ENZYME–LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF *OPISTHORCHIS VIVERRINI* INFECTION

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INTRODUCTION

Opisthorchiasis viverrini is an endemic disease of man in northeastern Thailand and parts of Laos, Cambodia (Sadun, 1955; Wykoff *et al.*, 1965; Harinasuta, 1969). A recent survey of the population of a village in the endemic area indicates that 90% harbored the parasites (Upatham *et al.*, 1984). The disease is chronic in nature; patients with light or moderate infections rarely produce significant symptoms (Viranuvatti and Stitnimankarn, 1972; Upatham *et al.*, 1984). Such chronic persistent infections can lead to cholangitis, and infection of the parasite has been epidemiologically and experimentally linked with cholangiocarcinoma (Sonakul *et al.*, 1978; Koompirochana *et al.*, 1978; Thamavit *et al.*, 1978; Schwartz, 1980; Flavell, 1981; Kurathong *et al.*, 1985).

Various immunological techniques have been established for serodiagnosis of *O. viverrini* infection, such as immunoelectrophoresis (Janechaiwat *et al.*, 1980), enzyme immunoassay (Feldheim and Knobloch, 1982) and passive haemagglutination (Sirisinha *et al.*, 1983). However, the value of immunological diagnosis in relation to the intensity of the parasite infection has not been determined. The purpose of this study is to investigate the diagnostic value of enzyme immunoassay in terms of its specificity and sensitivity, and the relationship between the antibody titer and the intensity of *O. viverrini* infection in humans.

MATERIALS AND METHODS

Patients: The study was conducted on two groups of patients. Group 1 consists of 135 patients from the areas endemic for *O. viverrini*, who were admitted to Ramathibodi hospital, Bangkok between May 1981 and April 1983. Their ages ranged from 17 to 77 years. All of them were born and lived in the endemic area in northeastern Thailand. Nine patients had biliary obstruction. Stool examination for *O. viverrini* infection was determined both by the Formalin-ether concentration technique (Ritchie, 1948) and Stoll's egg counting technique (Stoll, 1923). Some of these patients were also infected with other parasites (hookworm, *Trichuris trichiura*, *Ascaris lumbricoides*, protozoa).

Group 2 consists of 208 patients with other intestinal parasitic infections; negative for *O. viverrini* eggs in their stools, but positive for hookworms. Some were concomitantly infected with *Trichuris trichiura* and/or *Ascaris lumbricoides*. They were all natives of southern Thailand, where opisthorchiasis has not been reported, and therefore non-endemic. Their ages ranged from 18 to 56 years.

O. viverrini **antigen:** Adult worms were obtained from the liver of infected hamsters after 3 to 4 weeks of infection. The flukes were washed 3 times with phosphate buffered saline (PBS) pH7.4, ground in a glass tissue grinder and sonicated 6 times for 30 sec each. The parasite suspension was centrifuged

10,000 x g for 90 min at 4°C. The supernatant was collected, dispensed in small aliquots and stored in liquid nitrogen until used.

Enzyme Immunoassay: The indirect technique of enzyme linked immunosorbent assay (ELISA) was used in this study. The antigen was diluted with 0.05 M carbonate buffer pH 9.6 to a protein concentration of 2.5 µg/ml. Antigen solution 100µl, was added to each well of disposable polystyrene microtiter plates (Nunc, Denmark). After overnight incubation at 4°C, the plate was washed 3 times with 0.15 M PBS - 0.05% Tween 20 to remove the unbound materials. All serum samples were diluted 1:40 with the diluent (PBS - 0.05% Tween 20 containing 0.5% bovine serum albumin). Diluted serum 100 µl samples, 3 negative controls, 3 positive controls and diluent (used as blank) were applied to each well of antigen coated plate and incubated at 37°C for 30 min. After incubation the unbound materials were removed by washing the plate 5 times with washing buffer. Then, 100 µl of peroxidase conjugated rabbit immunoglobulins to human IgG (Dakopatts, Copenhagen) at a dilution of 1:2000 was added into each well, followed by 30 min incubation at 37°C, and washed 5 times with buffer. Enzymatic reaction was developed by the addition of 100 µl of O-phenylenediamine substrate solution containing hydrogen peroxide to the wells. The plate was incubated for another 30 min at 37°C, and the enzymatic reaction then stopped by addition of 100 µl of 6N sulfuric acid.

The yellow color developed in the solution is proportional to the amount of *O. viverrini* antibody which had bound to the well. The intensity of this color development was read against the diluent blank using a spectrophotometer at 490 nm. Specimens giving absorbancy values greater than 1.0 were considered as positive for *O. viverrini* antibody. The antibody titers were determined in all of the positive sera.

RESULTS

In the patients from the endemic area who did not have biliary obstruction, antibodies to adult *O. viverrini* were detected in 92.8% (77 out of 83 cases) of subjects with *O. viverrini* eggs in feces and 46.5% (20 out of 43 cases) of subjects without *O. viverrini* eggs in feces (Table 1). There was a significant difference ($p = 0.02$) in the percentage positive for antibody between the patients positive for *O. viverrini* only and those with *O. viverrini* plus other parasites. However, in patients without *O. viverrini* eggs in feces, there was no significant difference in the percentages positive for antibody between subjects with or without other parasites($p = 0.33$). The six patients with opisthorchiasis who were antibody negative had a very low number of *O. viverrini* eggs in their feces (3 cases could be detected only by concentration technique and the other 3 cases had an egg count in the range 0.08-0.15 eggs/mg of feces). In the group from the non-endemic areas, who were all infected with other parasites, the antibody could be detected in only 5 out of 208 cases (2.4%) (Table 1). The antibody titers in various groups are shown in Table 2.

The results of stool examination (Formalin-ether concentration technique and Stoll's egg counting technique) and the titer of antibodies to adult *O. viverrini* in sera of the 9 patients with biliary obstruction are shown in Table 3. It was observed that sera from 3 of the 4 patients with biopsy proved cholangiocarcinoma had very high titers of antibody, but *O. viverrini* eggs were detectable only by concentration technique.

Fig. 1 shows the relationship between the number of egg count and the antibody titers in the 126 patients from the endemic area with no biliary obstruction. There is a

Table 1

Stool examination and serological testing by ELISA of patients from endemic and non-endemic areas of opisthorchiasis.

Group	Total No. patients*	ELISA No. positive (%)
Endemic area		
A. *Opisthorchis* eggs positive*	83	77 (92.8)
Opisthorchis eggs only	22	18 (81.8)
Opisthorchis & other parasites	61	59 (96.7)
B. *Opisthorchis* egg negative*	43	20 (46.5)
Other parasite eggs	12	7 (58.3)
No other parasite	31	13 (41.9)
Non-endemic area		
Other parasite eggs	208	5 (2.4)

*Diagnosis by Stool examination; positive or negative *Opisthorchis viverrini* eggs in faeces.

Table 2

Antibodies to *Opisthorchis viverrini* by ELISA in sera of people from endemic and non-endemic areas.

	O. viverrini antibody titers (%)						
	<1:40	1:40	1:80	1:160	1:320	1:640	≥1:1,280
Endemic area							
A. *Opisthorchis* positive*							
Opisthorchis only	4** (18.2)	0	4 (18.2)	6 (27.3)	4 (18.2)	3 (13.6)	1 (4.5)
Opisthorchis and other parasites	2 (3.3)	2 (3.3)	2 (3.3)	10 (16.4)	14 (22.9)	11 (18.0)	20 (32.8)
B. *Opisthorchis* negative*							
Other parasites + ve	5 (41.7)	1 (8.3)	3 (25)	0	3 (25)	0	0
Other parasites – ve	18 (58.1)	3 (9.7)	3 (9.7)	3 (9.7)	2 (6.4)	2 (6.4)	0
Non-endemic area							
Other parasites positive	203 (97.6)	4 (1.9)	1 (0.5)	0	0	0	0

*Diagnosis by stool examination.
**Number of patients.

Table 3

Comparison of stool examination* for *O.viverrini* and detection by ELISA in 9 patients with biliary obstruction.

Case No.	Concentration technique	ELISA Antibody titers
1. Cholangiocarcinoma	none	1:160
2. Cholangiocarcinoma	few *Opisthorchis* eggs, hookworm	> 1:20,480
3. Cholangiocarcinoma	few *Opisthorchis* eggs	1:10,240
4. Cholangiocarcinoma	few *Opisthorchis* eggs, hookworm	1:2,560
5. Gall stone	hookworm	1:640
6. Sclerosing cholangitis	none	< 1:40
7. Acute pancreatitis with gall stone	none	1:640
8. Cancer head of pancreas	none	< 1:40
9. Gall stone	none	1:160

*Stoll's egg count negative in all cases

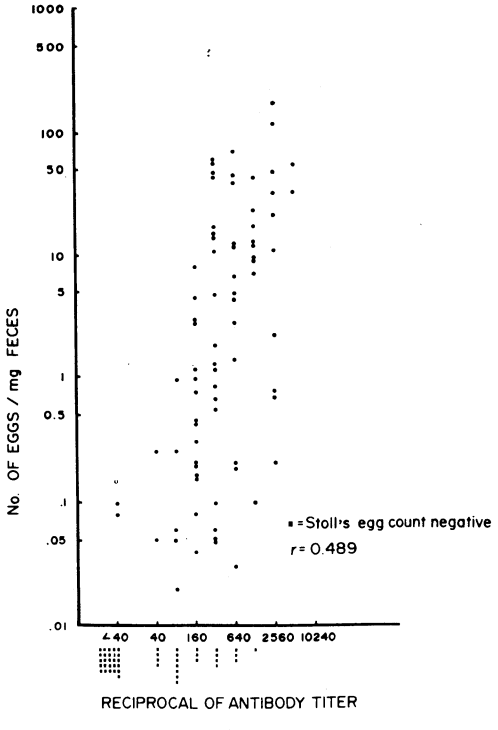

Fig. 1—Correlation between the titer of antibodies to *O. viverrini* and the number of eggs in feces.

positive correlation between the titer of antibodies to adult *O. viverrini* as detected by ELISA and intensity of *O. viverrini* infection based on Stoll's egg counting technique (correlation coefficent r = 0.489).

DISCUSSION

Antibodies to *O. viverrini* were rarely detected in patients from non-endemic areas. In subjects from the endemic area who did not have eggs in feces antibody titers were low, and uninfluenced by the presence or absence of other parasites. Thus, the positive reaction in the cases with out *O. viverrini* eggs in feces is not due to cross-reaction with hookworms, *Trichuris trichiura*, *Ascaris lumbricoides*, and other protozoan parasites. In previous studies (Chung *et al.*, 1956; Philipson and McFadzean, 1962; Janechaiwat *et al.*, 1980; Feldheim and Knobloch, 1982) cross-reaction between human liver flukes and other related parasites (e.g. *Clonorchis*, *Paragonimus*, *Schistosoma*, *Fasciola*) has been reported. However, the patients tested in these studies were

from the area endemic for opisthorchiasis, may have had low-level infection with *O. viverrini*. In addition,the presence of antibody may indicate both current and past infection.

The false negative rate for our ELISA test was 7.2% although the six patients concerned had, in fact, very low numbers of *O. viverrini* eggs in their feces. There was a positive correlation between the titer of antibodies to adult *O. viverrini* and the number of eggs in feces. Feldheim and Knobloch (1983) suggested that in cases with high antibody activity before chemotherapy, the antibody level may be a supplementary indicator of effective treatment. The usefulness of the antibody titer as a marker for evaluating the effectiveness of chemotherapy should be further investigated.

Opisthorchiasis patients with biliary obstruction could not be diagnosed by stool examination without the use of concentration technique, however these patients all had high titers of antibody to *O. viverrini* as demonstrated by ELISA.

SUMMARY

Antibodies to *O. viverrini* in the sera of people from endemic and non-endemic areas were investigated using indirect ELISA technique. For the patients from the endemic area, 92.8% who passed eggs in the stool were found to be positive for *O. viverrini* antibody. In addition, 46.5% of the people who did not pass eggs in the stool were also found to have low titer of *O. viverrini* antibody. On the other hand only 2.4% of the people from the non-endemic area with other intestinal parasite infections were found to have *O. viverrini* antibody in their sera. It was concluded that positive reaction of *O. viverrini* antibody is not cause by cross-reaction with other parasites but low liter of antibody is probably due to low-level or past infection.

There is a positive correlation between the titer of *O. viverrini* antibody and intensity of infection as indicated by number of eggs excreted per milligram of feces. Patients with a few *O. viverrini* eggs in feces, but biopsy-proved-cholangiocarcinoma had very high titer of antibody.

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REFERENCES

CHUNG, H.L., HOU, T.C. and WENG, H.C., (1956). Further studies on paragonimiasis complement fixation test and cross complement fixation reaction of sera of patients with paragonimiasis, clonorchiasis and schistosomiasis to different trematode antigens. *Chinese Med. J., 74* : 207.

FELDHEIM, W. and KNOBLOCK, J., (1982). Serodiagnosis of *Opisthorchis viverrini* infestation by an enzyme immunoassay. *Tropenmed. Parasit., 33* : 8.

FLAVELL, D.J., (1981). Liver-fluke infection as an aetiological factor in bile-duct carcinoma of man. *Trans. Roy. Soc. Trop. Med. Hyg., 75* : 814.

HARINASUTA, C., (1969). Opisthorchiasis in Thailand : a review. *In:* Proceedings of the Fourth Southeast Asian Seminar on Parasitology and Tropical Medicine, Schistosomiasis and other snail-transmitted helminthiasis. Harinasuta, C. (Ed), Bangkok, pp. 253.

JANECHAIWAT, J., THARAVANIJ, S., VAJRASTHIRA S., and CHAICUMPA, W., (1980).

The immunological diagnosis of human Opisthorchiasis and the humoral immune response to Opisthorchis infection in the hamster. *J. Med. Ass. Thailand.*, *63* : 439.

KOOMPIROCHANA, C., SONAKUL, D., CHINDA, K. and STITNIMANKARN, T., (1978). Opisthorchiasis : A clinicopathologic study of 154 autopsy cases. *Southeast Asian J. Trop. Med. Pub. Hlth.*, *9* : 60.

KURATHONG, S., LERDVIRASIRIKUL, P., WOUGPAITOON, V., PRAMOOLSINSAP, C., KANJANAPITAK, A., VARAWITHAYA, W., PHUPRADIT, P., BUNYARATVEJ, S., UPATHAM, S. and BROCKELMAN, W.Y., (1985). *Opisthorchis viverrini* infection and cholangiocarcinoma : A prospective, case-control study. *Gastroenterology*, (In press).

PHILLIPSON, R.F., and MCFADZEAN, J.A. (1962). Clonorchiasis, opisthorchiasis and paragonimiasis gel diffusion studies. *Trans. Roy. Soc. Trop. Med. Hyg.*, *56* : 13.

RITCHIE, L.S., (1948). An ether sedimentation technique for routine stool examination. *Bull. U.S. Army Med. Dept.*, *8* : 326.

SADUN, E.H., (1955). Studies on *Opisthorchis viverrini* in Thailand. *Amer. J. Hyg.*, *62* : 81.

SCHWARTZ, D.A., (1980). Helminths in the induction of cancer : *Opisthorchis viverrini*, *Clonorchis sinensis* and cholangiocarcinoma. *Trop. Geogr. Med.*, *32* : 95.

SIRISINHA, S., TUTI, S., VICHASRI, S. and TAWATSIN, A., (1983). Humoral immune responses in hamsters infected with *Opisthorchis viverrini*. *Southeast Asian J. Trop. Med. Pub. Hlth.*, *14* : 243.

SONAKUL, D., KOOMPIROCHANA, C., CHINDA, K. and STITINIMANKARN, T., (1978). Hepatic carcinoma with Opisthorchiasis. *Southeast Asian J. Trop. Med. Pub. Hlth.*, *9* : 215.

STOLL, N.R., (1923). An effective method of counting hookworm eggs in feces. *Amer. J. Hyg.*, *3* : 59.

THAMAVIT, W., BHAMARAPRAVATI, N., SAHAPHONG, S., VAJRASTHIRA, S. and ANGSUBHAKORN, S., (1978). Effects of dimethnylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini* infected Syrian golden hamsters. *Cancer Res.*, *38* : 4634.

UPATHAM, E.S., VIYANANT V., KURATHONG S., ROJBORWONWITAYA J., BROCKELMAN W.Y., ARDSUNGNOEN S., LEE P. and VAJARASTHIRA S. (1984).) Relationship between prevalence and intensity of *Opisthorchis viverrini* infection, and clinical symptoms and signs in a rural community in northeast Thailand. *Bull. WHO.*, *62* : 451.

VIRANUWATTI, V. and STITNIMANKARN, T., (1972). Liver fluke infection and infestation in South-east Asia. *In :* Progress in Liver Disease, Popper, H. and Schaffner, F. (eds), *4* : 537.

WYKOFF, D.E., HARINASUTA, C., JUTTIJADATA, P. and WINN, M.M., (1965). *Opisthorchis viverrini* in Thailand, the life cycle and comparison with *O. felineus*. *J. Parasit.*, *51* : 207.