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Affinity Purified Oval Antigen for Diagnosis of Opisthorchiasis Viverrini

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SUMMARY **Monoclonal antibodies (MAb) were raised against an oval antigen of the liver fluke *Opisthorchis viverrini* which is the causative agent of a parasitosis, i.e. opisthorchiasis in Thailand. The antibodies were used in an affinity column to purify the *O. viverrini* oval antigen from a crude extract of adult parasites by chromatography. The oval antigen was then used in a membrane (dot) ELISA for detecting antibodies in serum samples of parasitologically confirmed *Opisthorchis viverrini* infected individuals (adult parasites were found in stools after praziquantel treatment and salt purgation), as well as of individuals infected with other parasites and parasite-free controls. The MAb-based dot-ELISA using the affinity purified *O. viverrini* oval antigen revealed 100% sensitivity, specificity and accuracy for detecting *O. viverrini* infection. The test is simple, rapid and highly reproducible. Several samples can be tested at the same time without the requirement for special equipment or much increase in testing time; thus it is suitable for mass screening for *O. viverrini* exposure, especially in new endemic areas. Furthermore using serum specimens could increase patient and community compliance compared to the conventional parasitological survey which uses stool samples for the detection of *O. viverrini* ova, without treatment and subsequent salt purgation, this conventional method shows a low sensitivity and is also unpleasant to both the sample donors and the laboratory technicians which has historically shown a further negative impact on the final outcome.**

The current diagnostic method of liver fluke infection, *i.e.* opisthorchiasis caused by *Opisthorchis viverrini*, is by detecting the parasite ova in fecal samples. However, in light infections or when ova output is low or nil as a result of biliary obstruction, fecal examination for *O. viverrini* eggs may be falsely negative. This method is even more difficult in mixed parasitic infections (a common situation in parasite endemic areas such as northeastern Thailand), as ova of *O. viverrini* are not readily distinguishable from those of other harmless small intestinal flukes such as *Prosthodendrium molenkampi*, *Phaneropsolus bonnei* and *Haplorchis taichui*.[1] Special staining or electron microscopic examination of the parasite ova or finding of adult parasites in the stool obtained after Praziquantel treatment and salt purgation must be done for definite diagnosis, which are laborious and impractical, especially for a large scale survey of the prevalence of parasitic infections.

Alternative methods have been developed for diagnosis of opisthorchiasis viverrini. These include DNA hybridization, DNA amplification by PCR and antibody-based coproantigen detection using specific monoclonal antibodies. However, none of them has met the ideal criteria on adequate simplicity, specificity and sensitivity.[2-4] There is a need to develop a new screening test to overcome the above mentioned obstacles.

Surveillance of opisthorchiasis viverrini is still a high priority

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among the public health measures of Thailand. A five-year active parasite control program was launched by the Department of Communicable Diseases Control (CDC), Ministry of Public Health, Thailand during 1991 to 1996. At the end of the program, a nation-wide survey using Kato's thick smear[5] and modified Kato-Katz stool smear[6] methods for parasite ova detection and identification indicated a decreasing trend of opisthorchiasis in northeastern Thailand, where the infection has long been endemic, from 24.01% in 1991 to 15.3% in 1996.[7] However, the prevalence of the infection in the northern provinces had increased almost six times from 5.6% in 1991 to 32.6% in 1996.[7] Moreover, new endemic foci have been found in the central provinces of the country such as Lopburi (about 200 km north of Bangkok). Nevertheless, a large percentage of metacercariae found in fishes caught from fresh water reservoirs in the northern provinces, belonged to harmless intestinal flukes.[8] Also, purgated stools obtained from the northern inhabitants revealed a majority of adult heterophyid intestinal flukes, and only a minority of the liver fluke.[9] Thus, the question arises whether the parasite ova found in stools of the northern and central population in the 1996 survey were truly *O. viverrini* ova (which implies a high incidence of liver fluke infection) or rather ova of harmless small intestinal flukes misidentified as *O. viverrini* ova.

In this study, a specific antibody test has been developed for more definite determination of *O. viverrini* exposure. A monoclonal antibody affinity column chromatography was used for preparing a specific oval antigen of *O. viverrini* from crude extract of the parasite; the antigen was then used in a dot-ELISA for the serum antibody detection. Diagnostic sensitivity, specificity and accuracy of the assay were evaluated in comparison with, not only the *O. viverrini* ova detection in stools by a conventional method, but also with the definite finding of adult parasites and ova in purgated stools after Praziquantel treatment.

MATERIALS AND METHODS

O. viverrini antigens (OvAg)

O. viverrini adult worms were collected from bile ducts of hamsters which had been infected two-three months previously with 100 metacercariae of *O. viverrini*.[3] The metacercariae used for the hamster infection were dissected out from the muscles of experimentally infected fishes (*Pontius schwanenfeldi* and *P. altus*). The adult worms were washed thoroughly with several changes of normal saline solution (NSS) and once with distilled water (DW). They were ground in small volume of DW containing protease inhibitors (0.1 mM each of phenyl-methyl-sulphonyl-fluoride, and tosyl-amide-2-phenyl ethyl chloromethyl ketone and 10 mM ethylene-diamine tetraacetic acid) using a tissue grinder. The preparation was subjected to sonication at 20 kHz (MSE Ultrasonicator, England) at 4°C (3 × 5 minutes), then centrifuged at 10,000 × *g* at 4°C for 10 minutes. The supernatant was collected dialyzed against distilled water at 4°C overnight and the protein content was determined.[10] This crude extract of adult *O. viverrini* (OvAg) was aliquoted and stored at –20°C until use. For preparing *O. viverrini* oval (egg) antigen (EAg) for mouse immunization, living, uninjured adult flukes freshly collected from the hamster bile ducts were washed three times with sterile Hanks' balanced salt solution and once with sterile RPMI-1640 medium. After washing, the worms were cultured in a glass petridish containing sterile RPMI-1640 medium with 200 units per ml penicillin, 100 mg per ml streptomycin, 2 μg per ml fungizone and protease inhibitors at 37°C in a CO_2 incubator for 72 hours. The spent culture medium was collected every 6 hours and fresh medium was added to replace the harvested aliquot. The collected spent medium was centrifuged; the supernatant was collected, dialyzed against distilled water, and concentrated by Centriprep 30. This preparation represents an excretory-secretory (ES) antigen. The ova in the sediment were homogenized in a small volume of DW which contained the protease inhibitors using a tissue grinder. The preparation was sonicated at 20 kHz for 10 minutes at 4°C (MSE Ultrasonicator). The protein content of the oval (egg) antigen (EAg) was determined.[10]

Heterologous antigens (Table 1) were prepared from *Paragonimus heterotremus*,[11] *Trichinella spiralis* muscle larvae,[12] filariform larvae of *Strongyloides stercoralis*,[13] *Schistosoma* spp.,[14] third stage larvae of *Gnathostoma spinigerum*[15] and male and female *Angiostrongylus cantonensis*.[16] Intestinal parasites, *i.e. Echinostoma malayanum, E. ilocanum, E. revolutum, H. taichui, P. bonnei, Taenia saginata, Ascaris* spp., *Enterobius vermicularis* and human hookworms were obtained from stools of infected individuals after purgation. Extracts of the parasites were prepared in the same manner as *O. viverrini* adult

Table 1 Immunogen and homologous and heterologous antigens used for checking cross-reactivity of the antibodies produced by hybridomas

Name of parasite	Developmental stage		Source
	Adult	Larva	
TREMATODES			
LIVER FLUKES			
1. *Opisthorchis viverrini*	✓	-	Hamsters experimentally infected with metacercariae
2. *Opisthorchis viverrini* egg antigen	-	-	Collected from *in vitro* cultures of adult flukes
3. *Fasciola gigantica*	✓	-	Autopsy from cow
LUNG FLUKES			
4. *Paragonimus heterotremus*	✓	-	Cats experimentally infected with metacercariae
MEDIUM SIZED-INTESTINAL FLUKES			
5. *Echinostoma ilocanum*	✓	-	Stools of infected individuals after purgation
6. *Echinostoma malayanum*	✓	-	Stools of infected individuals after purgation
7. *Echinostoma revolutum*	✓	-	Stools of infected individuals after purgation
MINUTE INTESTINAL FLUKES			
8. *Haplochis taichui*	✓	-	Stools of infected individuals after purgation
9. *Phaneropsolus bonnei*	✓	-	Stools of infected individuals after purgation
BLOOD FLUKES			
10. *Schistosoma japonicum*	✓	-	Mice infected with cercariae obtained from infected snails
11. *Schistosoma mansoni*	✓	-	Mice infected with cercariae obtained from infected snails
12. *Schistosoma mekongi*	✓	-	Mice infected with cercariae obtained from infected snails
NEMATODES			
13. *Gnathostoma spinigerum*	-	3rd stage larvae	Livers of naturally infected eels
14. Human and dog hook worms	-	larvae	*In vitro* cultures of ova obtained from stools of infected children and dogs, respectively
15. *Strongyloides stercoralis*	-	filariform larvae	*In vitro* culture of rhabditiform larvae obtained from stools of infected children
16. *Trichinella spiralis*	-	✓	Experimentally infected mice
17. *Enterobius vermicularis*	✓	-	Stools of infected individuals after purgation
18. *Angiostrongylus cantonensis* males and females	✓	-	Experimentally infected rats
19. *Ascaris lumbricoides*	✓	-	Stool of patients
20. *Ascaris suum*	✓	-	Autopsy from pig
21. *Toxocara canis*	✓	-	Autopsy from stray dogs
22. *Dirofilaria immitis*	✓	-	Autopsy from stray dog's hearts
CESTODES			
23. *Taenia saginata*	✓	-	Stools of infected individuals after purgation
24. *Echinococcus granulosus*	-	-	Hydatid fluid
25. *Cysticercus cellulosae*	✓	-	Autopsy from hill tribe swine
26. *Hymenolepis nana*	✓	-	Stools of infected individuals after purgation
PROTOZOA			
27. *Toxoplasma* tachyzoites	✓	-	Experimentally infected mice
28. *Plasmodium falciparum*	✓	-	Blood stage cultures
29. *Entamoeba histolytica*	-	-	*In vitro* cultured trophozoites

worm antigen (OvAg). Other antigens, namely extracts of *Entamoeba histolytica, Toxoplasma* tachyzoites, *Fasciola gigantica, Dirofilaria immitis, Toxocara canis,* cysticercus, *Hymenolepis nana,* dog hookworms, *Ascaris lumbricoides, Echinococcus granulosus*, and *Plasmodium* spp. were gifts from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok and the Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen province, Thailand.

Ten BALB/c mice (6 weeks old) (Armed Forces Research Institute of Medical Sciences, Bangkok) were individually immunized intraperitoneally with 40 μg of EAg in 0.2 ml of NSS emulsified in complete Freund's adjuvant. Thereafter, three booster doses containing 80 μg of the same antigen in incomplete Freund's adjuvant were given to each mouse at 14 day intervals. Seven days after the last booster dose, individual mice were bled from retro-orbital plexi and the serum antibody titers were determined by an indirect ELISA against the homologous antigen. The mouse which had the highest antibody titer was selected as an immune splenocyte donor in cell hybridization and was given an intravenous booster with 40 μg of EAg in 0.1 ml of NSS. The other mice were bled and their sera were collected, pooled and used as a posi-tive serum (PS). Three days after an intravenous booster, the selected mouse was bled and the serum collected as an immune serum (IS), then the animal was sacrificed. Splenocytes of the mouse were hybridized with P3x-63-Ag8.653 myeloma cells.[17] The fused cells were suspended in hypoxanthine-azaserine selective medium and approximately 2.0×10^5 cells were distributed in 0.2 ml aliquots into wells of 96-well tissue culture plates. The plates were incubated in a humidified 5% CO_2 incubator at 37°C and the medium in tissue culture wells containing growing cells was changed at appropriate time. Spent culture media of all wells containing growing cells were screened for antibodies against the homologous antigen and for cross-reactivity against the heterologous antigens (Table 1) by the indirect ELISA. Cells from wells whose spent media reacted only to *O. viverrini* antigens, *i.e.* EAg, OvAg and ES antigen were subjected to cloning by a limiting dilution method. Specific hybridomas secreting monoclonal antibodies (MAb) reactive to only the homologous parasite antigens were subjected to an expanded growth. The spent media were collected, isotyped and the antibody titers at the stationary phase of cell growth were determined by the indirect ELISA. A representative clone of the so-produced hybridomas was selected for bulk production of MAb which were used for preparing an affinity column for capturing *O. viverrini* specific oval antigen from crude extract of adult parasites (OvAg).

SDS-PAGE and Western blot analysis (WB)

SDS-PAGE was carried out in a vertical slab gel apparatus (MiniPROTEAN, Bio-Rad Laboratories, USA) according to the system of Laemmli[18] A 4% acrylamide stacking gel and 12% acrylamide separating gel were used in the process. Western blot analysis (WB) was performed as previously described.[17]

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was performed as follows: wells in the microplates were coated individually with 100 μl of antigen diluted in carbonate-bicarbonate buffer, pH 9.6 (10 μg protein per ml). The plates were incubated at 37°C overnight. The unbound antigen was washed away using phosphate buffered saline (PBS), pH 7.4 containing 0.05% Tween-20 (PBST). The unoccupied sites in the wells were blocked by 200 μl of 1% bovine serum albumin (BSA) and 0.2% gelatin in PBS, pH 7.4 at 37°C for 1 hour. After washing as above, 100 μl of individual antibody preparations were added to appropriate wells. The blank wells with only PBS, pH 7.4 were included in each plate. The plates were incubated at 37°C for 1 hour, then 100 μl of 1:1,000 goat anti-human IgG-horseradish peroxidase conjugate (Dakopatt, Denmark) was added and incubated at 37°C for 1 hour. After the final wash, 100 μl of freshly prepared peroxidase substrate was added to each well. The enzymatic reaction was allowed to occur at room temperature in the dark for 30 minutes, then stopped by adding 50 μl of 1 N NaOH. The optical density (OD) of the content in each well was determined at 492 nm against the blank wells using an ELISA reader (Labsystem, Multiskan *Ex*, Finland). A result was declared positive when the read-out OD was ≥ 0.05. One indirect ELISA unit (EU) was the smallest amount of the antibody preparation that still gave a positive indirect ELISA result. The precision of the indirect ELISA was determined by testing the mouse positive serum (PS) on different occasions by different scientists. The OD were determined and percent coefficient of variation (% CV) was calculated. Reproducibility of the assay according to the criteria of Palmer and Cavallaro[19] was met.

Dot-ELISA was performed as previously described.[20] Three µl aliquots of affinity purified *O. viverrini* oval antigen were dotted (100 ng per dot) onto a nitrocellulose membrane (NCM) (Bio-Rad, USA) and air-dried. The NCM was submerged in 3% BSA for 1 hour to block the unoccupied sites. The NCM was then cut into individual squares; each square accommodated one spot of the dotted antigen. All squares were labeled appropriately and then placed individually in appropriate wells of a 24-well tissue culture plate containing diluted individual serum samples (1:400). The antigen-antibody reaction was allowed to take place for 30 minutes. All NCM squares were washed with PBS, pH 7.4 and then placed in a container of rabbit anti-human immunoglobulins (Igs)-alkaline phosphatase conjugate (Dakopatt, Denmark; diluted 1:1,000 in substrate buffer) followed by a substrate solution. Positive and negative controls (a pool of five sera of individuals from whom *O. viverrini* adult worms were found in stool after Praziquantel treatment and purgation and a pool of five sera of parasite-free individuals, respectively) were included in the test. Reproducibility of the dot-ELISA was determined by testing the same positive and negative sera on different occasions by different scientists. Correct results were obtained indicating high reproducibility of the assay.

Preparation of affinity-purified *O. viverrini* specific oval antigen

MAbIgG were prepared from a pool of spent culture media of the selected hybridoma by 50% ammonium sulfate precipitation and affinity chomatography on Protein A Sepharose 4 Fast Flow according to the techniques described by Moks *et al.*,[21] Uhlen *et al.*[22] and the instruction manual of Pharmacia LKB Biotechnology (Uppsala, Sweden). The MAbIgG were coupled to CNBr-activated Sepharose CL-4B (Pharmacia) according to the instruction of the manufacturer. In brief, pre-swollen Sepharose was equilibrated with coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, 0.5% Tween-80), pH 8.5. To each 2 ml volume of the packed gel, 4 ml of 2.5 mg per ml of the MAbIgG in the same buffer was added and mixed by rotating at 37°C for 2 hours then overnight at 4°C. The unbound MAbIgG were removed by low-speed (250 × *g*) centrifugation of the preparation at 4°C. The gel was washed with Tris-HCl buffer, pH 8.0 containing 0.5% Tween-20 by centrifugation until the supernatant contained no protein as detected by spectrophotometry at 280 nm. After the supernatant was removed, the gel was incubated in an excess volume of Tris-HCl, pH 8.0 containing 0.5% Tween-20 and 1% BSA on a rotator at room temperature for 2 hours. The gel was washed several times with Tris-HC1 buffer, pH 8.0 and twice with 0.01 M phosphate buffer, pH 7.0 (starting buffer). Three ml of crude extract of *O. viverrini* adult worms (6 mg OvAg per ml starting buffer) was mixed with the MAbIgG-coupled Sepharose and the mixture was rotated at 37°C for 2 hours. The preparation was centrifuged at 250 × *g*, 4°C for 10 minutes; the supernatant was removed and the gel was washed once with the starting buffer before being packed into a glass column (10 mm × 100 mm). The gel was washed thoroughly until the eluate contained no detectable protein by spectrophotometry at 280 nm. The *O. viverrini* antigen that bound with the MAb-IgG in the column was then eluted out with 0.1 M glycine-HC1 buffer, pH 2.0 (eluting buffer). The eluate was collected in 1 ml fractions and each fraction was immediately added with two drops of 1.0 M Tris-HCl, pH 8.5 (neutralizing buffer). The optical density of each fraction was determined at 280 nm in a spectrophotometer and the fractions which showed an OD above the blank (1.0 M Tris-HC1) were pooled, dialyzed against DW and lyophilized as the affinity-purified, specific oval antigen of *O. viverrini* (OvsAg).

Serum specimens

Two batches of serum samples were used in this study. A written and/or verbal informed consent was obtained from each subject before commencing the experiment.

Group 1 of the first batch included individual serum samples of 42 parasitology confirmed *O. viverrini* infected individuals. Not only did their stool samples reveal *O. viverrini* eggs by modified Kato-Katz method[6] at the time of serum collection, but also adult flukes were recovered from their stool after Praziquantel treatment (40 mg per kg body weight) and saturated magnesium sulfate purgation (45 ml orally 3 hours after the Praziquantel treatment).[1] Group 2 of this batch were 427 individuals infected with parasites other than *O. viverrini*. They were 84 patients with trichinellosis (*Trichinella spiralis* larvae were found in nurse cells of muscle biopsies),[12] 50 individuals with small intestinal fluke infections (ova and the adult flukes were found in stools), 48 individuals with strongyloidiasis (filariform larvae of *Strongyloides stercoralis* could be cultured from their stools

Table 2 Detailed classification of individuals whose serum samples were grouped in the first batch

Infection	Number of serum specimens	Diagnostic method(s)
Group 1		
Opisthorchiosis viverrini	42	*O. viverrini* ova in stools by modified Kato-Katz technique and *O. viverrini* adult worms in stools after praziquentel treatment and salt purgation
Group 2		
Trichinellosis	84	Patients whose muscular biopsies at the time of serum collection revealed encysted larvae of *Trichinella spiralis*[12]
Small intestinal fluke infection	50	Small intestinal fluke ova in stools by modified Kato-Katz method and adult worms in stools after praziquentel treatment and purgation.
Strongyloidiasis	48	Stool cultures were positive for filariform larvae of *Strongyloides stercoralis*[13]
Gnathostomiasis	47	Western blot analysis with the presence of a band at 24 kDa[15]
Schistosomiasis mekongi	32	*Schistosoma mekongi* ova in stools
Hookworm infection(s)	30	Hookworm ova in stools
Ascariasis	20	*Ascaris lumbricoides* ova in stools; adult worm in stools after anthelminthics
Echinostomiasis	20	*Echinostoma* spp. ova in stools by formalin-ether technique and Kato-Katz method
Taeniasis	20	Mature segments of *Taenia* spp. and adult worm in stools after Praziquantel treatment and purgation
Trichuriasis	20	*Trichuris* spp. ova in stools by formalin-ether technique and Kato-Katz method
Filariasis	13	Microfilaria in the blood smears
Paragonimiasis heterotremus	13	*Paragonimus heterotremus* ova in stools and /or sputum and a positive band in Western blot analysis at *Mr* 35,000[11]
Malaria	10	*Plasmodium falciparum* in blood smears
Amoebiasis	10	*Entamoeba histolytica* cysts in stools and serum antibodies to *Entamoeba histolytica* trophozoites[23]
Angiostrongyliasis	10	Western blot analysis of serum samples with the presence of a band at 31 kDa[16]
Group 3		
Normal sera (Thais)	52	Healthy individuals whose stool, sputum and blood samples were negative for parasites and parasite products and who have never been to an endemic area of *Opisthorchis viverrini*
Normal sera (Swedes)	30	Healthy Swedes whose stool, sputum and blood samples were negative for parasites or parasite products at the time of serum collection and who have never visited an endemic area of *Opisthorchis viverrini*; these samples were gifts from Dr. Marianne Fehlmann, Kalmar Hospital, Sweden

by polyethylene tube method),[13] 47 patients with gnathostomiasis (their serum samples contained antibodies to the 24 kDa specific antigen of *Gnathostoma spinigerum* infective larvae),[15] 32 individuals with schistosomiasis mekongi[14] (ova of *Schistosoma mekongi* were found in stool specimens), 30 individuals with hookworm infection (hookworm ova in stools), 20 individuals with taeniasis (proglottids and ova of the tape worm were found in stools), 20 individuals each with echinostomiasis, trichuriasis and ascariasis (ova of the respective parasites were found in stools), 13 individuals with filariasis (microfilaria in blood smears) and 13 individuals with paragonimiasis (ova of *Paragonimus heterotremus* were found in sputum and stool and their sera were positive for the 35 kDa band in Western blot analysis),[11] 10 individuals each with malaria (*Plasmodium falciparum* in blood smears), invasive amoebiasis (*E. histolytica* cysts in stool and serum antibody test positive)[23] and angiostrongyliasis cantonensis (a history of eating improperly cooked snail dishes and presence of serum antibodies to specific 31 kDa antigen in their sera).[16] Group 3 included 82 normal, parasite-free individuals; 30 of which were Swedes and 52 were Thais (Table 2).

The second batch of serum samples was obtained from 190 individuals. After an informed consent was signed, individual stool samples were screened for parasites/or parasite ova by modified Kato-Katz method.[6] Subjects were tentatively classified into four groups according to the results of the modified Kato-Katz method. Group 1, *O. viverrini* infected group, included 55 individuals whose stool samples revealed *O. viverrini* ova. Group 2, mixed infection of *O. viverrini* and other parasites, included 46 individuals whose stool samples revealed *O. viverrini* ova and other parasites or the ova, *e.g. Taenia* spp. (both proglottides and ova were found), ova of *Echinostoma* spp. and hookworms, and filariform larvae of *Stronglyloides stercoralis*. Group 3 included 40 individuals whose stool samples revealed parasites and/or ova of parasites other than *O. viverrini* ova; they were ova of *Echinostoma* spp., hookworms and *Trichuris trichiura* and filariform and rhabditiform larvae of *S. stercoralis*. Group 4 included 49 individuals whose stool samples revealed neither parasites nor ova of parasites by the modified Kato-Katz technique (normal). However, it is known that the diagnostic sensitivity of the modified Kato-Katz method is not 100%. Moreover, the so-interpreted *O. viverrini* ova might not definitely be the ova of *O. viverrini*, *i.e.* ova of small intestinal flukes misidentified as *O. viverrini* ova and *vice versa* or the mixture of the two. For definite diagnosis, adult parasites must be found in the hosts. Therefore, all of the 190 individuals were asked to take oral Praziquantel treatment (40 mg per kg body weight) followed 7 hours thereafter by saturated magnesium sulfate (45-50 ml orally). The purgated stool was collected from each individual and examined for adult parasites by sedimentation and sieve method.[1] Based on the sedimentation and sieve method, it was found that only 31 of 55 individuals originally classified in group 1 revealed adult *O. viverrini* and their ova (infected only with *O. viverrini*) while of the purgated stools of the remaining 24 individuals, 14 revealed only adult small intestinal flukes; thus, they were re-classified as group 3 and 10 individuals revealed mixed infection with *O. viverrini* and small intestinal flukes; thus they were re-classified as group 2. There were 16 individuals of group 4, who were originally classified as parasite-free individuals, whose purgated stool specimens revealed either few adult *O. viverrini* only (8 individuals; thus, they were re-allocated to group 1) or mixed adult *O. viverrini* and adult small intestinal flukes (8 individuals; thus, they were re-allocated to group 2). Therefore, there were only 33 individuals who were parasite-free (group 4) after intensive investigation of the purgated stools. Among the 46 individuals originally classified in group 2 (mixed infection of *O. viverrini* and other parasites), there were 21 individuals whose purgated stools revealed small intestinal flukes (not *O. viverrini*) and other parasites; thus, they were re-allocated to group 3. On the other hand, there were 22 individuals of group 3 whose purgated stools revealed mixed infection of *O. viverrini* and other parasites which is the criterion of group 2; thus, they were re-allocated to group 2. Detail classifications of the 190 individuals of the second batch are shown in Table 3. Serum samples were subsequently taken from all individuals for dot-ELISA which was subsequently performed by a different scientist who was not informed of the results of the stool examinations.

Statistical analysis

Diagnostic sensitivity, specificity and accuracy of the dot-ELISA using *O. viverrini* specific oval antigen were analyzed in comparison with the diagnosis by conventional parasitology and/or serological methods using the method of Galen.[24]

RESULTS

The immune mouse which

Table 3 Classification of 190 individuals whose serum samples were included in the second batch

Group	Classification	Method used for classification		
		Stool samples examined for parasites/parasite ova by modified Kato-Katz method	Praziquantel treatment and salt purgated stools examined by sedimentation and sieve method for adult parasites	
		Number	Number	Remarks
1	*O. viverrini* infection only	55	39	31 from originally classified group 1 and 8 from originally classified group 4 by modified Kato-Katz method
2	Mixed infection of *O. viverrini* and other parasite(s)	46	65	25 from originally classified group 2 + 10 from originally classified group 1 + 22 from originally classified group 3 and 8 from originally classified group 4 by modified Kato-Katz method
3	Infection by parasite (s) other than *O. viverrini*	40	53	18 from originally classified group 3 + 14 from originally classified group 1 and 21 from originally classified group 2 by modified Kato-Katz method
4	Parasite-free	49	33	Adult *O. viverrini* were found in purgated stools of 8 individuals who were originally classified in group 4; adult *O. viverrini* and other parasites were found in purgated stools of 8 individuals who were originally classified in group 4 by the modified Kato-Katz method. Thus, they were re-classified in groups 1 and 2, respectively

had the highest indirect ELISA titer (1:51,200) against the homologous antigen was selected as immune splenocyte donor. A total of 8.3×10^8 spleen cells were fused with approximately 8.3×10^7 myeloma cells. A total of 876 tissue culture wells (48%) revealed growing hybrids; spent culture fluids from 592 wells were screened for antibodies to *O. viverrini* antigens and the fluids from 196 wells were positive. Among them, fluids from 52 wells cross-reacted with extract of adult *Paragonimus heterotremus,* the lung flukes which are prevalent in Thailand. Fluids of only 12 wells of the remaining 144 wells gave high indirect ELISA optical densities; thus, the cells from these 12 wells were subjected to cloning. Five hybridomas secreting specific antibodies to *O. viverrini* antigens were established. They were clones 3D6, 1E4, 3E3, 6C2 and 6G11. The immunoglobulin (Ig) heavy chain isotypes of the MAb secreted by the individual clones were γ2a, μ, γ 1, μ and γ2a, respectively. All of the MAb carried kappa light chains. Fig. 1 shows antigenic specificities of the MAb secreted by the hybridomas in a Western blot analysis against SDS-PAGE separated-crude extract of adult *O. viverrini* (OvAg). The clone 3D6 was subjected to expanded growth for bulk production of MAb because the cells were healthy, grew fast and the Ig isotype was γ2a (IgG is known to have higher binding affinity to the antigenic epitope than IgM). Besides, at stationary phase of an *in vitro* growth, the spent medium of this hybridoma had an indirect ELISA titer at 1:1,024 against the homologous antigen which was the highest titer among all of the culture fluids of the five hybridomas. The MAb of clone 3D6 (MAb3D6) were used to prepare an affinity column for *O. viverrini* specific oval antigen (OvsAg). Fig. 2 illustrates anatomical location of specific epitopes of the MAb3D6 in the adult *O. viverrini* section by an immunoperoxidase staining. Specificity of the MAb3D6 to *O. viverrini* ova was confirmed.

Fig. 3 illustrates the SDS-PAGE separated-OvsAg stained by Coomassie Brilliant Blue and Silver stains.

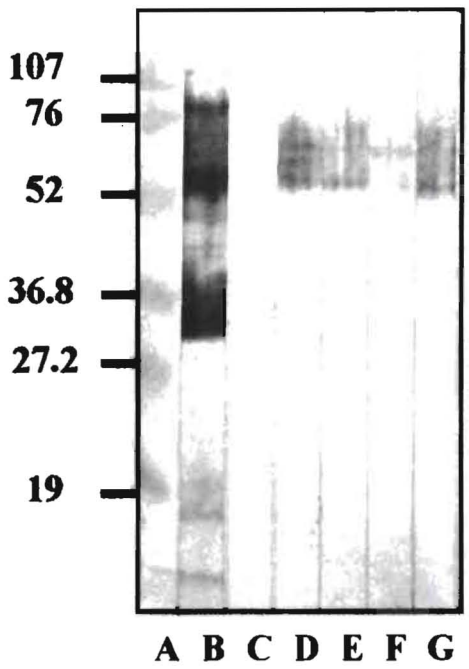

Fig. 1 Antigenic specificities of the monoclonal antibodies produced by hybridomas and immune mouse serum against (IS) SDS-PAGE separated-egg antigen (EAg) in Western blot analysis (WB). Lane A = Molecular weight markers ($\times 10^{-3}$), Lane B = WB of immune mouse serum, Lane C = WB of MAb 3D6, Lane D = WB of MAb 1E4, Lane E = WB of MAb 3E3, Lane F = WB of MAb 6C2, Lane G = WB of MAb 6G11.
Note that MAb3D6 did not react to the SDS-PAGE separated-oval antigen (EAg) in the WB although it was positive by an indirect ELISA at titer 1:1,024 against oval, ES and somatic antigens of *O. viverrini* and also reacted specifically with the parasite ova in an immunoperoxidase staining of the *O. viverrini* sections (Fig. 2).

The optimum concentration of OvsAg and the optimum dilution of sera for the dot-ELISA were determined by a checker board titration. Five serum samples each of groups 1 and 3 of the first batch were used in the titration as positive and negative control samples, respectively. It was found that the smallest amount of OvsAg which gave positive dot-ELISA to all five positive control serum samples (diluted 1:400) (parasitology confirmed *O. viverrini* infection) and negative to all five negative control serum samples of group 3 of batch 1 (also diluted 1:400), was 100 ng per a 3 µl-dot. An OvsAg concentration of 100 ng per dot of 3 µl and a serum sample dilution of 1:400 were used throughout the subsequent experiments.

At dilution 1:400, all sera of group 1 (*O. viverrini* infected individuals) of batch 1 were positive by the dot-ELISA while all samples of groups 2 and 3 of batch 1 (individuals with other parasitic infections and normal, parasite-free, respectively) were negative. Thus the dot-ELISA using OvsAg revealed 100% diagnostic sensitivity, specificity and accuracy.

Serum samples of all four groups of the second batch were tested for specific antibodies to *O. viverrini* by the dot-ELISA using the affinity purified *O. viverrini* oval antigen. It was found that all samples of group 1 (*O. viverrini* infected group) and group 2 (mixed infection of *O. viverrini* and other parasites) were positive while all serum samples of group 3 (individuals infected with parasites other than *O. viverrini*) and group 4 (parasite free individuals) were negative. Thus, the OvsAg-based dot-ELISA has 100% diagnostic specificity, sensitivity and accuracy. Examples

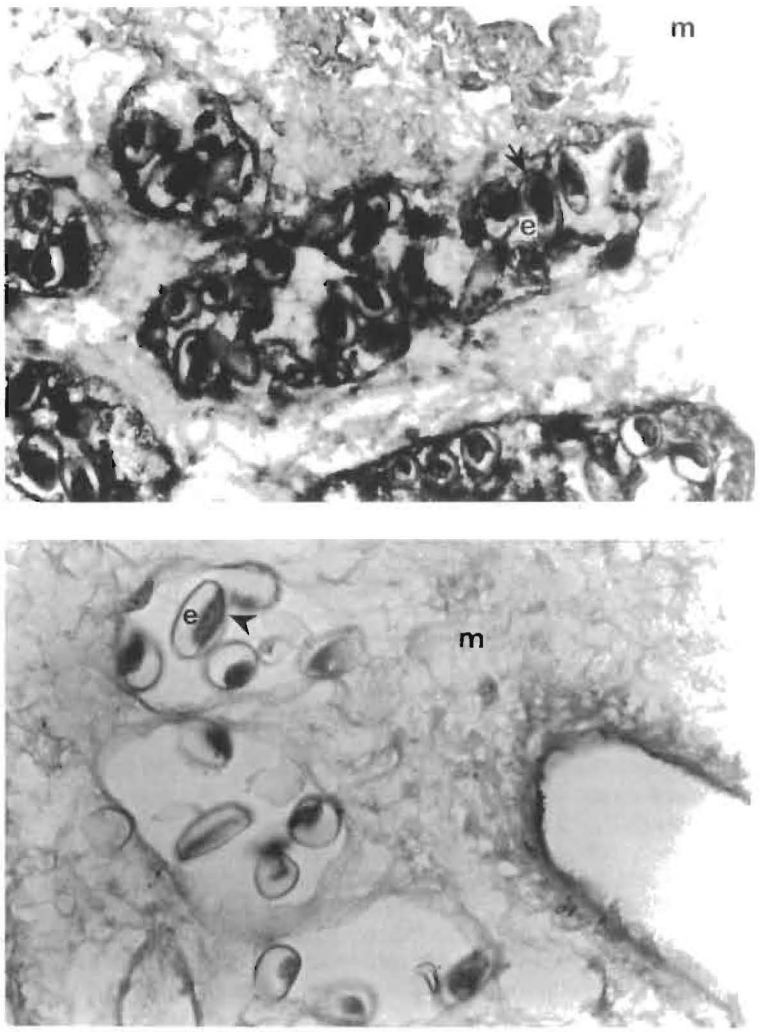

Fig. 2 Anatomical location of MAb3D6 epitopes in *O. viverrini* adult worm section as revealed by immunoperoxidase staining. A, ova in uterus were stained by MAb-based immunoperoxidase (arrow); B, control worm section showing unstained ova in uterus (arrow head).

of the dot-ELISA results are shown in Fig. 4.

DISCUSSION

In Thailand, it has been recognized that a liver fluke infection, *i.e.* opisthorchiasis, caused by *Opisthorchis viverrini*, is highly prevalent in the country, especially in the northeastern area. Infection is acquired by eating raw or undercooked fresh water Cyprinoid fish containing metacercariae which is an infective form of the parasite. Inhabitants of northeastern Thailand are usually infected since very young age (as early as 5 years old).[25] This is because of the eating habit of the traditional raw fish dishes called Koi-pla and Larb-pla, which is being practiced since generations. The ingested metacercariae excyst in the duodenum and the juvenile flukes migrate through the ampulla of Vater into the common bile duct, the extrahepatic biliary system and then into the intrahepatic bile canaliculi where they mature. The mature worms live in human bile ducts, by attachment to the biliary epithelium using their oral suckers, where they lay ova. Despite the detectable host immune responses to the parasite antigens, both locally and systemically, some patients may har-

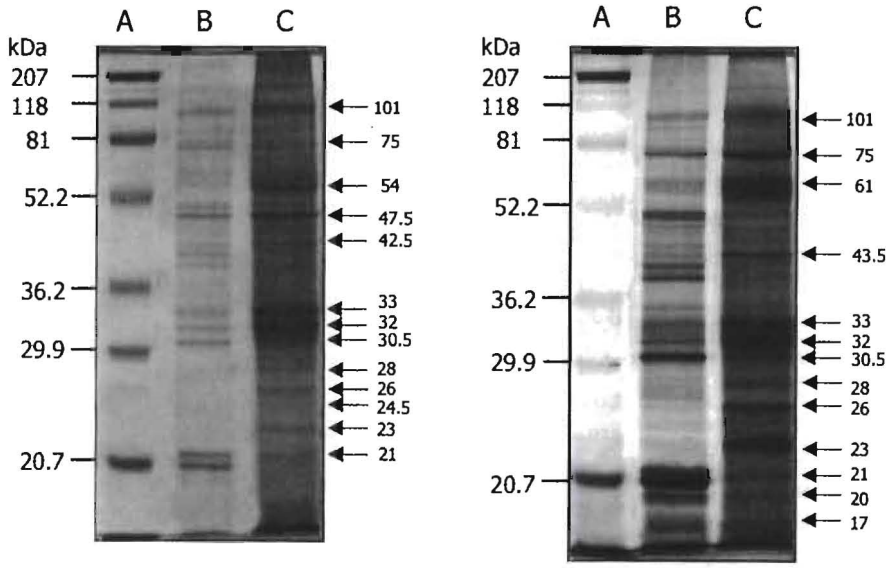

Fig. 3 SDS-PAGE separated-OvsAg (specific oval antigen of *O. viverrini* obtained from MAb3D6-affinity column chromatography) stained by Coomassie brilliant blue dye (left) and Silver stain (right).

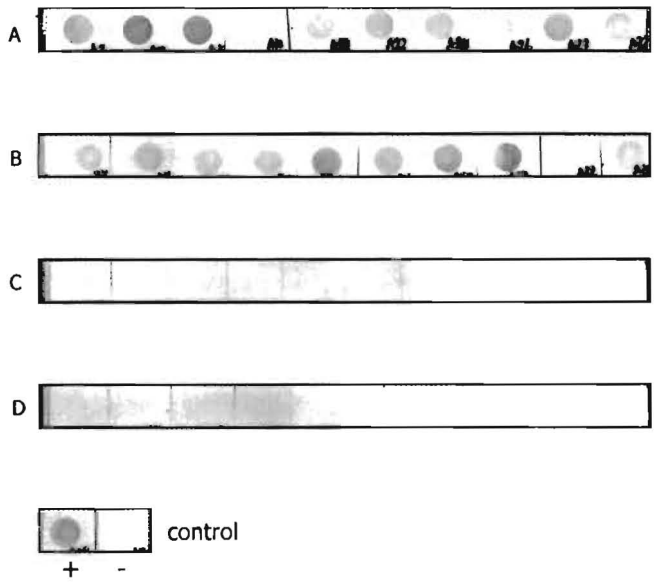

Fig. 4 Representatives of dot-ELISA results when OvsAg was used as antigen.
A and B, positive results appeared on antigen dotted NCM after the NCM were exposed to serum samples of groups 1 and 2, respectively, of the second batch; C and D, NCM showing negative results when the antigen dotted NCM were exposed to serum samples of groups 3 and 4, respectively, of the second batch.

bor thousands of adult flukes in the bile ducts at any one time suggesting that such acquired immune responses are not effective for parasite elimination, or that re-infection is common.

Although the majority of lightly infected individuals are symptomless, severe forms of opisthorchiasis are often present in case of prolonged infection with a heavy parasite burden, which is common in patients over 40 years of age.[26] The chronic, persistent, heavy infection (due to the parasite longevity and the continuation of re-infection) can lead to chronic relapsing cholangitis, obstructive jaundice and in a large proportion of cases eventually to fatal cholangio-carcinoma (CCA) which usually occurs during the 5th to 6th decade of life.[27]

Despite successful treatment of the infection by an effective drug, *i.e.* Praziquantel, and the implementation of an extensive health education program for promoting change in the habit of raw fish eating and ex-latrine defecation practices, the overall incidence of *Opisthorchis viverrini* infection in Thailand is not reduced. A nationwide parasite survey in 1996, using the conventional Kato thick smear and modified Kato-Katz techniques to detect parasite and parasite ova in stools revealed a higher incidence of opisthorchiasis in the country than in the year 1991.[7] Moreover, there are new endemic foci of the infection in the northern and central provinces. It is known, however, that the two methods used in the survey are not solely reliable regarding their sensitivity and specificity. A large percentage of individuals with a less than 20 adult *O. viverrini* worm burden, may reveal no parasite ova in their stool.[28] On the other hand, the ova found in stools may not belong to the *O. viverrini*, owing to the morphological similarity of the ova of this dangerous flukes and of the relatively harmless heterophyid flukes. For definite differential diagnosis and accurate quantification of the intensity of infection, it is necessary that the adult parasites are found in purgated stools and counted after Praziquantel treatment and salt purgation. Such measure is not only laborious, impractical in a large scale and unpleasant, it also usually does not gain much community compliance. Alternatives, *i.e.* a monoclonal antibody based-copro-antigen detection assay and DNA techniques, although specific, do not meet a satisfying sensitivity; also, the latter are laborious, expensive and may be hazardous. It is known, however, that during localization of the adult flukes in the host's biliary tracts, the adult parasites and their products irritate and damage the epithelium causing local inflammation[29] which potentiates accessibility of the parasite antigens, *e.g.* ES and oval antigens, to the host immune apparatus and ultimately elicit the host immune response(s). Detection of such adaptive immune response(s) in the host should be diagnostic of the parasitic infection/exposure. Several antibody detection assays have been developed for serum antibody detection of opisthorchiasis. However, most assays use crude extracts of adult parasites as antigen.[30] Thus, the assays are not specific because the serum antibodies so-detected might be incited by other organisms sharing similar heterogenetic (heterophile) antigens with *O. viverrini*.[31] Therefore, a specific antigen is needed for the antibody assay.

While production of a specific *O. viverrini* antigen by genetic engineering has been hampered by the carbohydrate nature of the specific antigen which does not readily allow expression of the antigen by the available techniques, like the conventional purification procedure of the antigen from crude extract of worms, *i.e.* chromatographic techniques, are laborious and expensive and might not be successful. Moreover, the yielded antigen is not only usually low in quantity but also subjected to variation in quality from batch to batch.

Monoclonal antibodies specific to a desired epitope obtained from hybridoma technology are excellent tools for purifica-tion of the parasite specific antigen by means of an affinity chromatography. Specific antigen can be prepared by passing a crude preparation of antigen through a specific MAb-based affinity column. After washing away the unbounds, the bound antigen can be eluted out under conditions which disrupt the interaction of the ligand with the MAb which have been immobilized by an insoluble matrix, such as Sepharose, in the column. After all of the specific antigen has been eluted, the affinity column is ready for recycling.

In this study, a hybridoma secreting specific MAb to *O. viverrini* oval antigen has been produced. Monoepitope specificity of the MAb was ascertained by testing the MAb with a large panel of heterologous parasite antigens. The MAb reacted specifically with only the antigens derived from the homologous parasite, *i.e. O. viverrini*, which included not only oval antigen, but also ES antigen and the crude worm extract. The crude worm extract (OvAg) is easier to prepare than the egg antigen (EAg). The MAb were, therefore, used to capture the respective epitopes from

OvAg and the yield of specific antigen (OvsAg) obtained was relatively high. The specific antigen was used as an immunodiagnostic reagent for specific detection of antibodies in sera of *O. viverrini* infected/exposed individuals using the simplest version of a sensitive enzyme-immunoassay, *i.e.* dot-ELISA. Perfect agreement of the results of adult *O. viverrini* fluke recovery after Praziquantel treatment and stool purgation followed by total stool sedimentation and sieve technique with the results of the serum antibody detection using the affinity-purified antigen was observed, indicating reliability of the latter for monitoring liver fluke exposure. The dot-ELISA is easy to perform, relatively less expensive, less laborious and more rapid (90 minutes) as well as more pleasant than the stool manipulation of the parasitological methods. Several samples can be tested at the same time without much increase in the test time which is suitable for a large scale survey. Most of all, it should gain higher compliance when serum samples are taken than when purgated stools are requested from a community. Concurrent testing for several different parasites using just a single serum sample per patient can also be performed by applying individual parasite specific antigens, prepared similarly to the OvsAg, on the same solid phase, *e.g.* a plastic comb or a nitrocellulose strip; then the dot-ELISA procedure can be completed in a similar manner as when testing for just one parasitic infection. It is recommended, therefore, that in an area where several parasitic infections are concurrently endemic, a more economical, multi-antibody test system based on individual specific parasite antigens prepared as described in the present communication should be implemented.

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