for the diagnosis of parasitic infection, including the caused by liver flukes. Therefore, the following study is an attempt to isolate several purified antigenic materials from *O. viverrini* and to assess the immunodiagnostic potential of these partially purified antigens.

MATERIALS AND METHODS

Opisthorchis viverrini antigens

Adult worms were collected from the liver of infected hamsters after four weeks of infection. The surface tegument of adult worms was prepared by extraction with non-ionic detergent (Triton X-100 solution containing 50 mM Tris HCl pH 7.4, 150 mM NaCl, 10 mM EDTA pH 7.0) at 5° C with continuous shaking for one hour. After surface tegument extraction, all adult worms were ground and homogenized in a glass tissue grinder and sonicated five times for 30 seconds each at 60 watts.

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The homogenate was centrifuged at 5,000 rpm for 30 min at 4° C. An aliquot of the supernatant designated as somatic *O. viverrini* antigens was further purified by Sephadex G-200 gel filtration chromatography, using phosphate buffered saline solution (PBS, pH 7.2, 0.15 M) as eluent. The fractions belonging to the same peaks were pooled, lyophilized, reconstituted with distilled water and stored at -70° C until use. The protein contents of each fraction of *O. viverrini* antigens were determined by Folin-Ciocalteau method. [8]

Human sera

Serum samples were obtained from three groups of inhabitants. Group 1 consists of the sera from 78 individuals from the endemic area and has been found positive for *O. viverrini* infection by stool examination. Group 2 consists of the sera from 70 individuals reside in Pang-Nga Province which is a non-endemic area for *O. viverrini.* Stool examination for this group of people revealed infection with other parasites, such as hookworm, *Trichuris trichiura* or *Ascaris lumbricoides.* Group 3 consists of the sera from 49 individuals reside in both non-endemic and endemic areas of *O. viverrini* which has been found negative for parasitic infection by stool examination. Stool examinations were performed by the method of Formalin-ether concentration technique [9] and Stoll's egg counting technique. [10]

Enzyme-Linked Immunosorbent Assay (ELISA)

The indirect technique of enzyme-linked immunosorbent assay for antibody to *O. viverrini* followed the method of Srivatanakul *et al.* [5] All fractions of the antigen were tested against the sera of groups 1, 2 and 3 individuals. A reference of the negative control was obtained by using a pool of sera from a group of individuals who reside in non-endemic areas, who never consumed raw fish and were found negative for *O. viverrini* infection by repeated stool examination. Pool sera from the groups of individuals with proved positive for *O. viverrini* infection by stool examination were used as the positive control.

RESULTS

The somatic antigen of *O. viverrini* was separated by gel filtration chromatography into three fractions, namely P_1, P_2 and P_3, respectively (Fig. 1). Therefore, *O. viverrini* antigens used in this study were composed of tegument extract, somatic extract, and P_1, P_2 and P_3 fractions of the somatic extract.

The relationship between the egg count and the antibody titer to each of five antigens of *O. viverrini* in 197 individuals with and without *O. viverrini* eggs in faeces are shown in Figs. 2 to 6. When the cut off for ELISA reading was made at the serum titer of 1:40, P_1, somatic extract and the tegument fractions gave 100% positive for all of the individuals with *O. viverrini* infection regardless of the number of parasite eggs in the faeces (Fig. 2,5,6). A rather poor sensitivity was obtained with P_2 and P_3 fractions (Figs. 3,4). When the relationship between the number of parasite eggs per gram of faeces and the reciprocal of the antibody titers were compared, the best correlation was observed with the tegument fraction (Fig. 6).

Table 1 shows the results of ELISA assay among the group of people with confirmed *O. viverrini* infection, a group of people with other intestinal parasitic infections (hookworm, *Trichuris* and *Ascaris)* and a group of people with negative stool examination. Although P_1 fraction was found to have 100% sensitivity, it also reacted with 52.8% of the sera from patients with other intestinal parasites and 28.5% with sera from

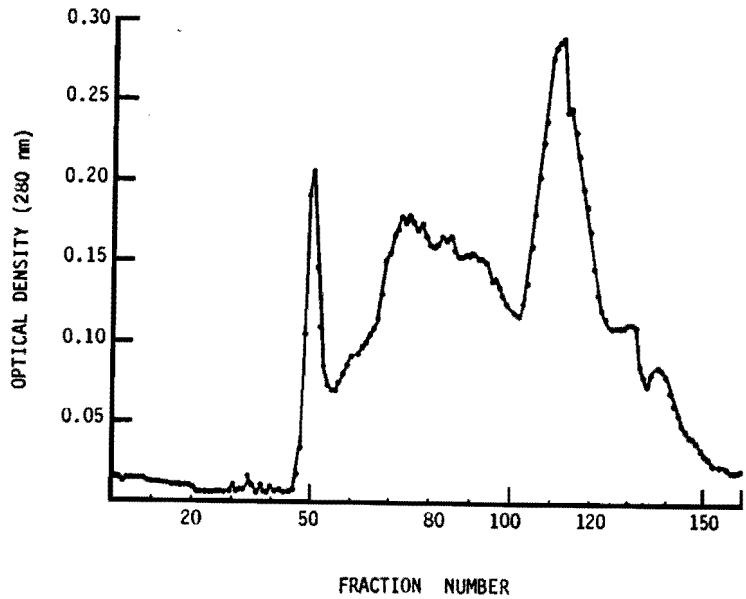

Fig. 1 Gel filtration chromatography of *O. viverrini* somatic antigens on Sephadex G–200 column (5.3x75.0 cm). Fractions of 3.0 ml with a continuous flow rate of 21 ml/h were collected.
Fraction 1 (P_1) = fraction number 45–55
Fraction 2 (P_2) = fraction number 64–100
Fraction 3 (P_3) = fraction number 102–124

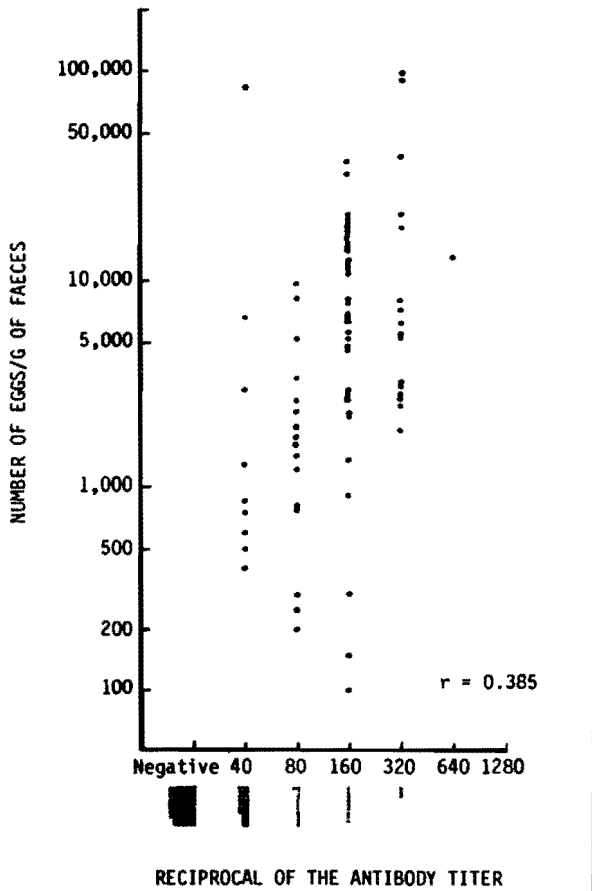

Fig. 2 Correlation between the titer of antibodies to P_1 fraction of *O. viverrini* antigens and the number of egg/G of faeces.

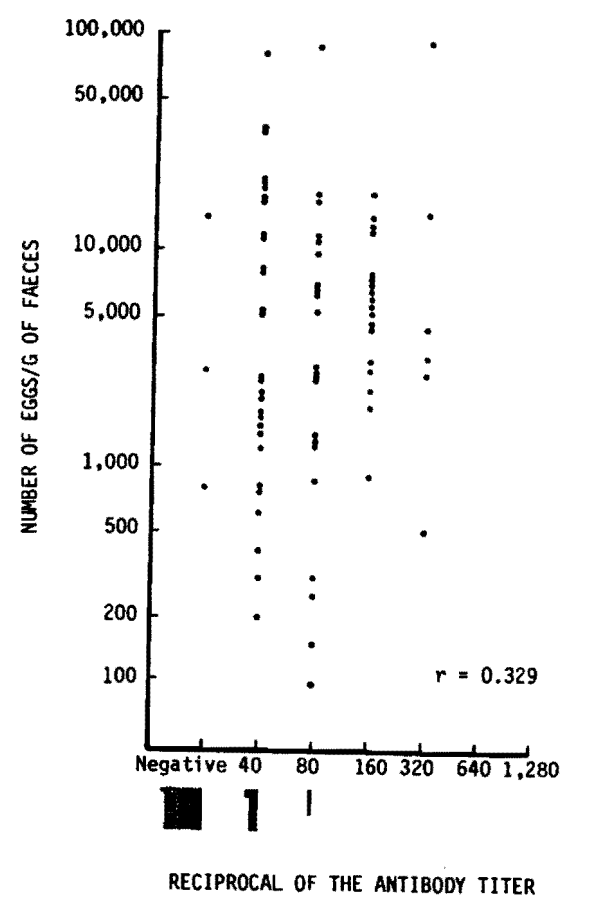

Fig. 3 Correlation between the titer of antibodies to P_2 fraction of *O. viverrini* antigens and the number of eggs/G of faeces.

Table 1. Results of ELISA assay among the group of individuals with confirmed opisthorchiasis, the group with other parasitic infections and the group with negative stool exam.

	Group of individual	Total number tested	No. Pos. ELISA with various antigens tested (%)				
			P1	P2	P3	Somatic	Tegument
I	Confirmed opisthorchiasis	78	78 (100)	75 (96.1)	65 (83.3)	78 (100)	78 (100)
II	Other parasitic infections	70	37 (52.8)	21 (30.0)	34 (48.5)	17 (24.2)	2 (2.8)
III	Negative stool exam	49	14 (28.5)	7 (14.2)	11 (22.4)	2 (4.0)	4 (8.1)

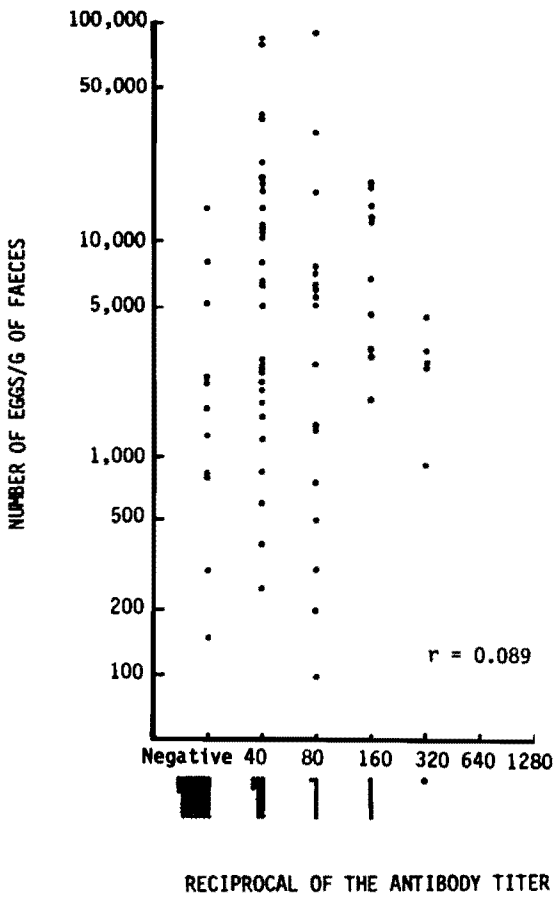

Fig. 4 Correlation between the titer of antibodies to P_3 fraction of *O. viverrini* antigens and the number of eggs/G of faeces.

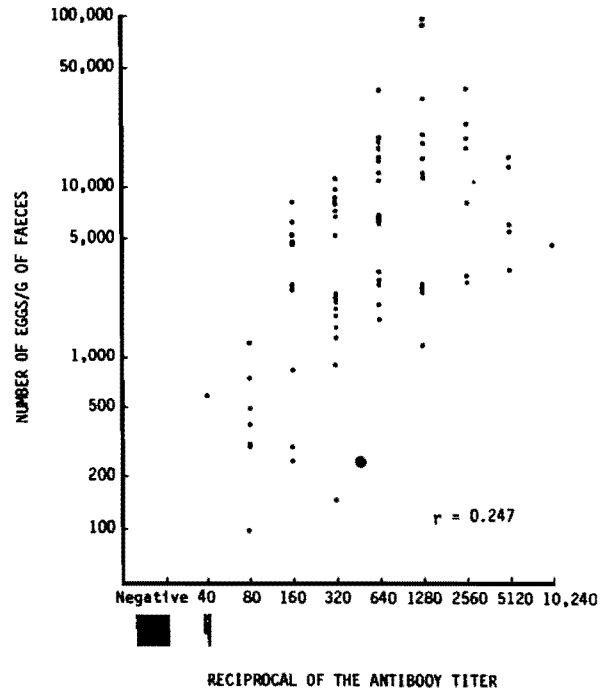

Fig. 5 Correlation between the titer of antibodies of somatic extract of *O. viverrini* antigens and the number of eggs/G of faeces.

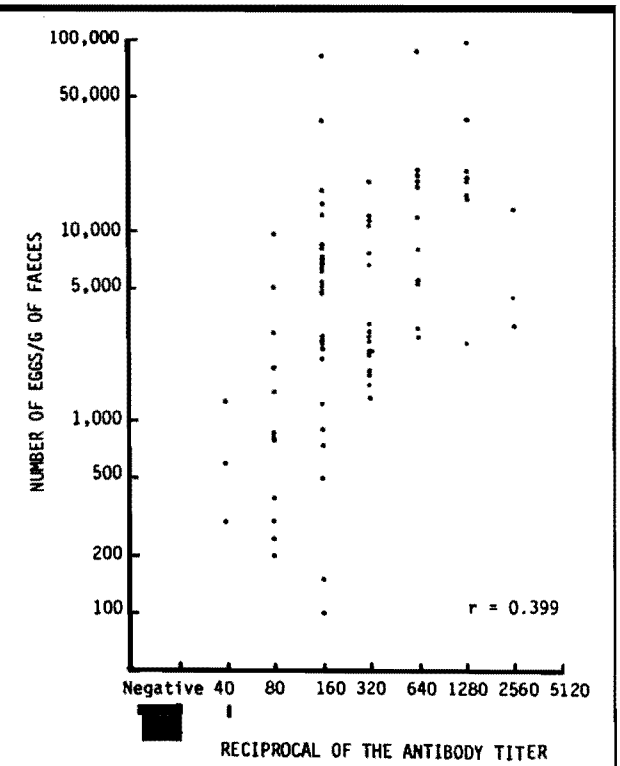

Fig. 6 Correlation between the titer of antibodies to the tegument fraction of *O. viverrini* antigens and the number of eggs/G faeces.

negative individuals. Somatic extract also exhibited high sensitivity, but its specificity was relatively low since it reacted with relatively large number of the sera from patients with other parasites. The integument fraction was the only fraction found to have the highest sensitivity and specificity. Among the five antigens shown to have positive correlation between the titer of antibodies and the intensity of *O. viverrini* infection based on Stoll's egg counting technique, the tegument extract was found to have the highest correlation (correlation coefficient, r = 0.399) (Figs. 2 to 6).

DISCUSSION

Antibodies to *O. viverrini* infection have been detected by various

immunological techniques, such as immunoelectrophoresis,[2,3,7] and ELISA.[4-6] With regard to ELISA assay, Feldheim and Knobloch[4] used a crude extract of *O. viverrini* and reported that high sensitivity was obtained but low levels of cross-reactivity with various species of trematodes and nematodes still occurred. Similar results were also obtained by Srivatanakul *et al.*[5], they reported that ELISA assay detected 92.8% of confirmed opisthorchiasis individuals but 46.5% of non-opisthorchiasis individuals also were found to give positive results.

In our study, we showed that by using partially purified antigens of *O. viverrini,* sensitivity and specificity of the ELISA assay can be improved. Among the five fractions of partially purified antigens used in this study, the tegument extract, P_1 antigen and somatic extract rendered 100% sensitivity. However, when specificity of the five antigens were tested, the tegument extract was found to have the highest specificity as indicated by the lowest cross-reactivity with antibody to other intestinal parasites (Table 1). the occurrence of false positive results of ELISA assay in the group of individuals with negative stool examination was probably due to the fact that the sera were obtained from heterogeneous groups of people from both non-endemic and endemic areas. Some of these individuals may have a very low parasite infection which cannot be detected by stool examination.

It is apparent that while the tegument extract, P_1 fraction and somatic extract have similar sensitivity, the P_1 fraction and somatic extract are less specific. The low specificity of P_1 fraction and somatic extract as compared to the tegument extract is probably due to the presence of non-specific epitopes in the first two antigens. Since hookworm, *Trichuris* and *Ascaris* are helminth parasites that have been found throughout Thailand, the test that shows less cross-reaction with these parasites would be the most preferable. Based on our results, it can be concluded that the tegument extract is the most suitable antigen to be used for immunological assay for the diagnosis of opisthorchiasis.

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