

SEROLOGICAL DIFFERENTIATION OF HUMAN SMALL FLUKE INFECTIONS USING *OPISTHORCHIS VIVERRINI* AND *HAPLORCHIS TAICHUI* ANTIGENS

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Abstract. Sera from 642 inhabitants of Vientiane Province (Laos) were examined by enzyme-linked immunosorbent assay (ELISA) using cytoplasmic and membranous antigens prepared from adult worms. Worms of *Opisthorchis viverrini* originated from liver of dissected cats, *Haplorchis taichui* were obtained from a stool specimen of a Laotian patient after praziquantel treatment. The sera were divided into five groups according to the intensity of infection expressed as egg count per gram of patients stool (EPG). Correlation between intensity of infection and the level of antibodies in serum was recorded. Reactions obtained using the cytoplasmic antigens were more sensitive and more specific compared to those with membranous antigens. Cross-reactions between antigens of both helminth species were found. Highly positive sera were examined using electroimmunotransfer blots (EITB) with cytoplasmic antigens of both species, which enabled the species differentiation. Antigens of both species yielded several shared fractions; however, differences between them were found: homologous sera reacted specifically with *O. viverrini* antigen in the area of 70 kDa and with *H. taichui* antigen in the area of 10 kDa.

Thirty-one of 122 tested sera had specific antibodies against *O. viverrini*, 77 sera against *H. taichui* and 14 sera against both species. The results confirmed our assumption about predominant occurrence of heterophyid flukes in the human population living in studied area, compared with the occurrence of opisthorchid flukes. Hence, serology seems to be helpful tool for correct diagnosis of small fluke infections.

INTRODUCTION

Opisthorchiasis is endemic in some parts of Southeast Asia and represents both medical and public health problems (Harinasuta 1969, Upatham 1988). Cases of human infection with heterophyid flukes are known to occur in this area as well (Manning *et al*, 1971; Kliks and Tantachamrun, 1974; Tantachamrun and Kliks, 1978; Radomyos *et al*, 1990). Differentiation of opisthorchid and heterophyid eggs is rather difficult with light microscopy; while typical features are visible in scanning electron microscopy, this is usually not used in routine examinations (Ditrich *et al*, 1990 a). During a field study in Vientiane Province, Laos, we found *O. viverrini* and several species of heterophyid flukes; *H. taichui*, *H. yokogawai*, *H. pumilio* and *Stellantchasmus*

falcatus in intermediate hosts and in cats serving as indicators of possible human infection with these parasites (Ditrich *et al*, 1990b; Giboda *et al*, 1991). On the basis of our results, we suspected that human infections caused by heterophyid flukes were common in this area.

The aim of this study was to elucidate this problem using two serological methods, the enzyme-linked immunosorbent assay (ELISA) and the electroimmunotransfer blot (EITB).

MATERIAL AND METHODS

Study population

Sera from 642 inhabitants of the Vientiane Province, Laos, mostly living on the banks of

Nam Ngum water reservoir were obtained during July-August 1989. Donors of sera were coprologically examined using concentration methods (Merthiolate-iodine formalin concentrate) and the intensity of infection of small flukes was estimated on the basis of egg count per gram of stool (EPG). This value served for grouping sera into five groups: without fluke eggs, lightly infected (1-999 EPG), moderately infected (1,000-9,999 EPG), heavily infected (10,000-29,999 EPG), and very heavily infected (more than 30,000 EPG) (Sadun, 1955). Sera from blood donors from České Budejovice, Czechoslovakia were used as a negative control. Serum samples of persons infected with various parasites served for the examination of the specificity.

Antigens

Adults of *O. viverrini* obtained from autopsied cats in Vientiane, and adults of *H. taichui* obtained from a stool sample of a heavily infected Laotian student after praziquantel therapy, served as the source of antigens (submitted).

Antigen preparation:

Adult flukes were rinsed twice in PBS, and placed into equal volume of 0.1 mM solution of phenylmethylsulfonyl fluoride (PMSF) in PBS and stored in -20°C . After thawing, worms were thoroughly homogenized and centrifuged for 30 minutes at 10,000 rpm at 0°C . Supernatant was stored at -60°C and used as cytoplasmic antigen. Equal volume of 0.1% solution of Triton X-100 in PBS was added to the sediment, mixed and sonicated 10×10 seconds with 30 second intervals with the amplitude 3, 5 μm peak to peak. Centrifugation was repeated and the supernatant was dialyzed to remove Triton and then used as the membranous antigen.

Enzyme-linked immunosorbent assay (ELISA)

All antigens used in ELISA were diluted in carbonate buffer to the standard 5 $\mu\text{g}/\text{ml}$ protein concentration and coated into microtitration plates, 150 μl per well. The serum samples, in the standard dilutions 1:200, 1:600, 1:1800 and 1:5400, were placed into the coated wells. Horseradish peroxidase conjugate SwAHuIg/Px (Sevac, Czechoslovakia) and acetate substrate solution with orthophenylenediamine was used

for detection. Color changes were read in the SUMAL PE 2 reader (Carl Zeiss Jena, Germany).

Analysis of antigens using electroimmunotransfer blots (EITB)

Sodium dodecyl sulphate electrophoresis in 5-15% gradient polyacrylamide gel (SDS-PAGE) was carried out according to the method of Laemmli (1970) for antigen separation. Then the proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell BA 85) following the procedure of Towbin *et al* (1979). The blots were blocked in 5% dried milk powder and incubated in human serum samples diluted 1:100. The anti-antibody peroxidase conjugate SwAHuIg/Px and the diaminobenzidine substrate solution was used as the detection system. Only sera highly positive in ELISA were used for EITB analysis. Sera from eight patients with known numbers and species of excreted flukes after praziquantel treatment served as standard samples (3 patients with only *O. viverrini*, 4 with *H. taichui*, and 1 with both species) (submitted). Six other sera from Laotians infected with *O. viverrini* determined on the basis of the typical muskmelon pattern of egg surface observed by scanning electron microscopy were used.

RESULTS

Membranous antigen fractions demonstrated lower sensitivity and specificity compared to cytoplasmic antigen. Titers obtained using the latter were one dilution higher in most cases. Results obtained using cytoplasmic fractions are presented in Table 1. In some extent, correlations between intensity of infection and the level of antibodies can be observed. Cross-reactions between both species antigens were frequent; higher titers were reached with the antigen of *O. viverrini* (Table 1). None of the control sera from blood donors reacted positively. Cross-reactions with sera from patients with high level of antibodies against *Schistosoma mansoni*, *Entamoeba histolytica*, *Leishmania donovani* and *Plasmodium falciparum*, and with sera from patients infected by common species of intestinal nematodes, were not observed. Two out of three sera from patients infected by *Fasciolopsis buski* reacted at a titer of 1:200. Protein analysis using SDS-PAGE showed a major doublet in the

Table 1
Relations of serological and parasitological results.

Fluke infection	ELISA			
	<i>O. viverrini</i>		<i>H. taichui</i>	
	% positive	GMRT	% positive	GMRT
Negative	52	502.4	45	442.1
Light	71	548.0	58	555.7
Moderate	81	729.6	73	541.6
Heavy	77	702.1	37	455.3
Very heavy	80	2097	60	1270

molecular weight area 16–17 kDa. This doublet is visible in an amidoblack stained blot (Fig 1). In this figure, immune replicas obtained with known sera are shown. Shared fractions were found especially in high molecular weight area over 94 kDa. However, there are sites reacting only with homologous sera: the distinct band in the area of 70 kDa in the case of *O. viverrini* antigen and two bands in the area of 10 kDa in the case of *H. taichui* antigen. Specificity of these bands was confirmed by all sera from patients with known etiological agents. According to the known pattern, all 122 highly positive sera could be divided into three groups: 31 of them had specific antibodies only against *O. viverrini* antigen, 77 against *H. taichui* antigen and 14 against both species simultaneously. Antibodies against *O. viverrini* predominated in the groups of heavy and very heavy infections. No correlations of the presence of specific antibodies with age group or sex were observed. All 12 highly positive sera from inhabitants of the village of Nanin situated on the bank of the Nam Ngum River demonstrated antibodies only against *H. taichui*.

DISCUSSION

Several attempts to use serological methods in the diagnosis of opisthorchiasis have been performed. Janechaiwat *et al* (1980) used immunoelectrophoresis and observed 76% positive

reactions with sera from patients suffering from opisthorchiasis. Wongratana-cheewin *et al* (1988) used ELISA with conjugates against immunoglobulin subclasses. They observed increases of specific IgA and IgG antibodies in infected patients and recorded moderate but statistically significant correlations between serum IgG antibody levels and severity of infection as judged from the quantity of eggs in the stool. Our results of ELISA were analogous to these.

Tantavanich *et al* (1988) used immunoblotting for analysis of *O. viverrini* antigen prepared from metacercariae and adults. They focused their attention to the high molecular weight areas (107, 132 and 190–200 kDa); however, distinct bands in the area of 74 kDa that could be identical with our 70 kDa protein are visible in their photographs of immunoblots obtained with antigens from adult worms. The authors showed specificity of immunoblots: in specific areas of their antigens reacted 18 out of 20 sera from patients with opisthorchiasis and no serum of patients with other helminthiasis. Wongratana-cheewin *et al* (1987) analyzed *O. viverrini* antigens using radioimmunoprecipitation. In case of adult somatic antigen, they described four components (20, 70, 89 and 116 kDa) reacting specifically with the homologous antibodies. Similar results were published by Wongratana-cheewin *et al* (1988), who proposed the use of 89 kDa protein present in somatic as well as in metabolic antigen of *O. viverrini* for immunodiagnosis.

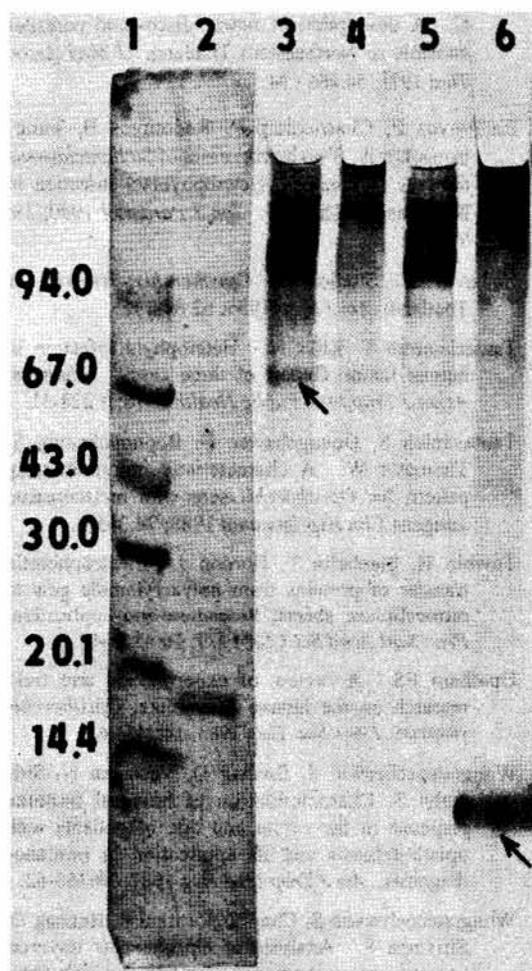


Fig 1 - EITB of soluble antigens of *Opisthorchis viverrini* and *Haplorchis taichui* antigens.

- 1: LMW standards, molecular weights in kilodaltons.
 - 2: *O. viverrini* antigen, amidoblack stain.
 - 3: *O. viverrini* antigen, serum of patient infected with *O. viverrini*.
 - 4: *H. taichui* antigen, serum of patient infected with *O. viverrini*.
 - 5: *O. viverrini* antigen, serum of patient with *H. taichui*.
 - 6: *H. taichui* antigen, serum of patient with *H. taichui*.
- Arrows show reactions with homologous sera.

Our results corresponded with most published data. Protein analysis confirmed major doublet 16–17 kDa very probably the glycoprotein localized in the surface of tegument (Wongratana-cheewin *et al*, 1988). The glycoprotein does not seem to have immunogenic properties

since no bands corresponded to this doublet in immunoblots. Similar doublet in the same area yielded *H. taichui* antigen. Differences in immunoblot pattern in *O. viverrini* and *H. taichui* enabled us to differentiate sera according to specific antibodies. The presence of antibodies against *O. viverrini* as well as against *H. taichui* in patients without eggs in stool indicates that not only current but also previous infection with this species can be serologically diagnosed. Considering a common occurrence of heterophyid flukes (*Haplorchis* spp, *Stellantchasmus falcatus*) in the area studied (Ditrich *et al*, 1990a; Giboda *et al*, 1991), the appearance of antibodies against *H. taichui* could not be explained by exclusive infection with this species. Our serological results confirmed the assumption about frequent occurrence of the flukes of the family Heterophyidae in inhabitants of study area. This study also revealed that, at least in some areas, human *Haplorchis* infections can be more frequent than *O. viverrini*. In some places, as in the village of Nanin, *Bithynia siamensis* snails (intermediate host of *O. viverrini*) were not found; conversely, many thiarid snails (intermediate hosts of haplorchiid flukes) were recorded in its vicinity (Ditrich, unpublished data). This corresponds with results of EITB that enable the identification of species-specific antibodies in sera from inhabitants of this village.

Our results serologically showed that human infections with intestinal flukes of the subfamily Haplorchiinae are common. This fact has great importance in evaluation of clinical symptoms of small fluke infections, in the dosage of anti-parasitic drugs, and in the epidemiology. Since identification of fluke species on the basis of eggs in stool samples is rather difficult and the possibility of determination of adult worms is limited, serological methods should be very helpful.

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REFERENCES

- Ditrich O, Giboda M, Sterba J. Species determination of eggs of opisthorchiid and heterophyid flukes using scanning electron microscopy. *Angew Parasitol* 1990a; 31:39.
- Ditrich O, Scholz T, Giboda M. Occurrence of some medically important flukes (Trematoda: Opisthorchiidae and Heterophyidae) in Nam Ngum water reservoir, Laos. *Southeast Asian J Trop Med Public Health* 1990b; 21:418-24.
- Giboda M, Ditrich O, Scholz T, Viengsay T, Bouaphanh S. Country report: Current status of food borne parasitic zoonoses in Laos. 1991; (this volume).
- Harinasuta C. Opisthorchiasis in Thailand: A review. Proceedings of the 4th Southeast Asian Seminar on Parasitology and Tropical Medicine, Schistosomiasis and other Snail-Transmitted Helminthiasis. Bangkok 1969:253-64.
- Janechaiwat J, Tharavanij S, Vajrasthira S, Chaicumpa W. The immunological diagnosis of human opisthorchiasis and the humoral immune response to *Opisthorchis* infection in the hamster. *J Med Assoc Thai* 1980; 63:439 - 46.
- Kliks M, Tantachamrun T. Heterophyid (Trematoda) parasites of cats in North Thailand, with notes on a human case found at necropsy. *Southeast Asian J Trop Med Public Health* 1974; 5:547-55.
- Laemli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680-5.
- Manning GS, Lertprasert P, Watanasirmkit K, Chetty C. A description of newly discovered parasites endemic to Northeastern Thailand. *J Med Assoc Thai* 1971; 54:466 - 74.
- Radomyos P, Charoenlarp P, Radomyos B, Tungtrongchitr A. Two human cases of *Stellantchasmus falcatus* (Trematoda, Heterophyidae) infection in Northeastern Thailand. *Jpn J Parasitol* 1990; 39: 7-11.
- Sadun EH. Studies on *Opisthorchis viverrini* in Thailand. *Am J Hyg* 1955; 62:81-115.
- Tantachamrun T, Kliks M. Heterophyid infection in human ileum: Report of three cases. *Southeast Asian J Trop Med Public Health* 1978; 9:228-31.
- Tantavanich S, Doungchawee G, Boonpucknavig S, Thamavit W. A characteristic immunoblotting pattern for *Opisthorchis* sera with metacercarial antigens *Clin Exp Immunol* 1988; 74:355-8.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and application. *Proc Natl Acad Sci USA* 1979; 76:4350-4.
- Upatham ES. A review of experimental and field research on the human liver fluke, *Opisthorchis viverrini*. *J Sci Soc Thai* 1988; 14:245 - 62.
- Wongratanacheewin S, Bunnag D, Vaeusorn N, Sirisinha S. Characterization of humoral immune response in the serum and bile of patients with opisthorchiasis and its application in immunodiagnosis. *Am J Trop Med Hyg* 1987; 38:356-62.
- Wongratanacheewin S, Chawengkirttikul R, Bunnag D, Sirisinha S. Analysis of *Opisthorchis viverrini* antigens by immunoprecipitation on polyacrylamide gel electrophoresis. *Parasitology* 1988; 96: 119-28.