PN-AAZ-824
5611
6.392

# PROGRESS REPORT NO. 3

# DEVELOPMENT OF METHODS FOR IMMUNODIAGNOSIS OF HUMAN LIVER FLUKE INFECTION

A RESEARCH PROJECT

USAID / PSTC PROGRAM

GRANT NO. 936-5542-G-00-6027-00

SUBMITTED BY

STITAYA SIRISINHA

DEPARTMENT OF MICROBIOLOGY

FACULTY OF SCIENCE MAHIDOL UNIVERSITY

BANGKOK THAILAND

Rec'd in SCI. DEC 7 1987

## PROJECT PROFILE

| | |
|---|---|
| Country | Thailand |
| Grant No. | 936-5542-G-00-6027-00 |
| Program | Program on Science and Technology Cooperation |
| Project Title | Development of Methods for Immunodiagnosis of Human Liver fluke Infection |
| Project Leader | Dr. Stitaya Sirisinha |
| Organization | Mahidol University |
| Co-investigators | Dr. Peter B. Billings<br>Dr. Watanalai Panbangred |
| Project Consultants | Dr. Sakol Panyim<br>Dr. Pornchai Matangkasombut |
| Authorized Officer | Dr. Natth Bhamarapravati<br>Rector, Mahidol University |
| Total Budget | US$150,000 |
| Project Duration | 3 years (May 1986 to December 1989) |
| Reporting Period | 5/1987 -10/1987 |
| Budget Allocation for this period | US$37970.63 (Baht 983900) |

## 1. Background

Liver fluke infection is a major health problem in Southeast Asia, the Far East, and Central and Eastern Europe, with at least 40 million people currently infected. In Thailand alone, it has been estimated that at least 7.5 million people (about 15% of the population) are infected with *Opisthorchis viverrini* and the prevalence in some districts is at least 90% or higher.

Attempts thus far to control the disease include interuption of life cycle, case detection and chemotherapy. These approaches with the exception of a trial mass chemotherapy with praziquantel in small villages are not satisfactory. Reinfection readily occurs after chemotherapy but can be reduced if such treatment is accompanied by health education aimed at changing food habits and improving sanitation and personal hygiene.

Currently the diagnosis of *O. viverrini* infection, based on the detection and identification of eggs in the feces, is tedious and time-consuming, and is reliable only under the supervision of experienced laboratory personel and therefore does not lend itself to wide application necessary for disease control programs in highly endemic areas where new cases alone overwhelm laboratory capacities. Moreover, cases with light infections and when there is biliary obstruction such as cholangiocarcinoma can result in false negative findings. The development of a simple, sensitive and specific test for antibodies to the parasites would provide a useful tool for epidemiological surveys, giving information on previous exposure to the parasite and, perhaps, some indication of pathological complications. The test can be

used to monitor the success of chemotherapy as well as the rate of reinfection. Serological tests for the detection of serum antibody including precipitation, agglutination and complement fixation have been used in the past. However, the results are not satisfactory as they lack sensitivity and specificity, and do not lend themselves readily to be used in small, poorly equipped laboratories. The development of enzyme-linked immunosorbent assay (ELISA) has reactivated the interest in developing serological methods for the diagnosis of liver fluke infections. In recent years, ELISA has been used in attempting to find suitable immunodiagnostic methods. However, due to the complexity of the antigens available, the test lacks specificity. An indirect immunofluorescent test has also been used but the technique cannot be readily performed in most laboratories in Thailand. The characterization and purification of parasite-specific antigens is hence a necessary starting point for establishing suitable systems with diagnotic potential. Moreover, some clues as to the possible immunological basis for protection may be obtained, thus allowing immunoprophylaxis or a way to enhance post-exposure immunity.

## 2. Objectives

The main objective of this project is to develop a simple, sensitve and specific immunoassay(s) for the diagnosis of human liver fluke infection caused by *Opisthorchis viverrini*. While the principal aim is to detect specific antibody in the serum and/or other body fluids (e.g., saliva and bile), a simultaneous

development of a method to detect specific parasite antigen(s) will also be carried out. The immunoassays to be developed should:

a. Be able to establish diagnosis is suspects including those with low intensity of infection.
b. Identify *O. viverrini* infection in patients with biliary obstruction and other complications.
c. Be reliably and extensively performed by laboratory personnel with minimal laboratory training.
d. Be able to monitor the efficacy of individual and mass anthelmintic treatment as well as reinfection.

The approach is to characterize, identify and purify relevant antigenic components by appropriate physicochemical and immunological methods using a battery of sera from infected individuals, rabbit polyclonal and mouse monoclonal antibodies. Initial steps in the attempt at production of relevant antigens by recombinant *E. coli* carrying genomic *O. viverrini* DNA fragments will also be made. Polyclonal and monoclonal antibodies together with corresponding relevant antigens developed will be used in the formulation of immunoassays.

Research activity

Activity 1 : Preparation of antigens

(A) *O. viverrini antigens*. The various *O. viverrini* antigens, including somatic, surface tegument and metabolic products

(ES) of adult worms, were prepared as described in details in Progress Reports No. 1 and 2. The parasites were obtained from naturally infected cyprinoid fish bought and caught in Khon Kaen province. The parasite cycle is being maintained in hamsters kept in the Animal Center of the Faculty of Science. Currently we have approximately 150 hamsers infected with 100 metacercariae each. These antigens were used for the various immunoassays as well as for screening of monoclonal antibodies.

(B) Schistosoma japonicum antigen. Crude somatic extract of S. japonicum was prepared from adult male and female worms obtained from albino Swiss mice infected with S. japonicum cercariae (kindly made availavble by Dr. Prasert Sobhon, Department of Anatomy, Faculty of Science, Mahidol Unviersity). The adult worms were homogenized and sonicated essentially the same as done for O. viverrini adults. The antigen will be used for analyzing specificity of the immunoassay systems being developed as well as for specificity screening of monoclonal antibodies.

(C) Nematode antigens. These nematode antigens will be used for specificity study mentioned in (B). Two tissue nematodes, namely Gnathostoma spinigerum and Angiostrongylus cantonensis, have been selected because both are available locally in the same general areas where O. viverrini is found and the infections are acquired by eating raw or poorly cooked food made from infected intermediate hosts (e.g., fish and snails). G. spinigerum third-stage larvae were prepared from eel livers bought from local markets in Bangkok. The livers were chopped

into small pieces and then digested overnight at 37°C in 1% acid pepsin solution. The larvae were picked from the digested liver one by one. After thorough washing, they were kept frozen until a sufficient number was available for antigen extraction. A. cantonensis third-stage larvae were obtained from snails (Australorbis glabratus) infected 3 weeks previously with infective first-stage larvae obtained from the feces of experimentally infected rats. The flesh of infected snails was chopped into small pieces and placed on a Baerman apparatus filled with warm (37°C) 1 % acid pepsin digesting solution pH 2-3. The larvae migrating down to the tip of the funnel were collected, washed and kept frozen until the time of antigen extraction. Both G. spinigerum and A. cantonensis third-stage larvae, and adult A. cantonensis from heart and lungs of infected rats were use for the preparation of crude somatic antigens by homogenization and sonication as described previously for O. viverrini antigens.

Activity 2: Collection of specimens from patients

We are now collecting serum, bile and saliva from patients with positive O. viverrini eggs in the stool. These were patients with biliary obstruction coming in for surgery at Khon Kaen University Hospital. Whereas hepatic bile was previously obtained by cannulation as described in Progress Report No. 2, gall bladder bile was collected in this group of patients. In some of these patients, saliva was also collected.

In addition to these opisthorchiasis patients, we also collected blood and sometimes saliva from O. viverrini negative

individuals who reside in the endemic area of Q. viverrini infection, either with or without infections by other intestinal helminths. In order to locate these individuals, a large number of patients attending the Outpatient Clinic of the University Hospital were screened by fecal examination for Q. viverrini eggs.

Activity 3 : Characterization of Q. viverrini antigens

The various Q. viverrini antigens, i.e., somatic extract, surface extracts and metabolic products of adult worms, somatic extracts of metacercariae and eggs, were characterized by radioimmunoprecipitation and SDS-PAGE as described previously in Progress Report No. 1. Because of its availability, detailed analysis was carried out with the somatic extract of adult worms. The relevant data on this section were analyzed and described in the manuscript which is now in press in Parsitology (paper #3 described in the last section of this report).

Briefly, the results showed that a prominent protein component that reacted with all sera from our patients has a molecular weight of 89,000 daltons. It is most likely specific for Q. viverrini as sera from animals experimentally infected with other parsites failed to react with this component. It should be mentioned that this 89 kd protein is the major component found in the in vitro maintenance fluid of adult worms and most likely represents one of the ES antigens of the fluke. Another prominent component is a low molecular weight doublet with $M_r$ of 16-17 K. This component represents approximately 50%

of the protein in the somatic extract and it most likely represents tegumental protein of the parasite. In addition to these 2 components, there are a few other minor components with specificity for O. viverrini. They are now being characterized.

Activity 4 : Testing of the immunoassay system

Using the optimal conditions otained as described previously in Progress Report No. 2, the enzmye-linked immunosorbent assay (ELISA) was tested to evluate its sensitivity and specificity. We found that it was possible to obtain positive ELISA readings of the serum from all O. viverrini-positive individuals. Under the same conditions, serum from patients with other parasitic diseases (including toxoplasma, filariasis, schistosomiasis, paragonimiasis) gave non-significant readings that were within a standard deviation of normal individuals. However, patients from the O. viverrini endemic area, but habouring other intestinal parasites gave slight to moderate readings. It is possible that the positive results may represent a true cross reactivity or an unrecognized subclinical previous infection by O. viverrini. In order to resolve this problem, we had to turn to testing serum from animals monoinfected with some of these parasites. Our limited data thus far obtained suggest that these positive results noted in humans may be associated with previous subclinical O. viverrini infection.

Although the antibodies can be detected in IgG, IgA and IgE fractions, a majority of antibody is associated with the IgG antibody isotype. The association of antibody primarily with the

IgG fraction is rather unexpected as we previously expected to see a closer asociation with the IgA isotype since this infection is totally a surface infection. Most other infections of the mucosal surfaces are associated with an IgA antibody response. In our study, the IgA antibody detection was not too satisfactory as the background ELISA reading was rather high compared with those of other antibody assays. The IgE antibody detection can also be used in diagnosis as it is highly specific. However, not all patients with opisthorchiasis gave positive ELISA results with IgE antibody. Therefore, if we can obtain a better antigen, it may be possible to test the IgE system. It is interesting, however, to see elevated total serum IgE in this infection, indicating that infection by O. viverrini can potentiate IgE production similar to observed with tissue helminth infections. Specific antibodies have also been detected in the bile of a number of patients with biliary obstruction. A more detail description of this finding has been written up (article #4 in the last section).

Activity 5 : Attempt at cloning O. viverrini genes

Because previous attempts during the last 8-10 months, as reported in Progress Report No. 2, have given negative results, several modifications have been incorporated into the work during the last 3-4 months.

(A) O. viverrini DNA preparation

Because the DNA preparation obtained by the protocol described in detail in the last progress report always had one

extra peak of unidentified material by gel electrophoresis, we therefore added one extra step in attempt to get rid of this unidentified material. In this experiment, 500-600 adult *O. viverrini* were homogenized and DNA extracted by the phenol-chloroform-ether method. The agarose gel electrophoresis of this material indicated that at this point about 700 μg of DNA was obtained. The preparation was then purified further by CsCl ultracentrifugation. All 700 μg DNA was dissolved in an appropriate solvent and then CsCl was added to a final concentration of 1 gm/ml. Then ethidium bromide was added to a final concentration of 800 μg/ml. The ultracentrifugation was carried out at 45,000 rpm at 20°C for 18-24 hr. The DNA band was collected by needle puncture technique and ethidium bromide was removed by isoamyl alcohol. The DNA was then ethanol precipitated at -20°C, centrifuged at 10,000 rpm and the precipitate was washed with 70% ethanol and subsequently dissolved in Tris-EDTA (TE) buffer.

(B) *Plasmid preparation*. Three plasmids, namely, pUC8, pUC9 and pUC12, were prepared essentialy as described previously for pUC12 in the last progress report. The p C12 was prepared from *E. coli* JM107 whereas the pUC8 and pUC9 were prepared from *E. coli* JM83 harbouring these plasmids. After the *E. coli* were grown until log phase, plasmids were prepared and finally purified by CsCl ultracentrifugation. The latter was performed at 45,000 rpm for 18-20 hrs at 20°C. The plasmid band was collected, washed, precipitated with absolute ethanol and then dissolved in TE solution.

(C) Construction of recombinant plasmids and transformation into E. coli JM 107

Slight modifications were made in order to increase the yield of recombinants. First, both the purified O. viverrini DNA and the three plasmid vectors were cut by restriction enzyme Pst I at 37°C for 1 hr. The average size of O. viverrini obtained was 4 kilobases. Secondly, the recombinant plasmid construction was performed using an O. viverrini DNA to plasmid DNA ratio of 10:1 instead of 4:1 used in the last report. The ligation was done using $T_4$ ligase in an overnight incubation at 15-16°C. The transformants were plated on LB-agar plate containing 100 μg/ml penicillin together with 200 mM isopropyl β-D-thiogalactopyranoside and 2% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. On this selection medium, the recombinants give colorless white colonies. On the overage 18-21% were found to be recombinants. However, about 17% of these white colonies resulted from vector deletion. It was estimated therefore that there were $2 \times 10^5$ white colonies from using 600 ng the recombinant plasmid DNA. To check the average size of the inserted DNA, white bacterial colonies were randomly picked for plasmid extraction and cut with EcoRI. The size of inserts were found to vary from 0.57 to 4.4 kb and 5 of 23 insert pieces contained an EcoRI site.

(D) Colony immunoassay

Our limited data suggest that detergent lysis of bacterial colonies may interfere with the immunoassay method in this system. Therefore, a different lysis method was used. The bacteria were blotted onto nitrocellulose paper (NC), grown as

previously described, and then lyzed by a non-detergent method (Bourgouin, C., Mol. Gen. Genetics 205:390-397, 1986). Briefly, the colonies on nitrocellulose paper were fixed in chloroform vapor for 15 min and then lyzed by lysozyme solution containing 3% BSA, 1 μg/ml DNase and 40 μg/ml lysozyme. This step was carried out for 2 hr at 37°C. The paper was washed to remove cell debris and then blocked overnight at 4°C with 5% non-fat powdered milk at pH 7.4. The colonies were then screened with polyvalent rabbit antiserum known to contain antibodies to various somatic antigens, egg antigens and tegumental antigens. Non-specific reaction with *E. coli* components was eliminated by affinity chromatography using *E. coli* lysate linked cyanogen bromide-activated Sepharose. Other procedures were essentially as described in Progress Report No. 2.

Using this colony immunoassay, none of the approximately 30,000 white clonies screened thus far gave positive results.

Activity 6 : Production of monoclonal antibodies

In the previous report, we detailed some of the evidence which led us to believe that the myeloma line we were using for our fusions was contaminated with mycoplasma. This particular strain of mycoplasma is associated with on extremely high thymine- (or pyrimidine-) nucleoside phosphorylase activity. As a consequence, it very rapidly depletes HAT medium of the essential protective component thymidine, and infected "wild-type" cells including hybridomas die in HAT after a short period of growth. Several strains of the same myeloma line were obtained from other laboratories in Bangkok, and most passed this

simple bioassay for mycoplasma--*i.e.*, co-culture with them or their supernatants did not render mycoplasma-free hybridomas susceptible to death in HAT. Two of these myeloma strains were used in fusions in our laboratory under the usual conditions. Although these myelomas gave hybridomas, the recovery of hybrid clones was relatively poor. In each case, a total of only about 100 fused cells or colonies were obtained, about $1/10^{th}$ to $1/20^{th}$ of that expected of a good myeloma under similar conditions. This yield probably reflects the unknown histories of these strains, total time in culture, minimum and maximum densities and particularly cloning, since the latter selects a homogeneous but as yet untested population, which either fuses well or poorly.

Rather than attempt a slow adaptation of these lines to our fusion conditions or further optimization of these conditions, we sought another strain of myeloma with a more known history. Ultimately such a line was obtained from Flow Laboratories in Australia. This strain of the 653 myeloma line has given us 10- to 20-fold better hybridoma yields in three fusions with different antigens and does not appear to need further adaptation/selection or optimization of fusion conditions .

One of these fusions with the new myeloma was with mice immunized with *Opisthorchis viverrini* somatic antigen. To conserve antigen and improve immunization, the soluble antigen was adsorbed onto nitrocellulose and about 25 and 35 μg were given in the primary and a single booster immunizations. The protein-adsorbed nitrocellulse was dissolved in dimethylsulfoxide to facilitate injection and mixed with a quantity of Freund's

complete or incomplete adjuvant such that it remained soluble during subcutaneous and intraperitoneal injection. Primary immunization was followed by a single booster after 1 month, and 2 months later, the best responder of 3 was given a single I.P. injection of 50 μg of soluble somatic antigen. Fusion was performed 3 days later under our original fusion conditions, i.e., before attempts to optimize these to our previous myeloma strains. The fusion was distributed between twenty 96-well culture plates. Almost all wells (ca. 2000 wells) exhibited growth and all were assayed by a replica plate ELISA when early wells reached 50-70% confluency. Between 2 assays, approximately 40 ELISA-positive wells were picked and transferred to 24-well plates for partial scale-up prior to freezing or cloning.

Many of these hybrids have been cloned once and positive clones re-frozen. However, we have now exhausted our current supply of soluble somatic antigen, and cloning for the remainder as well as repeat or sub-cloning will have to await the preparation of further antigen, which is underway at present. In the meanwhile, existing supernatants are being used in immunoprecipitation with radioiodinated antigen (an assay requiring very little antigen) to select the most suitable or desirable specificities and reserve precious antigen for their further cloning. At present, only a few hybridomas have been characterized as to their fine specificities by this procedure.

Problems

Monoclonal antibodies. Because of a limited supply of

antigen, mice were immunized using a complex mixture of soluble antigens from adult worms adsorbed onto introcellulose. The rationale for this was that it requires far less immunogen and considerably improves the response. This scheme may be useful when the total yield of hybrids is poor and the specific yield even lower -- 2 or 3 lines for assay, cloning and characterization. It would seem however, that combined with a more suitable myeloma giving a better fusion yield, this conservation of antigen at the immunization level is a false economy. Clearly, the majority of antigen is used in our ELISA; despite the fact that this is only 5 µg/ml, this represent, the equivalent of 1 immunization (25 µg) per assay plate. In the future, it may prove better to use more during the immunization to promote a more vigorous response and perhaps more affinity maturation. With a higher affinity, perhaps wells may be pooled by columns and rows and assayed checkerboard fashion, thus conserving antigen.

We anticipate that the problem of obtaining antigen will be lessened since a source of infected fish has been found and our infected hamster supply re-established. In addition, this will provide a source of more selectively enriched antigen such as ES antigen with a higher proportion of the parasite-specific 89 Kd polypeptide for use as immunogen and/or assay antigen.

Workplan for the next period

All activities initiated during this period will be refined further. The activity regarding the development of immunoassay has thus for reached a satisfactory stage and is ahead of other

activities, i.e., genetic manipulation of the parasite genome and production of monoclonal antibodies. With success in these 2 last mentioned activities, the work regarding improvement of the immunoassay method will be reinitiated. However, at the same time acquisition of the parasites, preparation of infected fish for the hamster infection and obtaining adult flukes from infected hamsters for antigen production (both somatic extract and *in vitro* maintenance culture fluid) are being continued to service the needs for other activities. Therefore, the activities that we will concentrate on during the next working period include genetic manipulation and gene cloning, and production and characterization of monoclonal antibodies. In addition to these experiments, we will also screen for repetitive DNA segments of *O. viverrini* in *E. coli*. This will be done by using the genomic *O. viverrini* as probe. The genomic DNA will be labeled by nick-translation using $\alpha$ -$^{32}$P-dATP and used in colony hybridization onto nitrocellulose filters containing recombinant clones. The clones with strong signal will be tested by Southern blot hybridization in order to get more information regarding the characteristics of each clone.

A reasonably large number of hybridomas positive by ELISA against some antigen present in the crude adult somatic antigen preparation have been produced. In so far as we do not have sufficient antigen to clone and subclone all of these, they will be assayed first by radioimmunoprecipitation to select the most suitable for further processing and characterization. This work which has already begun should provide extremely useful

information as to the fine specificities of the hybridomas at least to the point of polypeptide species, information which cannot be obtained by the ELISA.

In addition, we have a number of mice immunized by the same scheme, as well as animals primed with more selectively enriched antigen preparations. These animals will be used in additional fusions to increase the diversity of out repertoire of monoclonal antibodies while fine characterization of existing specificities is in progress. If antigen remains limiting, we will attempt to develop a method of pooling supernatants before using to minimize waste and enable more assays to be performed with the same amount.

Publications

Articles that have been and/or will be shortly published since the initiation of this project are listed below.

1. Sirisinha, S. Immunodiagnosis of human liver fluke infections. Asian Pacific J. Allergy Immunology 4:81-88, 1986 (reprints were submitted with Progress Report No. 2).

2. Sirisinha, S. and Wongratanacheewin, S. Immunization of hamsters against Opisthorchis viverrini infection. Southeast Asian J. Trop. Med. Pub. Hlth. 17:567-573, 1986 (reprints were submitted with Progress Report No. 2).

3. Wongratanacheewin, S., Chawengkirttikul, R., Bunnag, D. and Sirisinha, S. Analysis of Opisthorchis viverrini antigens by immunoprecipitation and polyacrylamide gel electrophoresis. Parasitology 96 (in press) 1988 (2 copies of a galley proof of the paper are enclosed).

4. Wongratanacheewin, S., Bunnag, D., Vaeusorn, N. and 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. (submitted for publication).

5. Sirisinha, S. Approaches to develop immunodiagnostic methods for liver fluke infection caused by Opisthorchis viverrini. Proceedings of USAID Science Award Grantees. July 1987.

Thesis

A portion of the research for this project has been conducted by a Ph.D. candidate in our laboratory (Mr. Surasakdi Wongratanacheewin). His thesis is entiled "Characterization of Humoral Immune Response in the Serum and Bile of Patients with Opisthorchiasis and Its Implication in Immunodiagnosis". He has now finished all requirement for the degree and will become a lecturer at the School of Medicine, Khon Kaen University, in Khon Kaen province where he will continue his research in conjunction with our group here in Bangkok.

Attending scientific meeting and presentation of paper

The principle investigator was invited to attend a 3-day scientific meeting on "Medical Microbiology Towards 2000; S.E. Asia-U.S.A. Perspectives and presented a paper entitled "Immunodiagnosis of human liver fluke infection caused by Opisthorchis viverrini". The meeting took place in Hong Kong on 29 May - June, 1987.

XA-AA2-824-A
#3 Proof of the paper for publication 56115
6. 392
Prog hyp /u/abs GS
H34 PAR Ms. No. 337 28.7.87 JBY

*Parasitology* (1988), 96, 000–000
*With 5 figures in the text*

# Analysis of *Opisthorchis viverrini* antigens by immunoprecipitation and polyacrylamide gel electrophoresis

S. WONGRATANACHEEWIN[1], R. CHAWENGKIRTTIKUL[1], D. BUNNAG[2] *and* S. SIRISINHA[1]*

[1] *Department of Microbiology, Faculty of Science, and Hospital for Tropical Diseases*[2], *Faculty of Tropical Medicine, Mahidol University, Bangkok* 10400, *Thailand*

(*Accepted* 29 *June* 1987)

SUMMARY

Antigenicity of adult *Opisthorchis viverrini*, metacercariae and eggs was investigated by radio-immunoprecipitation and polyacrylamide gel electrophoresis using sera from patients with opisthorchiasis and from hamsters experimentally infected with *O. viverrini* metacercariae. $^{125}$I-labelled aqueous somatic extract and *in vitro* metabolic products of adult worms, and the extracts of metacercarine and egg were immunoprecipitated, electrophoresed and autoradiographed and major antigens were characterized. Although components with $M_r$ > 116, 89, 78 and 20 kD appeared to be specifically associated with the somatic extract of adult fluke, the 89 kD protein was the most prominent component found in the *in vitro* culture fluid of adult worms and the metacercarial extract. Hamsters, experimentally infected with *O. viverrini*, also showed the presence of antibody reactive with the 89 kD component within 15 days of infection. The potential of this antigen in immunodiagnosis is discussed.

## INTRODUCTION

Liver fluke infections caused by *Opisthorchis viverrini*, *O. felineus* and *Clonorchis sinensis* are still a major health problem in Southeast Asia, the Far East, and Central and Eastern Europe, with many millions of people currently infected. Moreover, infections have been recently reported from several countries previously known to be free of the disease. The latter is attributed, at least in part, to increasing numbers of immigrants and refugees from countries in the endemic regions. Laboratory personnel in both endemic and non-endemic regions must be more aware of the possibility of *Opisthorchis* and *Clonorchis* infections. It is for this reason that a simple, reliable, less time-consuming and more objective method for diagnosis is needed. Current methods for the diagnosis of human liver fluke infection are based on the demonstration of eggs in stool of suspected individuals. However, in many circumstances, a false negative result is not uncommon, for example, in the case of light infection or biliary obstruction. Previous efforts to develop immunodiagnostic methods for human liver fluke infections were largely unsuccessful (Sirisinha, 1986). Several serological techniques have been employed to detect *O. viverrini* infection but considerable cross-reactivity with other helminthic infections remains to be eliminated (Janechaiwat, Theravanij, Vajrasthira & Chaicumpa, 1980; Feldheim & Knobloch, 1982; Sirisinha, Tuti, Vichasri & Tawatsin, 1983; Srivatanakul, Viyanant, Kurathong & Tiwaweeh, 1985; Boonpucknavig, Kurathong & Thamavit, 1986).

Analysis of the *O. viverrini* antigens is of importance for understanding the

* Reprint requests to Dr S. Sirisinha, Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

Rec'd in SCI. DEC 7 1987

host–parasite relationship, and for the development of sensitive and specific serodiagnostic assays. Little is currently known about *O. viverrini* antigens. It is reported that protein components of adult *O. viverrini* were highly complex, the most abundant component(s) representing 47% of total parasite protein having an apparent molecular weight ($M_r$) of 18000–19000 (Ruppel, Boonpucknavig & Hempelmann, 1985). By indirect immunofluorescence, sera from patients with opisthorchiasis were found to react strongly with the eggs and surface tegument of the parasite (Boonpucknavig *et al.* 1986). The purpose of the present study was to characterize and to identify *O. viverrini* components that may have potential in immunodiagnosis.

## MATERIALS AND METHODS

### *Preparation of* O. viverrini *antigens*

#### *Adult somatic antigens*

Adult *O. viverrini* were collected from hamsters experimentally infected with metacercariae obtained from the flesh of naturally infected cyprinoid fish (Sirisinha, Puengtomwatanakul, Sobhon, Saitongdee, Wongpayabal, Mitranonde, Radomyos, Bunnag & Harinasuta, 1984). After washing thoroughly, these flukes were homogenized at 4 °C in a small volume of 0·85% NaCl (saline) in the presence of 0·1 mM phenylmethylsulphonylfluoride (PMSF) and 0·1 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK). The suspension was then sonicated in an ultrasonic disintegrator (Soniprep 150, MSE Scientific Instruments, Manor Royal, England) set to operate at maximum amplitude at 1 min intervals for a total of 10 min at 4 °C. The sonicate was kept overnight at 4 °C before being centrifuged (Sorvall RC-5B, Dupont Instruments, Newtown, Conn.) at 10000 r.p.m. for 30 min. The supernatant fluid, referred to as somatic antigen, was kept in small aliquots at −20 °C.

#### *Adult ES antigens*

The metabolic products (ES) of adult *O. viverrini* were prepared from the *in vitro* culture fluid in which these flukes were maintained (Tuti, Vichasri & Sirisinha, 1982). Actively motile flukes, which had not suffered damage, were collected from the bile duct and gall bladder of experimentally infected hamsters and immediately washed in Hank's balanced salt solution (Gibco, Grand Island, N.Y.) containing penicillin (200 units/ml), streptomycin (200 μg/ml) and fungizone (2 μg/ml). In a typical experiment, flukes were maintained *in vitro* at 37 °C for 3 days in Eagle's basal medium (BME, Gibco: 0·1 ml/worm) in a loosely covered glass container under an atmosphere of 5% $CO_2$. The culture fluid was then centrifuged at 2000 r.p.m. for 15 min in a refrigerated centrifuge. The clear supernatant fluid was stored frozen at −20 °C until a sufficient quantity had been collected. The pooled supernatant culture fluid was thereafter concentrated in a nitrogen atmosphere by membrane ultrafiltration (PM 10, cut-off at 10 kD, Amicon, Lexington, Mass.). A large volume of saline was added to the concentrated fluid and it was reconcentrated by ultrafiltration twice more to get rid of residual culture medium.

#### *Egg antigens*

Adult flukes were homogenized, sonicated and centrifuged as described above. The pellet was largely intact eggs with traces of cellular debris, judging from microscopic examination. It was resuspended and allowed to sediment by gravitation several times

to reduce cellular debris. The preparation obtained as such was extensively homogenized at 4 °C until most eggs appeared to be ruptured. Soluble supernatant fluid obtained after centrifugation was used for SDS–PAGE analysis.

*Metacercarial antigens*

Metacercariae, individually picked from the peptic digest of infected cyprinoid fish, were washed several times in saline prior to being homogenized and sonicated as described for adult worm antigens.

### *Antisera to* O. viverrini *antigens*

Antisera containing antibodies reactive with *O. viverrini* antigens were obtained from the following sources.

*Patients with opisthorchiasis*

Human sera used in this study were obtained from patients attending the outpatient clinic of the Hospital for Tropical Diseases (Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). Examinations of stool samples for intestinal parasitic infections, together with blood and urinalysis, were routinely performed for all patients. Patients with signs and symptoms consistent with opisthorchiasis and whose stool examinations showed only *O. viverrini* eggs were selected for this study. Blood samples from these patients were allowed to clot and sera were kept frozen individually until analysed.

*Experimentally infected hamsters*

Blood was obtained from the ascending aorta of hamsters orally infected with 10 metacercariae of *O. viverrini* (Sirisinha *et al.* 1984; Puengtomwatanakul & Sirisinha, 1986). Groups of hamsters were sacrificed and blood taken 15 days, 1, 3 and 6 months after infection. Appropriate serum specimens were pooled and kept frozen at −20 °C until analysed.

### *Radio-immunoprecipitation of* O. viverrini *antigens*

Immunoprecipitation of $^{125}$I-labelled *O. viverrini* antigens by human and hamster immune sera was performed essentially as described by Ivarie & Jones (1979), using the Staphylococcal protein A precipitation technique. Antigens were radio-iodinated by the chloramine-T technique using a ratio of 2 $\mu$Ci radioactive iodine and 0·2 $\mu$g chloramine-T/$\mu$g protein (Greenwood, Hunter & Glover, 1963). Staphylococcal protein A used as immunoadsorbent was obtained commercially as Sepharose-conjugated protein A (Sigma, St Louis, MO). To minimize non-specific binding of radioactively labelled proteins to serum components and protein A, all labelled proteins, if not indicated otherwise, were pre-adsorbed by incubating the sample for 20 min at 4 °C with appropriate amounts of pooled normal serum (0·2 $\mu$l/$\mu$g protein) and protein A Sepharose. The pre-adsorbed labelled antigens were then immunoprecipitated with the appropriate immune sera. After washing, specifically bound antigens were dissociated from the complexes by boiling in sample buffer for 2·5 min, and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Mo

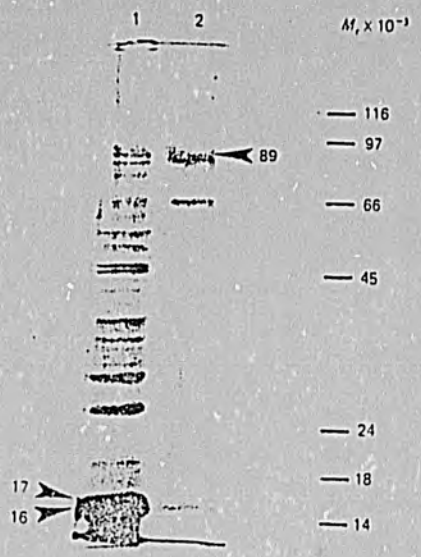


Fig. 1. SDS polyacrylamide gel electrophoresis of *Opisthorchis viverrini* antigens. Adult somatic extract (Lane 1) and metabolic product (ES, Lane 2) were electrophoresed and stained with Coomassie blue. Arrows indicate major components of the somatic extract (17–16 kD doublet) and of the ES (89 kD molecule). Relative molecular weights ($M_r$) of standard markers are shown.

### *Polyacrylamide gel electrophoresis (PAGE)*

Slab gel electrophoresis was performed in the presence of 1% (w/v) sodium dodecyl sulphate (SDS) as described by Laemmli (1970). A 10% (w/v) acrylamide solution was prepared and allowed to polymerize at room temperature for at least 3 h. A 4% (w/v) stacking gel was formed on the top 1 h before the samples were applied. Both the unknown and standard proteins were prepared in sample buffer containing 0·0625 M Tris–HCl, pH 6·8, 1% SDS, 10% (v/v) glycerol and with or without 5% (v/v) 2-mercaptoethanol and were heated in a boiling waterbath for 2·5 min. The gel was stained with Coomassie blue or periodic acid–Schiff (PAS), dried and exposed to Fuji X-ray film (Fuji Photo Film Co., Ltd, Japan) at −70 °C. The molecular weights of electrophoretically separated components were determined by comparing with standards as described elsewhere (Weber & Osborn, 1969; Segrest & Jackson, 1972).

### *Protein determination*

Protein concentrations of all *O. viverrini* preparations were estimated by a Folin method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as a standard.

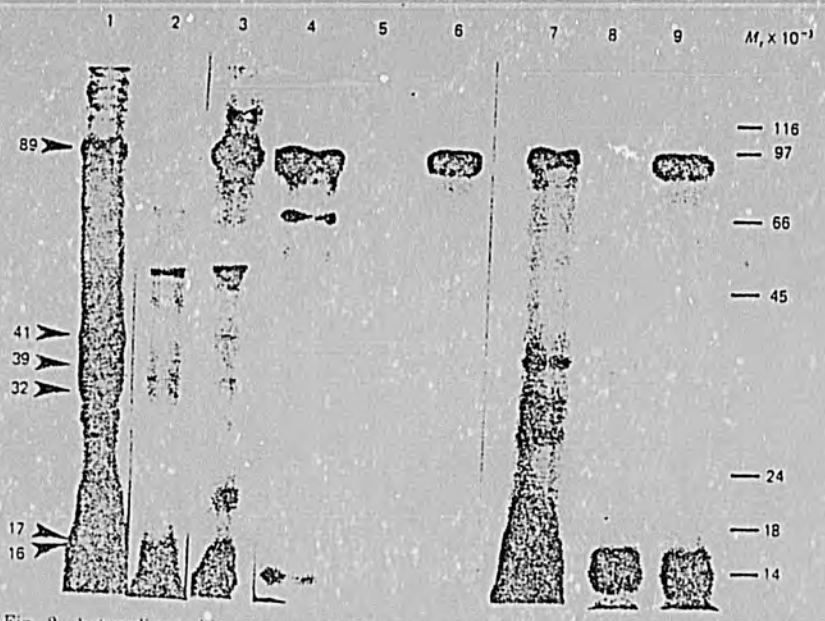


Fig. 2. Autoradiographic patterns of $^{125}$I-labelled *Opisthorchis viverrini* antigens. Adult somatic extract (Lanes 1–3), ES (Lanes 4–6) and metacercarial somatic extract (Lanes 7–9) were electrophoresed before (Lanes 1, 4 and 7) and after (Lanes 2, 5 and 8) being immunoprecipitated with pooled normal human serum and with pooled *O. viverrini*-positive ($Ov^+$) human serum (Lanes 3, 6 and 9). Arrows indicate components of adult somatic extract that were labelled strongly with $^{125}$I. The relative molecular weights ($M_r$) of standard markers are shown.

## RESULTS

### *Protein profiles of* O. viverrini

Coomassie blue staining of the SDS–PAGE patterns shown in Fig. 1 indicate the complexity of aqueous somatic extract of adult *O. viverrini*. More than 30 peptides with $M_r$ ranging from 12 to > 116 kD could be detected, regardless of whether electrophoresis was carried out under reduced or unreduced conditions. A densitometer tracing of the somatic extract showed that approximately 50% of the total protein was associated with a doublet having an $M_r$ of 17–16 kD (Fig. 1, Lane 1). Results from PAS staining and lectin binding (unpublished observations) indicated that the doublet is a glycoprotein. It is most likely that the glycoprotein doublet is localized in the surface tegument of the parasite as it was also a predominant component found in the Triton X-100 extract (unpublished observations).

Unlike the somatic extract, the metabolic products of adult flukes maintained *in vitro* in protein-free culture medium exhibited a simple SDS–PAGE pattern (Fig. 1, Lane 2). It consisted of a major component with an $M_r$ of 89 kD and a few minor components, one of which had the mobility of the glycoprotein doublet above. It should be noted that the 89 kD component was present in trace quantity only in the somatic extract.

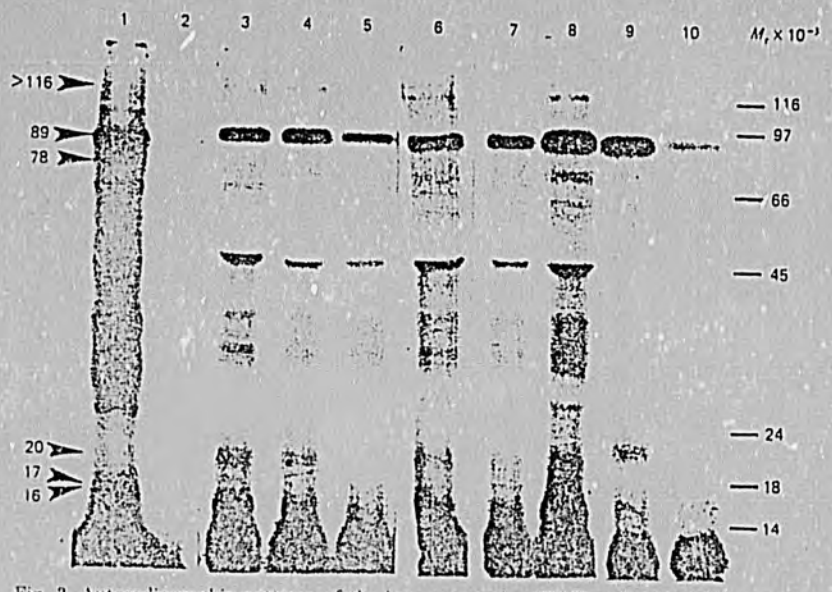


Fig. 3. Autoradiographic patterns of the immunoreactive 89 kD molecule. Adult somatic extract was labelled with $^{125}$I (Lane 1) and then immunoprecipitated with normal human serum (Lane 2) and with a panel of individual sera from *Ov*$^+$ patients (Lanes 3–8). Lanes 9 and 10 represent patterns of a paired serum from another *Ov*$^+$ patient before (Lane 9) and after treatment with praziquantel (Lane 10, 60 days post-treatment). See legend to Fig. 2 for all other explanations.

Because of the limited quantity available for study, the SDS–PAGE patterns of metacercarial and egg extracts had to be stained with the silver nitrate method (data not shown). The staining patterns of these two developmental stages were not as complex as that of the adult worm extract but were considerably more complicated than that of the ES. While the 89 kD peptide also represented a major component of the metacercaria, it was only a minor protein component in the eggs.

### *Characterization of* O. viverrini *antigens*

Autoradiographs of the various *O. viverrini* preparations labelled with $^{125}$I by the chloramine-T method are shown in Fig. 2. Similar to its protein staining pattern, the labelling pattern of adult somatic extract was highly complex but with major bands at positions 89, 41, 39–32, and 17–16 kD (Fig. 2, Lane 1). On the other hand, the labelling patterns of *in vitro* metabolic products (Fig. 2, Lane 4) and metacercarial somatic extract (Fig. 2, Lane 7) were considerably less complex, with a major band at the 89 kD position. The radio-immunoprecipitation results presented in Fig. 2 clearly demonstrated that the immunoreactive 89 kD component was present in all three *O. viverrini* preparations. A majority of sera from individuals with *O. viverrini* eggs in stools reacted also with the 17–16 kD doublet (Fig. 3). A weaker reaction was noted with components having an $M_r$ of >116, 78 and 20 kD.

Antibody reactive with the 89 kD component was consistently present in the sera of

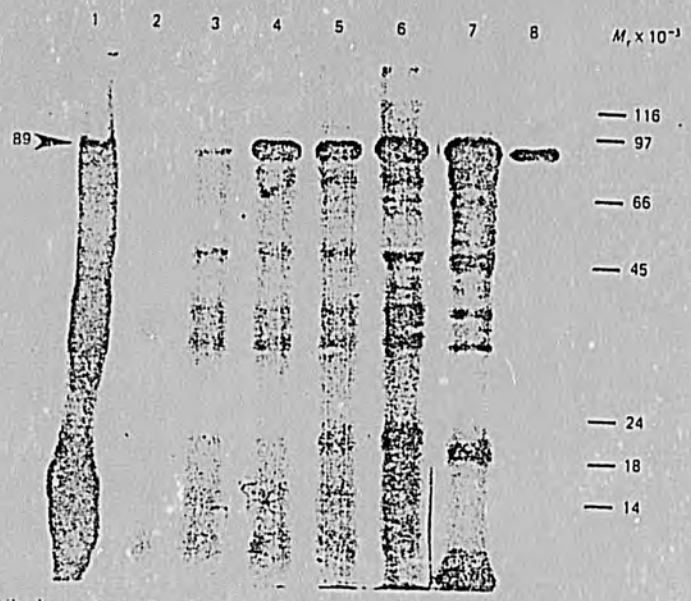


Fig. 4. Antibody against the 89 kD molecule in sera of hamsters with experimental opisthorchiasis. Shown are the autoradiographic patterns of $^{125}$I-labelled adult somatic extract (Lane 1) reacted with normal hamster serum (Lane 2) and with sera from animals infected for 15 days (Lane 3), 1 (Lane 4), 3 (Lane 5) and 6 (Lane 6) months. Lanes 7 and 8 represent patterns from a paired serum from an infected hamster before and after treatment with praziquantel, respectively. See legend to Fig. 2 for all other explanations.

all patients with opisthorchiasis, although the intensity of the band varied from one to another (Fig. 3). Moreover, the intensity of this band decreased considerably after the patients were treated with praziquantel (Fig. 3, Lane 10). Similarly, sera from hamsters experimentally infected with *O. viverrini* gave a strong reaction with this component (Fig. 4). The antibody to the 89 kD component could be detected as early as 15 days after infection and the intensity of the band increased markedly thereafter, but again declined following praziquantel treatment (Fig. 4, Lane 8). The 89 kD component therefore appeared to be specific for *O. viverrini* as it reacted only faintly or totally failed to react with sera from patients with other parasitic infections, for example, schistosomiasis, paragonimiasis and malaria (Fig. 5). Sera from animals experimentally infected with *Schistosoma mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongki*, *Paragonimus heterotremus*, *Angiostrongylus cantonensis* and *Gnathostoma spinigerum* also did not react with this component (data not shown).

## DISCUSSION

The data presented in this study confirmed and extended those reported by other investigators (Janechaiwat *et al.* 1980; Flavell, 1981; Flavell & Flavell, 1986) that several *O. viverrini* proteins could evoke a humoral immune response in the sera of infected man and animals. Using immunoelectrophoretic analysis, these investigators

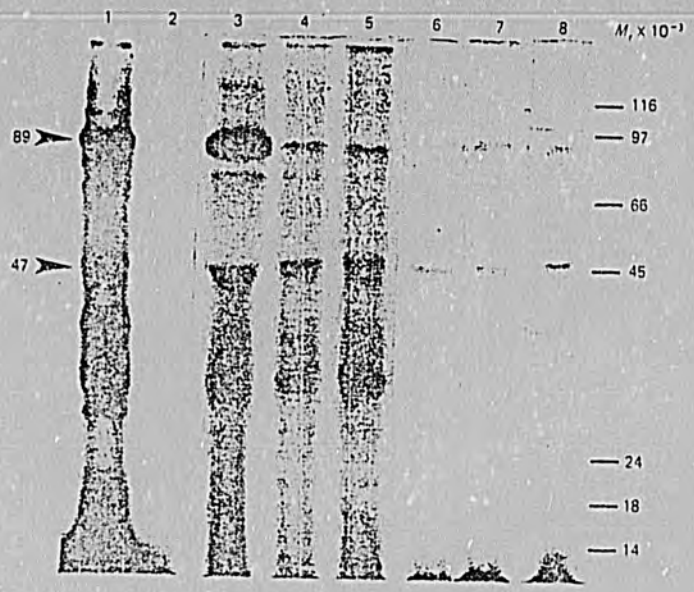


Fig. 5. Specificity of 89 kD molecule. Adult somatic extract was labelled with $^{125}$I (Lane 1) and then allowed to react with normal human serum (Lane 2), *Ov*$^+$ human serum (Lane 3) and sera from patients with *Plasmodium falciparum* (Lanes 4 and 5), *Schistosoma mansoni* (Lane 6) and *Paragonimus heterotremus* (Lanes 7 and 8). A significant reaction with the 89 kD molecule was noted only with the *Ov*$^+$ serum. The 47 kD component, on the other hand, reacted with a majority of sera tested.

demonstrated the presence of antibodies reactive with 4–7 different components in the aqueous extract of adult flukes, in the sera of patients with opisthorchiasis and of hamsters experimentally infected with *O. viverrini*. While several more components were found in the present study to be immunogenic by radio-immunoprecipitation and autoradiography, only 4–5 components appear to be specific for *O. viverrini*. At least one component, i.e., the 89 kD molecules, was not stage specific and could be demonstrated not only in the adult flukes but also in metacercaria.

It was of considerable interest to note that at least half of the total protein in the adult somatic extract was associated with a 17–16 kD glycoprotein doublet, a finding that is similar to the one reported previously by Ruppel *et al.* (1985). Together with the result from the radio-immunoprecipitation of detergent extracts (unpublished observations), it appeared that this doublet is associated with the surface tegument of adult flukes. However, it inconsistently, evoked immune responses in the sera of patients with opisthorchiasis and of hamsters experimentally infected with the parasites. Boonpucknavig *et al.* (1986) showed by an indirect immunofluorescence technique that approximately 75% of individuals whose stools were positive for *O. viverrini* eggs had antibodies reactive with the parasite tegument. Compared with other components, the relatively weak immunogenicity of this component may be related to its low molecular weight and/or its insoluble nature under natural infection.

Unlike the low molecular weight doublet, the 89 kD protein which was found to be

present in small quantities in adult worm somatic extract by the Coomassie blue staining SDS–PAGE pattern, appears to be highly immunogenic for man and animals. It reacted most strongly and consistently with all immune sera tested, which included sera from several individuals with opisthorchiasis, and sera from experimentally infected hamsters. Moreover, these sera also reacted with a similar molecular sized component present in metacercarial extract. Whether this immunoreactive component which seems to be present in the various developmental stages is antigenically identical or possesses only identical epitopes remains to be investigated.

Unlike the somatic extracts, the metabolic products of these adult worms were considerably less complicated. The major component was the 89 kD polypeptide that also reacted strongly with the various immune sera employed. It failed to be stained by PAS and therefore is presumably not a glycoprotein. Results of SDS–PAGE analysis with or without 2-mercaptoethanol indicated that it is composed of only a single peptide unit. Antibody to ES antigens, which mainly reacted in immunoprecipitation with this component, gave strong immunofluorescence with eggs and, to a lesser extent, with secretions of the reproductive and gastrointestinal systems (unpublished observations). Evidence for the presence of antibodies reactive with *O. viverrini* eggs had been noted previously in the sera of experimentally infected hamsters (Flavell, 1981; Sirisinha, Tuti, Tawatsin & Rattanasiriwilai, 1982) and of patients with opisthorchiasis (Boonpucknavig *et al.* 1986).

In addition to the 89 kD protein which reacted with all sera from individuals with positive stool examinations, there were a few minor components that reacted with some of these sera. For example, it appears from Fig. 3 that the component which also reacted with a majority of specimens from the patients migrated as a more diffuse band with an $M_r$ of 20 kD. Sera from infected hamsters, however, reacted more consistently with the >116 kD protein, particularly in those with chronic infection; this protein reacted also with some human sera. On the other hand, a majority of serum specimens from humans and animals infected with *O. viverrini* or with other parasites reacted with the 47 kD component of *O. viverrini*, suggesting that this molecule possessed epitope(s) that is ubiquitous among many parasitic helminths.

Altogether, the results presented in this study show that the 89 kD protein of *O. viverrini* is a potent immunogen and has potential as antigen for the immunodiagnosis of opisthorchiasis. Although its specificity has not yet been fully analysed, the data presented in this study showed that it is associated with *O. viverrini* as it neither reacted in immunoprecipitation with sera from patients with other parasitic infections nor with sera of animals experimentally infected with other parasites, including *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. mekongki*, *P. heterotremus*, *A. cantonensis* and *G. spinigerum*. Work is now in progress to isolate, characterize and test this component as a possible candidate for immunodiagnosis.

We would like to express our appreciation to Dr Peter B. Billings for valuable suggestions and discussion during the preparation of this manuscript. This investigation received financial support from the U.S. Agency for International Development (Grant No. 936-5542-G-00-6027-00) and Mahidol University.

REFERENCES

Boonpucknavig, S., Kuratho, S. & Thamavit, W. (1986). Detection of antibodies in sera from patients with opisthorchiasis. *Journal of Clinical and Laboratory Immunology* 19, 135–7.

Feldheim, W. & Knobloch, J. (1982). Serodiagnosis of opisthorchis viverrini infestation by an enzyme immunoassay. *Tropenmedizin und Parasitologie* 33, 8–10.

FLAVELL, D. J. (1981). The *in vitro* effects of serum on the adults, metacercariae and eggs of *Opisthorchis viverrini*. *Transactions of the Royal Society for Tropical Medicine and Hygiene* 75, 825–31.

FLAVELL, D. J. & FLAVELL, S. U. (1986). *Opisthorchis viverrini*: pathogenesis of infection in immunodeprived hamsters. *Parasite Immunology* 8, 455–66.

GREENWOOD, F. C., HUNTER, W. M. & GLOVER, J. S. (1963). The preparation of $^{131}$I labelled human growth hormones of high specific radioactivity. *The Biochemical Journal* 89, 114–23.

IVARIE, R. D. & JONES, P. P. (1979). A rapid sensitive assay for specific protein synthesis in cells and in cell-free translations: use of *Staphylococcus aureus* as an adsorbent for immune complexes. *Analytical Biochemistry* 97, 24–35.

JANECHAIWAT, J., THARAVANIJ, S., VAJRASTHIRA, S. & CHAICUMPA, W. (1980). The immunological diagnosis of human opisthorchiasis and humoral immune response to opisthorchis infection in the hamster. *Journal of the Medical Association of Thailand* 63, 439–47.

LAEMMLI, U. K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage $T_4$. *Nature, London* 227, 680–5.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. S. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265–75.

PUENGTOMWATANAKUL, S. & SIRISINHA, S. (1986). Impaired biliary secretion of immunoglobulin A in vitamin A-deficient rats. *Proceedings of the Society for Experimental Biology and Medicine* 182, 437–42.

RUPPEL, A., BOONPUCKNAVIG, S. & HEMPELMANN, E. (1985). Unusual protein pattern of *Opisthorchiasis viverrini*. *Journal of Helminthology* 59, 347–9.

SEGREST, J. P. & JACKSON, R. L. (1972). Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. *Methods in Enzymology* 28, 54–63.

SIRISINHA, S. (1986). Immunodiagnosis of human liver fluke infections. *Asian Pacific Journal of Allergy and Immunology* 4, 81–8.

SIRISINHA, S., TUTI, S., TAWATSIN, A. & RATTANASIRIWILAI, W. (1982). Lack of evidence for protective immunity in hamsters infected with *Opisthorchis viverrini*. In *Proceedings of the 25th Seameo-Tropmed Seminar, 19–25 October, 1982* (ed. J. W. Mak and H. S. Yong), pp. 91–100. Malaysia.

SIRISINHA, S., TUTI, S., VICHASRI, S. & TAWATSIN, A. (1983). Humoral immune responses in hamsters infected with *Opisthorchis viverrini*. *Southeast Asian Journal of Tropical Medicine and Public Health* 14, 243–51.

SIRISINHA, S., PUENGTOMWATANAKUL, S., SOBHON, P., SAITONGDEE, P., WONGRAYABAL, P., MITRANONDE, V., RADOMYOS, P., BUNNAG, D. & HARINASUTA, T. (1984). Alterations of the surface tegument of *Opisthorchis viverrini* exposed to praziquantel *in vitro* and *in vivo*. *Southeast Asian Journal of Tropical Medicine and Public Health* 15, 95–103.

SRIVATANAKUL, P., VIVANANT, V., KURATHONG, S. & TIWAWECH, D. (1985). Enzyme-linked immunosorbent assay for detection of *Opisthorchis viverrini* infection. *Southeast Asian Journal of Tropical Medicine and Public Health* 16, 234–9.

TUTI, S., VICHASRI, S. & SIRISINHA, S. (1982). Effect of culture media on production of excretory-secretory and egg output of *Opisthorchis viverrini in vitro*. *Journal of Parasitology* 68, 892–7.

WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* 244, 4406–11.

*Printed in Great Britain*

28

XN-AAA-229
B
5616

# PROGRESS REPORT NO. 4

# DEVELOPMENT OF METHODS FOR IMMUNODIAGNOSIS OF HUMAN LIVER FLUKE INFECTION

A RESEARCH PROJECT

USAID / PSTC PROGRAM

GRANT NO. 936-5542-G-00-6027-00

SUBMITTED BY

STITAYA SIRISINHA

DEPARTMENT OF MICROBIOLOGY

FACULTY OF SCIENCE MAHIDOL UNIVERSITY

BANGKOK THAILAND

MAY 3 1988

## PROJECT PROFILE

| | |
|---|---|
| Country | Thailand |
| Grant No. | 936-5542-G-00-6027-00 |
| Program | Program on Science and Technology Cooporation |
| Project Title | Development of Methods for Immunodiagnosis of Human Liver fluke Infection |
| Project Leader | Dr. Stitaya Sirisinha |
| Organization | Mahidol University |
| Co-investigators | Dr. Peter B. Billings<br>Dr. Skorn Mongkolsuk<br>Dr. Araya Dharmkrong-at |
| Project Consultants | Dr. Sakol Panyim<br>Dr. Pornchai Matangkasombut |
| Authorized Officer | Dr. Natth Bhamarapravati<br>Rector, Mahidol University |
| Total Budget | US$150,000 |
| Project Duration | 3 years (May 1986 to December 1989) |
| Reporting Period | 11/1987 -4/1988 |
| Budget Allocation for this period | US$18,320.08 (Baht 466,690) |

1. Background

Liver fluke infection is a major health problem in Southeast Asia, the Far East, and Central and Eastern Europe, with at least 40 million people currently infected. In Thailand alone, it has been estimated that at least 7.5 million people (about 15% of the population) are infected with *Opisthorchis viverrini* and the prevalence in some districts is at least 90% or higher.

Attempts thus far to control the disease include interuption of life cycle, case detection and chemotherapy. These approaches with the exception of a trial mass chemotherapy with praziquantel in small villages are not satisfactory. Reinfection readily occurs after chemotherapy but can be reduced if such treatment is accompanied by health education aimed at changing food habits and improving sanitation and personal hygiene.

Currently the diagnosis of *O. viverrini* infection, based on the detection and identification of eggs in the feces, is tedious and time-consuming, and is reliable only under the supervision of experienced laboratory personel and therefore does not lend itself to wide application necessary for disease control programs in highly endemic areas where new cases alone overwhelm laboratory capacities. Moreover, cases with light infections and when there is biliary obstruction such as cholangiocarcinoma can result in false negative findings. The development of a simple, sensitive and specific test for antibodies to the parasites would provide a useful tool for epidemiological surveys, giving information on previous exposure to the parasite and, perhaps, some indication of pathological complications. The test can be



31

used to monitor the success of chemotherapy as well as the rate of reinfection. Serological tests for the detection of serum antibody including precipitation, agglutination and complement fixation have been used in the past. However, the results are not satisfactory as they lack sensitivity and specificity, and do not lend themselves readily to be used in small, poorly equipped laboratories. The development of enzyme-linked immunosorbent assay (ELISA) has reactivated the interest in developing serological methods for the diagnosis of liver fluke infections. In recent years, ELISA has been used in attempting to find suitable immunodiagnostic methods. However, due to the complexity of the antigens available, the test lacks specificity. An indirect immunofluorescent test has also been used but the technique cannot be readily performed in most laboratories in Thailand. The characterization and purification of parasite-specific antigens is hence a necessary starting point for establishing suitable systems with diagnotic potential. Moreover, some clues as to the possible immunological basis for protection may be obtained, thus allowing immunoprophylaxis or a way to enhance post-exposure immunity.

## 2. Objectives

The main objective of this project is to develop a simple, sensitve and specific immunoassay(s) for the diagnosis of human liver fluke infection caused by *Opisthorchis viverrini*. While the principal aim is to detect specific antibody in the serum and/or other body fluids (e.g., saliva and bile), a simultaneous



32

development of a method to detect specific parasite antigen(s) will also be carried out. The immunoassays to be developed should:

a. Be able to establish diagnosis is suspects including those with low intensity of infection.
b. Identify O. viverrini infection in patients with biliary obstruction and other complications.
c. Be reliably and extensively performed by laboratory personnel with minimal laboratory training.
d. Be able to monitor the efficacy of individual and mass anthelmintic treatment as well as reinfection.

The approach is to characterize, identify and purify relevant antigenic components by appropriate physicochemical and immunological methods using a battery of sera from infected individuals, rabbit polyclonal and mouse monoclonal antibodies. Initial steps in the attempt at production of relevant antigens by recombinant E. coli carrying genomic O. viverrini DNA fragments will also be made. Polyclonal and monoclonal antibodies together with corresponding relevant antigens developed will be used in the formulation of immunoassays.

Research activity

Activity 1 : Preparation of antigens

(A) O. viverrini antigens. The various O. viverrini antigens, including somatic, surface tegument and metabolic products

(ES) of adult worms, were prepared as described in details in Progress Reports No. 1 and 2. Currently we have approximately 200 hamsers infected with 60 metacercariae each. These antigens were used for the various immunoassays as well as for screening of monoclonal antibodies and preparation of RNA an DNA.

(B) Schistosoma japonicum antigen. Crude somatic extract of S. mansoni was prepared from adult male and female worms obtained from albino Swiss mice infected with S. mansoni cercariae (kindly made availavble by Dr. E.S. Upatham, Department of Biology, Faculty of Science, Mahidol Unviersity). The adult worms were homogenized and sonicated essentially the same as done for O. viverrini adults. The antigen will be used for analyzing specificity of the immunoassay systems being developed as well as for specificity screening of monoclonal antibodies.

(C) Nematode antigens. These nematode antigens used for specificity study were prepared from Gnathostoma spinigerum and Angiostrongylus cantonensis as previously described in the Progress Report No. 3.

Activity 2: Collection of specimens from patients

We are now collecting serum, bile and urine from patients with positive O. viverrini eggs in the stool. These were patients with biliary obstruction coming in for surgery at Khon Kaen University Hospital. Whereas hepatic bile was previously obtained by cannulation as described in Progress Report No. 2, gall bladder bile was collected in this group of patients. In

some of these patients, saliva was also collected.

In addition to these opisthorchiasis patients, we also collected blood and sometimes urine and saliva from O. viverrini negative individuals who reside in the endemic area of O. viverrini infection, either with or without infections by other intestinal helminths. In order to locate these individuals, a large number of patients attending the Outpatient Clinic of the University Hospital were screened by fecal examination for O. viverrini eggs.

Activity 3 : Attempt at cloning O. viverrini genes

From previous report, we constructed the Opisthorchis viverrini DNA library in E. coli, using pUC 8, 9 and 12 as expression vectors. This library was screened with serum from rabbits immunized with Ov antigens and $^{125}$I-prot A in colony radioimmuno assay. In the Progress Report No 3 we screened about 30,000 white colonies, and in the present report we secrened another 30,000. However, these clones also gave negative result.

The genomic DNA digested with Pst I restriction enzyme which has 6 bp recognition sequence gave an average fragment length of around 4 kb. O. viverrini is eukaryotic and therefore likely having introns which may prevent expression of antigen in the E. coli. To resolve the problem, the restriction enzyme MSp I and Taq I were used. These enzymes have 4 base pairs recognition sequence that gave average fragment lenght of around 500 bp. To clone MSp I and Taq I fragments, the plasmid blue script pks was used by digesting with Cla I restriction enzyme which has

compatible ends to MSp I and Taq I. The plasmid pks was ligated to MSp I and Taq I fragments by enzyme T4 ligase at 15°C overnight and transformed into E. coli JM107.

The screening method was the same as described in the recent report excepted the O. viverrini-infected human serum and a new lot of higher titered rabbit anti-somatic antigen were used.

The results from screening of 10,000 white colonies of library in pUC and about 20,000 white colonies library in pks gave 6 positive clones on the autoradiogram. These positive clones, five came from pks library and one came from pUC library. All of these positive clones were separately grown in L-broth with 100 μg/ml of ampicillin and induced with 10 mM IPTG (Inopropyl β-D-thiogalactopyranonide) at log phase. The cell pellets were dissolved in sterile distilled water and added equal volumn of E-buffer (2% SDS, 5% Mercaptoethanol, 10% glycerol). The cell suspensions after mixed with E-buffer were boiled for 5 minutes or until the suspension become clear. The supernatant of the positive clones lysate (around 50 μl) of each samples in comparison with the lysate from pUC and pks in E. coli JM107 were loaded into 12% SDS-PAGE. The SDS-PAGE was western blotted onto nitrocellulose paper. The nitrocellulose paper was blocked with 5% skim milk in PBS pH 7.4 and incubated with infected human serum and followed with $^{125}$I-prot A. The autoradiogram of these clones showed three protein products of size smaller than lysozyme (which was the smallest marker on the gel), indicating molecular weight lower than 14,000.

Activity 4 : Production of monoclonal antibodies

Additional successful fusions were made during this period to increase the number of hybridomas with antibody specificity against O. viverrini antigens. In particular, we are attempting to direct the response more toward antigens which appear highly immunogenic in infected animals and patients, and which are also parasite-specific. The most appropriate antigen identified thus far, is the 89 Kd protein, found in most extracts and antigen preparations, but especially abundant in the Excretory-Secretory fraction. This antigen fraction concentrated from in vitro maintenance cultures of parasites has been used in successful fusions. This antigenic polypeptide is also present in lower proportion in somatic extracts, and more random approach has also been used.

We have attempted to develop a method of immunizing with much smaller amounts of antigen, in anticipation of the need to immunize with selected polypeptide bands from polyacrylamide gels. Previous results indicated that the response to nitrocellulose-adsorbed somatic antigen was excellent even with extremely small amounts. Adsorption appears to enhance the immune response, but exactly how much is not quite clear. DMSO used as solvent to facilitate i.p. or s.c. injection rapidly diffuses out of the Freund's adjuvant-DMSO-nitrocellulose mixture resulting in re-solidification as large irregular masses. These probably limit direct access to antigen by Ag-presenting cells. Thus, a large part of the adsorbed antigen could be physically embedded, not contributing to the response, or it could slowly be

released. We do not know how porous these solid forms are; however quite good responses have been obtained with total somatic antigen. In the most recent extension of this approach, the immunization with adsorbed antigen was combined with SDS gel electrophoresis and electrophoretic transfer (western blotting) onto the nitrocellulose. The bands of interest as immunogen--a closely spaced doublet of $M_r$ 89,000--were identified after transfer to introcellulose from a narrow preparative gel by reacting end strips with mouse antiserum to somatic antigen (i.e., by typical immunoblot reaction). The entire region of the nitrocellulose replica corresonding to 85-95 Kd was then excised and solubilized with DMSO. After being mixed with Freund's complete adjuvant, this material was used to prime BALB/c mice. Because of the generally poorer electrophoretic transfer of high MW polypeptide bands, a large proportion of the 89 Kd band was retained in he gel; each mouse probably received no more than 5 ug of this band. The remaining 89 Kd band, which failed to transfer from gel to nirocellulose, was stained with Coomassie Blue, cut out, crushed, mixed with FCA and also used to immunize (estimated by stain at about 10 ug/animal). By ELISA vs total somatic antigen, the resposne to the western blot 89 Kd region was good to excellent, while that to a higher amount of Coomassie-stained antigen embedded in acrylamide was only moderate. The best responder immunized with the blot 89 Kd band has been used in fusion after pre-fusion booster with crude total soluble somatic antigen. The expectation is that the primary response to the non-89 Kd "contaminants" will not involve

sufficient cells within the 3 days before fusion to be recovered in hybridomas. Again, we should be able to do the first hybridoma screen also with total somatic antigen by ELISA. Remaining animals were boostered with similar amounts of the corresponding antigens.

From earlier fusions with animals immunized with nitrocellulose-adsorbed total somatic antigen or with the 89 Kd-enriched ES antigen, we now have a reasonably large back-log of monoclonal antibodies remaining to be screened by radioimmunoprecipitation for fine specifiities.

## WORK PLAN FOR NEXT PERIOD

### (1) Production of monoclonal antibodies

Existing monoclonal antibodies need to be characterized and sorted according to polypeptide specificities. This will be done by relatively straight-foreward immunoprecipitation of $^{125}$I-iodinated somatic antigen using (rabbit) anti-mouse IgG prereacted with protein A-Sepharose. The gel dryer for analysis of radioimmunoprecipitates has recently been serviced to correct vacuum problems due to incomplete moisture entrapment, and a more effective dry-ice alcohol cold trap is being made loclly to prevent recurrence. Ambiguous cases of co-precipitation of antigen complexes may be resolved by western blotting, however this analysis used far too much precious antigen for general preliminary screening. The most promising monoclonals of each specificity will be used in immunofluorescence study for antigen localization on sectioned adult worms and on egg-enriched fractions to assess diagnostic utility.

(2) Gene cloning

The protein products shown to react with infected human serum will be used as an antigen in the ELISA to test for their sensitivity and specificity. The putative antigen clones will be used as DNA probe to search the O. viverrini cDNA library for a complete gene. This would permit a more definitive identification of the antigen.

PUBLICATION AND PRESENTATION OF THE RESULTS OBTAINED UP TO NOW INCLUDE

(1) Graduate students' theses

A portion of the research for this project has been conducted by a number of Ph.D. and M.Sc. candidates in the Department of Microbiology at the Faculty of Science, Mahidol University. These students include

1. Mr. Surasakdi Wongratanacheewin who has just received a Ph.D. degree and is now a lecturer at the School of Medicine, Khon Kaen University in Khon Kaen Province.

2. Miss Rasana Sermswan is a late-stage Ph.D. candidate and her work is concentrated on the gene cloning aspect of this project. She works primarily under the supervision of Drs. Skorn Mongkolsuk and Sakol Panyim.

3. Miss Suree Korbsrisate is an early Ph.D. candidate and will be working largely on O. viverrini genome under the supervision of Dr. Skorn Mongkolsuk.

4. Miss Sorujsiri Amornpunt is a second year M.Sc. student working on monoclonal antibodies specifically against the 89 kD



40'

component of O. viverrini. She works under the supervision of Dr. Suttipant Sarasombath of Siriraj School of Medicine (Mahidol Unversity) and Stitaya Sirisinha (Project leader).

5. Miss Nantana Utsakhit is a first year M.Sc. student working on monoclonal antibodies against various O. viverrini antigens. She works under the supervision of Dr. Peter B. Billings.

6. Miss Teeraporn Sureerug is a first M.Sc. student. She is characterizing biliary antibodies in patients with opisthorchiasis under Dr. Stitaya Sirisinha.

(2) Attending scientific meetings and presentation of papers

1. Paper entitled "Immunodiagnosis of human liver fluke infection caused by Opisthorchis viverrini" was presented at the conference on Medical Microbiology Towards 2000 in Hong Kong (29 May - 1 June, 1987).

2. Presenting a paper on "Approaches to develop immunodiagnostic methods for liver fluke infection caused by Opisthorchis viverrini" at the conference of USAID Science Research Award Grantees at Nakorn Pratom (24-26 July, 1987).

3. Participating in a symposium on "Opisthorchiasis" organized by Ministry of Public Health at Petchburi (6 August 1987).

4. Presenting a paper on "Biotechnology Research in the Study of Opisthorchiidal in Thailand at the Thailand-U.S.-SEAMEO-TROPMED Conference on Applications of Biotechnology on the Study of Animal Parasites and Their Vectors in Bangkok (23-25 Nov., 1987).



41

(3) Formal Publications

Articles that have been and/or will be shortly published since the initiation of this project are listed below.

1. Sirisinha, S. Immunodiagnosis of human liver fluke infections. Asian Pacific J. Allergy Immunology 4:81-88, 1986 (reprints were submitted with Progress Report No. 2).

2. Sirisinha, S. and Wongratanacheewin, S. Immunization of hamsters against Opisthorchis viverrini infection. Southeast Asian J. Trop. Med. Pub. Hlth. 17:567-573, 1986 (reprints were submitted with Progress Report No. 2).

3. Wongratanacheewin, S., Chawengkirttikul, R., Bunnag, D. and Sirisinha, S. Analysis of Opisthorchis viverrini antigens by immunoprecipitation and polyacrylamide gel electrophoresis. Parasitology 96:119-128, 1988 (2 copies of a galley proof of the paper were submitted with the Progress Report No. 3).

4. Wongratanacheewin, S., Bunnag, D., Vaeusorn, N. and 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. (In press) 1988.

5. Sirisinha, S. Approaches to develop immunodiagnostic methods for liver fluke infection caused by Opisthorchis viverrini. Proceedings of USAID Science Award Grantees. July 1987.

6. Wongratanacheewin, S., and Sirisinha, S. Analysis of Opisthorchis viverrini antigens: physicochemical characterization and antigen localization. Southeast Asian J. Trop. Med. Pub. Hlth. 18:511-520, 1988. (3 copies of the reprint are enclosed herewith).



42