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THE JOINT INTERNATIONAL TROPICAL MEDICINE MEETING 2006, AND THE 5th SEMINAR ON FOOD- AND WATER - BORNE PARASITIC ZOONOSES

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THE JOINT INTERNATIONAL TROPICAL MEDICINE MEETING 2006, AND THE 5th SEMINAR ON FOOD- AND WATER - BORNE PARASITIC ZOONOSES

Edited by

Stephen W King
Nick Walters
Suvanee Supavej

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FOREWORD

The Joint International Tropical Medicine Meeting 2006 (JITMM 2006), the 6th Asia-Pacific Travel Health Conference (6th APTHC), and the 5th Seminnar on Food- and Water-borne Parasitic Zoonoses (FBPZ) were held during 29 November - 1 December 2006 at the Miracle Grand Convention Hotel, Bangkok, Thailand with 734 participants from 34 countries.

The events were co-hosted by the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; the Asia-Pacific Travel Health Society; SEAMEO TROPMED Network; Parasitology and Tropical Medicine Association of Thailand, TROPMED Alumni Association; JSPS-Africa and Asia Research Platform; and FIBOZOPA.

The scientific programs of the JITMM 2006 and the 6th APTHC consisted of the 12th Chamlong-Tranakchit Harinasuta Lecture, 2 plenary sessions, 19 symposia, 9 free paper sessions, 1 session of the Best TropMed Presention Award, and 73 poster exhibits. The Chamlong - Tranakchit Harinasuta Lecture entitled **"Plague and pandemics"** was presented by Professor Nickolas J White, Chairman, Wellcome Trust Southeast Asian Research Unit and **"Travel medicine in the tropics: twists and turns"** presented by Professor Robert Steffen, Head, Division of Communicable Diseases, WHO Collaborating Center for Travellers' Health, Institute of Social and Preventive Medicine, University of Zurich, Zurich, Switzerland. The Scientific programs for the 5th FBPZ consisted of 1 keynote lecture, 6 symposia, 3 free paper sessions, 2 FIBOZOPA sessions, and 37 poster exhibits. The keynote lecture entitled **"Progress or regress?: biological aspects of food-borne parasitic zoonoses"** was delivered by Professor Yukifumi Nawa, Vice-President for Research and Planning, University of Miyazaki, Japan.

Forty-four full papers were submitted and 41 were published in Supplement 1 of the **Southeast Asian Journal of Tropical Medicine and Public Health 2007; Volume 38**.

Stephen W King
Nick Walters
Suvanee Supavej

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The Meeting organizers gratefully acknowledge the generous financial support of the following, without which these events would not have been possible.

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MALARIA: A RETROSPECTIVE STUDY IN HOSPITAL TENGKU AMPUAN RAHIMAH (HTAR), KLANG, SELANGOR, MALAYSIA (2004 - 2006)

I Jamaiah, M Rohela, V Nissapatorn, A Amriana, MN Sumaiyah, M Lila and A Norzawati

Department of Parasitology, Faculty of Medicine,
University of Malaya, Kuala Lumpur, Malaysia

Abstract. This retrospective study was carried out to determine the prevalence of malaria among patients admitted to Hospital Tengku Ampuan Rahimah, Klang, Malaysia, from January 2004 to May 2006. A total of 37 malaria cases were analyzed. Most cases occurred among foreigners, 81% (30 cases), while Malaysians constituted 19% (7 cases). Among foreigners, Indians constituted the majority, 40.5% (15 cases). Among Malaysians, most cases occurred among Malays, 16% (6 cases), followed by Indians, 3% (1 case). Males, 89% (33 cases), were more commonly affected. The majority of cases were within the 20-39 year age group (84%). Most cases occurred among laborers (24%). Two species of malaria parasites were reported, of which *Plasmodium vivax* constituted the most, 70% (26 cases), followed by *Plasmodium falciparum*, 30% (11 cases). In this study, 40.5% (15 cases) developed chloroquine resistance: six cases of *P. falciparum*, and nine cases of *P. vivax*. The most common complications were thrombocytopenia, 65% (24 cases), and anemia, 54% (20 cases), followed by jaundice, 32% (12 cases), and hepatosplenomegaly, 22% (8 cases). There were no reported deaths. This new source of malaria coming from foreigners must be given serious attention, as it has great potential of increasing malaria cases in urban Malaysia.

INTRODUCTION

Malaria (from medieval Italian, *mala aria*, or "bad air"; formerly called "ague," or "marsh fever") is an infectious disease that is widespread in many tropical and subtropical regions. It causes between one and three million deaths annually, mostly among young children in Sub-Saharan Africa. Malaria causes about 350-500 million infections in humans and approximately 1.3-3 million deaths annually (Campbell and Reece, 2005). This represents at least one death every 30 seconds. The vast majority of cases occur in children under the age of five years, and pregnant women are vulnerable (Greenwood *et al*, 2005). The death rate is expected to double in the next twenty years. Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals and/or the means to afford health care. Consequently, many cases are undocumented (Hull, 2006). Sub-Saharan Africa accounts for 85-90% of malaria fatalities, but it is also prevalent in northern South America and South and Southeast Asia (Layne, 2005).

Malaria remains an important public health issue in remote areas of Malaysia. Approximately 70% of the cases occur in Sabah, East Malaysia. The high morbidity is because this country is located within the equatorial zone, with high temperature and humidity, which is important for the transmission of this disease. It affects mainly the rural and semi-rural population, especially in the areas where clearing of jungles for development is going on (Palmer, 2002). Recently a new source of malaria has been introduced into the country. These cases were in immigrant workers (legal/illegal) and the large number of tourists coming into the country. The number of reported cases has increased nationwide, together with the increased incidence of drug resistance in Southeast Asia, which raises more concern among both health workers and clinicians (Sidhu and Ng, 1991; Leo *et al*, 1994; Moore and Cheong, 1995; Jamaiah *et al*, 1998, 2005).

Correspondence: I Jamaiah, Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: 603-7967-6618; Fax: 603-7967-4754
E-mail: jamaiah@umm.edu.my

This study was carried out to determine the number of malaria cases admitted to Hospital Tengku Ampuan Rahimah, Klang (HTAR), Klang, Malaysia, and to analyze the epidemiological data and complications.

MATERIALS AND METHODS

All cases of malaria admitted to HTAR, Klang, Malaysia, from January 2004 to May 2006 were analyzed. A total of 37 confirmed cases were reviewed and analyzed.

RESULTS

During the period from January 2004 to May 2006, 37 confirmed cases of malaria were admitted to HTAR, Klang, Malaysia. The majority of the infections occurred in the 20-39 year age group (Table 1), and this accounted for 84% (31/37) of all malaria cases studied. Malaria was much less common among females than males as there were only four cases of malaria infection reported in females. Foreigners from India represent 40.5% (15/37) of those infected, and Indonesians 24% (9/37). Among Malaysians, Malays constituted the most cases (16 %). There was only one case in an Indian, and no cases were reported among Chinese. Laborers represent the highest number of those infected with malaria, with 9/37 (24 %) cases, followed by those unemployed, 7/37 (19%), factory workers, 5/37 (14%), and unknown occupation, 4/37 (11%).

About 97 % of those infected had fever, and almost 70 % had fever associated with chills and rigors. Other major symptoms included vomiting (70 %), abdominal pain (59.5 %), urinary symptoms and headache (40.5 %), and others (51 %).

Plasmodium vivax was the predominant species (70%). *P. falciparum* constituted 30% of malaria cases. No patient was infected with *P. malariae* or *P. ovale*. More than half of the malarial patients developed complications of some medical importance (Table 2). The most common complication was thrombocytopenia (65%), followed by anemia (54%), and jaundice (32%). Most of the complications were caused by *P. vivax*.

The majority of patients were treated with a combination of chloroquine and primaquine for both *P. falciparum* and *P. vivax*. About 27% cases had chloroquine resistant strains and were treated with Fansidar.

DISCUSSION

In this study, the majority of the malaria cases

Table 1
Malaria distribution by age, sex, and race/ nationality (January 2004 - May 2006).

Age (year)	Race/Nationality																Total
	Malaysians						Foreigners										
	Malay		Chinese		Indian		Indonesia		India		Myanmar		Bangladesh		Pakistan		
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	
0 - 9																	0
10 - 19								1									1
20 - 29	1						3	1	8	1	1				1		16
30 - 39	3						3		4	1	2		1		1		15
40 - 49	1						1		1								3
50 - 59																	0
60 - 69					1												1
70 - 79	1																1
Total	6	0	0	0	1	0	7	2	13	2	3	0	1	0	2	0	37
%	16.2		0.0		2.7		24.3		40.5		8.1		2.7		5.4		

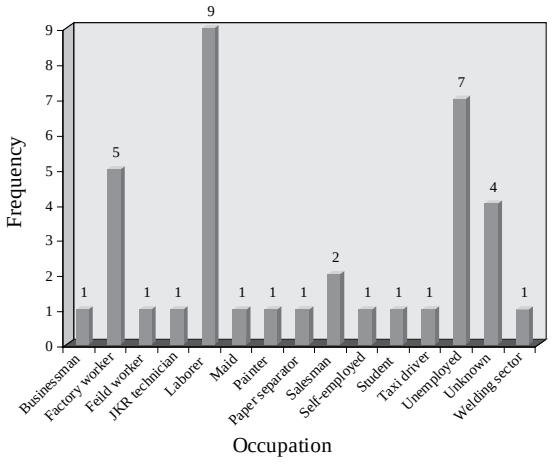

Fig 1- Distribution of malaria according to occupation.

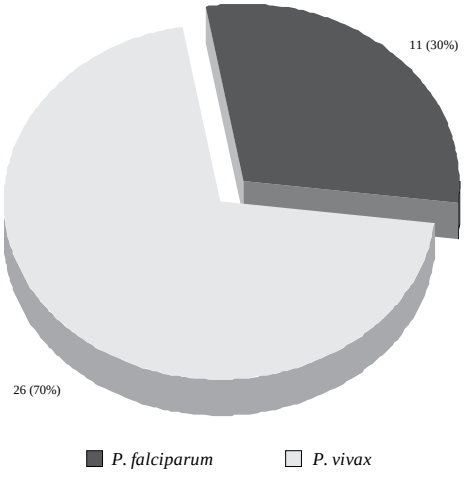

Fig 2- Distribution of malaria according to species.

occurred in the 20-39 year age group (84%). Most of the cases occurred in males (89%). Moore and Cheong (1995) and Jamaiah *et al* (1998; 2005) also reported similar findings. Foreigners from India constituted the most cases (40.5%) and were followed by the Indonesians (24%). Jamaiah *et al* (1998, 2005) reported that, among foreigners, Indonesians constituted the most cases. This could be because more Indians from India were recruited by the many plantations in Klang, Selangor, Malaysia. Moore and Cheong (1995) and Sidhu and Ng (1991) pointed out that the main problem in the future will be the increasing number of imported malaria in the main cities of Malaysia. Leo *et al* (1994) also reported that the majority of malaria cases in Singapore were due to imported cases. All foreign workers entering Malaysia must be screened for malaria to prevent resurgence of malaria in Malaysia. Among Malaysians, Malays constituted majority of the cases (16%). There were no cases reported among the Chinese.

Laborers constituted the highest number of those infected with malaria (24%). This was followed by those unemployed (19%). This was to be expected as most male foreign workers (legal/illegal) were employed as laborers in the various sectors.

The most common species in this study was *P. vivax* (70%), followed by *P. falciparum* (30%). Moore and Cheong (1995), Norhayati *et al* (2001), and Jamaiah *et al* (2005) also reported similar findings. Chuah (1985), Oothuman (1988), Sidhu and Ng (1991), Jamaiah *et al* (1998), and Koh *et al* (2004) reported *P. falciparum* to be the commonest species, followed closely by *P. vivax*. There were no reported cases of *P. malariae*, nor

Table 2
Complications of malaria according to species.

Complications	% cases	*P. falciparum* (%)	*P. vivax* (%)
Jaundice	32	32	68
Anemia	54	35	65
Hepatomegaly	13.5	20	80
Splenomegaly	5	50	50
Hepatosplenomegaly	3	50	50
Thrombocytopenia	65	21	79

cases of mixed infection.

Most of the malaria cases in this study presented with fever associated with chills, rigors, vomiting, and abdominal pain. Koh *et al* (2004) and Jamaiah *et al* (2005) also reported similar findings.

In this study, the two most common complications were thrombocytopenia (65%), followed by anemia (54%). Most of these complications were due to *P. vivax*. There were no reported cases of cerebral malaria or black water fever. Chuah (1985) reported that anemia was the most common complication. Jamaiah *et al* (1998) reported that cerebral malaria was the most common complication (40%), whereas in 2005 she reported that jaundice and anemia were the most common complications. Hepatomegaly, hepatosplenomegaly, and anemia were significantly associated with malaria (Norhayati *et al*, 2001). Koh *et al* (2004) reported a case of *vivax* malaria that was complicated by septicemic shock, and disseminated intravascular coagulopathy. Therefore, the presumption that *P. vivax* is a benign species must be revisited.

Most patients received a combination of drugs, chloroquine, and primaquine. This is because chloroquine and primaquine are the first-line drugs for treatment of *vivax* and uncomplicated chloroquine-sensitive *falciparum* malaria. The concept of combination therapy is based on the synergistic or additive potential of two or more drugs to improve therapeutic efficacy and also delay the development of resistance to the individual components of the combination. Chloroquine is used to alleviate symptoms and prevent spread. It is a blood schizonticidal and gametocytocidal drug. Primaquine is a tissue schizonticidal drug and is used to prevent relapses. We also reported chloroquine resistance in both species, six cases among *falciparum* malaria and nine cases among *vivax* malaria. These chloroquine resistant species were successfully treated with Fansidar.

Methods used to prevent the spread of disease, or to protect individuals in areas where malaria is endemic, include prophylactic drugs, mosquito eradication, and the prevention of mosquito bites. Insecticide-treated bed-nets (ITN) provide a simple but effective means of preventing malaria. One malaria vaccine, RTS S/AS02, has shown promise in endemic areas (Greenwood *et al*, 2005).

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RECOMBINANT EXPRESSION OF A TRUNCATED *TOXOPLASMA GONDII* SAG2 SURFACE ANTIGEN BY THE YEAST *PICHIA PASTORIS*

Yee Ling Lau[1], Mun Yik Fong[1], Radin Hisam Raden Shamilah[2] and Mohamed Zulqarnain[3]

[1]Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur; [2]Parasitology Unit, Institute for Medical Research, Kuala Lumpur; [3]Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia

Abstract. SAG2 is one of the major surface antigens of the intracellular protozoan parasite *Toxoplasma gondii*. In this study, we used the *Pichia pastoris* yeast expression system to produce a truncated form of the SAG2 and determined the serological characteristics of this recombinant antigen. We chose the *Pichia* system because of its high efficiency in expressing recombinant genes, and its ability to modify and secrete the recombinant proteins. Our strategy was to clone and express the part of the SAG2 gene that encodes the carboxyl half of the antigen. The recombinant antigen (recSAG2-C) secreted by the *Pichia* cells was harvested, and then evaluated in Western blot and enzyme-linked immunoassays (ELISA). Sixty human serum samples, including 45 from confirmed cases of toxoplasmosis, were tested against recSAG2-C. Results from the Western blot assay showed that recSAG2-C reacted with all 45 sera from the toxoplasmosis cases but none with the *Toxoplasma*-negative serum samples. Similar results were obtained for ELISA. These results indicated that recSAG2-C was specific for *Toxoplasma* antibody. To investigate the immunological characteristic of recSAG2-C, this recombinant antigen was injected subcutaneously into mice, and their serum was tested against total protein of *T. gondii*. It was observed that the serum specifically detected the native SAG2 (22 kDa) of *T. gondii*. This demonstrates that the recSAG2-C could evoke the production of antibody in mice, which readily recognized the native SAG2.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that can infect human and a host of warm-blooded animals (Hill and Dubey, 2002). In humans, *T. gondii* infection is mainly asymptomatic in immunocompetent adults, but immunodeficient individuals may suffer encephalitis, pneumonia, and disseminated infection. Congenital infection via transplacental transmission from mother to the fetus during pregnancy may lead to fetal and neonatal complications (Montoya and Liesenfeld, 2004).

The life cycle of *T. gondii* consists of an asexual phase that occurs in all hosts, and a sexual phase occurring only in the intestines of the definitive host, the cat (Frenkel *et al*, 1970). Four functionally distinct forms occur in the life cycle of *T. gondii*: tachyzoite, bradyzoite, merozoite, and sporozoite. In terms of infection, the tachyzoite is probably the most important, as it proliferates and disseminates rapidly within the host.

Routine diagnosis of *T. gondii* infection is based on serological detection of the antibody in a patient. Antigens used in these serological assays are usually prepared from *T. gondii* cells that are propagated in mouse or *in vitro* culture. Growing and maintaining this parasite is laborious, time-consuming, and expensive. Furthermore, such preparation usually contains extraparasitic materials and may result in interassay variability. Therefore, there have been efforts to produce pure antigens through safer means, such as recombinant DNA technology. The majority of these endeavors have focused on the major surface antigens of *T. gondii*, such as the SAG1 (p30) and SAG2 (p22) (Prince *et al*, 1990; Parmley *et al*, 1992; Harning *et al*, 1996; Chen *et al*, 2001). Most of these recombinant *T. gondii* antigens were produced using the *Escherichia coli* bacterial expression systems. However, such systems lack post-translational mechanisms to modify and fold the recombinant

Correspondence: Dr Mun Yik Fong, Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Fax: 603-7967 4754
E-mail: fongmy@um.edu.my

antigens into conformations similar to that of the native ones.

Pichia pastoris is harmless methylotrophic yeast that has been manipulated to generate recombinant proteins (Cereghino and Cregg, 2000; Daly and Hearn, 2005). This yeast is non-fastidious and can be cultured in a simple, inexpensive medium. Recombinant genes in *P. pastoris* can be induced to high level of expression. In addition, this eukaryotic yeast has post-translational mechanisms to modify the recombinant proteins into structures similar to their respective native counterparts.

Previous research work has shown that truncated forms of the recombinant SAG1 produced in *P. pastoris* were sufficiently immunogenic for serorecognition and protection in mice against lethal challenge with *T. gondii* tachyzoites (Biemans *et al*, 1998; Letourneur *et al*, 2001). In our present study, we constructed a truncated SAG2 gene, which upon expression in *P. pastoris*, produced and secreted only the carboxyl-terminal (C-terminal) half of the SAG2 antigen. We postulated that this truncated form of SAG2 would possess antigenic properties. Therefore, this recombinant antigen was evaluated by Western blot assay, ELISA, and *in vivo* experimentation.

MATERIALS AND METHODS

Parasite

Tachyzoites of the *T. gondii* RH strain were grown in MBDK cell monolayers, in RPMI supplemented with 10% fetal calf serum, at 37°C in a 5% CO_2 environment.

Bacterial strains and growth conditions

Escherichia coli TOP10F' was used as host for plasmid DNA manipulation experiments. In these experiments, this bacterial strain was grown in either Luria Bertani broth or on Luria Bertani agar, supplemented with zeocin (50 µg/ml) when appropriate.

Mice

Six- to eight-week-old female ICR mice, used for *in vivo* experiments, were obtained from the Animal Experimental Center, University of Malaya, Kuala Lumpur, Malaysia.

Human serum samples

The recombinant truncated SAG2 was tested in Western blot assays and ELISA with 60 human serum samples of the following categories: (A) IgG+ve, IgM-ve (15 samples); (B) IgG-ve, IgM+ve (15 samples); (C) IgG+ve, IgM+ve (15 samples); and (D) IgG-ve, IgM-ve (negative control, 15 samples). The serological statuses of these samples were initially determined by the Diagnostic Laboratory at the Department of Parasitology, University of Malaya. We reconfirmed the serological status (Table 1) using Captia™ *Toxoplasma gondii* IgG and *Toxoplasma gondii* IgM kits (Trinity Biotech, Ireland).

Polymerase chain reaction (PCR) of partial SAG2 gene

T. gondii genomic DNA was directly used as a template for PCR amplification as there are no introns within the SAG2 gene. The genomic DNA was extracted from the cell culture using a commercial kit (QIAGEN, Germany). The amplification was carried out using a primer pair consisting of the forward primer 5' ACTGAATTCAGCGAAAGGTCCTGCTACC 3'; and the reverse primer 5' CATGAATTC ACCTTGCCCGTGAGAGAC 3'. The primers were designed based on the published sequence of Prince *et a*l (1990). The EcoRI cutting site (GAATTC) was incorporated into the primers to facilitate splicing of the PCR fragment into the corresponding EcoRI site of the expression plasmid vector, pPICZαC (Invitrogen Corporation, USA). PCR was carried out in a typical 25 µl reaction mixture that contained 1 U *Taq* DNA polymerase (Fermentas Life Sciences, Canada). The PCR mixture was initially pre-heated at 95°C for 10 minutes before 30 cycles of amplification, which consisted of incubations at 94°C for one minute, 54°C for 1 minute, and 72°C for 2 minutes.

Recombinant plasmid construction

The amplified DNA fragment was digested with EcoRI and spliced into the corresponding cloning site in the pPICZαC. The recombinant plasmid was transformed into *E. coli* TOP10F'. Several

positive clones were selected and sequenced in a commercial laboratory to confirm the orientation and integrity of the partial SAG2 gene.

Transformation and expression of recombinant truncated SAG2 in *P. pichia*

Transformation of *P. pastoris* with the recombinant pPICZαC was carried out using the EasySelect™ *Pichia* Expression kit (Invitrogen Corporation, USA). Positive recombinant *P. pastoris* clones were selected for expression. A single recombinant *P. pastoris* colony was selected and inoculated into 10 ml of buffered complex medium containing glycerol (BMGY). The culture was grown at 28°C for 24 hours. The cells were harvested and resuspended in 50 ml of buffered complex medium containing methanol (BMMY). The culture was allowed to continue growing for 72 hours. Methanol was added every 24 hours, up to a final concentration of 0.5%, to induce expression of the recombinant SAG2 gene. Culture supernatant was collected at 12-hour intervals for protein extraction and analysis. Nonrecombinant *P. pastoris* host cells (X-33 strain) and X-33 transformed with parent vector pPICZαC (without insert) were similarly treated and analyzed as negative controls.

Protein extraction

Total protein was extracted from the culture supernatant by precipitation with 10% trichloroacetic acid. The protein precipitate was washed several times with acetone and finally resuspended in water.

SDS-PAGE and Western blotting

The harvested proteins were separated by SDS-PAGE and transferred by electroblotting to polyvinylidene difluoride (PVDF) membranes, (Bio-Rad Laboratories, USA). The proteins were probed with human serum samples (at 1:200 dilution). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgM or IgG (Bio-Rad Laboratories, USA).

ELISA for the detection of IgG and IgM

ELISA was carried out using the components of commercial kits (ELISA Ensemble for IgM detection, Alpha Diagnostic, USA; Protein Detector ELISA kit for IgG detection, KPL Inc, USA). Each well of a microtiter plate (Nunc, Roskilde, Denmark) was coated with 100 µl of 10 µg/ml of recSAG2-C in 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the antigen solution was removed and replaced with 100 µl of blocking buffer (1% BSA). The plate was incubated for one hour at room temperature. A serum sample, diluted 1:50 in 1% BSA, was added into each well (100 µl/well) and incubated for one hour at room temperature. The wells were then washed three times with PBS-T, followed by the addition of 100 µl antibody-enzyme conjugate (for the detection of IgG, alkaline phosphatase conjugate was used; horseradish peroxidase conjugate was used for IgM) and incubated for one hour at room temperature. The wells were then washed three times with PBS-T and incubated with the appropriate substrate solution for 30 minutes. Optical density (OD) was read at 655 nm and 450 nm for IgG and IgM detection, respectively.

Each sample was run in duplicate wells. The ELISA results were determined for each sample by taking the mean value of the two OD readings, minus the value of the blank. A sample was considered positive if the calculated OD was greater than the mean $+2\sigma$ for serum samples of 20 normal individuals.

Immunization of mice

Ten mice were injected subcutaneously with extracted protein (100 µg/mouse) derived from a recombinant *P. pastoris* culture. The mice were boosted with two further injections at five-day intervals. The negative control group consisted of mice that received total extracted protein of nonrecombinant *P. pastoris*. The sera of the mice from each group were examined for reaction with total protein of *T. gondii* tachyzoites in a Western blot.

RESULTS

The primers for PCR were designed to produce a DNA fragment that contained the C-terminal half of the SAG2 gene. This partial SAG2 gene was sequenced, and the data showed complete (100%) identity with the published

sequence of Prince *et al* (1990) (data not shown). The gene was spliced into the expression vector pPICZαC and transformed into *P. pastoris* host cells.

A time course experiment was carried out to determine the optimal time and conditions for maximum expression of the recombinant SAG2 gene. The recombinant clone showed high expression of a novel protein of ≈33 kDa (Fig 1) after 60 hours of induction with 0.5% methanol, which was absent in the control samples. Thus, this ≈33 kDa protein was likely the putative recombinant truncated SAG2. The identity of this truncated antigen was confirmed by a Western blot that was probed with a positive anti-*Toxoplasma* human serum (Fig 2). This recombinant antigen was designated as 'recSAG2-C.'

The recSAG2-C was further evaluated in Western blot assays using panels of human serum samples of categories: (A) IgG+ve, IgM-ve; (B) IgG-ve, IgM+ve; (C) IgG+ve, IgM+ve; and (D) IgG-ve, IgM-ve (negative control). The serological statuses of these samples were predetermined using commercial immunoassay kits (Table 1). The Western blot assays indicated that all 15 samples from categories A, B, and C reacted with recSAG2-C (five positive reactions from each category are presented in Fig 3a, 3b, and 3c, respectively). None of the serum samples from category D, which consisted of samples from non-toxoplasmosis individuals, reacted with recSAG2-C (Fig 3d, only five blots are shown).

In the ELISA, serum samples from 20 known, normal, healthy individuals were used to determine the cut-off optical density measurement for a positive value. All 15 samples from categories A, B, and C showed OD measurements higher than their respective cut-off values (Table 2). The specificity of the recSAG2-C was evident, as none of the sera in

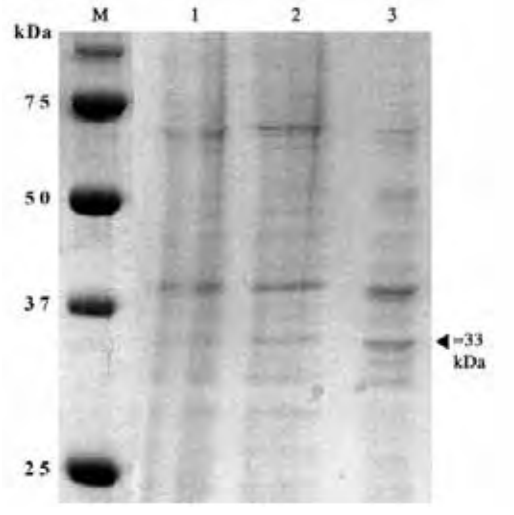

Fig 1- SDS PAGE analysis of total protein extracted from the culture supernatant of *P. pastoris*. Lanes 1 and 2 were loaded with total protein extracted from *P. pastoris* X-33 host cell and a nonrecombinant (*ie*, without SAG2 gene insert) *P. pastoris* clone, respectively. Lane 3 was loaded with total protein extracted from a recombinant *P. pastoris* clone after 60 hours of induction with 0.5% methanol, showing a novel ≈33 kDa protein (arrow). Lane M contained the protein molecular weight standard.

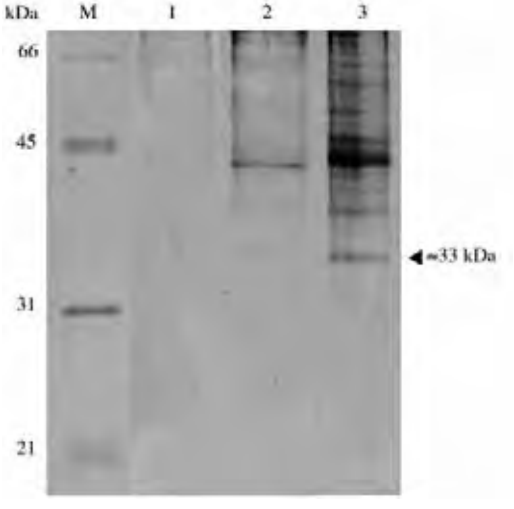

Fig 2- Preliminary Western blot analysis of total protein extracted from the culture supernatant of *P. pastoris*. Lanes 1 and 2 were loaded with total protein extracted from *P. pastoris* X-33 host cell and a nonrecombinant (*ie*, without SAG2 gene insert) *P. pastoris* clone, respectively. Lane 3 was loaded with total protein extracted from a recombinant *P. pastoris* clone after 60 hours of induction with 0.5% methanol. A positive anti-*Toxoplasma* human serum detected the novel ≈33 kDa protein in lane 3 (arrow), thus confirming the identity of the recombinant truncated SAG2 (recSAG2-C). Lane M contained the protein molecular weight standard.

Table 1
Serological status of human serum samples.

Category A (IgG +ve, IgM -ve)			Category B (IgG -ve, IgM +ve)			Category C (IgG +ve, IgM +ve)			Category D (IgG -ve, IgM -ve)		
Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR
1	2.01	0.03	16	0.25	1.23	31	3.67	1.15	46	0.40	0.11
2	1.14	0.16	17	0.31	1.47	32	3.86	1.53	47	0.38	0.28
3	1.93	0.42	18	0.58	1.25	33	4.20	1.13	48	0.40	0.16
4	1.66	0.15	19	0.21	2.29	34	3.26	1.72	49	0.42	0.12
5	1.55	0.15	20	0.66	1.19	35	3.03	1.40	50	0.46	0.35
6	1.78	0.58	21	0.45	1.13	36	2.78	1.27	51	0.45	0.34
7	1.45	0.49	22	0.29	1.18	37	3.53	1.12	52	0.45	0.11
8	1.34	0.46	23	0.32	1.15	38	3.16	1.13	53	0.56	0.02
9	1.79	0.25	24	0.87	2.19	39	3.30	2.75	54	0.61	0.01
10	3.30	0.47	25	0.71	1.28	40	3.83	1.12	55	0.57	0.25
11	3.65	0.21	26	0.36	1.56	41	2.63	1.99	56	0.68	0.06
12	2.37	0.71	27	0.21	1.15	42	2.89	1.18	57	0.40	0.11
13	3.92	0.45	28	0.34	1.11	43	3.77	1.30	58	0.38	0.28
14	3.45	0.46	29	0.33	1.10	44	2.90	1.18	59	0.70	0.14
15	2.19	0.34	30	0.16	1.10	45	3.45	1.74	60	0.56	0.25

[a] The samples were tested using commercial kits (Trinity Biotech, Ireland). Immune Status Ratio (ISR) values of >1.1 were recorded as positive for *Toxoplasma* antibody. Hence, samples 1-15 were positive for IgG (category A), samples 16-30 positive for IgM (category B), samples 30-45 positive for IgG and IgM (category C), and samples 45-60 were negative for both IgG and IgM (category D).

category D tested positive in the assay.

These results indicate that the recSAG2-C was specific for anti-*Toxoplasma* IgG and IgM antibodies in the human serum samples. The results also suggest that the C-terminal half of SAG2 possesses antigenic sites, or epitopes, which can evoke the production of IgM and IgG.

Results from the Western blots and ELISA indicate that the recSAG2-C has common epitopes with the native SAG2 of *T. gondi*i. Immunization of ICR mice was carried out to further evaluate the antigenicity of recSAG2-C. Two weeks after the final immunization, sera from the mice were used to probe the total protein of *T. gondii* tachyzoites. It was observed that sera from mice that received recSAG2-C reacted with the 22 kDa native SAG2 (Fig 4).

DISCUSSION

SAG2 is an attachment ligand that plays an important role in *T. gondii* invasion of host cells (Grimwood and Smith, 1996). Numerous attempts have been made to produce recombinant SAG2 using various expression systems. For example, recombinant SAG2 expressed in *E. coli* has been shown to be effective in detecting the IgG antibody to *T. gondii* in human patients with toxoplasmosis (Prince *et al*, 1990; Parmley *et al*, 1992). This challenges studies using animal models that suggest that the recombinant antigen provided only partial protection against lethal infection of *T. gondii* (Mishima *et al*, 2001). This lack of immunogenicity might be due to incorrect folding of the recombinant SAG2.

In this study, we exploited the recombinant

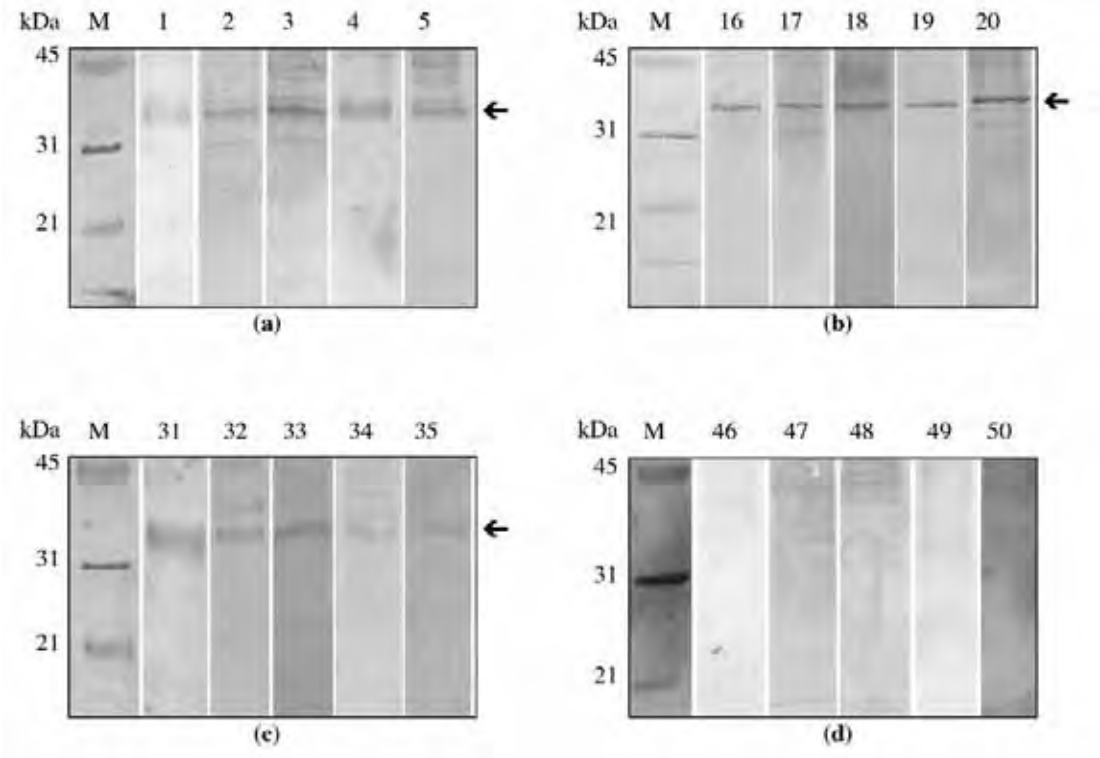

Fig 3- Detection of recSAG2-C with human serum samples. Each Western blot strip, containing the recSAG2-C, was tested with a serum sample from either category A (IgG +ve, IgM -ve), B (IgG -ve, IgM +ve), C (IgG +ve, IgM +ve) or D (IgG -ve, IgM -ve). Only five samples from each category are shown here. Strip numbers correspond to the sample numbers in Table 1. Strip M in each panel is the protein molecular weight standard. All samples from categories A, B and C (panels a, b, and c, respectively) reacted with the ≈33 kDa recSAG2-C (arrows). None of the samples in category D (panel d) showed positive reaction.

Table 2
Results of ELISA using recSAG2-C as antigen.

Category A (IgG +ve, IgM -ve)			Category B (IgG -ve, IgM +ve)			Category C (IgG +ve, IgM +ve)			Category D (IgG -ve, IgM -ve)		
Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR
1	1.66	0.45	16	0.22	0.91	31	1.63	1.22	46	0.26	0.11
2	1.29	0.32	17	0.43	1.34	32	1.40	1.34	47	0.32	0.18
3	1.00	0.12	18	0.12	1.25	33	0.86	0.98	48	0.38	0.03
4	0.80	0.40	19	0.33	2.00	34	0.82	0.99	49	0.44	0.23
5	0.87	0.13	20	0.45	1.87	35	1.96	1.12	50	0.25	0.23
6	0.82	0.11	21	0.44	1.05	36	1.20	0.89	51	0.39	0.13
7	1.94	0.18	22	0.29	1.05	37	1.19	0.99	52	0.23	0.32
8	1.21	0.22	23	0.35	0.70	38	1.44	1.23	53	0.17	0.24
9	0.99	0.32	24	0.22	1.93	39	1.49	1.12	54	0.42	0.36
10	2.42	0.17	25	0.23	1.31	40	1.17	0.92	55	0.24	0.12
11	1.32	0.45	26	0.43	0.83	41	1.43	0.89	56	0.42	0.22
12	1.31	0.46	27	0.38	1.69	42	1.41	0.97	57	0.22	0.35
13	1.96	0.23	28	0.29	0.98	43	1.47	1.23	58	0.23	0.12
14	1.07	0.11	29	0.19	1.54	44	0.91	0.87	59	0.18	0.15
15	1.85	0.17	30	0.17	0.61	45	1.91	0.90	60	0.31	0.19

Cut-off OD for positive IgG is 0.64; for IgM is 0.46

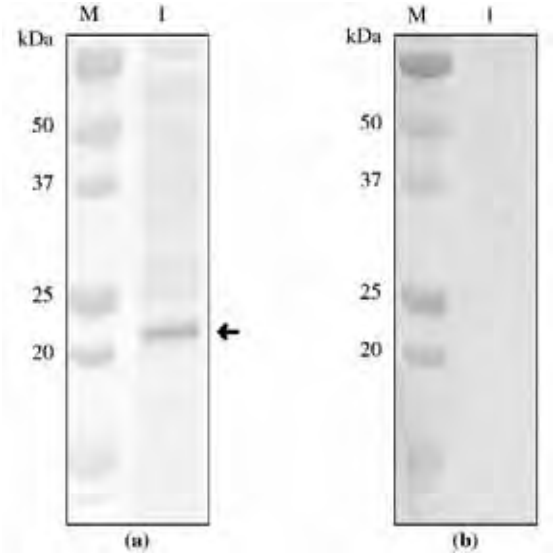

Fig 4- Reaction of mouse serum with total protein of *T. gondii* tachyzoites in a Western blot. In panel a, the blot was probed with serum from a mouse that was injected with recSAG2-C. The serum reacted with the native *T. gondii* SAG2, a protein of 22 kDa (arrow, lane 1). Serum of a mouse that received total cellular protein of a nonrecombinant *P. pastoris* did not react with any protein of *T. gondii* (panel b, lane 1). Lane M in each panel is the protein molecular weight standard.

system of the yeast *P. pastoris* to express and secrete the C-terminal half of SAG2 of T. gondii. Our reason for using a truncated SAG2 was primarily to increase its specificity by reducing any possible cross-reaction with surface antigens of other closely related protozoan parasites. The results of our studies indicated that the C-terminal half of SAG2 was sufficiently specific to detect human anti-*Toxoplasma* IgG and IgM antibodies. In addition, *in vivo* experiments suggested that recSAG2-C was sufficiently immunogenic to evoke antibody production against the native SAG2. Therefore, it would be worthwhile in future studies to examine the protective potential of recSAG2-C in immunized mice by challenging with live tachyzoites of *T. gondii*.

It was noted that the molecular mass of recSAG2-C was much larger than the expected truncated SAG2. However, this was not unexpected because some recombinant proteins that are produced in the *Pichia* expression system are known to be hyperglycosylated, causing significant increase in the molecular mass (Cereghino and Cregg, 2000; Letourneur *et al*, 2001). An increase in molecular mass was also found in our previous work on *P. pastoris* expression of a *Toxocara canis* worm antigen (Fong and Lau, 2004). There is also concern that glycosylation by the *Pichia* system may introduce glycans that negatively affect the specificity of diagnostic tests (van Oort *et al*, 2004). However, from this study, we can suggest that the difference in the size and extent of glycosylation did not significantly affect the antigencity and specificity of the recombinant SAG2. This was also observed in our work on *Pichia*-derived worm antigen (Fong and Lau, 2004).

As discussed above, we chose the *P. pastoris* expression system primarily for its high efficiency to produce recombinant proteins, and its ability to modify and fold the recombinant proteins into conformations that are similar to those of the native proteins. Another advantage of this expression system is the use of methanol as a component of the growth medium. Methanol plays two roles in this system. First, as a substrate for growth because *P. pastoris* is capable of utilizing methanol as the sole source of carbon. Second, methanol acts as an effective inducer in the expression of the foreign gene. This is unlike the *E. coli* expression system that requires expensive induction agents, such as IPTG or histidine. Therefore, the use of methanol can significantly reduce the cost of large-scale expression of the recombinant protein. In addition, the recombinant protein can be selectively secreted by *P. pastoris* into the growth medium. It has been estimated that recombinant protein makes up 80% of the secreted proteins, hence making harvesting and purification of the protein much easier.

In conclusion, our findings in this study have laid the groundwork for our further endeavor to produce a highly specific recombinant antigen that can be used for the development of an inexpensive seroassay kit for human toxoplasmosis.

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NOVEL DRUG COMPOUNDS AGAINST *NEOSPORA CANINUM* AND *TOXOPLASMA GONDII IN VITRO*

Jitbanjong Wiengcharoen[1,4], Ryan O'Hanley[2], Tanya Armstrong[2], Wayne Best[3], Yaowalark Sukthana[1,5] and RC Andrew Thompson[2]

[1]Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [2]Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Australia; [3]Epichem Pty Ltd, Murdoch University Campus, Australia; [4]Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok, Thailand; [5]Mahidol University International College, Nakhon Pathom, Thailand

Abstract. *Neospora caninum* has recently been identified as an important cause of abortion in cattle worldwide. This parasite is closely related to *Toxoplasma gondii*. To identify the drug compounds for potential use against both parasites *in vitro*, nine novel drug compounds were incubated with either parasite on microtiter plate. The number of extracellular tachyzoites and the quantities of Vero cells left in the wells after incubating with those nine drugs were compared to the conventional drug control, a combination of sulfadiazine 25µg/ml and pyrimethamine 0.1µg/ml. The most effective drugs against both *N. caninum* and *T. gondii* in this study were trifluralin analogues.

INTRODUCTION

Phylogenetic studies have shown that *Neospora caninum* is very closely related to *Toxoplasma gondii* although they cause quite different biological diseases (Dubey, 2003). Neosporosis is now considered as a major cause of abortion in cattle worldwide and it can also cause neurological symptoms in dogs whereas toxoplasmosis is more often associated with diseases in humans and sheep (Esteban-Redondo and Innes, 1997; Innes, 1997; Anderson *et al*, 2000; Dubey, 2003).

Little information is available on the efficacy of drugs for the chemotherapy of *N. caninum* infected animals even though sulfadiazine and pyrimethamine were found to be effective in some dogs at the early stage of neosporosis but there is no drug that can treat neosporosis in cattle at present (Lindsay *et al*, 1994; Thate and Laanen, 1998; Kim *et al*, 2002; Darius *et al*, 2004; Mui *et al*, 2005). The current usage of sulfadiazine and pyrimethamine for the treatment of toxoplasmosis also has many disadvantages (Mui *et al*, 2005).

Correspondence: Yaowalark Sukthana, Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2354-9100 ext 1833
E-mail: tmymv@mahidol.ac.th

The cell culture-based assays were performed in this present study to evaluate the novel drugs to determine which are effective to inhibit the growth of *N. caninum* and *T. gondii in vitro*.

MATERIALS AND METHODS

Microtiter plate assay

N. caninum (NC1 strain) and *T. gondii* (RH strain) were grown and maintained in Vero cells. For microtiter assays, the host cells were plated (104 cells/well) into flat-bottomed 96-well tissue culture plates with Dulbecco's modified eagle medium; with 10% fetal bovine serum, 1% L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin; at 37°C; and with 5%CO2 until a complete monolayer was visible under an inverted microscope. Monolayers were inoculated with 104 tachyzoites of *N. caninum* or *T. gondii* per well. Two hours post-inoculation, 50 µl of the medium was aspirated and replaced with the same volume of medium that contained either drug A, B, C, D, E, F, G, H, or I. All stock dilutions of the drugs were made in DMSO at a 10 mM or 20 mM concentration. The compounds were tested at a final concentration of 10 µM, 1 µM, and 0.1 µM in each well. Working dilutions were freshly prepared for each experiment in culture media. The drugs were incubated with the parasites for 72 hours. In each culture plate, three controls were included: (i) uninfected monolayer, (ii)

infected monolayers treated with a combination of pyrimethamine (0.1 µg/ml) and sulfadiazine (25 µg/ml), and (iii) untreated of infected monolayer (Derouin and Chastang, 1988; Mui *et al*, 2005). After a 72-hour incubation period, the number of extracellular tachyzoites in each well was assessed with a hematocytometer. The percentage of growth inhibition was calculated using the formula (Sarciron *et al*, 2002):

% growth inhibition = 100 - (100 x number of extracellular tachyzoites in treated well/number of extracellular tachyzoites in control well)

Crystal violet assay

Plates were visually monitored every day, and the assay was stopped when 90-100% of the untreated infected cells had lysed (three days post-inoculation). The crystal violet assay was performed as previously described with some modification (Linsay and Dubey, 1999; Zarubin *et al*, 2005). Briefly, the culture medium and extracellular tachyzoites were removed from wells. Adhering Vero cells were washed with 1xPBS and then fixed in 100% methanol for 5 minutes. The crystal violet solution was added and incubated at room temperature for 5 minutes. Cells were washed twice with 1xPBS. The 50% glacial acetic acid solution was added and incubated at room temperature for 1 hour. An ELISA plate reader operating at 595 nm was used to quantitate the amount of crystal violet present. The percentage of cell viability was calculated using the formula (Akca *et al*, 2003):

% cell viability = 100 x A1/A0
(When A1 is the OD of treated infected well and A0 is the OD of untreated uninfected well).

Cytotoxicity assay for Vero cells

To determine the cytotoxicity of these drug compounds against Vero cells, the MTT assay was used. Approximately 1 x 10^4 Verocell/well were applied to 96-well, microtiter plates that were treated with either drug at the final concentration of 10 µM and incubated at 37°C for 24 hours. The MTT assay was performed according to manufacturer's instructions. The absorbance was measured on an ELISA plate reader with a test wavelength of 595 nm and a reference wavelength of 620 nm. Cells treated with the medium only were used as the control.

RESULTS

Anti-protozoa activities of drug compounds determined by the percentage of growth inhibition

At 10 µM, drug B was the most effective drug against *N. caninum* in cell culture, whereas drug F was the most effective drug against *T. gondii* (Table 1).

Anti-protozoa activities of drug compounds determined by the crystal violet assay

Results of the crystal violet assay demonstrated that drug B was the most effective drug against *N. caninum*. Drugs F and G were also shown to inhibit *N. caninum* development. For *T. gondii* growth inhibition, drug F was the most effective drug, while drug B and H also demonstrated some effectiveness (Table 2).

Cytotoxicity test

None of the nine drug compounds showed any toxicity when used up to 10 µM. The morphology

Table 1
Percentage growth inhibition of the parasites after 72-hour incubation with each drug.

Drug (10 µM)	*N. caninum*	*T. gondii*
Drug A	60.8	70.8
Drug B	88.6	81.5
Drug C	78.5	79.2
Drug D	62.0	0.8
Drug E	78.5	77.7
Drug F	75.9	92.3
Drug G	79.7	59.2
Drug H	43.0	63.1
Drug I	45.6	53.1
Sulfadiazine and pyrimethamine (control)	84.8	88.5

Table 2
Vero cell viability of the drug test to *N. caninum* and *T. gondii*.

Drug	*N. caninum*			*T. gondii*		
	10 µM	1 µM	0.1 µM	10 µM	1 µM	0.1 µM
A	78.2%	71.7%	71.7%	53.8%	34.5%	40.6%
B	90.7%	86.6%	86.6%	89.0%	34.3%	31.7%
C	39.4%	68.3%	68.3%	17.5%	28.9%	31.9%
D	46.9%	79.1%	79.1%	27.8%	27.3%	49.6%
E	77.2%	83.7%	83.7%	31.1%	20.9%	33.1%
F	80.8%	84.0%	84.0%	91.7%	31.9%	37.1%
G	81.2%	90.5%	90.5%	66.0%	44.6%	38.6%
H	76.5%	84.3%	84.3%	83.0%	80.7%	82.8%
I	78.8%	77.7%	77.7%	74.4%	44.7%	36.8%
Sulfadiazine and pyrimethamine (control)		97.4%			99.0%	
Untreated uninfected Vero cells		100%			100%	

and growth rate of both treated and untreated Vero cells were not different (Fig 1).

DISCUSSION

In the present study, the cultured host cells, which were infected with *N. caninum* or *T. gondii*, were treated with various concentrations of nine drug compounds to examine the efficacy of drugs against *N. caninum* and *T. gondii* tachyzoites intracellular multiplication. A combination of sulfonamides and pyrimethamine has been well known as being effective in inhibiting the growth of *T. gondii* and *N. caninum* (Lindsay and Dubey, 1989; Lindsay *et al*, 1994). It also showed an excellent effect to inhibit the development of both *N. caninum* and *T. gondii* in our study. However, there are many disadvantages of this drug combination as it can block folic acid metabolism of host cells in long-term usage or at high doses, and it is associated with bone marrow suppression (Montoya and Liesenfeld, 2004; Mui *et al*, 2005). Moreover, pyrimethamine is teratogenic when used in pregnant woman, and it is not specifically approved for veterinary use (Toribio *et al*, 1998). Alternative drugs that are

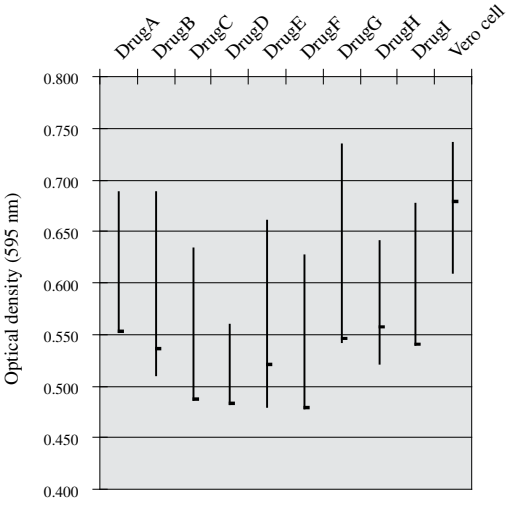

Fig 1- Effect of nine drugs on Vero cell *in vitro*. Cytotoxicity of drug compounds was measured by the MTT assay.

safer and more potent are needed. We examined nine new drug compounds that are effective in inhibiting the growth of other parasites *in vitro* in our laboratory (data not shown). Drugs B, F, G and H showed satisfactory effects to inhibit the development of *N. caninum* and/or *T. gondii*

in Vero cell culture. Drug B, F, G and H are trifluralin analogues. Trifluralin is used primarily as an herbicide on grass. It ranks as one of the five best-selling herbicides in the US (Extoxnet, 2001). It prevents weed growth by inhibiting root development through the interruption of mitosis.

These novel drug compounds might be of value in the prevention and treatment of neosporosis and toxoplasmosis in the future. For that purpose, antiprotozoal activity of these drugs in in vivo and the mechanism of action should be initiated.

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CRYPTOSPORIDIOSIS AMONG BIRDS AND BIRD HANDLERS AT ZOO NEGARA, MALAYSIA

YAL Lim, M Rohela and M Muhamat Shukri

Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract. This study was carried out at the Malaysian National Zoo to ascertain, not only the current prevalence rate in the birds, but also to determine the association between cryptosporidiosis in birds and the bird handlers. A total of 116 fecal samples from 71 species of birds were collected from six different locations in Zoo Negara, and eight fecal samples from bird handlers were also sampled. Results showed that the prevalence of *Cryptosporidium* oocysts in birds and bird handlers were 3.4% and 12.5%, respectively. The birds that were positive for cryptosporidiosis were Wreathed Hornbill (*Aceros undulatus*) and Great Currasow (*Crax rubra*) from the aviary, Bushy-crested Hornbill (*Anorrhinus galeritus*) from the bird house, and the Common Peafowl (*Pavo cristatus*) from the lake. Birds at the lake showed the highest percentage (12.5%) of positivity, followed by birds at the aviary (5.4%) and the birdhouse (2.8%). Result of the present study seemed to indicate that cryptosporidiosis might be spreading to other species of birds and to other locations in the zoo, which was not previously documented. This study also suggested the probable association of cryptosporidiosis among birds and their bird handlers. However, conclusions can only be drawn after the confirmation of speciation found in birds and bird handlers through molecular identification.

INTRODUCTION

Cryptosporidium is a ubiquitous enteric protozoan pathogen that infects humans, and domestic and feral animals, worldwide. It is an important causative agent of diarrheal disease in humans, leading to significant morbidity and mortality in both developing and developed countries. Cryptosporidiosis, a disease caused by *Cryptosporidium*, was first described in the ceca of chickens by Tyzzer in 1929 (Sterling and Arrowood, 1978; Current, 1989). Subsequently, a report in 1955 described structurally similar parasites in turkeys, and these parasites were later named *C. meleagridis* (Slavin, 1955). In 1986, Current *et al* (1986) isolated and described an organism from chickens and gave the name *C. baileyi* to the species.

Currently, there are 16 species of *Cryptosporidium* that have been identified as having different morphologies and hosts (Xiao *et al*, 2004). *Cryptosporidium* species that have been described in birds include *C. meleagridis*, *C. baileyi*, and *C. galli* (Awad-el-Kariem *et al*, 1997). Only *C. meleagridis*, which infects turkeys and parrots, is a known threat to humans. Nevertheless, *C. baileyi* is probably the most common avian *Cryptosporidium* species because it is able to infect chickens, turkeys, ducks, cockatiels, quails, and ostriches; whereas, *C. galli*, the latest addition to the family, infects hosts such as finches, domestic chickens, capercaillie, and pine grosbeaks (Xiao *et al*, 2004).

In birds, *Cryptosporidium* sp has not only been reported in chickens (Sterling and Arrowood, 1978), turkeys (Sréter and Varga, 1999), quails, pheasants, peafowl, junglefowl, ducks, geese, parrots, pinches, lovebirds, budgerigars, ostriches (Morgan *et al*, 2001), catercaille, and pine grosbeaks (Xiao *et al*, 2004); but also the wrinkled hornbill found in Malaysia (Rohela *et al*, 2005). Avian cryptosporidiosis can manifest as respiratory (Dhillon *et al*, 1994) and intestinal (Lindsay and Blogbum, 1990) diseases. In some cases, cryptosporidiosis might even manifest as renal disease, which can be fatal (Hoerr *et al*, 1986).

The association between cryptosporidiosis in animals and animal handlers is a topic of interest. In 1981, a case of cryptosporidiosis in an animal handler was reported (CDC,

Correspondence: Dr Yvonne Lim Ai Lian, Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: 603-7967 4748; Fax: 603-7967 4754
E-mail: limailian@um.edu.my

1982). The animal handler was a previously healthy 25-year-old male who had symptoms of cryptosporidiosis, such as nausea, low-grade fever, moderate abdominal cramps, anorexia, 5 to 10 watery-frothy bowel movements a day, and constipation. The diagnosis was confirmed by finding *Cryptosporidium* sp oocysts in the feces. Following this report, many similar cases of cryptosporidiosis were reported among animal handlers (Augus, 1983; Graczyk *et al*, 1996).

In 2004, a study on the occurrence of *Cryptosporidium* sp oocysts in the fecal samples of birds in Zoo Negara, Kuala Lumpur was conducted. The study showed 6 species of birds were infected with *Cryptosporidium* (Rohela *et al*, 2005). This present study is an expansion of the previous study. This study tries to ascertain not only the current prevalence rate in the birds but also to determine the association between cryptosporidiosis in birds and the bird handlers.

The main objectives of this study were to reevaluate the occurrence of *Cryptosporidium* sp oocyst in the fecal samples of birds in the Zoo Negara, Kuala Lumpur, Malaysia, and to determine the prevalence of cryptosporidiosis among bird handlers and its association with the occurrence of cryptosporidiosis in birds.

METHODOLOGY

Location of study

The study was carried out in the Kuala Lumpur National Zoo, located at Ulu Kelang, 13 km northeast of Kuala Lumpur. The birds at the National Zoo can be found at various places, such as the aviary, bird house, breeding area, animal show, children's world, and lake. All birds in the locations mentioned are caged except those in the lake. Birds housed in the aviary of various species that ranges from smaller birds, such as Lovebirds, to the larger birds, such as Common Peafowl and Hornbill. The birdhouse and the breeding area are places where the birds are quarantined before being sent to the aviary for visitors to view. They are also the places for the rearing the offspring. Children's world is designed especially for children to have a closer view of domesticated animals and birds, such as macaws and parrots. Birds appearing at the animal show are trained to perform stunts and entertainment activities. Storks and pelicans are the birds commonly found in the lake, where they are free to roam.

Sample collection

A total of 116 feces specimens from 71 species of birds were collected and kept in the fecal containers labeled according to the bird species and location. Fecal samples of birds were collected carefully to avoid any contamination with soil or other contaminants. Collection of fecal specimens and the identification of bird species from their respective locations were carried out in the morning with the assistance of the bird handlers. The specimens were returned to the Department of Parasitology, Faculty of Medicine, University of Malaya, and stored in the cold room at 4°C before processing.

Sixteen fecal containers were distributed to the bird handlers for fecal collection, but only eight containers (50%) were returned. The profile of the bird handlers, such as name, age, race, gender, and the location of work were recorded. All samples were returned to the laboratory to be processed.

Stool examination for *Cryptosporidium* oocysts

Direct smear and formalin ethyl acetate concentration technique were employed for all stool samples from both birds and bird handlers. The smears were stained using the Ziehl-Neelsen (acid-fast) stain. After staining, the slides were examined under 400x. Putative *Cryptosporidium* sp oocysts appeared as bright rose-pink spheres (5 ± 1 μm) on a bluish green background. The positive slides were examined again under 1,000x magnification to confirm the presence of *Cryptosporidium* oocysts. All slides positive for *Cryptosporidium* sp oocysts, using the Ziehl-Neelsen staining method were recorded.

RESULTS

A total of 116 fecal specimens from birds and eight fecal specimens from bird handlers were collected from the Kuala Lumpur National Zoo (Table 1 and Table 3). All specimens were

Table 1
List of birds and positive specimens for *Cryptosporidium* oocysts using Ziehl-Neelsen staining technique.

No	Common Name	Species	Location	No. of specimens taken	No. positive for *Cryptosporidium* oocyst
1	African Grey Parrot	*Psittacus erithacus*	BH	1	-
2	African Ground Hornbill	*Bucorvus leadbeateri*	BH	1	-
3	African Spoonbill	*Platalea alba*	A	1	-
4	All domestic chickens	*Gallus gallus*	CW	2	-
5	Barn Owl	*Tyto alba*	BH,AS	2	-
6	Barred Eagle Owl	*Bubo sumatranus*	CW,AS,BH	3	-
7	Black Hornbill	*Anthracoceros malayanus*	BH	1	-
8	Black Kite	*Mulvus migrans*	BH	1	-
9	Black Swan	*Cygnus atratus*	L	2	-
10	Blue and Yellow Macow	*Ara ararauna*	BH,AS,CW	3	-
11	Blue-breasted Quail	*Coturnix chinensis*	BH	1	-
12	Blyth's Hawk Eagle	*Spizaeus alboniger*	BH	1	-
13	Brahminy Kite	*Haliastur Indus*	BH,AS	3	-
14	Brown Barbet	*Calorhamphus fuliginosus*	BH	1	-
15	Buffty Fish Owl	*Ketupa ketupu*	BH	1	-
16	Bulbul	*Pyenonotus*	CW	1	-
17	Bushy-crested Hornbill	*Anorrhinus galeritus*	BH	1	1
18	California Quail	*Callipela californica*	BH	1	-
19	Cassowary		BH	1	-
20	Changeable Hawk Eagle	*Spizaeus cirrhatus*	AS	1	-
21	Common Peafowl	*Pavo cristatus*	L,AS,CW,	7	1
22	Crested Fireback Pheasant	*Lophura ignita*	BA,BH,A	3	-
23	Crested Serpent Eagle	*Spilornis cheela*	BA	1	-
24	Crestless Fireback Pheasant	*Lophara erythrophthalma*	BA,BH,A	3	-
25	Domestic Duck	*Anas domesticus*	A	1	-
26	Eclectus Parrot	*Eclectus roratus*	AS	1	-
27	Egyptian Goose	*Alopochen aegyptiacus*	L	1	-
28	Emu	*Dromalus novaehollandiea*	A	1	-
29	Golden Pheasant	*Chrysolophus pictus*	A	1	-
30	Great Argus Pheasant	*Argusianus argus*	A,BH,BA	3	-
31	Great Currasow	*Crax rubra*	A	1	1
32	Greater Hornbill	*Bucerros bicornis*	BH,AS,A	6	-
33	Greater Sulfur Crested Cockatoo	*Cacatua galerita*	BH	1	-
34	Green Jungle Fowl	*Gallus varius*	BH	1	-
35	Green-winged Dove	*Chalcophaps indica*	A	1	-
36	Green-winged Macow	*Ara chloroplera*	AS	1	-
37	Helmeted Guineafowl	*Numida meleagris*	A,CW	2	-
38	Hill Myna	*Gracula religiosa*	BH	1	-
39	Java Sparrow	*Padda oryzivora*	CW	1	-
40	Lesser Fish Eagle	*Ichthyophaga humilis*	BH	1	-

Table 1 (Continued)

No	Common Name	Species	Location	No. of specimens taken	No. positive for *Cryptosporidium* oocyst
41	Lesser Sulphur Crested Cockatoo	*Cacatua sulphurea*	CW	1	-
42	Little Corella	*Cacatua sanguinea*	CW	1	-
43	Lovebird	*Agopornis fisheri*	CW,BH,A	4	-
44	Magpie Robin	*Copsychus saularis*	BH	1	-
45	Malayan Peacock-Pheasant	*Polyplectron malacense*	BH,BA	2	-
46	Mandarin Duck	*Aix galericulata*	A	1	-
47	Milky Stork	*Mycteria leucocephala*	A	1	-
48	Moluccan Cockatoo/Salmon-Crested Cockatoo	*Cacatua moluecencis*	AS	1	-
49	Mountain Peacock-Pheasant	*Polyplectron inopination*	BH	1	-
50	Nicobar Pigeon	*Caloenas nicobarica*	A	1	-
51	Oriented Pied Hornbill	*Anthracoceros albirostris*	BH	1	-
52	Ostrich	*Struthio camelus*	Savanna	2	-
53	Painted Stork	*Mycteria cinerea*	L,BH	2	-
54	Pigeon	*Columba*	CW,AS	3	-
55	Pin-tailed Parrot Finch	*Erythuraprasima*	BH	1	-
56	Pink-backed Pelican	*Pelecanus rufescens*	A	1	-
57	Rainbow Lory	*Trichoglossus haematodus*	CW	1	-
58	Red Jungle Fowl	*Gallus gallus*	A	1	-
59	Rhinoceros Hornbill	*Buceros rhinoceros*	BH,A	3	-
60	Scarlet Macow	*Ara macoa*	CW	1	-
61	Spotted Billed Pelican	*Pelecanus rufescens*	L	1	-
62	Silver Pheasant	*Lophura nycthemera*	A,BH,BA	4	-
63	Spotted Wood-Owl	*Strix seloputo*	BH,AS	2	-
64	Storm's Stork	*Ciconia stormi*	L	2	-
65	Swan Goose	*Anser cygnoides*	L	2	-
66	Turkey	*Meleagris gallopavo*	CW	2	-
67	White Bellied Sea Eagle	*Haliaetus leucogaster*	BH,AS	2	-
68	White Cockcatoo	*Cacatua alba*	BH	1	-
69	White Rumped Shama	*Copsychus malabaricus*	BH	1	-
70	Wreathed Hornbill	*Aceros undulatus*	A,BH	2	1
71	Wrinkled Hornbill	*Aceros corrugatus*	A	1	-
		Total		116	4

A= Aviary; AS= Animal Show; BH= Bird House; BA= Breeding Area; CW= Children's World; L= Lake

collected according to their locations (Table 2). By using the Ziehl-Neelsen staining technique, four specimens (3.4%) from birds (Table 1) and one sample (12.5%) from a bird handler (Table 3) were positive. *Cryptosporidium* oocysts were identified as bright rose-pink spheres with the size of 5 ± 1 μm on a bluish green background.

The four positive samples for *Cryptosporidium* oocyst in birds were detected in the Great Currasow (*Crax rubra*) and Wreathed Hornbill (*Aceros undulatus*) (both located at the aviary), Common Peafowl (*Pavo cristatus*) (located at the

Table 2
Percentage of fecal bird specimens positive for *Cryptosporidium* oocysts according to location.

Location	Total no. of fecal specimens collected	No. of specimens positive for *Cryptosporidium*	Percentage positive
Animal show	13	-	0
Aviary	37	2	5.4
Bird house	35	1	2.8
Breeding area	5	-	0
Children's world	18	-	0
Lake	8	1	12.5

Table 3
Biodata of the bird handlers examined for *Cryptosporidium* oocysts.

Name	Age (years)	Gender	Race	Location	Result (present of *Cryptosporidium* oocysts)
J	26	M	Malay	Bird house	Negative
S	27	F	Malay	Bird house	Negative
M K	21	M	Malay	Bird house	Positive
M E	25	M	Malay	Bird house	Negative
Z	19	M	Malay	Bird house	Negative
N B H	20	F	Malay	Children's world	Negative
M Y	52	M	Malay	Children's world	Negative
M A M S	20	M	Malay	Children's world	Negative

lake), and Bushy-crested Hornbill (*Anorrhinus galeritus*) (located at the birdhouse) (Table 1). Only 14.3% (1 out of 7) of the specimens taken from the Common Peafowl, 50% (1 out of 2) of the specimens taken from the Wreathed Hornbills, and 100% (1 out of 1) of the specimen from Bushy-crested Hornbills and Great Currasows were positive for *Cryptosporidium* oocysts. Specimens from the bird handlers showed 12.5% (1 out of 8) was positive with *Cryptosporidium* oocysts (Table 3). Locations that were free from *Cryptosporidium* oocysts were the children's world, animal show, and the breeding area. Birds at the lake showed the highest percentage (12.5%) of positivity, followed by birds at the aviary (5.4%) and birdhouse (2.8%) (Table 2).

DISCUSSION

Cryptosporidiosis affects human and animals, which include reptiles, mammals, birds, amphibians, and fish. Studies have confirmed the presence of *Cryptosporidium* oocysts in pets, and domestic and wild animals (Sterling and Arrowood, 1978; O'Donoghue, 1995; Lim and Ahmad, 2001). Since the first detection of *Cryptosporidium* in birds in 1929 by Tyzzer (Sterling and Arrowood, 1978; Current, 1989), many studies have been conducted to determine the presence of *Cryptosporidium* oocysts in various species of birds. However, there are very few studies that have ascertain the naturally occurring cryptosporidiosis in wild birds.

Prevalence rate of 5.9% to 27.3% have been described in flocks in the United States (Lindsay and Blagburn, 1990).

A previous study carried out in 2004 at Kuala Lumpur National Zoo showed six species of birds were positive, with *Cryptosporidium* oocyst in their feces. The positive species included Wrinkled Hornbill (*Aceros corrugatus*), Great Argus Pheasant (*Argusianus argus*), Black Swan (*Cygnus atratus*), Swan Goose (*Anser cygnoides*), Marabou Stork (*Leptoptilos crumeniferus*), and Moluccan Cockatoo (*Cacatua moluccencis*). These birds were located in the aviary and lake, and the Moluccan Cockatoo was routinely used as a show bird (Rohela *et al*, 2005).

This present study showed a prevalence of 3.4% (4 out of 116) of *Cryptosporidium* sp in bird's feces. The prevalence rate has decreased when compared to the previous study that was conducted two years previously at the same sites. In this study, *Cryptosporidium* oocysts were detected in the feces of the Wreathed Hornbill (*Aceros undulatus*) and Great Currasow (*Crax rubra*) from the aviary, the Bushy-crested Hornbill (*Anorrhinus galeritus*) from the birdhouse, and the Common Peafowl (*Pavo cristatus*) from the lake.

Although the prevalence of *Cryptosporidium* sp in birds at the Kuala Lumpur National Zoo has decreased to 3.4% when compared to the previous study, the birds that were positive in this study were different from the ones that were positive in the previous study. The four types of birds that were positive with *Cryptosporidium* oocysts were not found to be positive before. This finding indicates that *Cryptosporidium* infection might be spreading to other species of birds in the zoo. In both the previous and present studies, birds that were found to be positive for *Cryptosporidium* were all asymptomatic. It is very likely that these birds were not quarantined or separated from the other birds that do not harbor the parasite because they did not present any symptoms thereby facilitating the transmission of *Cryptosporidium* oocysts to the other birds.

In terms of location, a comparison between the two studies showed that the aviary and lake were still contaminated with *Cryptosporidium* oocysts, whereas the location that was positive in 2004, the Animal Show, was negative for *Cryptosporidium* in this present study. Conversely, the location that was previously negative in 2004, the birdhouse, was positive in this present study.

Although there were three locations (*ie*, aviary, birdhouse, and lake) in this present study that were positive for cryptosporidiosis, the probability of birds acquiring cryptosporidiosis might be higher in the lake because birds could be infected, not only through contaminated food or close contact, but also through contaminated water. This postulation is confirmed with the findings of this study whereby the birds at the lake showed the highest percentage of positive samples for *Cryptosporidium* oocysts (12.5%), followed by the birds in the aviary (5.4%) and (2.8%) at the birdhouse. Birds at the animal show, breeding area, and children's world were negative for *Cryptosporidium* oocysts.

There are a few contributing factors to the high prevalence of avian cryptosporidiosis in the lake. Birds at the lake are not caged and therefore are free to mix with other birds or fly to any location. They may possibly fly out of the zoo compound. This will facilitate the transmission of *Cryptosporidium* to other areas inside or outside the zoo compound. The implication of birds in the lake being infected is great because the lake water may be contaminated with feces containing *Cryptosporidium* oocysts. This could be a source of transmission to other birds, animals, and humans who contact the contaminated water. Many studies as well as waterborne outbreaks have implicated contaminated water as a source of cryptosporidiosis (Current, 1989; O'Donoghue, 1995; Xiao *et al*, 2004). The findings of this study warrant future systematic study to investigate the quality of water from the lake in order to determine the association of waterborne cryptosporidiosis in a zoological context.

The occurrence of *Cryptosporidium* oocysts in the aviary is probably due to cross contamination. This is because the birds at the aviary are always being relocated from one cage to another. As an example, an asymptomatic infected bird from cage A might be transferred to cage B. Therefore, if cage A is not washed properly, it might still be contaminated with the feces of the previously infected bird that was transferred. When a new

bird, free of infection is placed in cage A, there is a chance for it to acquire the infection through the contaminated cage. This scenario enables the transmission of *Cryptosporidium* oocysts to other birds if it is not controlled properly.

Considering that the types of birds that were positive in this study are common in the wild (*eg*, wreathed hornbill is a common bird of lowland and hill forest of Borneo and Sumatra). It will be crucial to look also into the possibilities of conducting a similar study on the wild birds in Malaysia, to elucidate the role of wild birds in parasitic transmission.

This study discovered that one of the bird handlers who were stationed at the birdhouse was infected with cryptosporidiosis. This person not only managed the birds in the birdhouse, but he also managed the birds at the aviary and lake. As indicated earlier, all the locations where this bird handler was working were positive with infected birds. Therefore, it is difficult to conclude whether he acquired the infection from, or he was the source of infection to, the birds. This is because of the three avian *Cryptosporidium* sp (*eg*, *C. meleagridis*, *C. baileyi*, and *C. galli*), only *C. meleagridis* can infect both human and birds (Xiao *et al*, 2004). Only with the employment of molecular techniques can this dilemma be determined through speciation or genotyping.

In conclusion, a comparison between a study in 2004, and the present study, conducted in the birds at National Zoo, Kuala Lumpur, highlighted that there was a decrease in the prevalence rate in the birds. However, there seemed to be an indication that cryptosporidiosis might be spreading to other species of birds and to other locations in the zoo that was not previously documented. This study also discovered the possible association of cryptosporidiosis among birds and bird handlers. However, conclusions can only be drawn after the confirmation of speciation found in birds and bird handlers through molecular identification.

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CURRENT SITUATION OF *GIARDIA* AND *CRYPTOSPORIDIUM* AMONG ORANG ASLI (ABORIGINAL) COMMUNITIES IN PAHANG, MALAYSIA

AK Mohammed Mahdy, Johari Surin, YAL Lim and MS Hesham Al-Mekhlafi

Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract. A cross-sectional study on the distribution of *Giardia intestinalis* and *Cryptosporidium* species was conducted among the Orang Asli communities at Pos Betau, Pahang, Malaysia. Fecal samples were collected from 316 participants, age between 1-76 years (156 males and 160 females). The samples were examined using trichrome staining technique for *G. intestinalis*, and *Cryptosporidium* species were detected using modified acid-fast staining technique. Biodata were also collected through a pre-tested standard questionnaire. The overall prevalence rates were 17.1% and 4.1%, for giardiasis and cryptosporidiosis, respectively. The study indicated that there was a significant difference in the infection rate of *G. intestinalis* between age groups, with infections being higher in children ($p < 0.05$). However, cryptosporidiosis and giardiasis were not found to be gender biased. This study concludes that giardiasis and cryptosporidiosis are still public health problems in the Orang Asli communities in Malaysia, and special attention should be given to those in the high-risk groups.

INTRODUCTION

Human giardiasis and cryptosporidiosis have been recognized as the most common causes of protozoal diarrhea worldwide leading to significant morbidity and mortality in developing and industrialized nations (Marshall *et al*, 1997; Clark, 1999). Although foods and drinking water have been considered as the most common route of transmission of *G. intestinalis* and *Cryptosporidium*, person-to-person and zoonotic transmission of these protozoa may occur (Marshall *et al*, 1997; Monis and Thompson, 2003).

The ability of *G. intestinalis* and *Cryptosporidium* to survive for weeks to months in the environment and to withstand chlorine disinfection and filtration beside the low infective dose has defied water and health authorities. *Giardia* and *Cryptosporidium* have caused multiple waterborne outbreaks in developed and developing countries (Insulander *et al*, 2005). To date the biggest *Cryptosporidium* waterborne outbreak was the outbreak that occurred in South Milwaukee, USA that affected 403,000 persons, with more than 100 fatal cases (MacKenzie *et al*, 1994). Although livestock have been implicated as the source of waterborne outbreaks in Canada (Fayer *et al*, 2000) and UK (McLauchlin *et al*, 2000), genotyping the isolates in the Milwaukee outbreak has implicated human effluent as the source of water contamination causing the outbreak (Zhou *et al*, 2003).

In immunocompromised hosts, *Cryptosporidium* causes severe and life-threatening diarrhea. However, it induces self-limiting diarrhea in immunocompetent persons. In developing countries, cryptosporidiosis in early childhood may be associated with subsequent impaired physical development and cognitive function (Guerrant *et al*, 1999), even if the infection is asymptomatic (Checkley *et al*, 1998). Although giardiasis is most often asymptomatic, acute infection causes diarrhea and may be associated with the clinical manifestation of malabsorption. Chronic giardiasis in the children is usually associated with failure to thrive. A significant association between giardiasis and malnutrition has been documented (Gendrel *et al*, 2003).

In Malaysia, giardiasis has been reported as a predictor of malnutrition (Al-Mekhlafi *et al*, 2005). Although cryptosporidiosis is prevalent in Malaysia, it has not received much attention especially in communities, where the infection is still underestimated. To our knowledge, the

Correspondence: Professor Johari Surin, Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: 603-7967-4746; Fax: 603-7967-4754
E-mail: joharis@ummc.edu.my

last reported prevalence of *Cryptosporidium* infection among the Orang Asli was in 1997 (Lim *et al*, 1997). Thus, this study sought to determine the occurrence of *G. intestinalis* and *Cryptosporidium* among Orang Asli communities in Pahang, Malaysia.

MATERIALS AND METHOD

The study was conducted in Orang Asli villages in Pos Betau, Pahang, about 200 km from Kuala Lumpur. All villages are located near the river. Houses are made of wood or bamboo and basic amenities, such as electricity and piped water supply are provided free by the government. Most of the Orang Asli daily activities, such as swimming, playing, washing clothes and household items are carried out at the river. For some inhabitants, the river also doubles up as a "toilet" and the river water is used to clean themselves after defecation. During the period of study, no livestock were seen in the study area. However, many household pets, such as dogs, were noted.

A total of 316 Orang Asli individuals volunteered to participate in this study. Of these participants, 156 were males and 160 were females. The ages of the participants ranged from 1 to 76 with a median of 10 years. Stool samples were collected in screw-capped containers and preserved in polyvinyl alcohol. For the identification of *G. intestinalis*, fecal smears were made from the preserved stool, after centrifugation at 2,500 rpm for 5 minutes, and stained with trichrome. The smear was then examined by light microscopy under the magnification of 1,000x. A smear was reported as positive if *Giardia* cysts or trophozoites were detected. *Cryptosporidium* was diagnosed by using modified Ziehl-Neelsen staining technique. Biodata were collected via pretested standard questionnaire. Data were analysed using SSPS program for windows version 11.5. The associations between the prevalence and dependent variables were tested using Fisher's exact test and the significance was defined as $p < 0.05$. The study was approved by the research and ethics committee of Faculty of Medicine, University of Malaya, Malaysia.

RESULTS

The prevalence and distribution of *G. intestinalis* and *Cryptosporidium* are indicated in Table 1. The overall prevalence of *G. intestinalis* was 17.1%. There was a decrease in prevalence with increasing age. Children less than 15 years of age were at the highest risk ($p< 0.05$). Females had a higher infection rate compared with males; however, the difference was statistically non-significant.

Positive rate of *Cryptosporidium* was determined to be 11(4.1%) of the 271 examined. The highest prevalence of *Cryptosporidium* was among the 7-15 age groups. Females were also more infected than males. No significant association between *Cryptosporidium* infection and age or gender was noted.

DISCUSSION

During the past decade, *G. intestinalis* and *Cryptosporidium* have emerged as important pathogenic enteric protozoa. These parasites have gained great attention from public health authorities due to their significant association with childhood malnutrition, immunosuppressed patients especially those with AIDS (in case of *Cryptosporidium*), and their responsibility for several waterborne outbreaks in developed and developing countries. In 2004, WHO has added *Giardia* and *Cryptosporidium* into the 'Neglected Diseases Initiative,' which is a comprehensive approach to combat parasitic, viral, and bacterial diseases that impair the ability to achieve full potential and impair development and socio-economic improvements.

This study has determined the prevalence of *G. intestinalis* to be at 17.1%. This rate is generally in agreement with studies conducted among the Malaysian Orang Asli communities at different locations whereby prevalence of giardiasis among these people is usually above 15% (Lim *et al*, 1997; Al-Mekhlafi *et al*, 2005). Similar finding has also been reported in rural Malay communities (Norhayati *et al*, 1998). It would seem that factors influencing occurrence of giardiasis is related to living conditions rather than ethnicity. Che Ghani *et al* (1987) and Lai

Table 1
Prevalence of *G. intestinalis* and *Cryptosporidium* among Orang Asli population according to age and gender.

Age (years)	*G. intestinalis*			*Cryptosporidium*		
	No. examined	No. infected	Prevalence (%)	No. examined	No. infected	Prevalence (%)
≤ 6	17	4	23.5	14	0	0.0
7-15	239	48	20.1	204	9	4.4
≥ 16	60	2	3.3	53	2	3.8
Gender						
Male	156	26	16.7	136	5	3.6
Female	160	28	17.5	135	6	4.4
Total	316	54	17.1	271	11	4.1

(1992) have found that no significant differences in prevalence of giardiasis among major ethnic groups, such as Malays, Chinese, and Indians living in urban Malaysian areas. Furthermore, human giardiasis in Malaysia is more prevalent in rural communities (Norhayati *et al*, 1998; Al-Mekhlafi *et al*, 2005) than urban communities (Che Ghani *et al*, 1987; Lai, 1992). This could be attributed to the low socio-economic status and improper sanitation in rural areas.

The prevalence of *Cryptosporidium* was found to be 4.1% in this study. This result was consistent with a previous study on the prevalence of *Cryptosporidium* among Orang Asli community in Selangor (Lim *et al*, 1997). Several studies that had aim to determine the occurrence of cryptosporidiosis among children in pediatric wards in Malaysian hospitals indicated that the prevalence ranged from 0.9% to 11% (Ludin *et al*, 1991; Lai, 1992; Menon *et al*, 2001). In AIDS patients, cryptosporidiosis was 3.03% (Lim *et al*, 2005) and 23% (Kamel *et al*, 1994).

Our study indicated that children are at significantly higher risk of getting *Giardia* infection. This result was in agreement with previous studies (Lim *et al*, 1997; Norhayati *et al*, 1998; Laupland and Church, 2005). The possible reasons for this age-dependent pattern are probably related to children's behaviors (*eg*, sharing things among themselves, putting objects into the mouth, etc) and exposure to sources of fecal contamination because of their poor personal hygiene practices. Otherwise, the reason for higher infections in children may also be related to the lack of effective immunity. Children, in our study, had higher infection rates for cryptosporidiosis compared to adults. This finding was consistent with previous studies in several tropical countries, which confirmed that *Cryptosporidium* infection was commonly found in children (Samie *et al*, 2006). However, the differences were statistically non-significant.

Although females were slightly more likely to be infected with *Giardia* and *Cryptosporidium*, the difference was non-significant. Similar finding were also reported in Malaysia for *Giardia* infection (Lim *et al*, 1997; Norhayati *et al*, 1998; Al-Mekhlafi *et al*, 2005) and in South Africa for *Cryptosporidium* infection (Samie *et al*, 2006).

Humans may be the most possible potential source of *Giardia* and *Cryptosporidium* infection in Orang Asli communities, with direct transmission from person-to-person or indirectly though river water contamination. Sewage is usually discharged into the river, and some people may defecate in the river. Lim and Ahmad (2004) documented that 66.7% and 5.6% of river water samples, collected from a river located adjacent

to an Orang Asli community, were positive for *Giardia* cysts and *Cryptosporidium* oocysts, respectively. Zoonotic transmission should also be considered as numerous dogs have been noted living closely with human. Recent molecular epidemiological studies have implicated dogs as source of human infection with *Giardia* (Traub *et al*, 2004). Although livestock have been implicated in zoonotic transmission of *Cryptosporidium* (Fayer *et al*, 2000), they were not observed in this study location. In addition, domestic pets have rarely been implicated as a source of cryptosporidiosis.

It could be concluded that giardiasis and cryptosporidiosis are still public health problems and should receive more attention from public health authorities. Mass treatment should be combined with appropriate health education programs to prevent re-infection. The control of fecal disposal could be the most practical intervention to reduce the spread of giardiasis and cryptosporidiosis. Molecular tools should be applied to identify species and genotypes/ subgenotypes of these protozoans for better understanding of the epidemiology of these diseases.

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CYCLOSPORIASIS IN NEPAL

Jeevan B Sherchand[1], Jatan B Sherchand[1] and John H Cross[2]

[1]Institute of Medicine Teaching Hospital, Tribhuvan University, Kathmandu, Nepal;
[2]Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

Abstract. Nepal is one of the most highly endemic areas for cyclosporiasis in the world. Initial studies of the parasitosis in Nepal were among expatriates and tourists in the country. The present report, however, is a review of studies carried out since 1995 on the epidemiology of *Cyclospora cayetanensis* and infection in the Nepali populations. The parasitosis was found to occur mostly in children 2-11 years of age. Stools from several thousands of patients with diarrhea were examined, and approximately 6-30% were positive for oocysts of the parasite. The majority of patients were seen during the rainy season. Stool specimens from animals were examined, and oocysts were recovered from six chickens, two monkeys, three dogs, and five rats. PCR/RFLP analysis of oocysts from one monkey, one dog, and 2 chickens were positive for *Cyclospora* sp. Oocysts of *Cyclospora* were also found in sewage, a pond and two irrigation canals, and the washwater of cabbage, lettuce, mustard leaves, and basil. Basil may be an important source of infection since it is often eaten raw.

INTRODUCTION

Cyclospora cayetanensis is recognized as an important cause of diarrheal disease, and Nepal is considered one of the most highly endemic areas in the world. The disease was first recognized in Nepal among tourists and expatriate residents and reported at the time to be caused by cyanobacterium-like bodies (CLB) (Shlim *et al*, 1991). The epidemiology, clinical features, and treatment of the parasitosis were later studied in a similar type of patients (Hoge *et al*, 1995, 1996). Since little was known about cyclosporiasis in Nepali populations, studies were carried out from 1996 throughout 2004 on the transmission, distribution, and possible reservoir hosts of the parasite in Nepal.

METHODS

Populations throughout Nepal and patients seen at health care facilities were examined for infection with *C. cayetanensis*. Stool specimens from humans, animals, vegetable washwater, and various water bodies were examined for oocysts. The stool specimens were examined by direct microscopy (400×); leaves from vegetables were washed with distilled water, the washings centrifuged, and the sediment examined. Water samples were centrifuged and the sediment examined. Positive specimens were placed into 2.5% potassium dichromate and the organism examined periodically for sporulation.

Correspondence: John H Cross, Uniformed Services, University of the Health Sciences, Bethesda, Maryland 20814-4790, USA.
Tel: +(301) 295-3039; Fax: +(301) 295-1971
E-mail: jcross@usuhs.mil

RESULTS

The initial study was carried out at the Kanti Children's Hospital in Kathmandu, and the stools from 180 children with diarrhea were examined by direct microscopy for oocysts. Fifteen children, 2 months to 11 years-of-age were found to be positive (Cross *et al*, 1997). During 1995-1998, over 2,000 stools from Nepali as well as few expatriates with diarrhea were examined; approximately 30% were positive for oocysts. Most positive stools were from children 2-11 years of age (Sherchand *et al*, 1991). Over 4,000 stools were examined during 1997-2000, and over 22% were found to be positive (Sherchand *et al*, 2001). A number of stools from various districts of Nepal were examined, and many were found to contain *C. cayetanensis*-like oocysts indicating the widespread distribution of the parasitosis throughout the country. Additional stools from rural populations were examined during 2002-2003, and approximately 6% were positive (Sherchand *et al*, 2004). More recently (2005-2006), 1,842 stool samples from different areas of Nepal and health facilities were examined,

and approximately 8 % were found to be positive. Most of the infected people manifested diarrhea with the passage of 1-15 stools per day. The patients also reported abdominal discomforts, anorexia, fatigue, flatulence, and weight loss. Most patients responded to treatment with co-trimoxazole (Hoge *et al*, 1995). Although infections were detected throughout the year, the majority occurred during the rainy season, May to September.

Over 1,400 animal stools (chicken, pig, monkey, dog, cat, cow, buffalo, goat, rat, and pigeon) were examined, and cyclospora-like oocysts were recovered from six chickens, two monkeys, three dogs, and five rats (Sherchand *et al*, 2001, 2004). Oocysts from these animals were analyzed by PCR/RFLP. Cyclospora sp was demonstrated in monkey and dog samples, and *Eimeria* and *C. cayetanensis* were found in chicken samples (Chu *et al*, 2004).

During the rainy months, samples were collected from tap water, wells, ponds, irrigation canals, rivers, springs, as well as sewage. Cyclospora-like oocysts were recovered from sewage on 5 occasions, ponds once, and irrigation canals twice. Oocysts have also been recovered from chlorinated water during an epidemic of cyclosporiasis among a small British military detachment in Pokahara, Nepal (Rabold *et al*, 1994). Irrigation canal water is usually fecally contaminated, and these waters are used to irrigate crops and to keep vegetables fresh in the market. Over 300 samples of washwater from vegetables were examined for *C. cayetanensis* oocysts. The vegetables included cabbage, lettuce, cauliflower, spinach, green onion, radish, mustard leaves, and basil leaves. Oocysts were recovered from washwater of cabbage (5), lettuce (5), mustard leaves (3), spinach (3), and basil leaves (2). The oocysts recovered either were sporulated or were found to sporulate after several days in 2.5% potassium dichromate. Of interest is the finding of oocysts on basil leaves as basil is a holy plant in Nepal and considered an incarnation of God, and it helps purify eternal life. It is also considered a medicinal plant. The leaves of basil are usually eaten fresh without washing. Many of the people found infected with *C. cayetanensis* were vegetarians.

DISCUSSION

We have obtained a great deal of information on cyclosporiasis in Nepal; however, parasitosis remains an enigma. The disease is endemic in the Nepali population throughout the country, occurring mostly in the rainy season, May through September. Although most of the cases have been documented from a few medical facilities, it is not a reportable disease and therefore many more cases seen elsewhere have not been reported. The disease has been found primarily in children 2-11 years of age with relatively few reports in the older Nepali age groups.

One of the unknowns regarding cyclosporiasis is whether it is a zoonotic disease. *Cyclospora cayetanensis* oocysts have been reported elsewhere from the feces of ducks, chickens and dogs (Garcia-Lopez, 1996) and in chickens, monkeys, dogs and rats in Nepal (Sherchand *et al*, 2001, 2004). These infections, however, have been considered spurious as these animals are known to be coprophagic. In further studies, the PCR/RFLP analysis suggested the isolates from two dogs, one chicken, and one monkey from Nepal were *Cyclospora* sp (Chu *et al*, 2004). Experimental infections in animals and human volunteers have not been successful (Alfano-Sobsey *et al*, 2004). To understand fully the life cycle of *C. cayetanensis* and the pathology of the disease, it is imperative that an animal model be found.

Water from various sources has been suspected as a main source of infection with *C. cayetanensis* (Sterling *et al*, 1999; Cross *et al*, 2004). Untreated water has been implicated as a source of infection in Nepal, and oocysts were found in chlorinated water (Rabold *et al*, 1994). We have recovered oocysts from sewage, a pond, and irrigation canal waters (Sherchand *et al*, 2004). Fecally contaminated water is used in farm irrigation systems for vegetables and to keep vegetables looking fresh in markets. Other intestinal parasites such as *Ascaris lumbricoides* eggs, *Giardia lamblia* cysts, and *Cryptosporidium* sp oocysts have been recovered from pond and irrigation canal water. Furthermore, the drinking water supply is often contaminated with sewage water, especially in the Kathmandu valley. Many of the patients with cyclosporiasis report drinking untreated water.

Plants have been suspected as a source of infection (Sterling *et al*, 1999), but the organism is rarely isolated. Oocysts of the parasite have been found in the washwater of cabbage, lettuce, mustard leaves, and basil in Nepal (Sherchand and Cross, 2001), and fresh basil has been incriminated as a source of transmission of the parasite elsewhere (Lopez *et al*, 2001; Hoang *et al*, 2004). We have recovered *C. cayetanensis*-like oocysts from fresh basil washwater, and we consider this an important source of infection. In Nepal, basil is considered a "holy plant" by Hindu populations. It is also used in the treatment of respiratory disease. Basil is usually eaten raw. Although water is considered a source of infection, plants are probably contaminated with the organism during growth and irrigation with polluted water. The majority of the cases of cyclosporiasis among Nepali population have been found in vegetarians; therefore, the eating of raw vegetables is considered an important source of infection in the country.

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CHARACTERIZATION OF β-TUBULIN cDNA FROM A BENZIMIDAZOLE RESISTANT *STRONGYLOIDES STERCORALIS* ISOLATE

Wittaya Luadthong[1,2,3], Pewpan M Intapan[1,3], Thidarat K Prasongdee[1], Chaisiri Wongkham[1], Viraphong Lulitanond[1,3], Kanongwan J Imtawil[1] and Wanchai Maleewong[1,3]

[1]Faculty of Medicine, [2]Graduate School, [3]Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University, Khon Kaen, Thailand

Abstract. Previous reports have indicated that benzimidazole resistance in gastrointestinal nematodes is linked to a mutation in the β-tubulin codons 167 and 200. In our study, total RNA was isolated from an albendazole-resistant (ABZR) *Strongyloides stercoralis* filariform larval isolate, followed by reverse transcription PCR that was amplified using primers designed according to the alignment of the β-tubulin mRNA of *S. stercoralis* (GenBank accession No. AY 898942). The cDNA sequence of the β-tubulin gene of ABZR *S. stercoralis* larvae revealed 100% identity with the sequence of *S. stercoralis* (AY 898942). The polymorphisms at codons 167 and 200 encoded phenylalanine (Phe). The resistance mechanisms for benzimidazole in *S. stercoralis* were discussed in the light of these results.

INTRODUCTION

Strongyloidiasis is a serious threat to public health in tropical and subtropical areas (Grove, 1996). The disease is caused by infection with *Strongyloides stercoralis*, a nematode that infects several million people worldwide (Genta, 1989). The clinical spectrum of strongyloidiasis varies from asymptomatic infection, to mild symptomatic abdominal and skin diseases, to fatal disseminated infection in immune-suppressed patients (Grove, 1996; Pearson, 2002; Lewthwaite *et al*, 2005). Effective treatment is therefore important to prevent severe infection. Albendazole is the principal drug used to treat human strongyloidiasis (Horton, 2000). However, the efficacy of the treatment of strongyloidiasis with albendazole, a benzimidazole derivative, has been inconsistent and may be never complete (Horton, 2000; Nontasut *et al*, 2005; Singthong *et al*, 2006). Benzimidazoles inhibit the polymerization of the tubulin dimers that comprise microtubules, and as such obstruct vital cellular functions, including cell division (Dumontet and Sikic, 1999).

Many investigators have reported that nematodes that have infected humans may be resistant to benzimidazoles (Hoti *et al*, 2003; Albonico *et al*, 2004; Schwab *et al*, 2005), and the resistance has been associated with mutations in the β-tubulin (Elard *et al*, 1999). These mutations are supposed to change the structure of the β-tubulin protein, decreasing the β-tubulin-benzimidazole interaction that causes the antimitotic effect of the drug (Robinson *et al*, 2004). One point mutation in position 200 of the amino acid sequence is a phenylalanine (Phe) to tyrosine (Tyr) transition (Prichard, 2001). Another mutation in position 167 is a Phe-Tyr substitution (Silvestre and Cabaret, 2002). Robinson *et al* (2004) hypothesized that a number of mutations that caused substitution in a protein at a crucial junction between its N-terminal and intermediate domains may also confer resistance to benzimidazoles. Recently, the β-tubulin cDNA and genomic DNA of *S. stercoralis* have been cloned and sequenced (Melville *et al*, 2006). Here, we have sequenced β-tubulin cDNA from a benzimidazole resistant *S. stercoralis* isolate. This sequenced data were compared with the β-tubulin mRNA of *S. stercoralis* (GenBank accession No. AY 898942) in order to determine whether the genetic change causing the resistance would be present in other nematodes.

Correspondence: Pewpan M Intapan, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: 66-043-348387; Fax 66-043-202475
E-mail: pewpan@kku.ac.th

MATERIALS AND METHODS

Preparation of parasite RNA

An albendazole-resistant isolate of *Strongyloides stercoralis* infective-stage larvae was harvested from stool samples of a hyperinfected strongyloidiasis patient who did not respond to repeated treatment with albendazole. The subject received 800 mg albendazole (Zentel, GlaxoSmithkline Australia) per day, orally after meals, twice daily for three consecutive days; the same dose was repeated again seven days later. The drug efficacy was evaluated by parasitological examination using filter paper culture technique (Beaver *et al*, 1984) on day 14, after the second round of treatment. The surviving larvae were used for molecular analysis of βtubulin cDNA. Approximate 30,000 worms were pooled and extracted for RNA in Trizol reagent (Invitrogen, Carlsbad, CA) using the manufacturer's protocol.

RT-PCR and sequencing

Complementary DNA was generated using two pairs of gene-specific primers (Table 1) with the Robust II RT-PCR Kit (Finnzymes, Keilaranta, Espoo, Finland) following the manufacturer's instructions. All PCR products were sequenced in both directions by the dideoxynucleotide chain termination method, using the DYEnamic ET Dye terminator cycle sequencing Kit (Amersham Biosciences, Piscataway, NJ) and the MegaBACE DNA Analysis system (Amersham Biosciences). RNA sequenced data were searched against the GenBank data base using NCBI and BLASTN algorithms to assess their similarity to previously characterized βtubulin mRNA sequences. The alignment of the sequences was carried out using the ClustalW program (http://www.ebi.ac.uk/clustalW).

RESULTS

Four *S. stercoralis* β-tubulin primers (Ss22F1, Ss662F2, Ss703R1 and Ss1329R2) were designed from the *S. stercoralis* β-tubulin mRNA sequence (AY 898942) (Table 1). Primers Ss22F1 and Ss703R1, as well as primers Ss662F2 and Ss1329R2, were used as pairs in a one-step

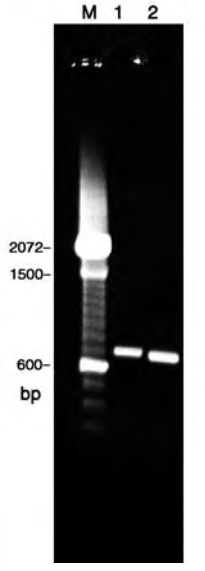

Fig 1- Agarose gel electrophoresis of products from *S. stercoralis* β-tubulin PCRs. Lane 1, RT-PCR with primers Ss22 F1 and Ss703R1; lane 2, RT-PCR with primers Ss662 F2 and Ss1329R2; lane M, 100 bp ladder.

Table 1
Primers employed in characterizing *S. stercoralis* β-tubulin cDNA.

Forward primers	Position (first)	Sequence
Ss22 F1	Ss cDNA Forward strand 1	ATGAGAGAAATTGTTCACGTCC
Ss662 F2	Ss cDNA Forward strand 641	GAACACTCAAGCTTAGTTCACC
Reverse primers	Position (last)	Sequence
Ss703R1	Ss cDNA Reverse strand 723	GAGGCATGTAGTTACACCAGA
Ss1329R2	Ss cDNA Reverse strand 1350	CTTCAGCTATTCCTCTTCAGCA

Ss = S. stercoralis

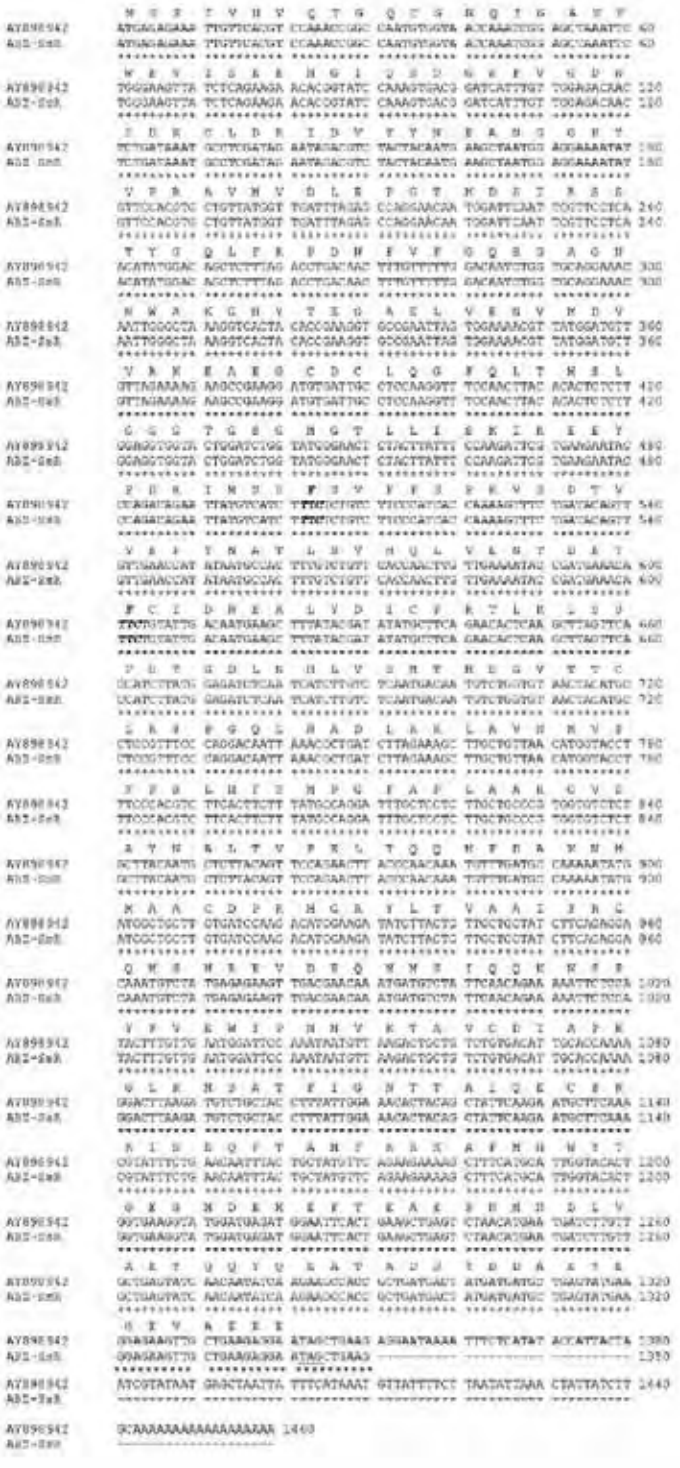

Fig 2- Alignment of a benzimidazole-resistant *S. stercoralis* β-tubulin sequence (ABZ-SsR) and mRNA of a *S. stercoralis* sequence (GenBank accession No. AY 898942). Codons corresponding to amino acids 167 and 200 are emphasized with bold and italic text. Identity between the two sequences is indicated by asterisks. Stop codon is indicated by underlined text.

reverse transcription PCR to specifically generate and amplify *S. stercoralis* β-tubulin cDNA from a total parasite RNA. The PCR reactions yielded a visible product by agarose gel electrophoresis (Fig 1). An amplicon cDNA of 723 bp was obtained in the Ss22F1-Ss703R1 cycle, and an amplicon cDNA of 710 bp was obtained by Ss662F2-Ss1329R2. The combined coding sequence from the two amplicons, the DNA sequence, and the deduced amino acid sequence revealed 100% identity with the *S. stercoralis* β-tubulin mRNA sequence (AY 898942) (Fig 2). The codons 167 and 200 encoded Phe.

DISCUSSION

The β-tubulin is the target of the benzimidazoles, which are broad-spectrum anthelmintics used to control parasitic helminthes in ruminants (Pape *et al*, 1999). Here, we described the sequenced data of β-tubulin cDNA of a benzimidazole-resistant *S. stercoralis* isolate by direct sequencing of the PCR products. The hypothesis was that this could help to detect resistance because *Strongyloides* also likely has a single β-tubulin gene in the *S. ratti* genome (Melville *et al*, 2006). However, the present molecular analysis indicated that codons 167 and 200 encode Phe. This evidence suggests that this polymorphism is not the only reason for the development of benzimidazole resistance in *S. stercoralis*. The development of resistance also possibly evolved through other mechanisms. Different modes of inheritance are probably responsible for the development of resistance in different populations (Prichard, 2001). The low cure rate of *S. stercoralis* may be attributable to the habitat of these worms, the gastrointestinal tract, where the active drug concentration is low (Melville *et al*, 2006). Other reasons for treatment failures are autoinfection, dissemination, and a variation in the activity of the drug efflux. Several mechanisms of drug resistance are reported in veterinary helminths, such as (1) a change in the molecular structure of the target molecule of the drug such that the drug ineffectively recognizes the target, (2) modification of metabolism resulting in inactivated or completely removed drugs, (3) changes in the drug distribution to the target, and (4) an extension of the target genes to overcome the drug action (Wolstenholme *et al*, 2004).

We will continue this investigation with a single worm PCR, genotyping the site that is associated with benzimidazole-susceptibility and resistance in different worm populations. This method can be used to document the resistance status of parasite populations. This result possibly leads to a better understanding of the biology of the parasite: why they become resistant and how the development of resistance could be controlled.

ACKNOWLEDGEMENTS

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EGG POSITIVE RATE OF *ENTEROBIUS VERMICULARIS* IN CHILDREN IN A RURAL AREA OF PHICHIT PROVINCE, THAILAND

Saengchai Nateeworanart[1], Apichat Vitta[2] and Urat Pimolsri Lee[2]

[1]Department of Medical Technology, Faculty of Allied Health Sciences, [2]Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand

Abstract. The pinworm *Enterobius vermicularis* is a cause of enterobiasis or oxyuriasis that causes anal itching and enuresis among school-age children, and genital inflammation and irritation in women and girls. The objective of this study was to determine the egg positive rate of pinworm infection in students of a rural area in Phichit Province, from January to December 2005. Cellophane tape method was used to identify the infection of pinworm. Of 298 students examined, 56 (18.7%) were found positive for pinworm eggs. The rate of boys (19.0%) found positive for pinworm was nearly as high as that of girls (18.5%). The results of the present study indicated that pinworm infection was highly prevalent, and there is a need to control it in the study area.

INTRODUCTION

The pinworm, *Enterobius vermicularis*, is one of the most common parasites of man, particularly in schoolchildren. It is characterized by an esophagus with a posterior bulb. The worms are most abundant in the cecum and appendix. Humans are generally thought to be the only host, but these worms have been reported in a few other primates, for example, chimpanzees and gibbons (Noble *et al*, 1989). The nocturnal migration of the female worm to the host's anus to lay eggs frequently leads to severe irritation. Most cases are asymptomatic. However, anal or vaginal pruritus, abdominal pain, constipation, or diarrhea can occur. Children are more commonly infected than adults are, presumably because they are less fastidious in matters of personal hygiene (Paingjai *et al*, 1992).

Phichit is situated in the northern region of Thailand and is located 345kms to the north of Bangkok. The number of inhabitant of Phichit was 559,000 in 1992. The provincial economy is based on agriculture. The important economic crops are rice, corn, green pea, and various kinds of tropical fruit.

Correspondence: Saengchai Nateeworanart, Department of Medical Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok 65000, Thailand.
Tel: 66 (055) 261000 ext 6358
E-mail: saengchai_n@yahoo.com

The objective of this study was to determine the egg positive rate of pinworm infection in students of a rural area of Phichit Province, from January to December 2005.

MATERIALS AND METHODS

A survey of *Enterobius vermicularis* was carried out in six districts (Tap Khlo, Dong Charoen, Taphan Hin, Sam Ngam, Pho Thale, and Bueng Na Rang) in Phichit. The 290 children in these areas, age 1-12 years, who were recruited for this study, had verbal informed consent from their parents. The children were clarified for using cellophane tape, as described by Beaver *et al* (1984). The results were analyzed with respect to gender using the chi-square test.

RESULTS

A total of 56 (18.8%) of the 298 samples were positive for pinworm egg. The egg positive rate ranged from 0% to 33.3%, by location (Table 1). The egg positive rate among boys (19.0%) was not significantly different to that of girls (18.5%).

DISCUSSION

The overall infection rate was 18.8% (56/298), in which the egg positive among boys (19.0%) was not significantly different to that among girls (18.5%). Three hundred seven

Table 1
Egg positive rates of *Enterobius vermicularis* among children living in Phichit

District	Boys No. positive/ No. examined (%)	Girls No. positive/ No. examined (%)	Total No. positive/ No. examined (%)
Tap Khlo	7/30 (23.3)	7/40 (17.5)	14/70 (20)
Dong Charoen	4/30 (13.3)	3/26 (11.5)	7/56 (12.5)
Taphan Hin	0/15 (0)	4/16 (25)	4/31 (12.9)
Sam Ngam	10/31 (32.3)	7/20 (35)	17/51 (33.3)
Pho Thale	0/14 (0)	0/8 (0)	0/22 (0)
Bueng Na Rang	10/43 (23.3)	4/25 (16)	14/68 (20.6)
Total	31/163 (19.0)	25/135 (18.5)	56/298 (18.8)

(18.5%) of the 1,661 samples were positive for *E. vermicularis* eggs. The results of the present study were similar to other findings in Thailand. Using the same method, other studies found that the infection rates were 15.5% and 21.5%, in Mae Chaem, Chiang Mai and Bang Khun Thian, Bangkok, respectively (Saksirisampant *et al*, 2004; Changsab *et al*, 2000). High prevalence of 41.6 % was found in hill tribe children Mae Suk Sub-district and Karen hill tribe villages in Chiang Mai (Chaisalee *et al*, 2004). In the Republic of Korea, the egg positive rate ranged from 0% to 59.3% by location on western and southern coastal regions (Park *et al*, 2005).

The prevalence of enterobiasis greatly depends upon the socioeconomic situation, and on personal hygiene and habits. A lack of personal hygiene and close contact between people encourage the spread of *E. vermicularis*. Other factors, such as playing on the floor, nail biting, failure to wash hand before meals, and living in non-apartment dwellings, have been associated with the prevalence of enterobiasis (Sung *et al*, 2001). In this respect, kindergarten and school-based mass control activities are likely to be more effective than individualized treatment.

Enterobiasis is a disease with usually mild symptoms, such as perianal itching and dermatitis; it is asymptomatic in most adult who have low worm burdens. In children, particularly those who have heavy worm burdens, neurological symptoms including nervousness, restlessness, irritability, and distraction may occur, which may affect child development (Beaver *et al*, 1984; Cook, 1994; Song *et al*, 2003). Rarely, ectopic infections in the pelvic area or urinary tract of women can occur.

Egg positive rates in our study were rather low, which may result from using a single test. Goldsmith and Heyneman (1989) suggested that three tests will detect 90%, and five tests will detect 99%. Use of the cellophane tape method must be repeated to get the real prevalence in these communities.

Effective chemotherapeutic regimens have been developed and used for decades; however, the control of enterobiasis is difficult because of frequent reinfection and a short life cycle (Lee *et al*, 2001). Repeated health education concerning improved personal hygiene, regular inspections, and mass chemotherapy with appropriate anthelmintics are essentially required to control enterobiasis among children living in Phichit.

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THE SPREAD OF ANGIOSTRONGYLIASIS: THE GLOBETROTTING RAT LUNGWORM

John H Cross

Uniformed Services University of the Health Sciences, Bethesda, MD, USA

Abstract. *Angiostrongylus cantonensis* is recognized as one of the major causes of eosinophilic meningitis. The dissemination at one time was believed to be by the spread of the snail *Achatina fulica*. Indeed this intermediate host may have been involved with the early spread in Asia; however, the spread of the parasite to other places probably has been due to the movement of rats. Public health authorities should be made aware that angiostrongyliasis might become a problem.

THE SPREAD OF ANGIOSTRONGYLIASIS

Angiostrongylus cantonensis is recognized as one of the major causes of eosinophilic meningitis. The nematode was first reported from China in 1935 (Chen, 1935) and the first human infection was from Taiwan in 1945 (Nomura and Lin, 1945). Eosinophilic meningitis was reported occurring in the Pacific Islands for a number of years (Bailey, 1948), but the etiology was not known until the parasite was found in the brain of a mental patient in Hawaii (Rosen *et al*, 1962). This was followed by reports of the diseases in Southeast Asia and the Pacific Basin. The parasitosis is now being reported worldwide (Cross, 2004).

In the rat definitive host, the parasite is located in the pulmonary arteries. Eggs produced by the female worms hatch, and the larvae migrate to the intestine and pass in the feces. Molluscan intermediate host acquire the infection, and the larvae develop into the infective third stage. When the intermediate molluscan host is ingested by rats, the larvae are digested out of the tissues, migrate to the central nervous system, develop into young adults that migrate to the pulmonary arteries, and become sexually mature. When humans ingest an infected intermediate or paratenic host, the released larvae migrate to the CNS and cause disease. The parasites usually die in the CNS; however, occasionally they migrate to the eye and rarely to the lung where they can become sexually mature (Cross, 1987).

A number of *Rattus* and *Bandicota* species are known definitive host of *A. cantonensis*. *Rattus norvegicus* and *R. rattus* are the rats most commonly infected internationally, followed by regional rats such as *R. argentiventer, R. exulans, R. jalorensis*, and *R. rattus alexandrinus* (Bhaibulaya, 1982).

Various species of mollusc serve as an intermediate host. The giant African land snail, *Achatina fulica*, is considered a major source of infection in Asia and the Pacific Basin, and many other terrestrial and aquatic molluscan species can also be intermediate hosts. Species of aquatic snails (Ampullariidae) imported from South America are evolving as important host in China and Taiwan. Other snails such as *Pila* spp, *Bradybaena similaris*, and *Subulina octona* and slugs such as *Vaginulus plebeius*, *Veronicella leydigi* and *Deroceras laeve* are also known vectors of the parasite. A great number of other mollusc species have been shown experimentally to be susceptible to infection (Richards and Merritt, 1967) and aquatic snail *Biomphalaria glabrata* has been used in the laboratory to maintain the life cycle (Alicata and Jindrak, 1970; Bhaibulaya, 1982). Other animal species are known as paratenic or transport host for the parasite. The land planarians are accidental hosts; aquatic and terrestrial crabs, prawns, and shrimps have been associated with epidemics. Frogs, toads, and monitor lizards are paratenic host and

Correspondence: John H Cross, Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4790, USA.
Tel: (301) 295-3139; Fax: (301) 295-1971
E-mail: jcross@usuhs.mil

are reported to be sources of human infection.

The spread of *A. cantonensis* throughout the world initially was attributed to the dissemination of the great African snail from Madagascar to Mauritius, and on to India, Sri Lanka, Malaysia, China, Taiwan, Southeast Asia, the Pacific Islands, and Australia. The snail, along with the parasite, may have been transported around the Pan-Pacific area during and after World War II (Kliks and Palumbo, 1992). Mead (1961) has depicted the spread with dates of dispersal of the *A. fulica* in the nineteenth century. Alicata and Jindrak (1970) subsequently speculated that the spread of *A. cantonensis* was by the snail as it was transported to various parts of the world (Fig 1).

Since 1962, angiostrongyliasis has been reported primarily from Asia and the Pacific Basin. The disease was later reported from Africa in 1970 (Kliks and Palumbo, 1992). The first report of the parasite and the disease in the Western Hemisphere was from Cuba (Pascual *et al*, 1981; Aguiar *et al*, 1981). Rats and local molluscs were found infected. The parasite was later reported from the Dominican Republic, Haiti, Jamaica, Puerto Rico, and the Bahamas. The disease demonstrated serologically was also reported from Guadalupe (Cross, 2004). *Rattus* spp and local molluscs have also been found infected in the Caribbean Islands. *Achatina fulica*, however, has not been implicated. The means of transmission of the parasite is not known in the islands, and infection in humans is believed to be due to the accidental ingestion of an intermediate or paratenic host.

The parasite was not reported in rats or molluscs in the United States until Campbell and Little (1988) found rats in New Orleans, Louisiana, infected. They did not find infected molluscs but were able experimentally to infect local snails with the parasite. During the 1960s and 1970s, rats in New Orleans were examined routinely for parasitic infection, but *A. cantonensis* was never found. The authors speculated that infected rats arrived in New Orleans by ship and introduced the nematode to the area in the 1980s.

The first report of possible spread *A. cantonensis* in the Americas was a report of fatal meningoencephalitis in a howler monkey in the Audubon Park and Zoological Gardens in New Orleans (Gardiner *et al*, 1990). The same paper reported infection in a white handed gibbon in the Ardastra Gardens and Zoo, Nassau, Bahamas. In 1999, Aguilar *et al* (1999) reported angiostrongyliasis in seven other monkeys

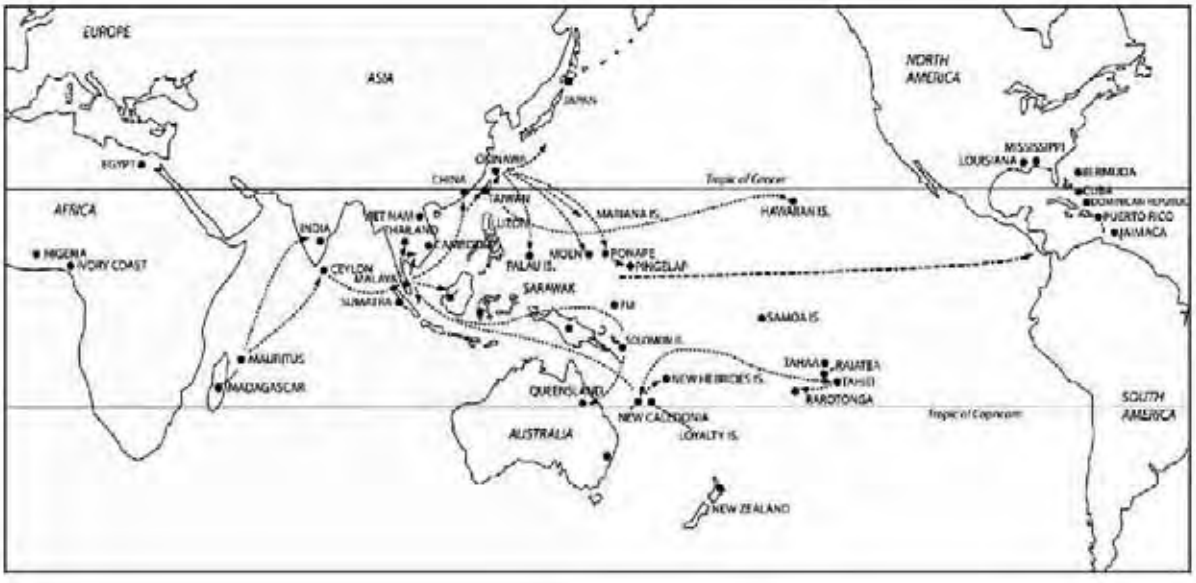

Fig 1- Geographical distribution of murine and human angiostrongyiasis in the tropical and subtropical regions. Arrows indicate theoretical easterly migration of the parasite. (Modified from Alicata and Jindrak, 1970).

in the New Orleans Zoo. The parasite was also found in snails and slugs after the second report in the New Orleans Zoo. A human case of angiostrongyliasis was reported from an 11 year old boy in New Orleans who acquired the infection by ingesting a snail on a dare from his sister (New *et al*, 1995). A miniature horse from Picayune, Mississippi, was reported infected with the parasite and at the same time *R. norvegicus* trapped in Baton Rouge, Louisiana, was found infected (Costa *et al*, 2000). In 2002, a lemur from the New Iberia Louisiana Zoo was found with a CNS infection, and infection was found in a wood rat and possums from Baton Rouge, Louisiana (Kim *et al*, 2002). A second human infection was reported from Louisiana in a man who ate two raw legs from a green tree frog on a dare (Cuneo *et al*, 2006). Further evidence of the spread of *A. cantonensis* in the United States was a report in a white-handed gibbon in the Miami Metrozoo in Miami (Duffy *et al*, 2004). An epidemic of eosinophilic meningitis caused by *A. cantonensis* was reported in 12 medical students who ate a common meal of Caesar salad in Jamaica (Slom *et al*, 2002). Symptoms were experienced few days after returning to the United States and serological diagnoses made. There have been reports of finding the parasite in *Rattus* spp and snails in Jamaica, and a fatal case was documented in a young male with worms found in the brain and lung (Lindo *et al*, 2002).

COMMENTS

Angiostrongylus cantonensis and eosinophilic meningitis, due to infection with the nematode, have now been reported globally. The dissemination at one time was believed to be by the spread of the snail, *Achatina fulica*. Indeed this intermediate host may have been involved with the early spread in Asia; however, the spread of the parasite to other places probably has been due to the movement of rats. Rats easily gain access to ships and it is likely that infected rats "jump ship" at various ports. Many species of snails are susceptible to infection and rats passing larvae in the feces can easily be picked up by local snails and slugs, thus is establishing the parasite (Kliks and Palumbo, 1992). Cargo being shipped from endemic areas may harbor rats and molluscs, and drug trafficking may have been involved. Without question, the parasite will spread and propagate, especially in the warmer parts of the world. The southern United States should be concerned. The intermediate, definitive, and paratenic hosts are available and are susceptible to infection. Two human infections have been reported from Louisiana and Public Health authorities should be made aware that angiostrongyliasis might become a problem.

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STUDY OF EOSINOPHILIC MENINGITIS IN HO CHI MINH CITY, VIETNAM

Le Thi Xuan, Pham Thi Hai Men and Pham Thi Le Hoa

Faculty of Medicine, University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam

Abstract. This hospital-based study aimed to describe the predominant clinical manifestations and epidemiologic details of cases with eosinophilic meningitis at the Hospital for Tropical Diseases in HCMC during January 2002 to January 2006. Of the 56 cases collected during this time, 49 (87.5%) were positive for IgG of *Angiostrongylus cantonensis*. The majority of cases occurred in the 15-50 age group, which accounting for 73% of the total cases. Males were affected three times more frequently than females. The occupation distribution showed that 34% were farmers and 36% were laborers. Patients came from different provinces in the south of Vietnam, including 54% from Mekong Delta. Those who had previously eaten raw snails accounted for 46% of cases. The incidence was notably high (75%) from September to March. The incubation period ranged from 1-43 days, with an average of 17 days. The predominant clinical manifestations were headache (91%) and fever (89%). Meningeal signs were detected in only 44% of cases. Cranial nerve palsies were detected in 45% of cases. Neurological disorders were found more often in cases of late admission. Eosinophilia was observed in 92% of cases. Parasitic meningitis, especially *Angiostrongylus* meningitis, is emerging in Vietnam. Early diagnosis was difficult due to the lack of meningeal signs. Immunoblot and IgG subclass antibodies should be used for screening that is more sensitive and more specific to confirm human cases.

INTRODUCTION

Since *Angiostrongylus cantonensis* was initially discovered by Chen from the pulmonary artery of the brown rats in Guangzhou in 1935, many cases of *Angiostrongylus* meningitis have been reported in the world (Liang, 1986). Other pathogens, such as *Strongyloides*, *Cysticercosis*, and *Toxocara*, sometimes caused meningitis. *Gnathostoma spinegerum* has also been reported, mainly in Southeast Asia, related to raw food. In Vietnam, eosinophilic meningitis was first reported in humans before the 1970s.

During the recent years, the number of cases of eosinophilic meningitis has increased in the Hospital for Tropical Diseases, Ho Chi Minh City. They often had been transferred from a community or local medical center. In the cases of late diagnosis, serious complications and some neurological sequella were experienced for several years afterwards. The difficulty in diagnosing these cases was due to the unremarkable clinical symptoms, the lack of epidemiological awareness, and the unavailability of serological tests. This study aimed to define the epidemiological factors and risk habits related to exposure, the main clinical signs, and the serological patterns of cases with eosinophilic meningitis in the south of Vietnam.

Correspondence: Dr Le Thi Xuan, Department of Parasitology, Faculty of Medicine, University of Medicine and Pharmacy HCMC, 217 Hong Bang Street, 5th District, Ho Chi Minh City, Vietnam.
Tel: 84-8-8558411; Fax: 84-8-8552304
E-mail: lexuan02@hcm.vnn.vn

MATERIALS AND METHODS

Fifty-six in-patient cases were admitted from January 2002 to January 2006 with fever, headache, eosinophils in CSF >10% white cell count (or CSF eosinophils >10 cell/mm3), and a positive ELISA test for one of the following pathogen: *A. cantonensis, G. spinigerum, T. canis* or *C. cellulosae*. Among them, 42 were men, age range from 2-71 years (average age was 29.4 ± 16.2). For every case, information about exposure or risk behavior, the incubation time, epidemiologic details, and clinical symptoms and signs were recorded. Serologic test were done to detect antibody to 4 parasites as above at the Hospital for Tropical Diseases (*G. spinigerum, T. canis*, or *C. cellulosae*) and at the School of Medicine, Ho Chi Minh City (*A. cantonensis*). An MRI was performed in cases with local neurological signs.

RESULTS

Epidemiological factors

Among the 56 cases, 74% were farmers or laborers. The ratio between male and female gender was 3/1. Seventy-three percent of the cases ranged in age from 15-50 years; especially among this age group, the male/female ratio was about 5/1 (34/7). Forty-six percent of cases (26 cases) had previously eaten raw snails, and 17% cases (9 cases) had used raw fish. However, among the cases from An Giang, 84% (16/19) had histories of eating raw snails. The incubation period ranged from 1-43 days (17 ± 12.5 days).

The number of cases increased sharply after September, reaching a peak in November, and decreasing dramatically thereafter until January, right before the end of annual rainy seasons and flooding time in the Mekong Delta river region. Seventy-five percent of the cases were detected during September to March.

Clinical presentations

Mild to severe headache and mild fever were the most commonly symptoms (91% and 89%). Nausea or vomiting was founded in 54% of cases. Meningeal (Kernig's or Brudzinski's) signs were seen only in 46%. In cases with late admission, there were focal neurologic signs (45%): with Bells' palsy (16%), and with diplopia or eyeball abduction disorder (29%). Paraplegia or quadriplegia was seen in 7% of cases and hemiplegia in 5%. Local or general paresthesia (scorching or burning sensation) and/or local muscle pain was reported in 13% and 14% of cases, respectively. Macular or papular rashes made fugitive appearances in 9% of cases. Abnormal neurological presentations, with focal lesion seen in cerebral or spinal cord (13/34 cases, 38%), were seen in cases of late admission.

Fig 1- Locations of study sites.

Laboratory test

Ninety-three percent of cases had eosinophilia and 64% of cases had moderate to high eosinophil counts (> 1,500 cell/mm3) in their blood. The CSF showed mild increases of protein and eosinophilia, and mildly increased CSF pressure. The IgG antibody to *A. cantonensis* was detected in 49/56 cases (87.5%). The IgG antibody to *G. spinigerum* was detected in 14 cases (25%). Of these latter cases, 11/14 tested positive for the IgG antibody for both *Angiostrongylus* and *Gnathostoma*. The test for the IgG antibody to *A. cantonensis* and *G. spinigerum* in the cerebrospinal fluid were done in 27 cases; of which, 16/27 cases (60%) tested positive for *A. cantonensis* and 5/27 cases (18%) for *G. spinigerum*.

DISCUSSION

Parasitic meningitis is more common in tropical areas, such as the Pacific and Southeast Asia regions. Some pathogens cause meningitis during their migration inside their accidental hosts, such as *Gnathostoma* and *Toxocara*. Cysticercosis more often causes intracerebral-specific lesions with special changes in magnetic resonance. *A. cantonensis* was the leading causes of meningitis worldwide and in Southeast Asia. It is necessary for *Angiostrongylus* to stay in the brain before subsequently migrating to the lung and completing their next cycle in other hosts. Eggs from infected rats infected snails

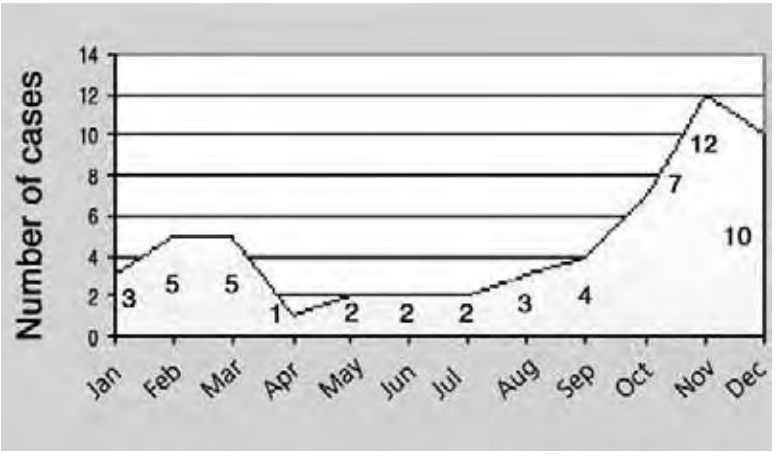

Fig 2- Annual trend of cases.

and other mollusk intermediate hosts. Their larvae are transmitted to humans as accidental hosts to continue their life cycle. *A. cantonensis* causes meningitis with a predominance of eosinophils, tortuous tracts of various sizes in the brain and spinal cord surrounded by an inflammatory reaction and degenerating neuron, and granulomatous response to the dead parasite and the non-specific vascular reactions including, thrombosis, rupture of vessel, arteritis, and aneurysm formation (Nye *et al*, 1970). In our hospital, the disease was sporadic during the time of study, but increased precipitously in 2005.

In this study, most of the cases were among the 15-50 age group (73%), male (70%), and did manual work (74%). Half of them had eaten raw snails, and 21% had drunk water from rivers. This subpopulation was likely to have leisure time sharing suppers with raw or improperly cooked snails and drink (Châu *et al*, 2003). The cultural background and eating habits have been known as causative for the disease among different populations and geographic areas (Sato and Otsuru, 1983).

The number of case was especially high in An Giang and Dong Thap, along the Mekong Delta River and on the Cambodia border, but not in other provinces downstream of the Mekong River. We did not have any investigation to determine whether or not there was an existing endemic of disease in rats or other animals in these two provinces.

An outbreak, reported in Wenchou City, China, also appeared during October to November in 1997 that was related to *Angiostrongylus* and *Ampullaris gigas* snails (Xue *et al*, 2000). Another study on *Gnathostoma* in Ho Chi Minh City reported an increased rate and level of infection in eels during the rainy season (Xuan and Rojkittigul, 2000). In this study, the number of cases increased from September, one month after the rains. It is likely that rain and flood changed the environment suitably for snails and fish. These environmental factors enabled the opportunity for humans to be exposure and infected, thus explaining the increased cases in man in rainy season.

Meningeal signs were only found in half of the cases, so that eosinophilia and headache were the only modest clues for investigating the cerebrospinal fluid. The IgG antibody to *A. cantonensis* was founded in 87.5% of cases, compared with *G. spinigerum* (25% of cases). The IgG antibody to *A. cantonensis* in the CSF was also predominant, as well as predominant in the blood, suggesting that *Angiostrongylus* was the main pathogen in this study.

in conclusion, parasitic meningitis, especially *Angiostrongylus* meningitis, is emerging in Vietnam. Early diagnosis should be undertaken, based on the presence of headache, epidemiological details (raw snail consuming, local cases), and eosinophilia. The infection statuses in animals need to be investigated further

in the future. Immunoblot and IgG subclass antibodies to parasite in blood and cerebrospinal fluid should be used for more sensitive screening and more specific confirmation in human cases.

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PREVALENCE OF *FASCIOLA* SPP INFECTIONS OF SHEEP IN THE MIDDLE AWASH RIVER BASIN, ETHIOPIA

EF Ahmed[1], K Markvichitr[1], S Tumwasorn[1], S Koonawootrittriron[1], A Choothesa[2] and S Jittapalapong[3]

[1]Department of Animal Science, Faculty of Agriculture; [2]Department of Physiology, Faculty of Veterinary Medicine; [3]Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand

Abstract. Ovine fascioliasis plays an important role of the major constraints to small ruminant production in Ethiopia. The objectives of this study were to assess the prevalence of *Fasciola* spp infections of sheep in Middle Awash River Basin, and to compare *Fasciola* spp. The fecal samples (3,697)) were collected and tested using the ethyl-acetate centrifugation technique to identify eggs of *Fasciola* spp. The overall prevalence of fascioliasis was 13.2%. The results demonstrated that *Fasciola* spp infection was higher among Afar (13.5%) than blackhead breed (9.1%). Infection rates for the different age groups were found to vary significantly ($p<0.001$). Infection rates for sheep with poor body condition (19.5%) were higher than sheep with good body condition (2.8%). With regard to the seasonal factors, the highest infection rate was observed during the cool season (6.9%), and lowest infection rate was recorded during the main rainy season (0.8%). To control the disease in this area, appropriate preventive control strategies have to be designed to reduce the impact of the disease on sheep production.

INTRODUCTION

Production of sheep for meat, milk, wool, hair, skin, and manure is an attractive agricultural enterprise for Ethiopian farmers because of the relatively low cost of breeding stock, the high productive rate of sheep, and the source of cash income. Sheep require minimal inputs and maintenance costs to live in various conditions, from desert to humid rainforest (Gatenby, 1991). In Ethiopia, sheep are the dominant livestock, providing up to 63% of cash income and 23% of the food subsistence value obtained from livestock production (Zelalem and Fletcher, 1993). The sheep population of the country is estimated to be 25.5 million (Central Statistical Authority, 2004). Despite the large size of the sheep population, the productivity per animal and the contribution of this sub-sector to the national economy is relatively low. Endo-parasitic infections, malnutrition, and management problems are known to be the main factors that affect productivity. The various species of gastro-intestinal and pulmonary nematodes, trematodes, and cestodes are known to be prevalent in Ethiopia (Bahiru and Ephraim, 1979; Bekele *et al*, 1981, 1982; Brook *et al*, 1985). As previously reported (Bergeon, 1968; Scott and Goll, 1977; Yilma, 1985; Yadeta, 1994), fascioliasis is one of the major parasitic disease that causes immense economic losses in livestock productivity.

Fascioliasis is caused by *Fasciola*, commonly referred to as liver flukes. Fascioliasis is a widespread parasitic disease of sheep, cattle, and occasionally humans. *Fasciola hepatica* and *F. gigantica* were commonly implicated. While *F. hepatica* has a worldwide distribution, but predominates in the temperate zones and cool areas of high altitude in the tropics and sub-tropics (Troncy, 1989). *F. gigantica* is mostly located in tropical areas (Urquhart *et al*, 1994). The geographical distribution of *F. hepatica* and *F. gigantica* is determined mainly by the distribution patterns of the snails that have a role as intermediate hosts (Pantalouris, 1965; Soulsby, 1982; Hall, 1986). In Ethiopia, both species co-exist at different altitudes (Graber, 1975).

The prevalence and distribution of fascioliasis varies from 11% in the Rift Valley to100% in the central highlands of Ethiopia (Erich, 1983).

Correspondence: Dr Sathaporn Jittapalapong, Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok10903, Thailand.
Tel/Fax: 662-942-8438
E-mail: fvetspj@hotmail.com or fvetspj@yahoo.com

Fascioliasis was widespread, particularly in the north and the west of the Great Rift Valley, which divides the country into two parts of unequal size (Malone and Yilma, 1999).

In Ethiopia, the annual losses due to ovine fascioliasis were estimated at 48.4 million Ethiopian Birr (1 US$ = 2.07 ETB) per year, of which 46.5, 48.8, and 4.7% were due to mortality, productivity (weight loss and reproductive wastage), and liver condemnation at slaughter, respectively (Ngategize *et al*, 1993).

In Afar National Regional State, especially in Awash River basin, fascioliasis is the most important disease of sheep production. The main reason is that the Awash River and its tributaries create a favorable environment for the growth and multiplication of snails as intermediate hosts by providing moisture, from flooding during rainy season and from the irrigation schemes during the dry season. Unfortunately, the data regarding the prevalence and distribution of fascioliasis in sheep and other ruminant species are fragmented or not well documented. Therefore, seasonal coprological studies were designed to investigate the prevalence of *Fasciola* spp infections in this area and to associate the infection with breed, sex, and age.

MATERIALS AND METHODS

Description of the studied areas

Investigation of *Fasciola* spp infections was carried out from January to December 2005 in three districts (Gewane, Bure Mudaytu, and Amibera) of Middle Awash River Basin in Afar National Regional Sate. The sheep population was estimated at 24,308, 22,823, and 44,290 in Amibera, Gewane, and Bure Mudaytu Districts, respectively (Afar Forestry Action Program, 1998). The Middle Awash River Basin is located between latitude 8° to 10° N and longitude 38° to 42° E; and situated at lower altitudes, ranging from 850 m above sea level in the Melka Werer area to 550 m in the Gewane area. The areas are 270 to 365 km from Addis Ababa, the capital of Ethiopia. Gewane, Bure Mudaytu, and Amibera were selected because they are wet and low lying along the irrigated and flooding areas of Awash, an ecology which has the potential for transmission of ovine fascioliasis.

On the flood paths and near the Awash River, the area is covered with the acacia trees (*Prosopis juliflora*), while the rest of the area is covered with shrubs, bushes, or grass. After the introduction of irrigated agriculture in this area, it modified the ecology. Currently, these areas are cultivated for cotton, banana, and oil seeds. The majority of the population search for better grazing and watering sites for their livestock. The climate of the area is normally hot and dry. The rain falls between July and September, with a brief rainy period during March and April. The mean total annual rainfall of the areas ranges from 663.7 to 687.8 mm. The mean minimum and maximum temperature ranges between 33.2˚C to 42.8˚C, and 19.6˚C to 26.7˚C, respectively; the average humidity is around 50.4% to 52%.

Sample collection

A total of 3,697 fecal samples were collected from nine selected sites of the three districts: 3,465 from Afar and 232 from Blackhead local sheep breeds, respectively; 2,845 samples were taken from females and 852 were taken from males. Age groups were classified as adult, young, and lamb.

Using a plastic glove, five grams of fresh fecal matter were collected from the rectum or during defecation. The samples were stored in plastic bags, well labeled, and placed in icebox until used. Coproscopic examinations were performed to detect *Fasciola* eggs using the standard sedimentation technique. The age of the animals was recorded by interviewing stockowners and using dental formula (Gatenby, 1991) for analysis. The study sites were randomly selected, the distribution of samples was the proportion of one herd per one study site, and all sheep in the herd were sampled. Samples were collected on a seasonal basis.

The seasons are traditionally classified as *kerma* (July-September), which is the long rainy season; *sugum* (March-April), which is the short rainy season; *hagai* (May-June) which is the hot-dry spell; and *gilal* (October-February) which is the cool season. *Gilal* is sometimes interrupted by rains in January and February and known as *dedda*.

Scoring of the body condition of animals was conducted during sample collection, according to the method described by Thompson and Meyer (1994).

Data analysis

Data were analyzed with a nested design using the chi-square test to analyze the relationships between sex, age, breed, and body condition. Infection rates, based on age and the seasonal variations in the prevalence of fascioliasis, were analyzed by the Pearson's correlation coefficient (Putt *et al*, 1988).

RESULTS

Of the total, 489 (13.2%) were positive for *Fasciola* spp parasites (Table 1). Several factors were associated with a higher prevalence of liver fluke infection. Geographically, the *Fasciola* spp prevalence in the three districts ranged from 11.3 % in Bure Mudatyu to 17.4% in Amibera

Table 1
Factors associated with *Fasciola* spp infections of sheep in Middle Awash River Basin of Afar Regional State, Ethiopia.

Factors	Category	No. examined	No. positive (%)
District	Gewane	1,712	223 (13.0)[a]
	Bure Mudaytu	1,296	146 (11.3)[a]
	Amibera	689	120 (17.4)[b]
Sites	Gewane:		
	Egele	721	95 (13.2)[a]
	Gebaya Bora	482	66 (13.7)[a]
	Galela Dora	509	62 (12.2)[a]
	Bure Mudaytu:		
	Debel	659	86 (13.1)[a]
	Beadafore	411	41 (9.9)[b]
	Gelalu	226	19 (8.4)[b]
	Amibera:		
	Aleysumalie	297	50 (16.8)[a]
	Ambash	174	29 (16.7)[a]
	Awash Sheleko	218	41 (18.8)[a]
Breed	Afar	3,465	468 (13.5)[a]
	Blackhead	232	21 (9.1)[a]
Sex	Male	852	114 (13.4)[a]
	Female	2,845	375 (13.2)[a]
Age	< 1 year	444	36 (8.1)[a]
	1-2 year	819	89 (10.9)[a]
	> 2 year	2,434	364 (14.9)[b]
Season	Cool dry	951	257 (6.9)[a]
	Short rainy	825	153 (4.1)[b]
	Hot dry	933	49 (1.3)[c]
	Main rainy	988	30 (0.8)[c]
Body condition	Poor	2,306	450 (19.5)[a]
	Good	1,391	39 (2.8)[b]
Total		3,697	489 (13.2)

Different superscripts within subgroup represent statistical significance of different prevalence ($p < 0.05$).

($p < 0.001$). The high incidence of *Fasciola* spp infections was found at Awash Sheleko (18.8%) of Amibera District, Debel (13.1 %) of Bure Mudatyu District, and Gebaya Bora (13.7%) of Gewane District.

There was an insignificantly higher prevalence of *Fasciola* spp infections among the Afar breed (13.5%) as compared with the Blackhead (9.1%) breed ($p > 0.05$). The prevalence of *Fasciola* spp among male sheep (13.4 %) was higher than that among females (13.2 %), but this difference was not statistically significant. A higher *Fasciola* spp infection prevalence ($p < 0.001$) was found among sheep of >2 years (14.9 %) when compared with sheep of 1-2 years (10.9 %) or < 1 year old (8.1 %).

Higher seasonal prevalence of ovine fascioliasis in the study areas was found during cool season (6.9 %), followed by the short rainy period (4.1%). During hot season, most animals were returned from the wet grazing areas to the farms nearby the Awash River. Animals were possibly infected at this time. From November, the prevalence of the disease increased and clinical signs were observed. During rainy season, animals moved from place to place in search of grazing areas. Because of this, the prevalence was relatively low.

The prevalence of ovine fascioliasis in animals of different body conditions was indicated that animals with a poor body condition were more highly infected than animals with a good body condition ($p < 0.001$).

DISCUSSION

Comprehensive knowledge of parasite ecology is crucial to sustainable control because parasites interact differently with hosts in specific climatic, managerial, and production environments (Almeria and Uriate, 1999; Waller, 1999; Papadopoulos *et al*, 2003). Our data indicated that the exposure of domestic sheep to *Fasciola* spp infections in Middle Awash River Basin in Afar National Regional Sate was common, with an overall prevalence of 13.2%. The results concurred with Graber (1975), who noted that fascioliasis was rare in the Rift Valley areas, Ethiopia. In contrast to our findings, Michael *et al* (2005) have reported prevalence rates of 56.3% for ovine fascioliasis in the Upper Awash River Basin. This difference may be due to different agro-ecological conditions, traditional pasture management practices, the pattern of movement of the animals from grazing near water logged areas, and agricultural irrigation practices during rainy season.

The variation in prevalence between the different locations was also likely due to the differences in landscape, such as swampy areas, and agricultural irrigation practices. During the rainy season, the amount of the rainfall flooding Awash River created a favorable condition, which favored the development of the intermediate host (snail) and the transmission of the diseases. Irrigation based on agricultural practice and the swampy areas were important ecologies for the continuity of the lifecycle of fascioliasis. Similar findings were previously reported (Graber, 1975; Urquhart *et al*, 1994; Michael *et al*, 2005; Solomon, 2005).

Climate conditions, particularly rainfall, were frequently associated with differences in the prevalence of *Fasciola* spp infection because this was suitable for intermediate hosts like snails to reproduce and to survive longer under moist conditions. The Middle Awash River Basin has a rainy season for five months, which facilitates parasitic survival in such an environment. Moreover, the flooding areas were found to have a significant influence on the risk of *Fasciola* spp infection since this enhanced a predisposing factor for many snails to complete their life cycles.

The difference in *Fasciola* spp infection between sheep breeds was previously reported (Pralomkarn *et al,* 1997). In Ethiopia, sheep are usually reared under non-intensive conditions, whereby animals may be brought out to graze and wander freely.

Solomon (2005) has suggested that fascioliasis equally affect both sexes. In this study, a higher prevalence of parasitic infection was not associated with sex ($p > 0.05$). However, although not statistically significant, males actually had a higher infection prevalence than females. This might be because all the animals were also grazing similar pastureland.

Although climatic conditions are consistent throughout the Middle Awash River Basin area, the prevalence of *Fasciola* spp in sheep varied slightly, from 11.3% in Bure Mudaytu District to 17.4% in Amibera district. Thus, these geographical differences in prevalence might be due to agricultural irrigation practices and the time spent by animals grazing near the Awash River. The intensity of infection is reportedly related to the availability of the intermediate hosts; thus, better snail control and separate grazing for different age groups would likely reduce the infection rate and the prevalence of fascioliasis among sheep in Ethiopia.

Our study indicated that sheep in Middle Awash River Basin were usually infected with *Fasciola* spp parasites. Economic evaluations consistently show that major losses due to parasitism affected animal production rather than mortality; and in Middle Awash River Basin, parasitism could influence the productivity, morbidity, and mortality of these animals (Githigia *et al*, 2001). Parasite-nutrient interactions were probably exacerbated by the effects of poor nutrition and management practices, which lead to decreased efficiency in feed utilization.

Young animals had a lower prevalence of *Fasciola* spp infections in this study. This finding was consistent with other reports, and it was not surprising because naive kids have maternal immunity. Higher infection rates were found in adults in other age groups ($p < 0.05$). Based on this finding, it can be suggested that the higher exposure risk of adults may be due to physiological differences, such as stress, pregnancy, lambing, inadequate nutrition, and infectious diseases. Similar results were reported by Ayalew (1994).

Hunter (1953) observed that a well-fed animal was not in trouble with worms, and usually a poor diet resulted in more helminth infections. Furthermore, helminthes also led to a loss of appetite and poor utilization of food, which results in a loss of body weight. Hawkins and Morris (1978) demonstrated that weekly growth rates of wool and live weight decreased with increasing fluke burdens in sheep.

In conclusion, the *Fasciola* spp infected sheep examined in this study harbored parasites and acted as reservoirs for transmission. Observed differences in the prevalence of parasitic infections between districts were probably due to differences in management systems. A strategic control program must be launched to prevent the increase of parasites in the environment and to avoid heavy contamination of the pasture by fecal eggs. Traditional pasture management (*eg*, restriction of animals during rainy season around Awash River) should be encouraged. Awareness must be created among stockowners regarding fascioliasis.

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THE MITOGENIC EFFECT OF *OPISTHORCHIS VIVERRINI* EXCRETORY/ SECRETORY PRODUCT AND ITS ACTIVATED SIGNAL TRANSDUCTION PATHWAYS

Chanitra Thuwajit, Kusumawadee Utispan, Chanisa Chuaysri and Peti Thuwajit

Department of Biochemistry, Faculty of Medicine, Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen, Thailand

Abstract. Our group has studied the responses of fibroblast cell line, NIH-3T3, to *O. viverrini* ES product using a non-contact co-culture. The results indicated a marked increase in cell proliferation with either absence or presence of serum in the media compared with the co-culture without parasites. The ES product increased cell proliferation by stimulating the expression of phosphorylated retinoblastoma (pRB) and cyclin D1, the key proteins in driving cells through the G1/S transition point of the cell cycle. In addition, the whole gene expression data from cDNA array indicated 239 genes with 2-fold and more up-regulated by *O. viverrini* ES product compared to those in cells without exposure to the parasitic product. This finding may clarify, in general, how this parasitic product affects human epithelium during cholangiocarcinogenesis. The understanding of the exact signal transduction pathways activated by *O. viverrini* ES product particularly in hyperproliferative fibroblasts will provide a novel target for chemoprevention and treatment of fibrosis in this cancer.

INTRODUCTION

Opisthorchis viverrini infection or opisthorchiasis remains a major public health problem in Thailand, especially in Southeast Asia, including northeastern Thailand, Lao PDR, Vietnam, and southern China (Sithithaworn and Haswelll-Elkins, 2003). The highest incidence is in the northeastern part of Thailand, with the rate of 188 per 100,000 (Sriamporn *et al*, 2004). The morbidity of *O. viverrini* infection does not directly relate to the parasite itself but relates to the carcinogenic capability of the parasites. The International Agency for Research on Cancer (IARC) has accepted that *O. viverrini* is a risk factor for bile duct cancer or cholangiocarcinoma (IARC, 1994). The mechanism by which this parasite causes the normal bile duct epithelium to transform into cancer cells involves several mechanisms (Watanapa and Watanapa, 2002). Three main irritations, including mechanical, immunological and biochemical, caused by the parasites have been proposed and demonstrated by many investigations. The mechanical process activated by *O. viverrini* sucker causes the epithelial cells to be expelled and then induces hyperproliferative cells. This effect has been demonstrated both in animal and human models (Sripa, 2003). For the immunological process, a significant degree of humoral and cell mediated immune responses to the parasite can be detected both in patients and animal models (Wongratanacheewin *et al*, 2003). Although the pathology of parasites by their direct contact and indirect suppressed immune response have been clearly shown as described, the effect of the biochemical substance released from the parasites has not been demonstrated. This may be the limitation of the studies in animal models or even in *O. viverrini*-infected people; the problem of eliminating direct contact and isolating the immune responses. We have designed an *in vitro* model to demonstrate the effect of *O. viverrini* excretory/secretory (ES) product on cells. The mechanisms by which the parasites initiate activation of cell proliferation and the activated intracellular signal transduction pathways have been proposed in this review.

Correspondence: Chanitra Thuwajit, Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel/Fax: 66 (043) 348386
E-mail: tchani@kku.ac.th, cthuwajit@yahoo.com

MITOGENIC EFFECT OF *O. VIVERRINI* ES PRODUCT

The non-contact co-culture technique using double-chamber culture plates was used to culture

cells and adult parasites together under the same conditions but with no direct contact. The double-chamber culture plate is composed of lower and upper chambers in the same cavity. The cells that we used were the mouse fibroblast cell line, NIH-3T3. We used these plates in the lower chamber, as a model, because of their sensitivity to the external stimuli. The 1.5-2 month-old parasites were then added in the upper chamber of the culture plate. The porous membrane of the upper chamber allows only the *O. viverrini* ES product to pass through the membrane and act on the cells in the lower chamber (Fig 1). After a two-day co-culture, an increased cell proliferation was observed in the co-culture treatment with parasites when compared to the control without parasites (Thuwajit *et al*, 2004) (Fig 2). This effect could be observed in media with either the presence or absence of a serum supplement.

The significant increase of viable NIH-3T3 cell numbers when co-cultured with viable parasites in serum-free medium indicated that ES product from *O. viverrini* might act as the growth factor, similar to supplementation with calf serum, which can induce the NIH-3T3 cell to proliferate. However, the efficacy of the ES product was not equal to serum (Fig 2). An explanation might be that, first, the concentration of growth stimulant in ES product is lower than that in serum. Secondly, the pathways that ES product used to control cell proliferation are different from those used by serum. The numbers of dead cells, including apoptotic cells, were measured, and the results showed no differences of cell death in co-culture

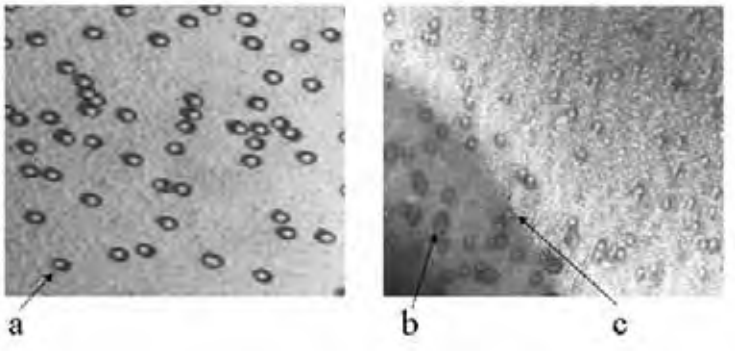

Fig 1- The porous membrane of the double-chamber culture plate comparing the pores sizes (a), *O. viverrini* eggs (b) and a part of an adult parasite (c).

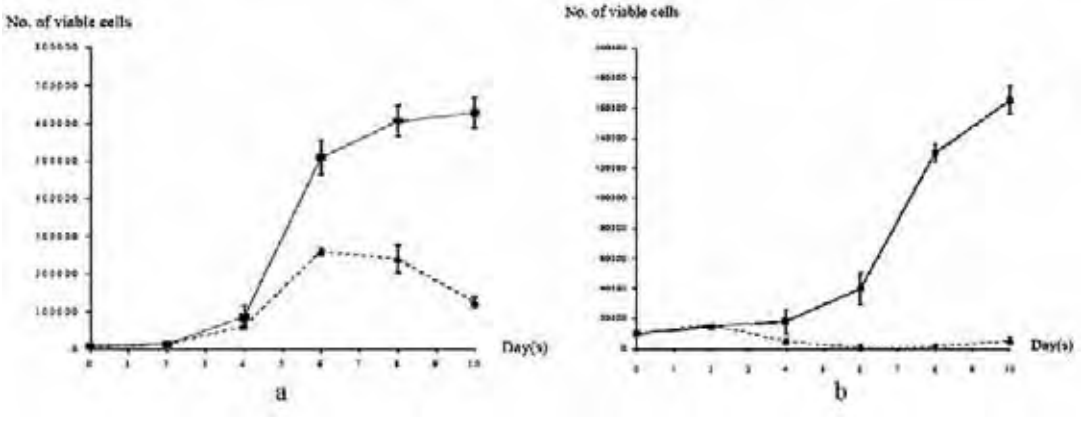

Fig 2- Growth curves of cells treated with and without adult *O. viverrini*, either cultured in media containing serum (a) or without serum (b).

compared to the non-co-culture ones (Thuwajit *et al*, unpublished data). An increased expression of cyclin D1 and phosphorylated Rb was observed in cells of both serum and ES product treatments. The similar expression profiles of pRB and cyclin D1 were previously shown in human fibroblasts treated with serum (Thuwajit *et al*, 2004). In conclusion, ES product from *O. viverrini* seems to act as growth stimulant(s) that can induce fibroblast cell proliferation. The increase in cell proliferation can be explained by the capability of ES product to stimulate the entrance of cells through G1/S transition point into cell cycle by over-expression of pRB and cyclin D1.

Many studies have demonstrated the effects of substances released from several parasites. For *Fasciola hepatica*, cathepsin L has been reported for its capacity to inhibit lymphocyte cell proliferation (Prowse *et al*, 2002). This facilitates the parasite to mask the immune response and help parasites to stay in the body. Moreover, mucin was also detected in the secretory product of *F. hepatica*. This can help the parasite to move thorough the tissues (Barnal *et al*, 2004). The secreted product of *Acanthocheilonema viteae*, a filarial nematode, has been demonstrated to have an anti-inflammatory effect via the reduction of cytokine production from macrophages (Goodridge *et al*, 2001). The larval stage of *Taenia taeniaeformis* could also induce gastric mucosa cell proliferation in SCID mice leading to gastric mucosal hyperplasia (Lagapa *et al,* 2002). For *O. viverrini*, there have been a few reports on the ES product and its effect (Sripa 2003; Thuwajit *et al*, 2004). The reason why parasites release such mitogens is still unclear. We believe that, first, the intended effect of the parasites may be to induce host cell proliferation as their source of energy, nutrients, and so forth. Second, the mitogens may be synthesized aimlessly but are a part of the molecules that act like mitogens.

MORPHOLOGICAL CHANGE OF *O. VIVERRINI*-TREATED FIBROBLAST

In addition to the cell proliferation induction of *O. viverrini* ES product, this product can also induce changes in NIH-3T3 cell shape (Fig 3). These results indicated that the loss of cell-to-cell contact in the refractive shape, after exposure to ES product, could allow cells to proliferate continuously; whereas, those with normal flat fibroblastic shape did not. The refractive shape NIH-3T3 can be observed in both types of media, either with or without serum, used in the non-contact co-culture systems. This might indicate that the shape change phenomenon was induced by the ES product, not by either the presence or absence of serum. The same shape change of NIH-3T3 cells was previously presented as the result of *h-ras* over-expression that could induce collapse of the actin filament that resisted the antiproliferation signals mediated by cell-cell contact and led to the increased cellular proliferation (Ritter *et al*, 1997). Moreover, *raf*-1 could induce NIH-3T3 cell proliferation (Kerkhoff and Rapp, 1997). We performed real time RT-PCR of *h-ras* and *raf*-1 genes, and the result demonstrated an increased *h-ras* expression level but not significantly in cells treated with *O. viverrini* ES product, while *raf*-1 expression was decreased (Fig 4). Since their actions involve the capability to induce other proteins to be phosphorylated (Woods *et al*,

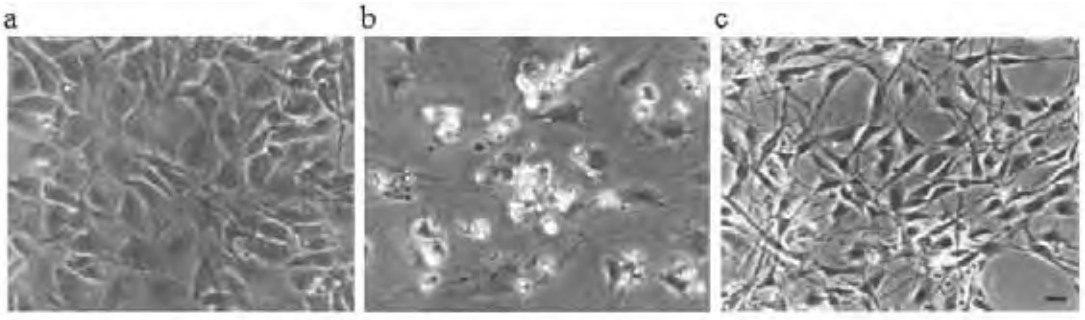

Fig 3- NIH-3T3 cells cultured in different conditions as detected by inverted microscopy. Cells in serum containing medium (a), serum-free medium (b) and co-cultured with parasites (c) (scale bar = 10 μm).

2001), these kinase enzymes may not be needed to increase the mRNA expression level. The kinase activity measurement will be of great interest to explore. The cascade of *h-ras* after growth factor stimulation is to send the signal to the downstream molecules, *raf*-1, and then activate either the MAPK-dependent pathway (Janulis *et al*, 2001) or MAPK-independent pathway (Laird *et al*, 1999). They may activate or increase expression levels in parasitic product induced fibroblasts.

EXPRESSION PROFILE IN *O. VIVERRINI* ES PRODUCT TREATED CELL

To understand the intracellular mechanism parasites used to activate cell proliferation, whole gene expression profiles were investigated using a mouse cDNA array (Thuwajit *et al*, 2006). A total of 15,000 genes and ESTs were tested. *O. viverrini* caused widespread alteration in gene expression. Most of the two-fold up-regulated expression genes have a cell proliferation-related function. They were categorized in groups as proteins that play roles in energy and metabolism, signal transduction, protein synthesis and translation, matrix and structural function, transcription control, cell cycle, and DNA replication. Only the signal transduction genes will be focused on in this review paper.

Growth factors or mitogens usually activate cells through signal transduction pathways. To know which intracellular signal transduction pathway *O. viverrini* utilized, the signal transduction genes were focused and categorized as corresponding to the stimulations. We selected the pathways stimulated by either PDGF or EGF, PDGF, EGF, and TGF-β. Regarding both the cDNA array and a validated real time RT-PCR, the most up-regulated expressions are *tgf*β 1*i*4 and *eps* 8, which are the representatives of TGF-β- and EGF-stimulated signal transduction pathways, respectively (Thuwajit *et al*, 2006). In addition, the receptors for PDGF and EGF are classified in different types as tyrosine kinase and serine/threonine kinase receptors. The cDNA array data represented the up-regulated expression level of these two types of receptors (increases around 1.59-4.42 fold). All the data support the possibility that TGF-β and/or EGF signal transduction pathways will be utilized by the parasites to activate cell proliferation.

ROLE OF THE TGF-β ASSOCIATED SIGNAL TRANSDUCTION PATHWAY IN *O. VIVERRINI* ES PRODUCT-INDUCED CELL PROLIFERATION

Though both TGF-β and EGF have been proposed as candidate signal transduction pathways activated by *O. viverrini* ES product, the highest up-regulation level was observed

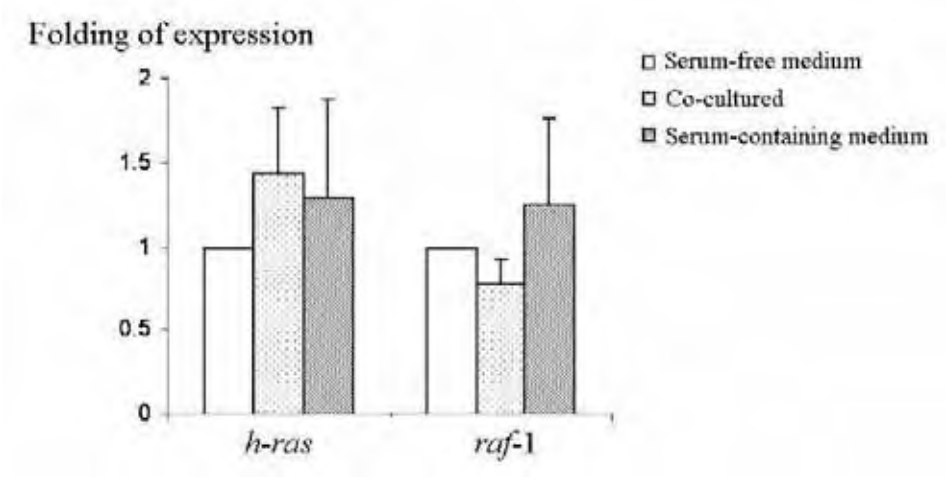

Fig 4- Expressions of *h-ras* and *raf*-1 genes in cell treated with *O. viverrini* compared to those in media with or without serum (using the level of gene expression in serum-free medium = 1).

in the pathway of TGF-β. Moreover, our study demonstrated a statistically significant increased Smad 4 mRNA in the same fashion as *tgf*β 1*i*4 (Fig 5). Because Smad-4 is a molecule in the TGF-β signal transduction pathway (Lutz and Knaus, 2002), it is possible that *O. viverrini* ES product-induced cell proliferation is through this pathway. It has been shown that TGF-β can induce fibroblast proliferation and is the major cause of hyperproliferative and hyperactive fibroblasts causing the fibrosis in many pathological conditions (Javelaud and Mauviel, 2004). TGF-β stimulates cell proliferation via the activation of either the Smad-dependent or the Smad-independent pathway according to cell types (Kaminska *et al*, 2005). For fibroblast cell proliferation, the Smad-dependent pathway has been proposed to play important role (Flanders, 2004). Given these above results and the published data, it may be of great interest to propose the importance of the TGF-β signal transduction pathway in *O. viverrini* induced cell proliferation. Through using Smad-dependent or -independent pathways of TGF-β, the most important upstream molecule in this signal transduction pathway is TGF-β receptor type II (TBRII) (Lutz and Knaus, 2002; Kaminska *et al*, 2005). In order to know whether the TGF-β signal transduction pathway plays a role in *O. viverrini* ES product induced cell proliferation, RNAi was used to knockdown the expression of TBRII on the cell membrane of fibroblasts before treatment with the parasitic product. The real time RT-PCR data demonstrated the successfulness of TBRII siRNA treatment with a statistically significantly decreased expression level of TBRII (Fig 6). Cell proliferation induced by *O. viverrini* ES product was decreased in TBRII siRNA treated cells compared to those with a normal expression level of TBRII (Fig 6). This result may confirm the possibility that fibroblasts are activated by *O. viverrini* ES product to increase cell proliferation using the TBRII-mediated signal pathway. Though the exact molecules existing in the parasitic product have yet to be identified, it has been proposed from this present finding that these pathway entities may be a component of the molecules acting like TGF-β. However, further experiments will be needed for a definitive conclusion.

ROLE OF ES PRODUCT ON HUMAN FIBROBLASTS

Using mouse fibroblasts as a model to study the effect of *O. viverrini* ES product has both advantages and disadvantages. The sensitive response of the NIH-3T3 mouse fibroblast cell line to external stimuli is the most important advantage. However, mouse fibroblast may respond to the same stimulus differently from human fibroblast cells. The human fibroblast cell line derived from normal colon was used to confirm the similarity of response between mouse and human cells. The human fibroblast cells were cultured with adult parasites in double-chamber culture plate. The

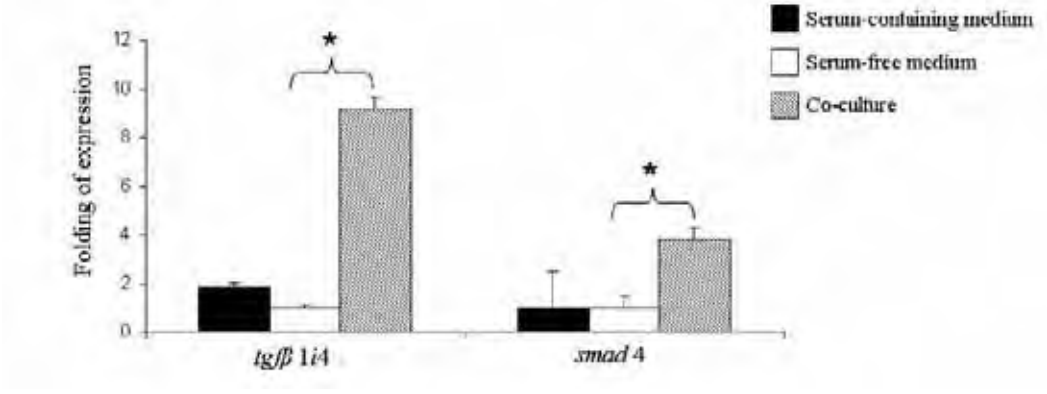

Fig 5- The expression levels of TGRb 1*i*4 and Smad 4 genes in cells exposed to *O. viverrini* ES product compared to those treated in serum-containing and serum-free media (* p-value <0.05) (using the level of gene expression in serum-free medium = 1).

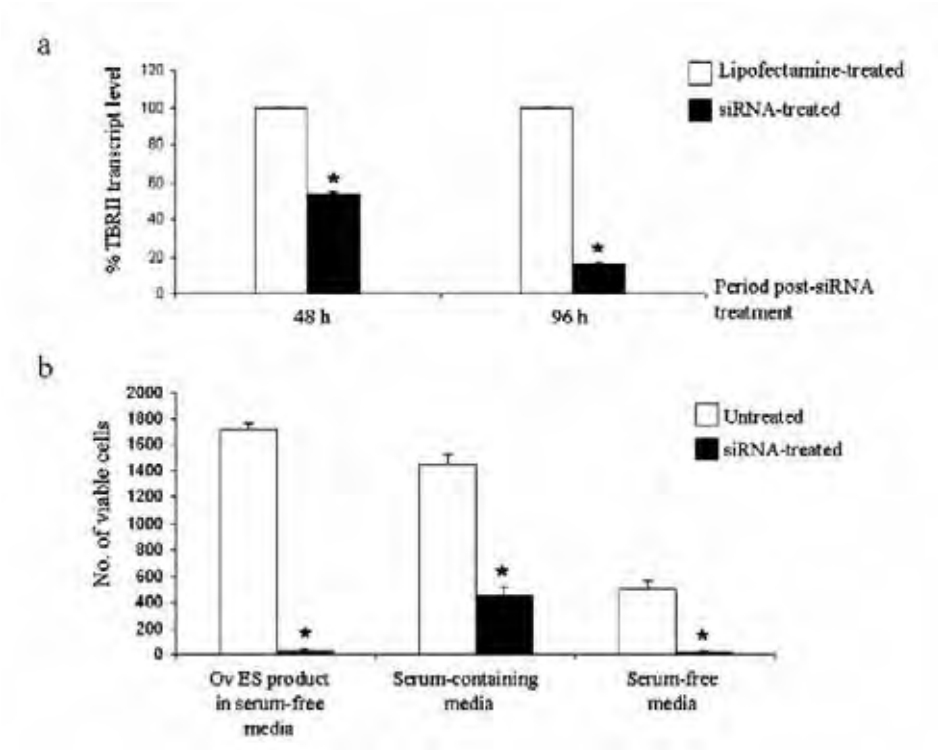

Fig 6- Real time RT-PCR of TBRII in fibroblast cells with and without treatment with TBRII siRNA (a) and the growth activated by *O. viverrini* ES product compared between cells with and without treatment with TBRII siRNA (b) (* p-value<0.05).

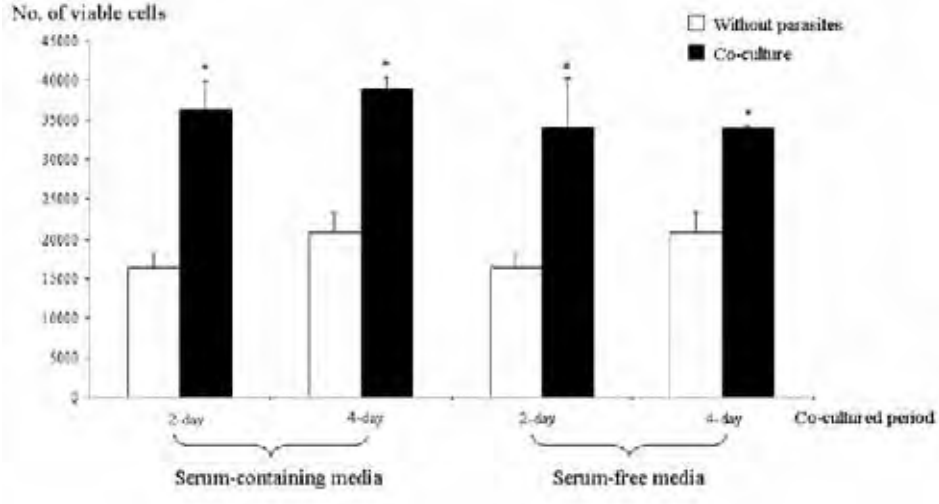

Fig 7- Cell proliferation effect of *O. viverrini* ES product on human fibroblast cell line (* p-value <0.05).

induction of human fibroblast cell proliferation was observed in both 2- and 4-day co-culture periods, with statistical significance (Fig 7). The cell proliferation induction was observed in conditions of both using media with and without serum. However, further experiments are needed to confirm these observations, it may imply that the *O. viverrini*-activated signal transduction

pathways detected in mouse fibroblasts can be similar or the same in human fibroblasts as well.

We have proposed the mechanism of *O. viverrini* ES product-induced fibroblast cell proliferation as an additive mechanism to a variety of demonstrated mechanisms, that is, mechanical and immunological processes induced by the parasites. *O. viverrini*-activated fibroblasts themselves are not enough to play only one important role in this process; however, the two-way interaction between the activated fibroblasts and the bile duct epithelium has been proposed and demonstrated in many kinds of carcinoma, including colon, prostrate, and breast (Olumi *et al*, 1999; Maffini *et al*, 2003; Nakagawa *et al*, 2004). The most abundant forms of fibroblasts in cancer tissues are called myofibroblasts (Kunz-Schughart and Knuechel, 2002). They are characterized by increased cell proliferation and collagen production. Our data indicated that *O. viverrini* ES product could induce cell proliferation and cDNA; microarray data also demonstrated incremental collagen production (Thuwajit *et al*, 2006).

CONCLUSION

We think that chronic infection by *O. viverrini* causes exposure of the bile duct epithelium and the surrounding fibroblasts to the parasitic product. For the epithelium, the increased cell proliferation causes vulnerable, incorrect DNA-containing cells to propagate and, if the damage occurs at oncogene sites or tumor suppressor genes, cancerous cells will result. For fibroblasts, *O. viverrini* ES product caused the hyperproliferative cells that may then secrete many substances, especially several growth factors. These mitogens activated fibroblasts themselves as the autocrine stimulus and acted on bile duct epithelium as the paracrine initiator. Taken all together, the proposed roles of activated-fibroblasts, that is, *O. viverrini* ES product-activated fibroblast during cholangiocarcinogenesis should be considered.

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EFFECT OF CURCUMIN ON THE INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) AND ANTIOXIDANT ENZYME EXPRESSION IN HAMSTERS INFECTED WITH *OPISTHORCHIS VIVERRINI*

Butsara Kaewsamut[1,3], Somchai Pinlaor[1,3], Thidarut Boonmars[1,3], Tuanchai Srisawangwong[1] and Puangrat Yongvanit[2,3]

[1]Department of Parasitology, [2]Department of Biochemistry, [3]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Abstract. Opisthorchiasis, caused by infection with the liver fluke *Opisthorchis viverrini*, is one of the major risk factors for cholangiocarcinoma (CCA). The imbalance between free radicals and antioxidant defense mechanisms causes oxidative and nitrative stress, leading to pathophysiological changes characteristic of the disease. A chemopreventive agent, such as curcumin, used to prevent inflammation may decrease the severity of the disease. The expression of inducible nitric oxide synthase (iNOS) and antioxidant enzymes were investigated in the livers of *O. viverrini*-infected hamsters with and without curcumin treatment using RT-PCR analysis. These results suggest that curcumin suppresses the expression of iNOS, whereas it enhances the expression of the antioxidant enzyme genes SOD1, CAT and GPx resulting in the inhibition of nitrative stress. Therefore, curcumin may be used as a chemopreventive agent to reduce the severity of opisthorchiasis as well as to prevent the development of CCA.

INTRODUCTION

Chronic inflammation is implicated in the pathogenesis and carcinogenesis of certain types of human cancers, including Opisthorchiasis viverrini-associated cholangiocarcinoma (CCA) (IARC, 1994; Ohshima and Bartsch, 1994; Coussens and Werb, 2002). Opisthorchiasis is caused by *O. viverrini* infection, the primary risk factor for cholangiocarcinoma (CCA) (IARC, 1994). The highest prevalence of *O. viverrini* infection and CCA in the world has been reported in northeast Thailand. Although praziquantel, an effective drug for opisthorchiasis, is widely used (Pungpak *et al*, 1998), the incidence of CCA is still a major public health problem in this region. This may be due to reinfection, which frequently occurs after praziquantel treatment in endemic areas because of the local diet of raw freshwater fish, the intermediate host (Saowakontha et al, 1993).

Intervention using a chemopreventive agent may reduce the severity of opisthorchiasis. At present, chemopreventive agents such as curcumin, a yellow pigment of the turmeric plant, has demonstrated properties involved in the suppression of tumor promotion through the inhibition of inflammation and antioxidant formation (Duvoix *et al*, 2005). Curcumin can also increase both bile secretion and production (Deters *et al*, 2000). This compound, a common spice in Thailand, may be used as an agent of choice for the reduction of pathogenesis characteristic of opisthorchiasis. Our experiment was designed to study the effect of curcumin on the pathogenesis of opisthorchiasis in an animal model that focused on the expression of inducible nitric oxide synthase (iNOS) and antioxidant enzymes-such as Cu/Zn-superoxide dismutase (SOD1), Mn-superoxide dismutase (SOD2), catalase (CAT) and glutathione peroxidase (GPx) using semi-quantitative RT-PCR analysis. The anticipated outcome of this research may provide insight into a new therapeutic approach for opisthorchiasis as well as a strategy for reducing the risk of developing CCA.

Correspondence: Somchai Pinlaor, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: +66-43-348-387; Fax: +66-43-202-475
E-mail: psomec@kku.ac.th

MATERIALS AND METHODS

Parasites

Opisthorchis viverrini metacercariae were isolated from naturally infected fish by pepsin digestion (Pinlaor *et al*, 2004). Briefly, cyprinoid fish were minced and digested in artificial pepsin-

HCl solution, sedimented several times in normal saline solution. Finally, the metacercariae of *O. viverrini* were collected under a dissecting microscope. Only viable cysts were used to infect the animals.

Curcumin was purchased from Sigma, and was mixed with the normal pellets to a concentration of 0.25% curcumin.

Animal and experimental design

Random bred male Syrian hamsters (obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University), 6-8 weeks of age and 90-150 g body weight, were used in the study. The hamsters were divided into four groups (5 animals/group): Group I: the normal hamster control group fed normal pellets; Group II: the normal hamster fed curcumin-supplemented pellets; Group III: the hamsters infected with 50 *O. viverrini* metacercariae and fed normal pellets; and, Group IV: the hamsters infected with 50 *O. viverrini* metacercariae and fed curcumin-supplemented pellets. All of the hamsters were kept at room temperature in the animal house and fed their designated diet with water given *ad libitum*.

On days 7, 14, 21, 30, 60, and 90, hamsters from Groups I and III were sacrificed, while hamsters in the curcumin-treated group were sacrificed on days 30 and 90. Samples of blood and liver (at the hilar region) were collected from each sacrificed animal and used for this study. The study was approved by the Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

Histopathological study

A histopathological study was performed using hematoxylin and eosin staining of paraffin sections (Sripa and Kaewkes, 2000). Briefly, the liver tissues from the hilar region were fixed in 10% buffered formalin overnight then processed conventionally. The sections were then cut in 6-μm slices and stained with hematoxylin and eosin.

RT-PCR for the expression of iNOS and antioxidant enzyme genes in the liver of *O. viverrini*-infected hamsters with and without curcumin treatment

Primer design for semi-quantitative RT-PCR and sequence analysis. The iNOS primer was designed from GenBank (Accession No. AJ863052), *Mesocricetus auratus* partial mRNA for iNOS, iNOS, sense CAG CTT GGA GTT CAC CCA GT, and iNOS antisense CCA CTC GTA TTT GGG ATG CT (169 bp). The gene-specific primers of antioxidant genes (SOD1, SOD2, CAT, and GPx) were designed based on the published sequence of *Mus musculus* and *Rattus norvegicus* mRNA.

The deduced nucleotide sequences were aligned using the Clusta W software (http://www.

Table 1
Sequences of primers used and expected size of amplified fragments.

Genes	GenBank Accession numbers	Sequence 5'→ 3' Upper line: forward primer Bottom line: reverse primer	PCR product size (bp)
SOD1	NM_017050.1	F5' CGGATGAAGAGAGGCATGTT 3' R5' CACCTTTGCCCAAGTCATCT 3'	165
SOD2	NM_017051.2	F5' CCGAGGAGAAGTACCACGAG 3' R5' GCTTGATAGCCTCCAGCAAC 3'	174
GPx	NM_030826.2	F5' GGTTCGAGCCCAACTTTACA 3' R5' CGGGGACCAAATGATGTACT 3'	152
CAT	NM_012520.1	F5' TTGACAGAGAGCGGATTCCT 3' R5' AGCTGAGCCTGACTCTCCAG 3'	179
MG3PDH	M32599	F5' GGCATTGTGGAAGGGCTCAT 3' R5' GACACATTGGGGGTAGGAACAC3'	218

ebi.ac.uk/clustaw/). Searches for homologies were done using the Fasta3 software (http://www.ebi.ac.uk/fasta3/). General "homology" searches were done using the Blast software from the NCBI home page (BLASTn, http://www.ncbi.nlm.nih.gov./BLAST/).

The expected sizes of the amplified fragments are presented in Table 1. In addition, the primer pairs for endogenous controls (glyceraldehyde-3-phosphate dehydrogenase, G3PDH) was designed based on the published sequence (Boonmars *et al*, 2005).

RNA preparation. Total RNA was isolated from each of the hamster livers using TRIZOL® reagent (Invitrogen), according to the manufacturer's instructions. Approximately 150 mg of hamster liver was dissected and immediately dipped into the reagent. The isolated RNA was further processed (Boonmars *et al*, 2005). Briefly, the total RNA was treated with DNase (5 units of RQ1 RNase-Free DNase, Promega), and 100 units of ribonuclease inhibitor (Recombinant RNase Inhibitor, Promega) in a buffer (containing, 400 mM Tris-HCl, 100 mM NaCl, 60 mM $MgCl_2$, and 20 mM dithiothreitol, pH 7.5).

Total RNA was then extracted with phenol/chloroform, precipitated with ethanol, and dissolved in RNase-free water (100 µl). The extracted RNA was reverse-transcribed into cDNA using Oligo (dT) 15 primers (Promega). The PCR reaction mixture comprised 2 µl of reverse transcription products (1:10 diluted), 2 µl of 10x PCR buffer, 2 µl of deoxynucleoside triphosphate (2.5 mM dNTP, each), 2 µl of primer pairs (10 pmol), 0.08 µl of *Ex Taq* polymerase (5 U/µl, Takara, Shuzo, Japan), and distilled water was added to a final volume of 20 µl. PCR analysis was performed using a Thermocycler (GeneAmp®, PCR system 9700), in which all the target genes remained in the exponential phase for 30-40 cycles.

PCR reactions were conducted: 94°C for 3 minutes; and 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds for SOD1, SOD2, GPx; whereas, iNOS and CAT were performed for 40 cycles. The resulting PCR products were visualized on 2% agarose gel. Gel images were digitally captured, and the fragment intensity was determined using the Scion image (Herolab, Wiesloch). All reactions for the standard control and the experimental sample were performed in triplicate. The amount of iNOS and antioxidant genes (*viz*, SOD1, SOD2, and GPx) were calculated relative to the expression of the control gene, G3PDH. Fragment identity was confirmed after cloning into home-made T-vector followed by sequencing using the respective gene specific primers with the Cy5 label primer (Applied Biosystems) on MegaBACE™ 1000 DNA analysis System (Pharmacia).

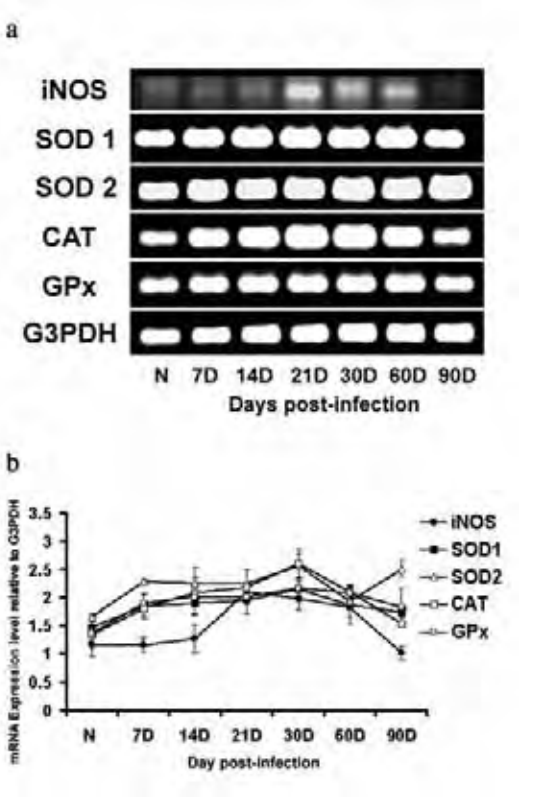

Fig 1- RT-PCR analysis of the expression of iNOS and antioxidant enzyme genes in the liver of *O. viverrini*-infected hamsters. The representative gel image for iNOS, antioxidant enzyme genes and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression in *O. viverrini*-infected hamsters and in normal hamsters is shown in 2% agarose gel (a). The expression level is represented by the ratio of the intensity of the sample gene relative to G3PDH (b) of three hamsters. The data comprise means ± SD for three hamsters per group. N = normal hamsters, D = day.

RESULTS

The mRNA expression profiles of iNOS, SOD1, SOD2, CAT, and GPx

The time-interval profiles of mRNA expression of iNOS, SOD1, SOD2, CAT and GPx in the liver of *O. viverrini*-infected hamsters are shown in Fig 1. The specific band of G3PDH had unchanged intensity throughout the time-course study (Fig 1a). The specific bands of iNOS, SOD1, SOD2, CAT, and GPx were also observed throughout the time-course study. Semi-quantitative RT-PCR analysis showed that the respective profiles of SOD1, CAT, and GPx gene expression in the liver of *O. viverrini*-infected hamsters gradually increased on day 7, reached a peak on day 30, and tended to decrease by day 90 (Fig 1b). In addition, iNOS expression reached its peak on day 21; but the expression profile of SOD2 reached two peaks, one on day 30 and another on day 90.

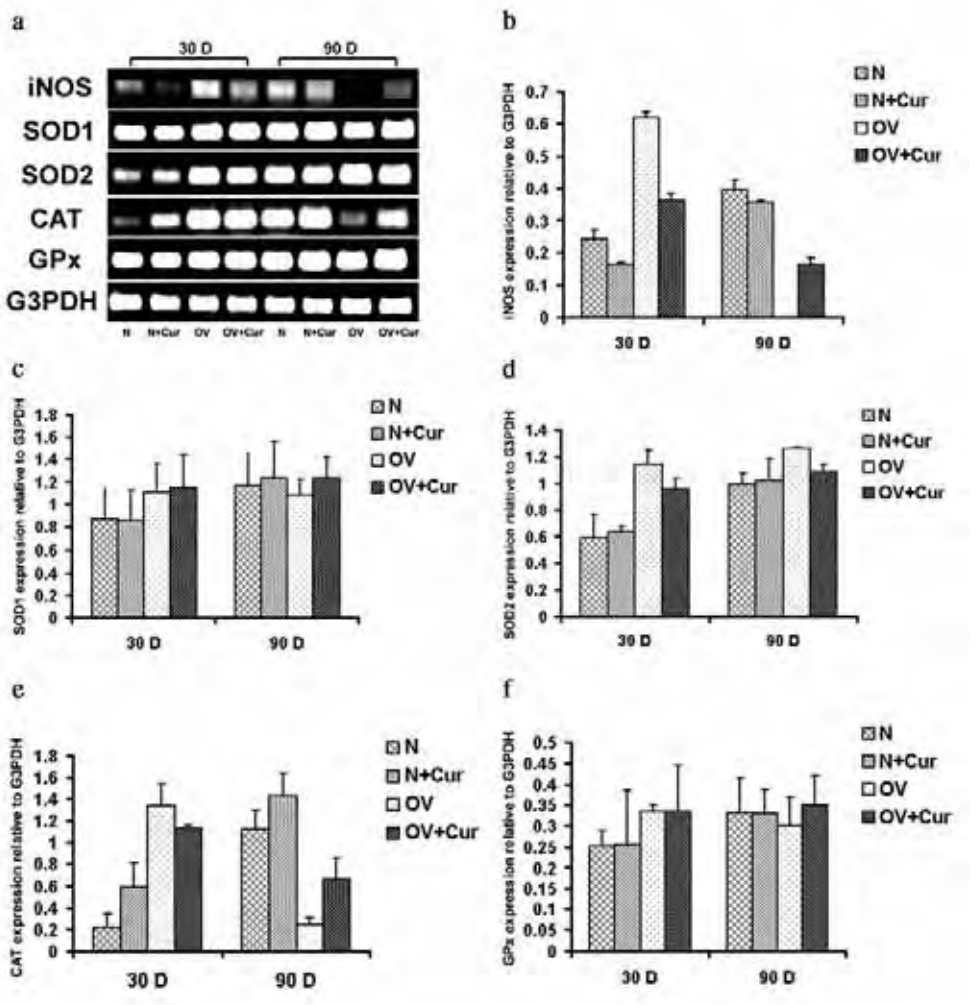

Fig 2- RT-PCR analysis of the effect of curcumin on the mRNA expression of iNOS and antioxidant enzyme genes in the liver of *O. viverrini*-infected hamsters. The representative gel image for iNOS, antioxidant enzyme genes and G3PDH expression in *O. viverrini*-infected hamsters supplemented with curcumin are shown in 2% agarose gel (a). The expression level is represented by the ratio of the intensity of the sample gene relative to G3PDH (b) in three hamsters. N = normal hamsters treated with normal pellets, N+Cur = normal hamsters treated with curcumin, OV = *O. viverrini*, OV+Cur = *O. viverrini*-infected hamsters and administration with curcumin.

Effect of curcumin on the mRNA expression of iNOS, SOD1, SOD2, CAT and GPx

The effect of curcumin on the mRNA expression of iNOS, SOD1, SOD2, CAT, and GPx in the liver of *O. viverrini*-infected hamsters is shown in Fig 2. The intensity of specific band of G3PDH on days 30 and 90 was the same (Fig 2a). The specific bands for iNOS, SOD1, SOD2, CAT, and GPx were also observed on days 30 and 90. The expression of iNOS was lower (0.37-fold) in the curcumin-treated group than the 0.62-fold in the *O. viverrini*-infected group on day 30 (Fig 2b). The expression of SOD2 was lower (1.00-fold on day 30 and 1.08-fold on day 90) in the curcumin-treated group than in the *O. viverrini*-infected group (1.20-fold on day 30 and 1.26-fold on day 90) (Fig 2d). By contrast, SOD1 expression was higher (1.28-fold) in the curcumin-treated group than the 1.13-fold in the *O. viverrini*-infected group on day 90 (Fig 2c). CAT expression was higher (0.66-fold) in the curcumin-treated group than the *O. viverrini*-infected group (0.24-fold) on day 90 (Fig 2e). GPx expression was higher (0.35-fold) in the curcumin-treated group than the in the *O. viverrini*-infected group (0.30-fold) on day 90 (Fig 2f).

Effect of curcumin on the histopathological changes

The effects of curcumin on the histopathological changes in the liver of *O. viverrini*-infected hamsters are shown in Fig 3. Curcumin did not significantly reduced the accumulation of inflammatory cells around the bile ducts on day 30 compared with the untreated group (Fig 3). The thickening of the walls of the gallbladder and extrahepatic bile ducts was less on day 90 in the curcumin-treated group when compared to the untreated group (Fig 4).

DISCUSSION

The present study is the first report on the effect of curcumin on the pathological changes caused by opisthorchiasis through the iNOS and antioxidant enzyme gene expression. Curcumin seems to reduce the accumulation of inflammatory cells around the bile ducts and to decrease the thickness of the gallbladder wall and extrahepatic bile duct in experimental opisthorchiasis. The mRNA expression of iNOS and SOD2 was decreased by curcumin treatment, whereas the antioxidant enzyme genes, such as SOD1, CAT, and GPx, increased the level of mRNA expression after curcumin treatment. We therefore hypothesize that curcumin suppresses the expression of iNOS, whereas it enhances the expression of antioxidant enzyme genes (SOD1, CAT, and GPx) resulting in the inhibition of nitrative stress and reduces the severity of opisthorchiasis.

In *O. viverrini*-infected animals, increases in

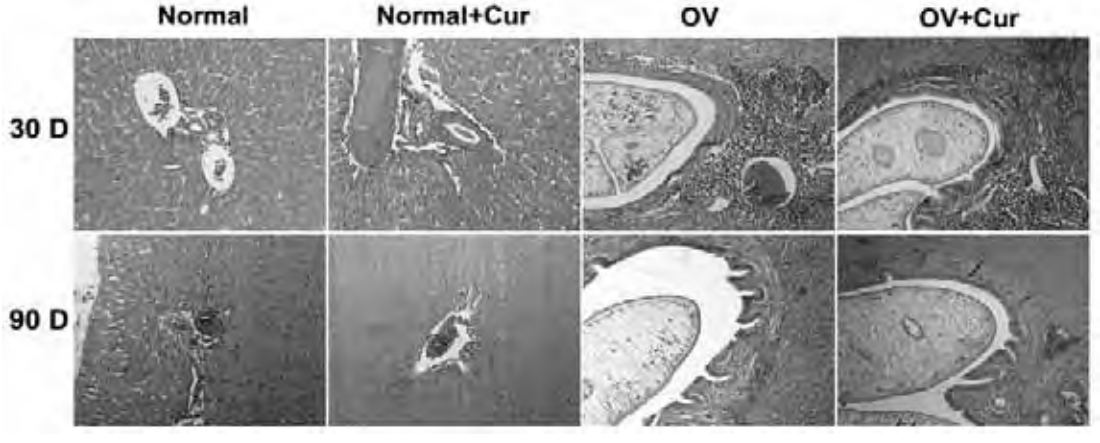

Fig 3- Histopathological studies of the livers of untreated and curcumin-treated hamsters. The histopathological changes in *O. viverrini*-infected hamster livers stained using hematoxylin-eosin. Original magnification is 40x. D = day, Cur = curcumin, OV = *O. viverrini*, OV+Cur = *O. viverrini*-infected hamsters and administration with curcumin.

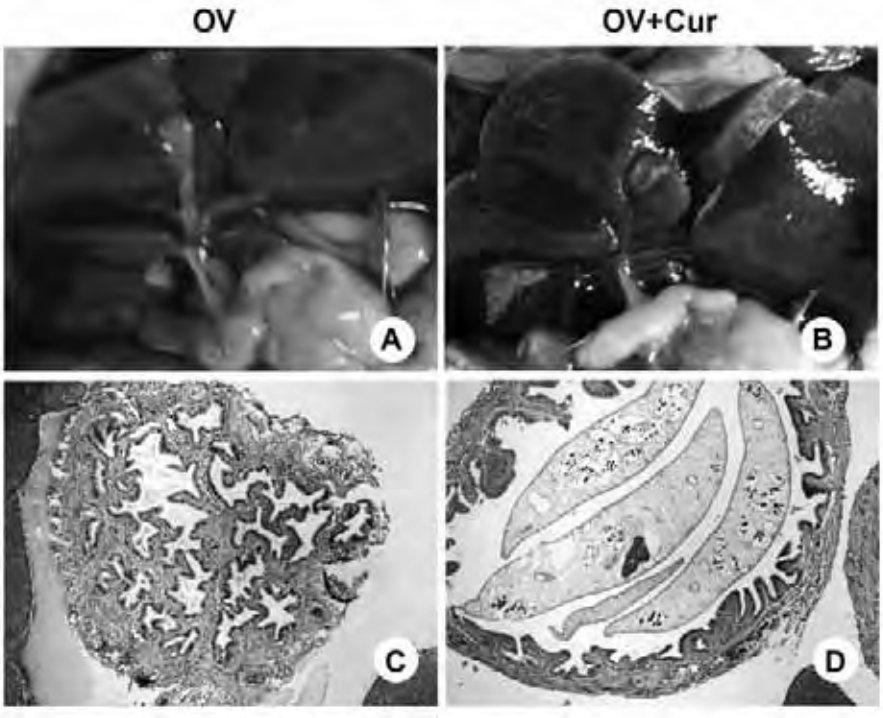

Fig 4- Effect of curcumin on the extrahepatic bile ducts and the gallbladder of untreated and curcumin-treated hamsters. Presented here are the gross appearance (in the first row) and histopathological changes (in the second row) of the extrahepatic bile duct and the gallbladder of *O. viverrini*-infected hamsters (A, C) and curcumin-treated *O. viverrini*-infected hamsters (B, D) on day 90. Original magnification is 40x. OV = *O. viverrini*, OV+Cur = *O. viverrini*-infected hamsters and administration with curcumin.

the mRNA expression of iNOS and the antioxidant enzyme genes (*ie*, SOD1, SOD2, CAT, and GPx) were associated with the accumulation of inflammatory cells around the bile duct, as with previous reports (Bhamarapravati *et al*, 1978; Sripa and Kaewkes, 2000; Pinlaor *et al*, 2004). The expression of iNOS is directly related to nitric oxide (NO) production. Over-expression of iNOS leads to increased damage to NO-mediated biomolecules (*ie*, oxidative and nitrative DNA damage) (Pinlaor *et al*, 2003). Similarly, iNOS-associated NO production has been reported in mice infected with *Schistosoma mansoni* (Brunet *et al*, 1999) and *Trichinella spiralis* (Garside *et al*, 2000). Excess expression of iNOS leading to a large amount of NO production is therefore involved in the pathology of various parasitic infections (Brunet *et al*, 1999; Garside *et al*, 2000). iNOS-mediated DNA damage through the oxidation and nitration reaction in hamster infected with *O. viverrini* may be a risk factor for the development of CCA (Pinlaor *et al*, 2004). Therefore, iNOS expression may increase nitrative stress and result in the pathological changes characteristic in opisthorchiasis.

The host defense against cell injury via oxidative and nitrative damage is through the induction of antioxidant enzyme expression. Our results showed that the expression of SOD1, CAT, and GPx gradually increased, reached their peaks on day 30, and then tended to decrease by day 90. This was perhaps because the first line of antioxidant enzyme defense to the overproduction of superoxide anion (O_2•-) is the SODs (such as SOD1 and SOD2) which catalyze the O_2•- into hydrogen peroxide (H_2O_2) and O_2 to protect biomolecules from injury (Kim *et al*, 2005). GPx and CAT mRNA expression is the second order of induction to catalyze H_2O_2 into a less toxic substance, that is, H_2O and O_2. Changes in the antioxidant enzyme gene expression in the liver and in the phospholipid structure of

the cell membrane is accompanied by raising the liver damage in rats infected with *Fasciola hepatica* (Kolodziejczyk *et al*, 2005). However, over-expression of SOD1 and SOD2 also partially protected liver injury from the increasing reactive oxygen species production. Therefore, the increase in the expression of antioxidant enzymes may act as a useful mechanism in the host to prevent oxidative and nitrative stress triggered by *O. viverrini* infection.

The decrease in mRNA expression on day 30 for iNOS, and on days 30 and 90 for SOD2, in the present experiment suggests that curcumin might play a role in inhibiting the constitutive NF-kappaB and IKK activities (Aggarwal *et al*, 2005; Surh *et al*, 2001). The inhibiting effect of curcumin on iNOS and SOD2 may be the result of the suppression of nuclear factor kappaB translocation (Karin and Greten, 2005). Curcumin can also reduce the iNOS mRNA in the livers of lipopolysaccharide-injected mice (Mabbott *et al*, 1998) and inhibit NO generation in mice infected with *Leishmania* (Chan *et al*, 2005). By contrast, curcumin can enhance the expression of SOD1, CAT, and GPx. The increase in the expression of antioxidant enzyme genes may have a beneficial effect as a host defense mechanism by reducing the severity of opisthorchiasis. This idea is supported by the observation that curcumin increased the mRNA expression of SOD1, CAT and GPx on day 90. The inflammatory cells around the bile ducts seemed to be decreased on day 30, and the extrahepatic bile duct had reduced thickening wall on day 90. Similarly, curcumin increases activities of SODs and CAT enzymes thus preventing oxidative damage in the cataracts of rats (Padmaja and Raju, 2004). Curcumin also reportedly reduces the severity of diseases in mice infected with malaria (Reddy *et al*, 2005) and coccidian infection in chickens (Allen *et al*, 1998).

In conclusion, the results of our experiment indicate that curcumin may (1) reduce the pathogenesis of opisthorchiasis through the suppression of iNOS and SOD2, and (2) enhance the expression of SOD1, CAT, and GPx thereby preventing oxidative and nitrative stress. Our results suggest that curcumin may have a beneficial effect on opisthorchiasis and be a useful chemopreventive treatment agent for prevention of *O. viverrini*-associated CCA development.

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ROLE OF MATRIX METALLOPROTEINASE (MMP)-2 AND MMP-9 IN THE PATHOGENESIS OF HAMSTER OPISTHORCHIASIS: EFFECT OF PRAZIQUANTEL TREATMENT

Suksanti Prakobwong[1,3], Somchai Pinlaor[1,3], Thidarut Boonmars[1,3], Tuanchai Srisawangwong[1], Chaisiri Wongkham [2,3] and Yusuke Hiraku[4]

Departments of [1]Parasitology, [2]Biochemistry, [3]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand; [4]Department of Environmental and Molecular Medicine, Mie University Graduate School of Medicine, Japan

Abstract. To clarify the role of matrix metalloproteinases (MMPs) in the pathogenesis of opisthorchiasis and the effect of the treatment with praziquantel (an anti-parasitic drug), we examined MMP-2 and MMP-9 activities, using gelatin zymography and hydroxyproline assay, in hamsters infected with *O. viverrini* for 21 days (acute) and 4 months (chronic) compared with those in infected hamsters following praziquantel treatment for 1, 3, and 6 months. The activities of these MMPs were higher in the hamsters with acute infection than in those with chronic infection after drug treatment. The plasma hydroxyproline level increased in a time-dependent manner and was correlated with the thickening of periductal fibrosis in *O. viverrini*-infected hamsters. Praziquantel treatment decreased the plasma hydroxyproline concentration in *O. viverrini*-infected hamsters. The profile of the hydroxyproline level of the acute infection group was lower than that of the chronic infection group, suggesting MMP-2 and MMP-9 are associated with resorption of the periductal fibrosis triggered by *O. viverrini* infection, but that praziquantel treatment may cause a decrease in tissue resorption of periductal fibrosis.

INTRODUCTION

Opisthorchiasis, caused by infection with *Opisthorchis viverrini*, is the major risk factor for cholangiocarcinoma (CCA) development (IARC, 1994) and is still a major health problem in northeast Thailand. *O. viverrini* infection induces several pathological changes (hyperplasia, bile duct proliferation, granulomatous formation, and periductal fibrosis), in both human and animal studies (Bhamarapravati *et al*, 1978; Harinasuta and Harinasuta, 1984). The severity of the disease is dependent on the duration and frequency of infection (IARC, 1994). Periductal fibrosis is the most prominent histological feature during chronic infection in the animal model (Pinlaor *et al*, 2004) and it persists after one-week praziquantel treatment (Pinlaor *et al*, 2006). Irreversible fibrosis may result in primary sclerosing cholangitis (PSC), which leads to CCA development (Boberg *et al*, 2002). Therefore, the molecular mechanism of fibrosis in opisthorchiasis-associated cholangiocarcinogenesis needs to be elucidated.

Periductal fibrosis results from a relative imbalance between synthesis and degradation of extracellular matrix (ECM) proteins that play important roles in the pathogenesis of several diseases including carcinogenesis. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that specifically degrade ECM component (Visse and Nagase, 2003). The MMPs are produced as zymogens (pro-MMPs) that must be removed during activation. Proteolytic degradation of ECM is an essential feature of tissue remodeling of connective tissue under physiological and pathological conditions (Gomez *et al*, 1997). Therefore, an analysis of the role of various MMP activities may provide basic information in opisthorchiasis-associated CCA.

The gelatinases MMP-2 and MMP-9 are capable of digesting components of connective tissue matrix and several types of collagen,

Correspondence: Dr. Somchai Pinlaor, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: +66(043) 348 387; Fax: +66 (043) 202 475
E-mail: psomec@kku.ac.th

which is a major component of the ECM. MMP-2 and MMP-9 contribute to tissue injury in experimental models, are strongly associated with the formation of granulomatous fibrosis in angiostrongyliasis (Hsu *et al*, 2005), and are associated with the recovered stage in CCl4-induced tissue damage (Knittel *et al*, 2000). Although the role of MMPs in opisthorchiasis is not clear, these MMPs appear to contribute to the fibrosis induced by *O. viverrini* infection.

The expressions of MMP-2 and MMP-9 in the plasma of hamsters infected with *O. viverrini* were analyzed using gelatin zymography in order to elucidate the tissue remodeling in opisthorchiasis, before and after drug treatment in the variably induced injuries, between acute and chronic infection. Hydroxyproline, a major component of collagen, in the plasma was assessed by spectrophotometer. The periductal fibrosis was studied by staining paraffin sections with Masson trichrome.

MATERIALS AND METHODS

Experimental animals

Four- to six-week-old male, Golden hamsters were housed under conventional conditions and fed a stock diet and given water *ad libitum*. Metacercariae of *O. viverrini* were isolated from cyprinoid fish under a dissecting microscope after artificial pepsin digestion as described by Pinlaor *et al* (2004). Five hamsters were put in each group then infected by oral inoculation with 50 *O. viverrini* metacercariae. Animals were sacrificed on days 21, 51, 111, 120, 150, 201, 210, and 300 post-infection. In addition, normal hamsters were used as a control group and sacrificed at these time points.

In the praziquantel treatment groups, after the hamsters had been infected with the metacercariae for 21 days (for the acute phase) and 4 months (for the chronic phase), a single dose of praziquantel (Biltricide, Bayer) 400 mg/kg body weight, suspended in 2% chemophore EL (Sigma, St Louis, MO) was given orally. The animals were sacrificed 1, 3, and 6 months after receiving this treatment. The control group of *O. viverrini*-infected hamsters received only 2% chemophore solution.

Samples at the hilar region of the liver and EDTA blood were collected. After centrifugation at 5,000*g* for 5 minutes, plasma was collected and stored at -80°C until assayed.

The Animal Ethics Committee of the Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand approved this study.

Gelatin zymography

Hsu *et al* (2005), described substrate-specific zymography for determination of gelatinolytic activities of MMP-2 and MMP-9 in plasma. Briefly, 5 µl of plasma was diluted in 20 µl Tris-buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl and 5 mM CaCl2) and 25 µl sample buffer [500 mM Tris-HCl, pH 6.8, 17.4% (w/v) glycerol, 4% sodium dodecylsulphate and 0.01% bromophenol blue]. An aliquot of this solution (6 µl) was loaded onto the 7.5% (mass/volume) sodium dodecylsulphate-polyacrylamide gels co-polymerized with 0.1% gelatin (Sigma). Stacking gels were 4% (mass/volume) polyacrylamide gels and did not contain gelatin substrate.

Gel electrophoresis was performed using a mini-gel system apparatus (Bio-Rad). Electrophoresis was performed in running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS) at room temperature at 100 V for 50 minutes. The gels were washed three times at room temperature for 30 minutes in distilled water containing 2.5% Triton X-100, and then twice at room temperature for 10 minutes in distilled water. After incubation in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, 10 mM $CaCl_2$, 0.02% Brij-35, 0.01% NaN_3) at 37°C for 20 hours, the gels were stained with 0.25% Coomassie brilliant blue R-250 (Sigma) for 1 hour and finally de-stained in 15% methanol/7.5% acetic acid. After de-staining, zones with enzymatic activity appeared as non-stained zones.

The quantitative analysis of the gelatinolytic enzyme was performed with a computer-assisted imaging densitometer system (Scion image, Scion Corporation). The 80 kDa MMP-9 and 66 kDa MMP-2 (active forms) and 92 kDa pro-MMP-9 and 72 kDa pro-MMP-2 were measured and compared with a standard protein marker (Amersham). Mean MMP activities

for each sample were derived from duplicate experiments.

Hydroxyproline assay

The ECM content was measured as a major protein component in the plasma by hydroxyproline assay. The hydroxyproline content in the plasma was modified from Reddy and Enwemeka (1996). Briefly, duplicate sets containing 50 µl sample plasma, 20 µl 10 N NaOH and 30 µl water were hydrolyzed in screw-capped tubes in boiling water for 3 hours. A hydrolysate solution was taken and supplemented with 100 µl chloramine T solution. Samples were incubated at room temperature for 20 minutes. Then, 200 µl freshly prepared Ehrlich's reagent was added to the tubes and incubated in a 65°C water bath for 20 minutes. Samples were transferred to a 96-well plate and read for absorbance at 540 nm. Hydroxyproline (Sigma) was used as the standard.

Histology

The periductal fibrosis was assessed by Masson's trichrome staining of 5 µm-thick paraffin sections.

Statistical analysis

Data were presented as means ± SE. The *t*-test was used to compare between groups, while ANOVA was used to compare three or more groups. Statistical analyses were done using SPSS version 11. A p-value of < 0.05 was required for statistical significance.

RESULTS

Histopathological changes in hamster opisthorchiasis and effect of praziquantel treatment

The profile of fibrotic changes in the liver was evaluated using Masson's trichrome (Fig 1). An increase in periductal fibrosis was observed around bile ducts in a time-dependent manner, as inflammatory cells decreased (Fig 1a). The periductal fibrosis in the chronic infection group (4 months) was thicker than that of the acute infection group (21 days). After praziquantel treatment, the thickness of periductal fibrosis in the acute infection group tended to decrease. However, periductal fibrosis in the chronic infection group tended to persist from 1 month to 6 months after drug treatment (Fig 1b).

Hydroxyproline content in the plasma of hamsters infected with *O. viverrini* and effect of praziquantel treatment

The plasma hydroxyproline level increased in a time-dependent manner in *O. viverrini*-infected hamsters (Fig 2). A significant increase in plasma hydroxyproline content was observed between day 51 and 300, whereas the content was unchanged in the uninfected control groups. The plasma hydroxyproline level was associated with the thickening of periductal fibrosis in the *O. viverrini*-infected groups.

After praziquantel treatment, the hydroxyproline content gradually decreased compared with the infected control groups whether acute or chronic (Fig 3). The hydroxyproline content the chronic infection group was significantly higher than that of the acute infection group ($p < 0.05$). The profile of the hydroxyproline level in the acute infection group was also significantly lower and closer to a normal level than that of the chronic infection group ($p < 0.01$).

MMP-2 and MMP-9 activities in hamster opisthorchiasis: effect of praziquantel

The time-course study of MMP-2 and MMP-9 activities in the plasma of hamsters infected with *O. viverrini* is shown in Fig 4. Gelatin zymography revealed that the active MMP-2 (66 kDa) and MMP-9 (80 kDa) were observed in the plasma at all time points (Fig 4a). In the *O. viverrini*-infected control group, the activity of MMP-9 (80 kDa) reached its highest intensity on day 51 then decreased until day 300. Active 80-kDa MMP-9 was stable from day 210 to day 300, although pro-MMP-9 (92 kDa) was also prominent. MMP-2 (66 kDa) activity reached its highest intensity on day 111 then was stable until day 300 (Fig 4a). In addition, the intensity of pro-MMP-2 (72 kDa) was unchanged compared to the normal control group. The significant increase in MMP-9 (80 kDa) activity was observed on day 21 and 51 compared with normal hamsters ($p < 0.05$ and $p < 0.01$, respectively) (Fig 4b).

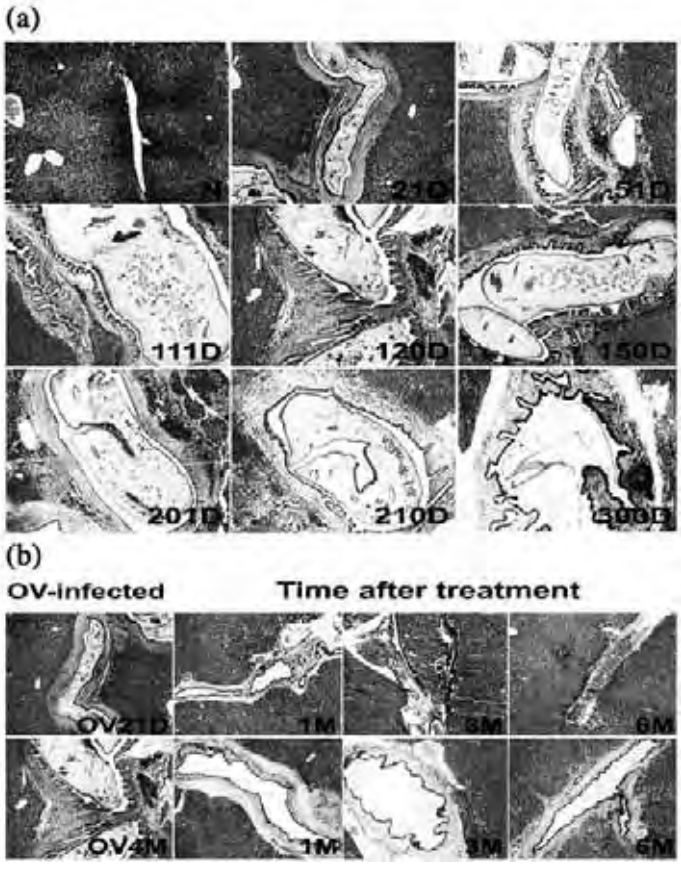

Fig 1- Histopathological features of hamster opisthorchiasis and effect of praziquantel treatment during acute (OV 21 days) and chronic (OV 4 months) infection. The periductal fibrosis was stained with Masson's trichrome. The dilatations of bile ducts and the thickness of periductal fibrosis increase with the duration of *O. viverrini* infection (a). After praziquantel treatment, the thickening of the periductal fibrosis in the 21-day infection groups tends to N = normal hamster, D = day, M = month decrease compared with those of the 4-month infection groups (b). The magnification is 40x.

MMP-2 and MMP-9 activities in groups treated with praziquantel after acute *O. viverrini* infection are shown in Fig 5. The activity of MMP-9 (80 kDa) decreased after praziquantel treatment from 1 month to 6 months, whereas the activity of MMP-2 (66 kDa) increased to 3 months then decreased after reaching 6 months (Fig 5b). By contrast, the intensity of pro-MMP-9 (92 kDa) was highest at 6 months after treatment. In addition, pro-MMP-2 (72 kDa) was stable after praziquantel treatment (Fig 5a).

MMP-2 and MMP-9 activities in groups with praziquantel treatment after chronic *O. viverrini* infection are shown in Fig 6. The activities of MMP-2 (66 kDa) and MMP-9 (80 kDa) decreased after praziquantel treatment, although MMP-2 activity was slightly increased at 1 month. The significant increase in MMP-9 (80 kDa) activity was observed at 1 and 3 months compared with normal hamsters (Fig 6b). By contrast, the intensity of pro-MMP-9 (92 kDa) was highest at 6 months after drug treatment. In addition, pro-MMP-2 (72 kDa) was stable from 1 to 6 months after praziquantel treatment (Fig 6a).

DISCUSSION

We first demonstrated MMP-2 and MMP-9 activities in *O. viverrini*-infected hamsters and then showed their relationship with respect to liver fibrosis resorption after praziquantel treatment. Praziquantel decreased the activities

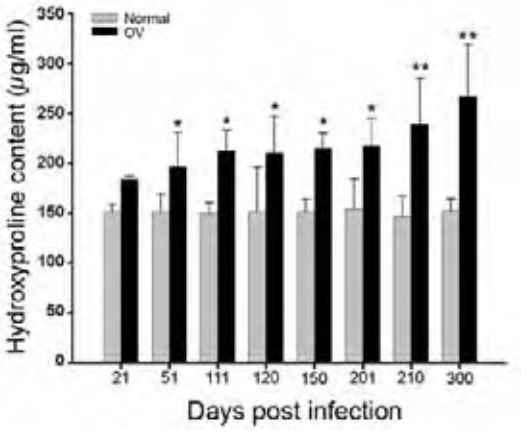

Fig 2- Hydroxyproline content in the plasma of *O. viverrini*-infected hamster compared with normal hamsters. The hydroxyproline level in the plasma increased in a time-dependent manner. A significant difference is observed on day 51 and throughout the study compared with the normal control groups. The *t*-test is used to compare between *O. viverrini*-infected and normal hamsters. Data are presented as mean ± SE of five animals. *=p < 0.05, ** = p < 0.01, and *** = p < 0.001.

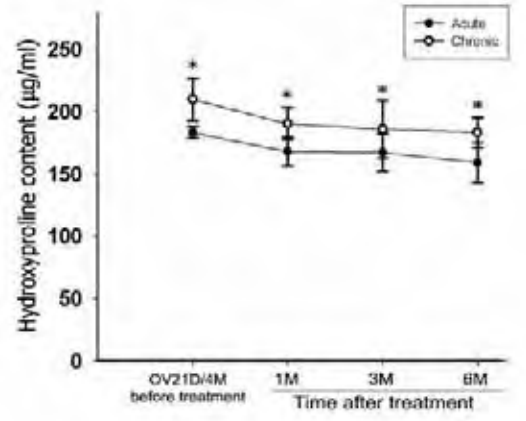

Fig 3- Effect of praziquantel treatment on hydroxyproline content in the plasma of hamsters infected with *O. viverrini* for 21 days and 4 months. After praziquantel treatment of the 21 days (acute) and 4 months (chronic), hydroxyproline levels tend to decrease from 1 to 6 months. The hydroxyproline content after drug treatment for the chronic groups is significantly higher than that of the acute groups ($p < 0.05$). The profile of hydroxyproline after drug treatment for the chronic groups is also significantly higher than that of the acute groups ($p < 0.01$). The *t*-test is used to compare between the acute and chronic groups at each time point and a one way ANOVA for comparisons of hydroxyproline profiles. The data are presented as means ± SE of five animals. * = $p < 0.05$.

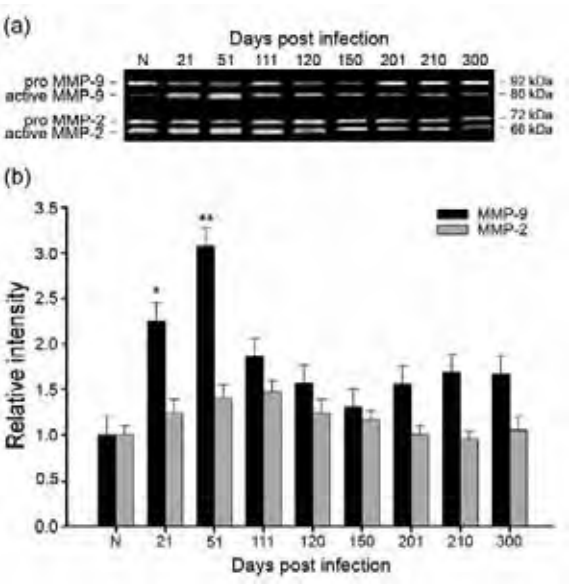

Fig 4- The profiles of MMP-2 and MMP-9 activities in the plasma of hamster opisthorchiasis. MMP-2 and MMP-9 activities were analyzed by gelatin zymography. MMP-9 (92 kDa and 80 kDa) and MMP-2 (72 kDa and 66 kDa) appear at all time points (a). The intensity of 80 kDa MMP-9 and 66 kDa MMP-2 (active form) reaches its maximum on day 51 and 111, respectively (b). The intensity of 72 kDa pro-MMP-2 is unchanged, whereas 92 kDa pro-MMP-9 gradually increases on day 51 then decreases until day 150. Afterward, its activity gradually increases and reaches a plateau (a). The relative intensity of the 80 kDa MMP-9 in the infected groups is significantly higher on day 21 and 51 post-infection compared with the normal control hamster (b). The *t*-test is used to compare between the infected and normal hamsters. Data are presented as mean ± SE of five animals. N = normal control hamster, * = $p < 0.05$ and ** = $p < 0.01$.

of both MMP-2 and MMP-9 during the acute and chronic phases of *O. viverrini* infection. We hypothesized that MMP-2 and MMP-9 might play roles in the pathogenesis of opisthorchiasis through inflammation, and that praziquantel treatment decreases tissue resorption of periductal fibrosis.

Our hypothesis was apparently supported by evidence of MMP-9 and MMP-2 activity being at their highest on days 51 to 111 post-infection, respectively. Moreover, the inflammatory cells

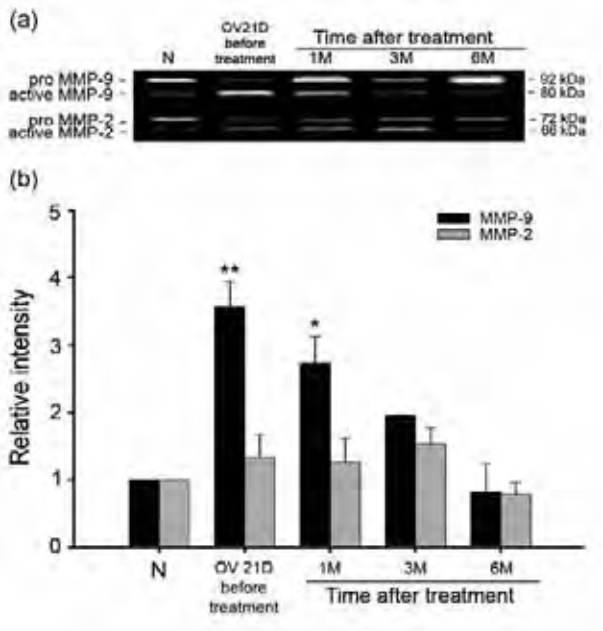

Fig 5- Effect of praziquantel treatment on MMP-2 and MMP-9 activities in plasma of *O. viverrini*-infected hamsters for 21 days. MMP-2 and MMP-9 activities were analyzed by gelatin zymography. The intensity of 80 kDa MMP-9 and 66 kDa MMP-2 (active form) gradually decreases from 1 to 6 months after drug treatment, whereas the intensity of 92 kDa pro-MMP-9 gradually increases and reaches its maximum at 6 months after drug treatment (a). The relative intensity of 80 kDa MMP-9 is significantly higher on day 21 post-infection and 1 month after praziquantel treatment compared with normal control hamsters (b). The *t*-test is used for a comparison between infected and normal hamsters (N). Data are presented as means ± SE of five animals. * = $p < 0.05$ and ** = $p < 0.01$.

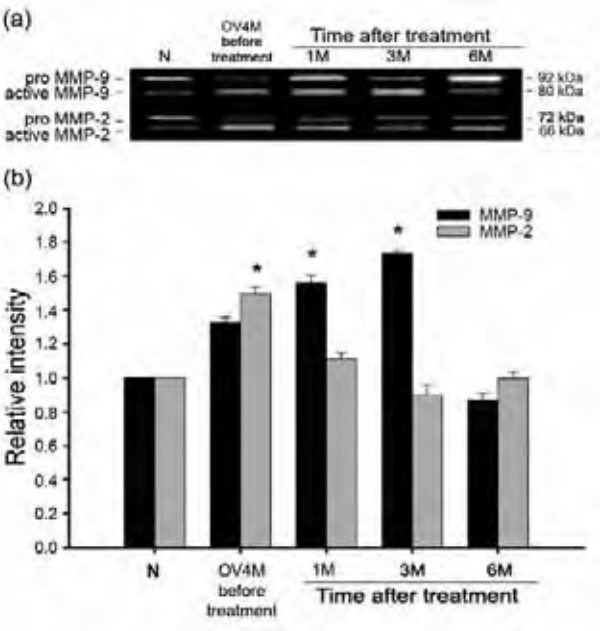

Fig 6- Effect of praziquantel treatment on MMP-2 and MMP-9 activities of *O. viverrini*-infected hamsters for 4 months. MMP-2 and MMP-9 activities were analyzed by gelatin zymography. The intensity of 80 kDa MMP-9 and 66 kDa MMP-2 (active form) gradually decreases, whereas 92 kDa pro-MMP-9 gradually increases (a). The relative intensities of 66 kDa MMP-2 and 80 kDa MMP-9 are significantly higher on 4 months post-infection and 1 and 3 months after praziquantel treatment, respectively, compared with normal control hamsters (b). The *t*-test is used to compare between infected and normal hamsters. Data are presented as means ± SE of five animals. N = normal control hamster, * = $p < 0.05$.

were prominent in the same period. By contrast, pro-MMP-9 showed the most prominent intensity during chronic infection that was associated with the accumulation of periductal fibrosis, supported by the finding of hydroxyproline persistence in the plasma in the chronic phase and a correlation with periductal fibrosis.

Perhaps MMPs activities are based on a balance between pro-enzyme activation and inhibition by tissue inhibitors of MMPs (TIMPs) (Gomez *et al*, 1997). TIMPs promote the progression of periductal fibrosis through inhibition of matrix degradation (Iredale, 1997). Our study may elucidate a possible mechanism for the accumulation of periductal fibrosis during chronic infection, as previously proposed by Pinlaor *et al* (2004). Therefore, MMP-2 and MMP-9 may play roles in tissue remodeling triggered by infection with *O. viverrini* and could therefore serve as markers of inflammation-associated opisthorchiasis rather than the fibrosis stage of chronic infection.

Praziquantel decreased the MMP-2 and MMP-9 activities and decreased collagen content in the liver after drug administration. Our results showed that the MMP-2 and MMP-9 activities gradually decreased after praziquantel treatment. Similarly, the hydroxyproline level tended to

decrease and was associated with the periductal fibrosis. Thus, we cannot exclude the activity of other MMPs in the resorption of periductal fibrosis triggered by *O. viverrini* infection.

After praziquantel treatment, the profile of MMP-9 activity in the acute phase (21-day infection) was higher than that of the chronic phase (4-month infection). This result is supported by the profile of the hydroxyproline level of the acute phase group, which was lower than that of the chronic phase group. Periductal fibrosis, as demonstrated by Masson trichrome, also helps to explain the hydroxyproline level.

Our results suggest that in chronic infection, MMPs may show a slow resorption of periductal fibrous tissue after drug treatment. Mice infected with *Schistosoma mansoni* showed a slow resorption of liver fibrous tissue following praziquantel treatment (Singh *et al*, 2004). The irreversible fibrosis may predispose to relative risk of primary sclerosing cholangitis, leading to CCA development. This hypothesis is supported by a previous study showing that irreversible fibrosis was not affected by drug treatment (Mairiang *et al*, 1993).

Early treatment (starting at 21 days post-infection) has yielded better resorption than treatment at 4 months post-infection. Our results are similar to those of rabbits infected with *Clonorchis sinensis* that showed that liver changes caused by acute clonorchiasis in the first two weeks were reversible if treated while chronic biliary epithelial changes were irreversible (Lee *et al*, 1987). Thus, to prevent the risk of opisthorchiasis-associated CCA development, early treatment should be recommended to minimize the remaining periductal fibrosis in opisthorchiasis.

Based on our results, MMP-2 and MMP-9 appear to be associated with the inflammation-mediated opisthorchiasis, and that praziquantel treatment, at the early stage, may enhance tissue resorption of periductal fibrosis. Other MMPs and TIMPs may play important roles in the tissue resorption of periductal fibrosis triggered by *O. viverrini* infection. Therefore, plasma MMP-2 and MMP-9 could be useful markers of opisthorchiasis and be used for the evaluation of the efficacy of praziquantel treatment.

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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF *PARAGONIMUS HETEROTREMUS*, THE CAUSATIVE AGENT OF HUMAN PARAGONIMIASIS IN INDIA

T Shantikumar Singh[1], Hiromu Sugiyama[2], Achariya Rangsiruji[3] and K Ranjana Devi[4]

[1]Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Sikkim, India; [2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; [3]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok Thailand; Department of Microbiology, Regional Institute of Medical Sciences, Imphal, Manipur, India

Abstract. In order to identify the causative species of human paragonimiasis, we performed a combined morphological and molecular investigation on the metacercariae and *Paragonimus* eggs isolated from the freshwater crab host, *Potamiscus manipurensis*, and sputum specimens of a patient, respectively. Experimental infection of laboratory animals with the metacercariae resulted in the isolation of adult worms that were morphologically identified as *P. heterotremus*. Molecular characterization based on polymerase chain reaction and DNA sequencing of the metacercariae and *Paragonimus* eggs from the sputum specimens yielded identical ITS2 sequences. Results of phylogenetic analyses of the ITS2 region suggested that Indian *P. heterotremus* is nested within the *P. heterotremus* clade; the Indian population is less closely related to other members within the clade.

INTRODUCTION

Paragonimus species hitherto reported in Asia number 17, of which *P. westermani* is the most common cause of human paragonimiasis (Miyazaki, 1974). *Paragonimus heterotremus* was first described in rats in Guangxi, China (Chen and Hsia, 1964). The first human paragonimiasis due to *P. heterotremus* in the world was reported by Miyazaki and Harinasuta (1964). This species is considered medically more important than other species in Thailand, Lao PDR, Vietnam, and some parts of China where man and mammals serve as naturally infected final hosts (Miyazaki and Harinasuta, 1964; Doanh *et al*, 2005). In Manipur in India, a recently recognized endemic area, *P. westermani* was presumed to be the etiological agent of human paragonimiasis (Singh *et al*, 1982;1993). However, no scientific study supported this speculation nor was able to determine which lung fluke species occurred in Manipur until recently. A joint Indo-Japan research on *Paragonimus* and paragonimiasis in Manipur resulted in the identification of *Potamiscus manipurensis*, a freshwater crab species, as the second intermediate host of at least three *Paragonimus* species, including *P. heterotremus*.

In this study, further investigation on the determination of etiological agents of human paragonimiasis was performed by nucleotide sequencing of the ITS2 region on *Paragonimus* (Sugiyama *et al*, 2002). The study also aimed to determine the phylogenetic relationships of the Indian species with other *Paragonimus* found in various geographical areas in Asia.

Corresponence: T Shantikumas Singh, Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, Sikkim, India.
E-mail: shantikumar_singh@rediffmail.com

MATERIALS AND METHODS

Parasite material

Metacercariae harvested from freshwater crab host, *Potamiscus manipurensis*, which were collected from Luwangsangbam Matai in Imphal East and Motbung in Senapati Districts both in Manipur State were used for morphological study, laboratory animal infections, and molecular study. Adult worms as well as immature worms that were recovered from the experimentally infected puppies and albino rats were used for morphological identification. *Paragonimus* eggs were collected from sputum specimens of a patient in Senapati District. All materials, metacercariae, adult worms, and eggs were preserved in equal

proportions in 70% ethanol and 10% formalin until utilized. Morphological features of both fresh and preserved metacercariae and borax-carmine-stained worms were examined under microscope.

DNA isolation, amplification and sequencing

DNA samples were prepared from individual metacercariae and eggs. The ITS2 region of the nuclear ribosomal DNA was amplified by PCR and sequenced as described previously (Sugiyama *et al*, 2002). The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward: Bowels *et al*, 1995) and A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse: Blair *et al*, 1997).

Sequence and phylogenetic analyses

The Indian *Paragonimus* ITS2 sequences were aligned with other *Paragonimus* sequences obtained from the GenBank database and an outgroup (*Fasciola hepatica*; Table 1), using the Clustal X program (Jeanmougin *et al*, 1998). Maximum parsimony analysis was conducted with the branch-and-bound algorithm using PAUP* (version 4.0b) (Swofford, 1998). The robustness of tree(s) inferred from the analysis was evaluated using bootstrap analyses with heuristic searching (Felsenstein, 1985).

RESULTS

Characteristics of metacercariae, eggs, and adult worms

The metacercariae (Fig 1) were oval to suboval in shape. The inner cyst measured 163 to 215 µm (av = 196 µm) in the long axis and 133 to 188 µm (av = 162 µm) in the transverse axis. The thickness of the inner wall was 4.2 to 10.4 µm (av = 6.3 µm) on the side and 10.4 to 27.1 µm (av = 18.2 µm) at the pole. The oral sucker, provided with a stylet, was smaller than the ventral sucker.

Paragonimus eggs (Fig 2), golden-yellow in color, oval shaped, and operculated, measured 89-100 µm (av = 92 µm) in length and 47-58 µm (av = 50 µm) in width. The eggshell thickness was almost uniform in 22 (63%) and discernible at the nonoperculated end in 13 (37%). The

Table 1
GenBank accession numbers of *Paragonimus* species and *Fasciola hepatica*.

Species	Origin	Accession No.
P. heterotremus	Thailand	AF159603
P. heterotremus	China	AY618758
P. heterotremus	India	AB308377, AB308378
P. skrjabini	China	AY618752
P. miyazakii	China	AY618741
P. westermani	Thailand	AF159604
Fasciola hepatica	Australia	AB207148

Fig 1- *P. heterotremus* metacercariae: average longitudinal diameter 196 µm and average transverse diameter 162 µm.

Fig 2- Morphological characteristics of eggs discharged from a patient. Size of eggs: av length = 92 µm, av width = 50 µm.

widest transverse diameter was located at the middle 28 (80%), at operculated half 6 (17%), and at nonoperculated half 1(3%).

The borax-carmine-stained worms (Fig 3) that were recovered from the experimentally infected puppies showed singly spaced cuticular spines, oral suckers (385-500 µm) that were much larger than the ventral suckers (260-300 µm), and the ovaries and testes that were delicately branched. The vitellaria were not seen in immature worms. The morphological features of metacercariae, eggs, and worms conform to the features of *P. heterotremus*.

Fig 3- *P. heterotremus* adult worm recovered from the experimentally infected puppies showed delicately branched ovary and testes and the oral sucker was much larger than the ventral sucker.

Sequence and phylogenetic analyses

Molecular characterization, which is based on PCR and DNA sequencing of the metacercariae (accession No. AB308377) and eggs (AB308378), yielded identical ITS2 sequences. The alignment of the ITS2 region of six taxa of *Paragonimus* and its outgroup was 378 bp in length. Twenty-four characters (6.3%) were phylogenetically informative. A single most parsimonious tree (Fig 4), with a length of 144 steps, was obtained from a maximum parsimony analysis of the informative characters with 1,000 bootstrap (BS) replicates. Fit measures of the tree were as follows: consistency index (CI) = 0.951, retention index (RI) = 0.811, and rescaled consistency index (RC) = 0.771. The phylogenetic tree revealed that Indian *P. heterotremus* is nested within *P. heterotremus* clade (BS = 99%), which includes *P. heterotremus* from Thailand and China. The Indian population is however, less closely related to other members of the clade.

DISCUSSION

Although India is the first country from whence *P. westermani* was first described by Kerbert in 1878, from a Bengal tiger, very little attention has been given to this parasite because human paragonimiasis was never considered a public health problem. In India, there was no record of an autochthonous human case of paragonimiasis, although *P. westermani* infection was described in many mammals.

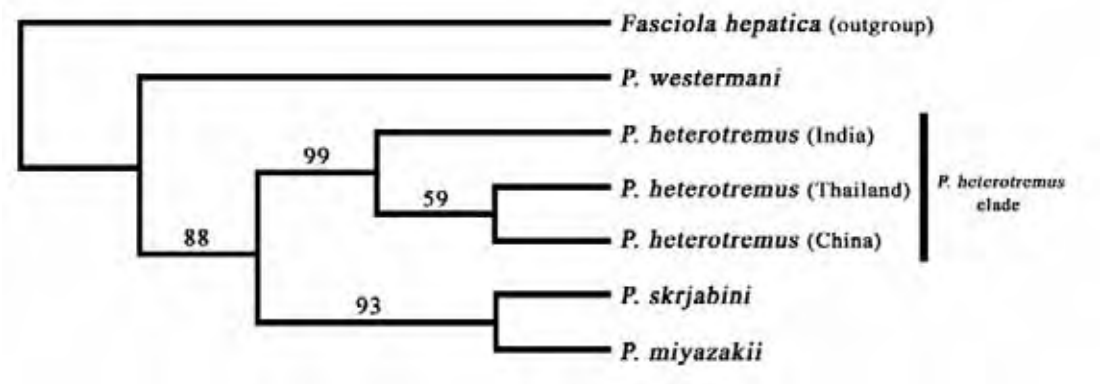

Fig 4- Single most parsimonious tree with a length of length 144 steps, based on parsimony analysis of the informative characters of the ITS2 region. Fit measures of the tree: CI = 0.951, RI = 0.811, RC = 0.771. Numbers above the branches indicate bootstrap values (%).

Evidence of infection with lung flukes of the genus *Paragonimus* in wild mammals has often been reported in India (Gaur *et al*, 1980; Rao, 1935; Srivastava, 1938; Singh and Somvanshi, 1978; Parihar and Shrivastava, 1988; Sano *et al*, 1994). The authors described *P. westermani* as the causative agent, based on the morphology of the eggs in the fecal specimens only or sections of worms and worm cysts in the lungs obtained on autopsy or postmortem examination of the animals. In the absence of detailed morphological descriptions of the adult worms, it was not possible to identify the species by examination of histopathological sections of the worm or worm cyst in the tissue and eggs in the feces. *P. westermani* was also reported to be the causative agent of human paragonimiasis in Manipur, based on the morphology of the eggs seen on microscopy examination of the sputum specimens of the patients (Singh *et al*, 1982). Therefore, doubts prevailed as to whether or not *P. westermani* was actually the only species infecting mammals and humans in India. Singh and Vashum (1994) first described the *P. heterotremus* adult worm from the biopsy specimen of a subcutaneous nodule in a 10-year-old boy in Imphal, Manipur. No other information on the *Paragonimus* species causing human paragonimiasis has been available in India.

The occurrence of *P. heterotremus* in freshwater crab, *Barytelphusa lugubris*, in an endemic area of paragonimiasis in Arunachal Pradesh was reported by Narain *et al* (2003). However, the morphological features of the metacercariae and adult worms, as described by these authors require further confirmation. In addition, it may not be safe to assume that this species is the causative agent of human paragonimiasis without morphological and molecular characterization of the parasite material recovered from the patient.

Recently, molecular analysis of any one of the developmental stages of the parasite has proved to be highly sensitive, and specific techniques are required to confirm the parasite species and its relationship with other species occurring elsewhere in the world. Technique is of importance in the identification of *Paragonimus* species, which can be made from the eggs in clinical specimens. Adult worms are rarely recovered from the patient, and hence not available for morphological identification and molecular characterization. The results of the present study confirmed that *P. heterotremus* was the causative agent of human paragonimiasis in Manipur, India.

Phylogenetic analysis indicated that all *P. heterotremus* species that originate from Vietnam, Thailand, and China form a distinct group (Le *et al*, 2006). However, our study revealed that the Indian species, although situated within the *P. heterotremus* group, is distantly related to the Chinese and Thai species.

This species has been identified as significant cause of human paragonimiasis in Southeast Asia, and endemic in South/Southwest China, Thailand, Lao PDR, and Vietnam (Blair *et al*, 1997; De *et al*, 2000; Doanh *et al*, 2005; Waikagul and Yoonuan, 2005). Morphometric and molecular characterization of the *Paragonimus* species are important for epidemiological, ecological, and taxonomic studies. This knowledge will also help in the control and treatment of paragonimiasis.

Potamiscus manipurensis, the natural second intermediate crustacean host of *P. heterotremus*, was found to contain metacercariae of *P. skrjabini* (Singh *et al*, 2006), and possibly two more species as well. The metacercariae of *P. skrjabini* were most frequently isolated from the freshwater crabs in some localities in Manipur State, where patients of pulmonary as well as cutaneous paragonimiasis have been reported. The possible relationship of *P. skrjabini* with human paragonimiasis in these localities is now under investigation.

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NEW FORM OF *PARAGONIMUS WESTERMANI* DISCOVERED IN THAILAND: MORPHOLOGICAL CHARACTERISTICS AND HOST SUSCEPTIBILITY

Hiromu Sugiyama[1], Yasuyuki Morishima[1], Sutheewan Binchai[2], Achariya Rangsiruji[2] and Punsin Ketudat[2]

[1]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; [2]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand

Abstract. During an intensive field survey for *P. westermani* in southern Thailand, a new form of *Paragonimus* metacercariae was isolated. In this study, we referred to this new form as *P. westermani*-like, as it was almost identical to *P. westermani* in shape. To investigate the susceptibility of feline host to *P. westermani*-like, as well as its morphology at the adult stage, we inoculated the peritoneal cavity of a cat with 60 *P. westermani*-like metacercariae. Morphological examination revealed that the adult *P. westermani*-like recovered from the lungs had a six-lobed ovary, a spermatozoa-filled seminal receptacle, and singly spaced cuticular spines. These findings indicated that the morphological features of *P. westermani*-like were fundamentally identical to those of *P. westermani* (diploid type) at the adult stage. The susceptibility of feline hosts to *P. westermani*-like was different from that of *P. westermani*. To determine the proper taxonomic status of *P. westermani*-like, we have been investigating the phylogenetic relationships between *P. westermani*-like and *P. westermani* in southern Thailand.

INTRODUCTION

Paragonimus westermani is widely distributed in Asia (Miyazaki, 1991). Individuals from different geographical regions (or countries) show variations in animal and/or human susceptibility, although they share almost identical morphological features at both the adult and metacercarial stages (Blair *et al*, 1998). This implies that they form a complex of cryptic species (Blair *et al*, 1997).

During an intensive field survey for *Paragonimus* in southern Thailand (Rangsiruji *et al*, 2005), we collected another form of *Paragonimus westermani* metacercariae from freshwater crabs, *Phricotelphusa aedes*. These crabs simultaneously acted as the second intermediate host of *P. westermani*. Metacercariae of newly isolated *Paragonimus* were almost identical to those of *P. westermani* in shape, but were much smaller. For descriptive purposes, we refer to this new form as *P. westermani*-like.

Correspondence: Hiromu Sugiyama, Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel: +81-3-5285-1111; Fax: +81-3-5285-1173
E-mail: hsugi@nih.go.jp

In this study, we inoculated a cat with *P. westermani*-like metacercariae isolated from freshwater crabs, *Phricotelphusa aedes*, in order to identify the susceptibility of feline hosts. The morphological characteristics of *P. westermani*-like at the adult stage were also compared with those of *P. westermani*.

MATERIALS AND METHODS

Identification of freshwater crabs

The captured freshwater crabs, belonging to the family Potamidae, were identified as *Phricotelphusa aedes* according to the method of Naiyanetr (1988).

Isolation of *Paragonimus* metacercariae

Between January and May 2003, we collected 922 freshwater crabs, *Phricotelphusa aedes*, from mountain streams in the Phanom District of Surat Thani Province, Thailand. We examined the crabs for metacercariae, as described previously (Rangsiruji *et al*, 2005). Isolated metacercariae were placed on glass slides and gently pressed under a coverglass for morphological observation and measurement.

Worm recovery from test animal

We inoculated the peritoneal cavity of a cat

with 60 *P. westermani*-like metacercariae. The cat was then treated with prednisolone (20 mg/kg) at 7-day intervals and was necropsied 148 days after inoculation. We examined the whole body of the cat for worms, as described previously (Sugiyama *et al*, 1984). Recovered worms were pressed between two glass slides, fixed in 70% ethanol, stained with borax carmine, and mounted with Canada balsam for morphological observation and measurement.

DNA amplification and sequencing of ITS2 region

We prepared DNA samples from individual *P. westermani* and *P. westermani*-like metacercariae (five metacercariae each). The ITS2 region of the nuclear ribosomal DNA was amplified by PCR and sequenced, as described previously (Sugiyama *et al*, 2002). The primers used were 3S: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3' (forward: Bowels *et al*, 1995) and A28: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' (reverse: Blair *et al*, 1997). We aligned and compared sequences using GENETYX-WIN software (ver 7.0, Software Development, Tokyo, Japan).

RESULTS

New crab intermediate host of *Paragonimus* in southern Thailand

We captured 922 freshwater crabs (Fig 1) from mountain streams in the Phanom District of Surat Thani Province. The crabs were positive for *P. westermani* metacercariae; this is the first report of this crab species serving as a second intermediate host of *P. westermani*. *P. westermani*-like metacercariae were also isolated from the same crab species captured at the same sites.

Morphology of *P. westermani*-like metacercariae from crabs

We isolated 89 *P. westermani*-like metacercariae from the crabs. All were spherical in shape and had thin walls (Fig 2). The thickness of the cyst wall in 30 specimens ranged from 4-14 μm, with an average of 8.7 μm. The longitudinal and transverse diameters of the cyst ranged from

Fig 1- Freshwater crabs, *Phricotelphusa aedes*, which serve as the second intermediate host of both *P. westermani* and *P. westermani*-like in southern Thailand.

Fig 2- Photomicrograph of fresh *P. westermani* metacercaria. Bar is 100 μm.

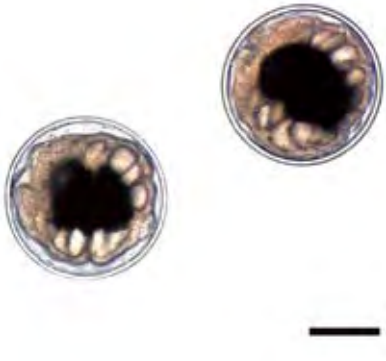

Fig 3- Photomicrograph of fresh *P. westermani*-like metacercariae. Bar is 100 μm.

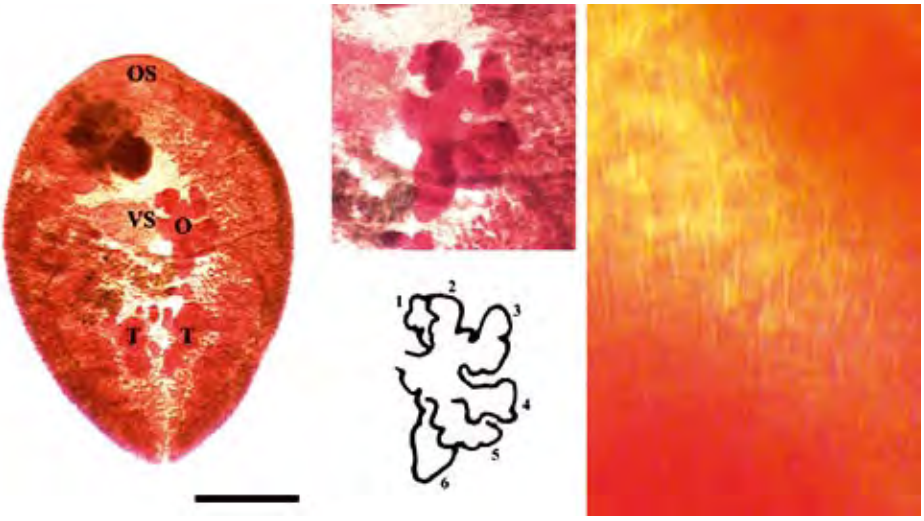

Fig 4- Adult worm of *P. westermani*-like from a cat inoculated with metacercariae. The worm had six-lobed ovary and singly spaced cuticular spines.

```
Pw1 ------------------------------------------------------------ 060
Pw2 TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA 060
PwL ............................................................ 060

Pw1 ----------------ATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pw2 TCGACATCTTGAACGC.............................................. 120
PwL ............................................................ 120

Pw1 TCGGCTTATAAACCATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT 180
Pw2 ............................................................ 180
PwL .........G...T.............................................. 180

Pw1 GATCTCCCCAATCTGGTCTTGTGCCTGTGGGGTGCCAGATCTATGGCGTTTCCCTAACAT 240
Pw2 ............................................................ 240
PwL ..........................................G......C.......... 240

Pw1 ACTCGCGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG 300
Pw2 ............................................................ 300
PwL .....G...................................................... 300

Pw1 GCTCAGTAAATGATTTATGTGCGCGTTTCGCTGTCCTGTCTTCATCTGTGGTTCATGTTG 360
Pw2 ............................................................ 360
PwL .......G.T..G..............C.............................T...... 360

Pw1 CGCGTGGTCTGCGTTCGATGCTGACCTACGTATGTGCCATGTGGTCCATTCTTCTGACCT 420
Pw2 ............................................................ 420
PwL ..............................................T.......C....... 420

Pw1 CGGATTAGACGTGAGTACC---------------------- 463
Pw2 ...................CGCTGAACTTAAGCATATCACTAA 463
PwL .....C..................................... 463
```

Fig 5- Aligned sequences of the ITS2 region from *P. westermani* strain Thailand (AF159604, Pw1), *P. westermani* (Pw2) and *P. westermani*-like (PwL). Identical bases are represented by dots. Hyphen indicates missing data. Numbers refer to nucleotide sequence length.

212-252 μm and from 204-240 μm, respectively, with an average of 227 x 221 μm.

The metacercariae of *P. westermani*, also isolated from the same crab hosts, were spherical in shape and had thick walls (Fig 3). The thickness of the cyst wall in five specimens ranged from 19-37 μm, with an average of 28.2 μm. The longitudinal and transverse diameters of the cysts ranged from 458-510 μm and from 438-501 μm, respectively, with an average of 492 x 480 μm.

Morphology of an adult worm

On postmortem examination of the test cat, 148 days after inoculation, 13 worms were recovered; 2 from the lungs (being paired in the worm cyst), 2 from the pleural cavity, and 9 from the liver. The worms from the lungs and pleural cavity were identified as either adults (one each, with eggs in the uterus) or pre-adults (without eggs), while the worms from the liver remained in the juvenile stage.

The size of the adult worm from the lung was 3.95 mm in length and 2.83 mm in width. The transverse diameters of the oral and ventral suckers measured 504 μm and 500 μm, respectively. The adult worm had a six-lobed ovary and singly spaced cuticular spines (Fig 4). The seminal receptacle was filled with spermatozoa.

ITS2 sequence analysis

The ITS2 region was amplified from DNA samples of individual *P. westermani* and *P. westermani*-like metacercariae using the consensus primers 3S and A28. Sequence analysis of the PCR products revealed that the aligned ITS2 region was 463 bp in length for both *P. westermani* and *P. westermani*-like samples. Pairwise comparison of the sequences showed 13 (2.8%) nucleotide differences (Fig 5). Similarity searches of the nucleotide databases GenBank/EMBL/DDBJ revealed that the ITS2 sequences of *P. westermani* were identical to those found in the databases under the accession number AF159604 for the *P. westermani* strain Thailand. However, the sequences of *P. westermani*-like did not exhibit a striking similarity to any of those found in the databases.

DISCUSSION

In this study, we observed adult *P. westermani*-like samples obtained from a cat that was inoculated with the metacercariae. The adult had an ovary that was simply divided into six lobes, a seminal receptacle filled with spermatozoa, and cuticular spines arranged singly. These morphological features at the adult stage are in good agreement with the description of *P. westermani* (Thai strain) (Sugiyama *et al*, 2001; Binchai *et al*, 2005). With regard to the morphology of metacercariae, other than the size, the features of *P. westermani*-like were almost identical to those of P. *westermani*. Therefore, it can be concluded that *P. westermani*-like should be classified as *P. westermani*, or as one of the members (a cryptic species) of the *P. westermani* complex (Blair *et al*, 1997), based on the anatomical similarities.

We investigated the susceptibility of feline hosts to *P. westermani*-like by experimental infection, and compared the results with those of *P. westermani*. From the cat experimentally infected with *P. westermani*, worms were detected only in the lungs or pleural cavity. The worms recovered were identified as adults or at least pre-adults (Binchai *et al*, 2005). In contrast, as shown in this study, juvenile *P. westermani*-like lodged predominantly in the liver, while some matured into adults in the pleural cavity or lungs. These findings suggested that the susceptibility in cats differed between *P. westermani* and *P. westermani*-like. The susceptibility of feline hosts to *P. westermani* was also examined using worms from Malaysia (Habe *et al*, 1996). About half of the worms recovered were identified as juvenile worms, but the principal domicile of the juveniles was not the liver but the skeletal muscles.

Molecular comparison based on ITS2 sequences revealed that there were a few nucleotide differences (2.8%) between *P. westermani* (*P. westermani* strain Thailand) and *P. westermani*-like. Therefore, in order to determine the proper taxonomic status of *P. westermani*-like, we need to investigate the detailed phylogenetic relationships between *P. westermani*-like and *P. westermani*. In terms of the susceptibility of *P.*

westermani-like, information regarding host-parasite relationships, particularly relating to the first intermediate hosts, is required. Studies into these issues are currently underway (Binchai *et al*, 2007).

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MOLECULAR SYSTEMATICS OF A NEW FORM OF *PARAGONIMUS WESTERMANI* DISCOVERED IN THAILAND

Sutheewan Binchai[1], Achariya Rangsiruji[1], Punsin Ketudat[1], Yasuyuki Morishima[2] and Hiromu Sugiyama[2]

[1]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok Thailand; [2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan

Abstract. This study aimed to clarify evolutionary relationships of *P. westermani*-like with other members of *Paragonimus* in Asia. The parsimony method was employed in molecular analyses of the second internal transcribed spacer (ITS2) region of nuclear ribosomal DNA and the partial cytochrome *c* oxidase subunit I (COI) region of mitochondrial DNA. A single most parsimonious tree obtained from the ITS2 region revealed two important groups within *P. westermani* complex that is based on geographical origins. From this study, it is evident that *P. westermani*-like is either placed well within the *P. westermani* complex or is located close to the complex. Since a significant genetic variation was observed between Thai *P. westermani* and *P. westermani*-like, further investigation on the specificity of first intermediate hosts should be carried out to determine a proper taxonomic status of *P. westermani*-like.

INTRODUCTION

Paragonimus westermani is widely distributed in Asia (Miyazaki, 1991). In Thailand, *P. westermani* metacercariae were reported in the central and southern parts of the country (Miyazaki, 1982; Kawashima *et al*, 1989). During our field survey, a new form of *P. westermani* metacercariae was discovered. The metacercariae obtained were almost identical to *P. westermani* metacercariae, except the size was smaller; thus, they were provisionally named *P. westermani*-like. Studies concerning the morphology of adult worms and susceptibility of feline hosts to *P. westermani*-like carried out by Sugiyama *et al* (2007) indicated that the adult worms resembled a diploid-type *P. westermani*, but the susceptibility in cats differed between *P. westermani* and *P. westermani*-like. This present study aimed to characterize genetically *P. westermani*-like as well as to clarify its phylogenetic relationships with other members of *Paragonimus* in Asia using nucleotide sequences of the ITS2 region of nuclear ribosomal DNA and a portion of the mitochondrial COI gene.

Correspondence: Achariya Rangsiruji, Department of Biology, Faculty of Science, Srinakharinwirot University, Sukhumwit 23, Bangkok 10110, Thailand.
Tel: 66 (0) 2664 1000 ext 8403;
Fax: 66 (0) 2260 0128
E-mail: achariya@swu.ac.th

MATERIALS AND METHODS

Parasite materials

Metacercariae of *P. westermani* and *P. westermani*-like were obtained from the waterfall crab, *Phricotelphusa aedes*, which were collected in Phanom District, Surat Thani Province. The metacercariae of other Thai *Paragonimus* species were harvested as follows: P. bangkokensis from *Ranguna smalleyi* (Phanom District, Surat Thani Province); *P. harinasutai* and *P. heterotremus* from *Larnaudia larnaudii* (Kaeng Khoi District, Saraburi Province) and *P. siamensis* from *Sayamia germaini* (Na Di District, Prachin Buri Province).

DNA sequencing and amplification

Total genomic DNA was prepared from individual metacercariae following Sugiyama *et al* (2002). The ITS2 region of nuclear ribosomal DNA and a portion of the mitochondrial COI gene were amplified by PCR and sequenced using primers 3S (forward: 5'-GGTACCGGTGGATCACTCGGCTCGTG-3'; Bowels *et al*, 1995), A28 (reverse: 5'-GGGATCCTGGTTAGTTTCTTTTCCTCC GC-3'; Blair *et al*, 1997), JB3 (forward: 5'-TTTTTTGGGCATCCTGAGGTTTAT-3'; Bowels *et al*, 1995) and JB 4.5 (reverse: 5'-TAAAGAAAGAACATAATGAAAATG-3'; Bowels *et al*, 1995), respectively. The PCR cycle consisted of three major steps: 98° C for 5 seconds to denature DNA, 55° C for 10 seconds

for primer annealing, and 72˚ C for 10 seconds for primer extension. The cycle was repeated 30 times, followed by a final extension at 72˚ C for 1 minute.

Sequence and phylogenetic analyses

Sequence alignments were carried out using Clustal X program (Jeanmougin *et al*, 1998) with additional sequences of *Paragonimus* species and *Fasciola hepatica* (outgroup) from GenBank database. The GenBank accession numbers of all sequences employed are shown in Table 1. Phylogenetic trees were reconstructed using maximum parsimony analysis with a branch-and-bound algorithm. Alignment gaps were treated as missing data; all characters were assigned equal weight. The reliability of internal branches of the trees was assessed using the bootstrap method (Felsenstein, 1985), with 1,000 replicates. All phylogenetic analyses were performed using PAUP* version 4.0b (Swofford, 1998).

RESULTS

Metacercariae of *Paragonimus* species employed in this study were shown in Fig 1.

Sequence characteristics

ITS2. The actual length range of the ITS2 region of the ingroup was 359-363 bp. The alignment of this region of 15 taxa of *Paragonimus* species and its outgroup was 378 bp in length, with 10 sites of insertion or deletion. Out of 378 total characters, 230 (60.8%) were constant, 92 (24.4%) were parsimony-uninformative and 56 (14.8%) were parsimony-informative. Sequence divergence between ingroup and outgroup taxa obtained from pairwise distance analysis ranged from 37.6-41.9% but within the ingroup the sequence divergence range was 0-13.7%. The mean G+C content of all taxa was 55.5%, and transition/transversion ratio was 2.60.

COI. The actual length of the partial COI region of the ingroup was 381 bp. The alignment of this region for all 15 taxa under study was 384-bases long, with only one site of deletion. From 384 characters, 241 (62.8%) were constant, 38 (9.9%) were parsimony-uninformative, and 105 (27.3%) were parsimony-informative. The sequence divergence between ingroup and outgroup taxa was computed using pairwise distance analysis, and ranged from 22.2-34.7%;

Table 1
GenBank accession numbers of *Paragonimus* and *Fasciola* used in this study.

Species	Origin	ITS2	COI
P. westermani	Hyogo, Japan	U96907	U97205
	Minchin, China	U96907[a]	AY140681
	Haenam, Korea	AF333278	AF333281
	Karapai, Taiwan	U96908	AY140673
	Philippines	U96910	U97213
	Malaysia	U96909	U97211
	Central Thailand	AF159604	U97212
	Southern Thailand	AB354216	AB354224
P. westermani-like	Thailand	AB354218	AB354225
P. macrorchis	Thailand	AF159608	AF159598
P. heterotremus	Thailand	AB354221	AB354229
P. harinasutai	Thailand	AB354220	AB354226
P. bangkokensis	Thailand	AB248091	Ab354228
P. siamensis	Thailand	AB354222	AB354231
Fasciola hepatica	Australia	AB207148	AF216697

[a]sequence identical to *P. westermani* from Hyogo, Japan (Blair *et al*, 1997).

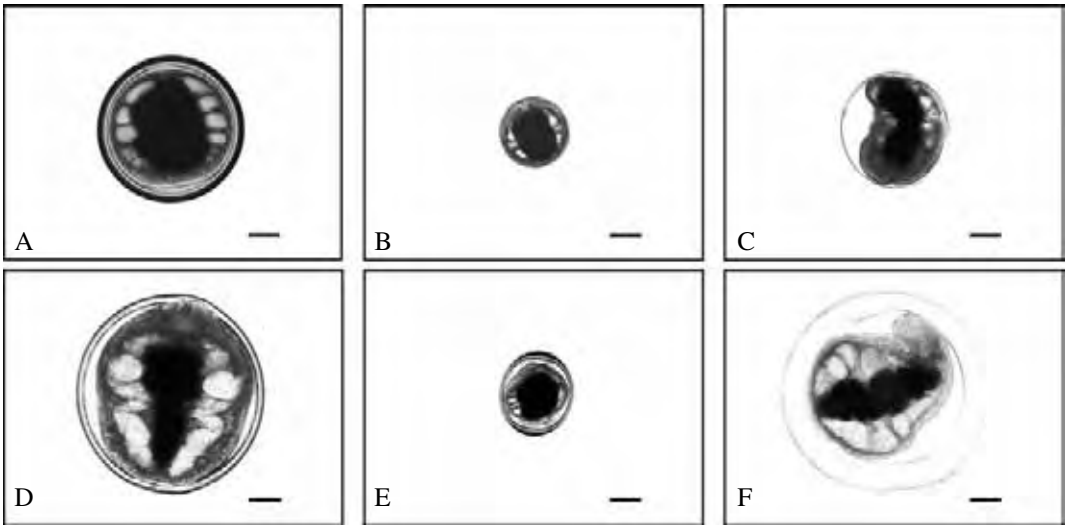

Fig 1- Metacercariae of *Paragonimus* species A: *P. westermani*, B: *P. westermani*-like, C: *P. bangkokensis*, D: *P. harinasutai*, E: *P. heterotremus* and F: *P. siamensis*. Scale bar indicates 100 μm.

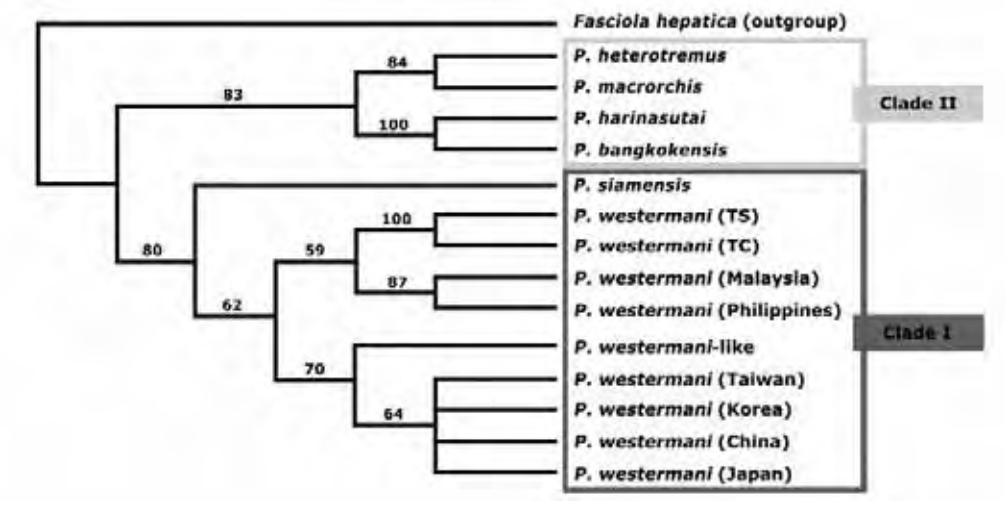

Fig 2- Single most parsimonious tree of length 191 steps based on parsimony analysis of the informative characters of the ITS2 region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. *P. westermani* (TS) = *P. westermani* from southern Thailand. *P. westermani* (TC) = *P. westermani* from central Thailand.

whereas, within the ingroup, it ranged from 0.3-25.3%. The mean G+C content was 44.0%, and transition/transversion ratio was 3.85.

Phylogenetic analyses

ITS2. A single most parsimonious tree (Fig 2) of length 191 steps was obtained based on parsimony analysis of the informative characters with 1,000 bootstrap replicates. Fit measures of the tree were as follows: consistency index (CI) = 0.9058, homoplasy index (HI) = 0.0942, retention index (RI) = 0.8393, and rescaled consistency index (RC) = 0.7602. The phylogenetic tree comprised two clades: clade I, including the *P. westermani* complex and *P. siamensis* (bootstrap value (BS) = 62%), and clade II, including other Thai *Paragonimus* species (BS = 83%). Within the *P. westermani* complex, two groups of organism can be obtained based on geographical distribution. The first group

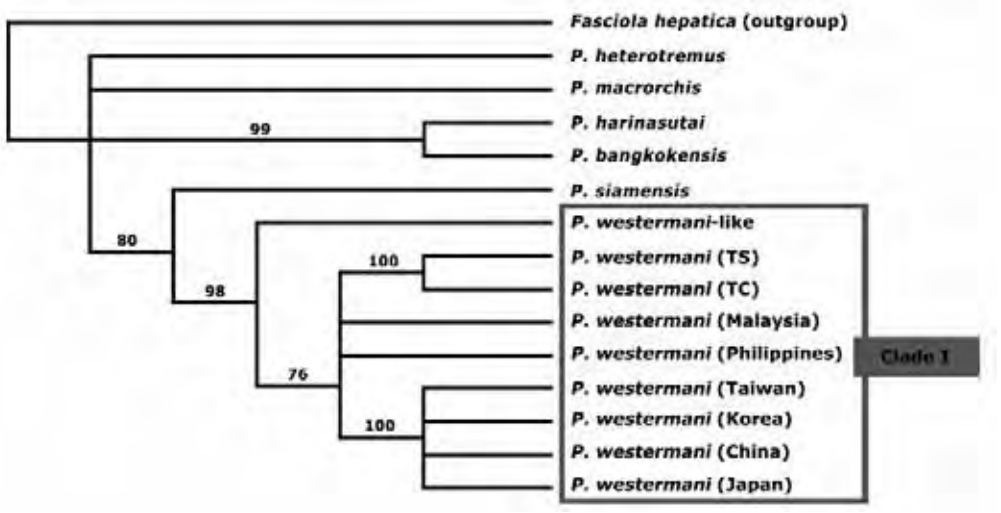

Fig 3- Strict consensus tree derived from 10 equally parsimonious trees of length 319 steps based on parsimony analysis of the informative characters of the partial COI region. Numbers above the branches are bootstrap values (%) of 1,000 replicates. *P. westermani* (TS) = *P. westermani* from southern Thailand. *P. westermani* (TC) = *P. westermani* from central Thailand.

contains *P. westermani* from Southeast Asia (BS = 59%), while the second group contains *P. westermani* from East Asia and *P. westermani*-like from Thailand (BS = 70%).

COI. A strict consensus tree (Fig 3) was derived from 10 equally parsimonious trees of 319 steps long, based on parsimony analysis with 1,000 bootstrap replicates. Fit measures of the tree were as follows: CI = 0.6364, HI = 0.3636, RI = 0.6822, and RC = 0.4341. The tree inferred from the partial COI region showed a single clade with strong bootstrap support of 98%. This clade forms a complex of *P. westermani* from Southeast and East Asia (BS = 76%). *Paragonimus westermani*-like is excluded from the complex and designated as a sister group.

DISCUSSION

The alignment of the ITS2 region of *Paragonimus* species and its outgroup was 378 bp in length which was similar to those of other digeneans such as *Schistosoma* (398 bp; Bowles *et al*, 1995) and *Fasciola* (364 bp; Mas-Coma et al, 2001). The level of sequence variation between *P. westermani*-like and *P. westermani* (1.39-4.0%) was close to the intraspecific variation within *P. westermani* from different geographical origins (0-3.41%). Intraspecific variation in the ITS2 region was also observed in other digeneans, including *Schistosoma* (Agatsuma *et al*, 2001) and *Fasciola* (Adlard *et al*, 1993).

The numbers of the variable characters of the partial COI (143 characters) and the ITS2 (148 characters) sequences were almost equal. However, this region of the COI gene exhibited approximately two-times more informative characters (27.3%) than the ITS2 region (14.8%). Nonetheless, a remarkably large amount of homoplasy was observed in the COI data (HI = 0.3636) as compared to the ITS2 data (HI = 0.0942).

From this study, the phylogenetic tree inferred from the ITS2 region showed that *P. westermani* formed a complex of cryptic species and could be divided into two groups as previously reported (Blair *et al*, 1997, 1998). The first group comprises *P. westermani* from Southeast Asia (Thailand, Malaysia, and the Philippines), and the second group composes of *P. westermani* from East Asia (Taiwan, Korea, China, and Japan), which was closely related to *P. westermani*-like. In contrast to the ITS2 tree, the phylogenetic tree reconstructed from the COI region revealed that *P. westermani*-like is excluded from the complex and

designated as a sister group. Thus, it is evident that *P. westermani*-like is either well placed within the *P. westermani* complex (ITS2 data), or it is located close to the complex (COI data). However, since the protein-coding gene (COI) is uder selective constraint while the non-coding ITS region is not, this suggests that the spacer is free to diverge and evolve with a rate that is close to the neutral rate of sequence evolution. In addition, due to such a high level of homoplasious characters present in the COI data, the tree inferred from the ITS2 data would be more reliable. This result of *P. westermani*-like being classified as one of the members of the *P. westermani* complex was strongly supported by the morphological characters of the adult worms (Sugiyama *et al*, 2007).

Since the susceptibility of feline hosts to *P. westermani*-like was found to be different from that of Thai *P. westermani* (Sugiyama *et al*, 2007) and a significant genetic variation was also observed between them, further investigation on the specificity of first intermediate hosts should be carried out to determine the proper taxonomic status of *P westermani*-like.

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ASSESSMENT OF THE EFFICACY, SAFETY, AND TOLERABILITY OF PRAZIQUANTEL AND TRICLABENDAZOLE IN THE TREATMENT OF PARAGONIMIASIS

VY Belizario[1], AO Mallari[1], WU de Leon[2] and AC Lucero[1]

[1]National Institutes of Health, University of the Philippines Manila; [2]College of Public Health, University of the Philippines, Manila, the Philippines

Abstract. This was a community-based, double-blind, randomized, controlled therapeutic trial undertaken at the municipality of Roxas to determine the efficacy, safety and tolerability of triclabendazole (10 mg/kg, single dose) versus praziquantel (25 mg/kg, three times daily) for three days at 30, 60, and 90 days posttreatment. This study concludes that triclabendazole administered at 10 mg/kg single dose has comparable efficacy, safety, and tolerability with praziquantel 25 mg/kg given three times a day for three days.

INTRODUCTION

Pulmonary paragonimiasis is a parasitic infection caused by lung flukes of the genus *Paragonimus*, of which *P. westermani* is the most common species infecting humans. It is acquired through ingestion of metacercariae, which are found in freshwater crabs and crayfish. Once ingested, the metacercariae are released, develop in the gut, and then migrate to other organs, most commonly to the lungs. Paragonimiasis manifests as fever, chest pain, cough, hemoptysis, dyspnea, and night sweats. The most sensitive, reliable method of diagnosis is the identification of *Paragonimus* eggs in the sputum, stool, or pleural fluid (Beaver *et al*, 1984).

Paragonimiasis is endemic in identified rural communities in the Philippines. These endemic areas are in the provinces of Mindoro, Camarines, Sorsogon, Samar, Leyte, Davao, Cotabato, and Basilan. There has been a re-emergence of cases of pulmonary paragonimiasis; such that, more recently, the provinces of Davao Oriental, and Zamboanga del Norte have been added to the list of endemic areas (Cabrera, 1979; Belizario and Malte, 2004).

Correspondence: VY Belizario, National Institute of Health , University of the Philippines Manila, 625 Pedro Gill Street, Ermita, Manila 100, The Philippines.
Email: vbelizar@yahoo.com

The drug of choice for pulmonary paragonimiasis is praziquantel at 25 mg/kg, three times daily, for three days (Johnson *et al*, 1985). It causes flaccid paralysis of the fluke by blocking calcium homeostasis. It also damages the fluke's tegument, which makes it susceptible to host defense mechanisms (Pearson and Guerrant, 1983). Cure rates for praziquantel have been reported to approximate 100% (Calvopina *et al*, 1998); however, in rural communities, patient compliance has been demonstrated to be negatively influenced by adverse drug reactions and the need to take the drug for three consecutive days (Calvopina *et al*, 2003).

Compliance of patients to the prescribed medication contributes significantly to the control of paragonimiasis. Compliance, in turn, is dictated by the availability, ease of administration, safety, and tolerability of medications.

Triclabendazole is a benzimidazole compound with the following selective actions against trematodes; it produces metabolites that block ATP production by inhibiting protein synthesis, and inhibits microtubule formation. Triclabendazole targets both mature and immature flukes (Laburte, 1999). Based on limited published literature, it is a highly effective drug, with an efficacy rate comparable with praziquantel. Moreover, patients who were treated with triclabendazole reported clinical tolerance to its adverse effects that is superior to that of praziquantel. Gastrointestinal symptoms were much less common, and no serious adverse events were noted in patients

treated with triclabendazole (Calvopina *et al*, 1998, 2003).

Because of the above findings, it is valid to explore the use of triclabendazole, with its documented efficacy and tolerability, in endemic areas to improve the control of paragonimiasis. This study was undertaken to determine the efficacy, safety, and tolerability of triclabendazole, using the following criteria: (1) improvement in clinical symptoms and physical findings; (2) cure rates or absence of lung fluke ova in the sputum at 30, 60, and 90 days posttreatment; and (3) occurrence of adverse drug events.

MATERIALS AND METHODS

Study site and patient selection

This double-blind, randomized, controlled, therapeutic trial was conducted at the Municipality of Roxas in Zamboanga del Norte Province, from December 2005 to March 2006. The site was chosen because it was known to be endemic for paragonimiasis, with a prevalence rate of 27.2% in 2002 (de Leon and Piad, 2005). Moreover, the Roxas Municipal Health Office reported that paragonimiasis ranked as the fourth-leading cause of morbidity in 2003 (Roxas RHU, 2003).

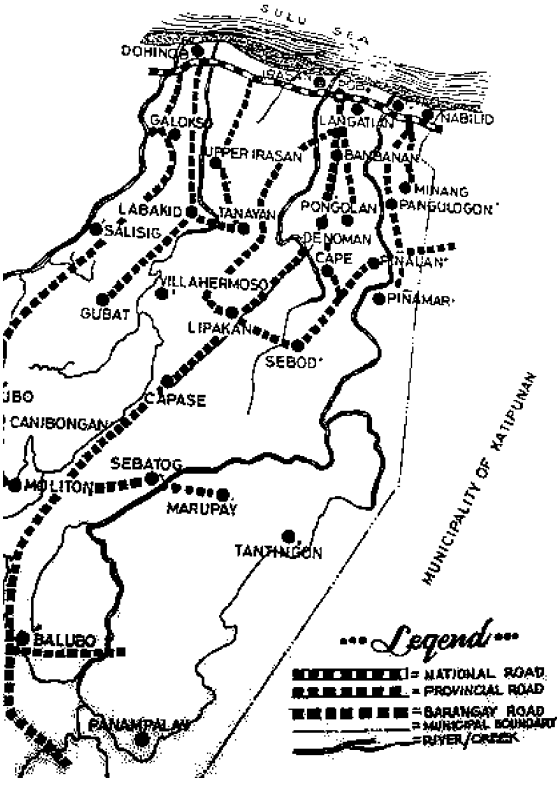

Fig 1- Map of municipality of Roxas, Zamboanga del Norte (Roxas RHU, 2003).

Roxas Municipality has a total land area of 27,082.28 hectares, mostly with hilly to mountainous topography. One-third of the municipality is situated along a 10-kilometer coastline. Four wide rivers (Dohinob Daku, Dohinob Diut, Tangian, and Piao) and three prominent creeks (Irasan, Langatian, and Minang) traverse this municipality (Roxas RHU, 2003). The location of the barangays surveyed in the study relative to these rivers and creeks is described in Fig 1. *Kampays*, or freshwater crabs, which abound in these natural bodies of water, serve as the intermediate hosts of *Paragonimus*.

Roxas Municipality comprises 31 barangays, 26 of which are dependent on agriculture, while the remaining five depend on marine-related livelihood. Health needs are provided by a main health center located within the Poblacion area, and nine barangay health stations are dispersed throughout the municipality. Roxas has 53 traditional birth attendants (38 trained and 15 untrained) and 33 active barangay health workers (BHWs) (Roxas RHU, 2003).

Currently, Roxas is a third-class municipality; that is, having an average annual income, during the last three calendar years, of 21,000,000-27,000,000 pesos. Farmers comprise 62% of the working population (Roxas RHU, 2003). The *kampays*, which are readily available from rivers, are frequently eaten raw, a delicacy with low costs. Informal interviews with the participants in the study provided information that this cultural practice was popular in the municipality.

The sample size required for the study was 72 (36 patients for each treatment group) and was estimated based on the assumption that the cure rates for the two treatment groups will be the same, with an allowable difference of 5%. Power Analysis and Sample Size (PASS, version 1.0) software was used to verify the calculated sample size.

The following inclusion criteria were applied

in the study: presence of chronic productive cough lasting for at least four weeks, or history of anti-TB treatment without observable clinical improvement; positive *Paragonimus* eggs on sputum examination; patients with the following characteristics were excluded from the study; age less than 15 years; pregnancy; history of acute or chronic disease of the liver or kidney; and history of drug hypersensitivity.

Treatment designation

Patients who were enrolled in the study were randomly assigned to either praziquantel or triclabendazole treatment groups. The patients who belonged to the praziquantel group were given praziquantel 25 mg/kg, three times daily, for three days; while those included in the triclabendazole group received triclabendazole 10 mg/kg, in a single dose.

Triclabendazole (Egaten®) was provided by Novartis International AG, (Switzerland), while praziquantel (Distocide®) was provided by Shin Poong Pharmaceutical (Korea). All drugs were procured by the World Health Organization (WHO) and forwarded to the University of the Philippines Manila (UP Manila).

Patient and parasitologic assessment

Baseline clinical assessment, which focused on history-taking and chest auscultation, was done on the patients. The patients were monitored for the occurrence of adverse drug events at 30 minutes, 24 hours, 48 hours, and 72 hours post-treatment. Patients who experience severe adverse events (SAE) were to be reported promptly to the Clinical Safety and Epidemiology, Novartis Healthcare Philippines. Compliance to the drugs was subjectively assessed through direct inquiry of the patients.

Sputum specimens were assessed for color and consistency; and were processed using 3% sodium hydroxide (NaOH) and centrifuged at high-speed setting for five minutes, after which, the resulting sediment was examined. A reference microscopist validated all positive specimens and re-read all negative specimens.

Statistical analysis

Results of the clinical and parasitologic assessments were encoded using EpiInfo (v 6) software. Re-encoding was done to ensure accuracy of data entry. Accordingly, actual values were used to describe efficacy, safety, and tolerability data. Fisher's exact probability test was utilized to compare and determine significant differences between the parasitologic and clinical cure rates of praziquantel and triclabendazole.

An intention-to-treat approach was applied in this study to account for the dropouts in both treatment groups. Dropouts were assigned the worst possible outcome, described as follows: no improvement in reported symptoms, no resolution of previous abnormal chest examination findings, and positive *Paragonimus* egg in sputum specimen.

Ethical considerations

The study was undertaken in accordance with Good Clinical Practice Guidelines and the Declaration of Helsinki, and was approved by the Technical and Ethical Review Board, Research Implementation and Development Office (RIDO), College of Medicine, UP Manila. For ethical reasons, all patients screened with documented pulmonary paragonimiasis were given treatment.

RESULTS

Baseline findings

A total of 378 patients qualified for sputum examination. Of the 378 patients screened, 56 had positive *Paragonimus* eggs in their sputum, or paragonimiasis prevalence rate of 14.8%. Nine patients, found to be infected, were excluded from the study. Reasons for their exclusion included failure to indicate informed consent, anticipated difficulty in follow-up due to distance of place of residence, and physical disability.

Of the 47 patients enrolled in the study, 23 were randomly assigned to the praziquantel group, and 24 were assigned to the triclabendazole group. Thirty participants were < 45 years of age, while the remaining 17 participants belong to the ≥ 45 years age group. The gender distribution was nearly equal, with 24 males and 23 females. Almost half (42.6%) of the participants resided in Barangay Sibatog (Table 1).

Table 1
Paragonimus infection rates and distribution of study participants, by barangay.

Barangay	Number surveyed	No. positive for *Paragonimus* (%)	No. participants included in study (%)
Sibatog	86	24 (27.9)	20 (42.6)
Marupay	58	9 (15.5)	9 (19.1)
Piñalan	55	8 (14.5)	9 (19.1)
Piñamar	49	4 (8.2)	3 (6.4)
Pangologon	25	3 (12.0)	3 (6.4)
Tantingon	30	5 (16.7)	1 (2.1)
Sebod	12	3 (25.0)	2 (4.3)
Others: Balubo, Canubongan, Capasi, Coribongon, Denoman, Gubot, Moliton, Panapaloy	63	0 (0)	0 (0)
Total	378	56 (14.8)	47 (12.4)

Chronic productive cough was the most common clinical manifestation elicited during history-taking, with all participants in both treatment groups complaining of it. Baseline abnormal breath-sound findings were minimal for both treatment groups. On auscultation, only five patients in the praziquantel group and six patients in the triclabendazole group were documented to have crackles/rales. Wheezing was noted in one patient in each of the treatment groups.

Sputum submission and clinical re-examination during follow-up were completed for 30 and 60 days post-treatment. However, on day 90, four patients in the praziquantel group and five patients in the triclabendazole group were unavailable for physical examination. Moreover, four patients in the praziquantel group and three patients in the triclabendazole group failed to submit sputum specimens on day 90.

Treatment efficacy

For the clinical response to treatment based on change in symptoms from initial assessment to day 90 follow-up, all except one patient in the praziquantel group and all patients in the triclabendazole group reported an improvement relative to their previous complaint of chronic productive cough. All patients in the praziquantel group and all but two patients in the triclabendazole group reported an improvement relative to the initial complaint of weight loss. All patients in both treatment groups reported improvements relative to initial complaints of hemoptysis, dyspnea, fever, and night sweats during day 90 follow-up. The difference in improvement in symptoms on day 90 between the two treatment groups was not statistically significant (Table 2).

Crackles/rales resolved in three-out–of-five patients in the praziquantel group. Of the six patients with crackles/rales at day 0 in the triclabendazole group, crackle/rales resolved in three patients. The remaining three patients in the same group dropped out of the study. Wheezing in one patient in the praziquantel group resolved at day 90, while another patient with wheezing in the triclabendazole group was lost to follow-up. Nonetheless, the difference in the resolution of abnormal chest findings on day 90 between the treatment groups was not statistically significant (Table 3).

For parasitologic cure, the number of infected patients rapidly dropped from an initial 23 to only five patients for the praziquantel group, while 11 out of 24 patients remained infected in the triclabendazole group on first follow-up (day 30). On day 60, the patients with *Paragonimus* infection further reduced to two in the praziquantel

group, while 11 patients were still infected in the triclabendazole group. On day 90, one patient in the praziquantel group remained infected, while six patients still had positive sputum findings in the triclabendazole group (Fig 2). The cure rate of praziquantel was 96.0%, while that of triclabendazole was 75.0%, on day 90 post-treatment. Intention to treat analysis showed no significant difference in the cure rates between praziquantel and triclabendazole (Fisher's exact probability = 0.29).

Compliance

All patients in the praziquantel group reported completing their intake of all tablets provided for the prescribed three-day period. All patients in the triclabendazole group took the drug provided on the first day of treatment.

Safety and tolerability

A total of sixteen patients reported adverse events that were assessed to be mild (Table 4). Of the 16 patients, nine belong to the praziquantel group, while seven belong to the triclabendazole group. There was no serious adverse event reported or observed.

Headache was the most common complaint of patients 30 minutes post-treatment in both groups. Dizziness was more frequently reported in the praziquantel than in the triclabendazole group.

Table 2
Status of reported symptoms, by treatment group, days 0 and 90.

Reported symptoms	Praziquantel				Triclabendazole				Fisher's exact probability[a]
	Day 0	Day 90			Day 0	Day 90			
		No. improvement	With improvement	Lost to follow-up		No. improvement	With improvement	Lost to follow-up	
Chronic productive cough	23	1	18	4	24	0	19	5	1.00[b]
Hemoptysis	16	0	13	3	22	0	17	5	1.00[b]
Dyspnea	11	0	9	2	12	0	9	3	1.00[b]
Fever	8	0	5	3	8	0	7	1	0.57[b]
Weight loss	11	0	9	2	13	2	6	3	0.10[b]
Night sweats	15	0	14	1	16	0	12	4	0.33[b]

[a]Fisher's exact probability computed using data applied with intention to treat analysis
[b]not statistically significant

Table 3
Status of abnormal chest findings, by treatment group, days 0 and 90.

Abnormal chest findings	Praziquantel			Triclabendazole			Fisher's exact probability[a]
	Day 0	Day 90	Lost to follow-up	Day 0	Day 90 follow-up	Lost to	
Crackles/ Rales	5	2	0	6	0	3	1.00[b]
Wheezes	1	0	0	1	0	1	1.00[b]

[a]Fisher's exact probability computed using data applied with intention to treat analysis; [b]not statistically significant

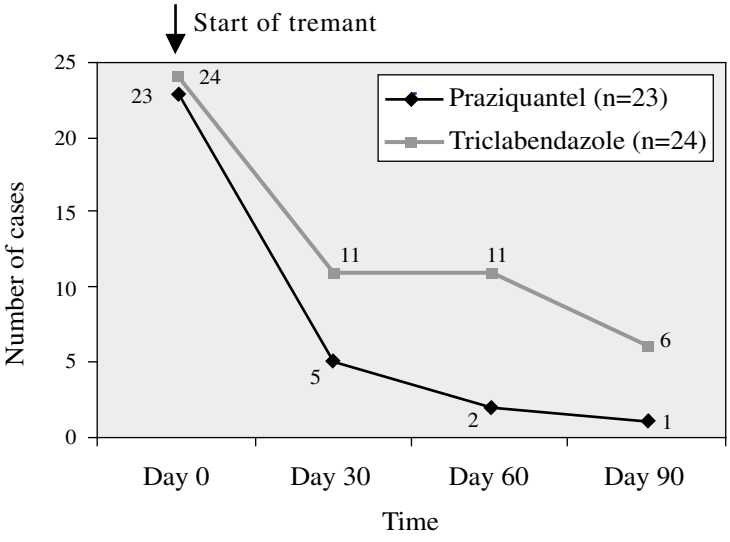

Fig 2- *Paragonimus* infection according to treatment group from day 0 to day 90.

Table 4
Adverse drug events and status, 30 minutes up to 24 hours posttreatment, according to treatment groups.

Adverse Event	Praziquantel					Triclabendazole				
	No. of reported adverse events	Status of adverse event				No. of reported adverse events	Status of adverse event			
		Re-solved	Im-proving	Con-tinuing	Wor-sening		Re-solved	Im-proving	Con-tinuing	Wor-sening
Headache	4	2	2	0	0	5	2	2	0	0
Dizziness/vertigo	4	4	0	0	0	1	1	0	0	0
Nausea/vomiting	1	1	0	0	0	0	0	0	1	0
Chest pain	0	0	0	0	0	1	0	0	0	0
Fever	0	0	0	0	0	0	0	0	0	0
Anorexia	0	0	0	0	0	0	0	0	0	0
Abdominal pain	0	0	0	0	0	0	0	0	0	0
Diarrhea	0	0	0	0	0	0	0	0	0	0
Allergic reaction/ urticarial	0	0	0	0	0	0	0	0	0	0
Others	0	0	0	0	0	0	0	0	0	0
Total	9	7	2	0	0	7	3	2	1	0

One patient complained of nausea/vomiting in the praziquantel group, while another reported chest pain in the triclabendazole group.

Headache resolved in the two patients from each of the treatment groups within 24 hours post-treatment. The other two patients from each group, who complained of headache, noted continuing improvement within 24 hours post-

treatment. The status of headache in one patient in the triclabendazole group was not determined. Nausea/vomiting resolved in all patients from both treatment groups who complained initially of it. Chest pain persisted but resolved within 24 hours in a patient in the triclabendazole group.

DISCUSSION

The prevalence rate of paragonimiasis in the municipality, as determined in the study, was 14.8%. This was much lower when compared with the previous prevalence rate of 27.2% in the area, in 2002 (de Leon and Piad, 2005). It should be noted that, in that particular study, surveillance was conducted only in Barangay Sibatog, which has the highest infection rate among the barangays. As such, the prevalence rate in that study would likely be higher than the one found in this study. Another important implication of this current prevalence rate is the probable ineffectiveness of the existing control program to curb paragonimiasis in the municipality. Possibly, residents in the municipality remain infected or they become continually re-infected after being previously cured. The former possibility may reflect inaccessibility to medical care due to topographical limitations imposed by the area, while the latter may reflect the difficulty of changing eating practices through health promotion and education.

The prevalence rate in Roxas is comparable to that of other areas in the country. As early as 1974, the prevalence rate of paragonimiasis was found to be high, at 12.5% in the province of Leyte (Cabrera and Fedival, 1974). In 1992, *Paragonimus* eggs were found in the sputum smears of patients from Basilan who were notable due to their lack of response to anti-TB medications (Infante *et al*, 1992). In Sorsogon Province, the municipality of Irosin had a prevalence rate of 16.3%, while that of the municipality of Casiguran was 25% (Belizario *et al*, 1997a,b;1998). In Davao del Norte Province, a project team from the DOH found an infection rate of 18.8% among 320 subjects (Zuazula et al, 2000).

The study confirmed the efficacy of praziquantel against paragonimiasis. The rapid decrease in the number of infected patients from day 0 to day 90 and the marked improvements on symptoms reported by patients validate the efficacy of praziquantel, which agrees with previous studies (Johnson *et al*, 1985; Calvopina *et al*, 1998). However, for community-based control of paragonimiasis, the use of praziquantel poses problems because compliance by patients with the drug would be discouraged by the three-day dosage requirement as well as the frequency of the adverse events due to it (Calvopina *et al*, 2003). In this study, compliance, which was assessed subjectively, was satisfactory; however, it was not validated through an objective means.

Triclabendazole was shown to have a comparable efficacy with praziquantel in this study. Clinical improvement in triclabendazole was comparable with praziquantel. It must be noted, however, that the time of resolution of the symptoms was not determined in this study. Therefore, there are no findings to compare the immediacy with which either drug accorded symptomatic relief.

For parasitologic cure, although the praziquantel group showed a more rapid drop in infection rate and a smaller number of infected patients relative to the triclabendazole group, the statistical difference between the two cure rates was not significant. This finding is in agreement with a previous similar trial (Calvopina *et al*, 1998). The 10 mg/kg dose used in this study, which was proven effective in significantly reducing fluke burden in patients, was similar to the dose tested in the previous trials on triclabendazole in the treatment of paragonimiasis (Ripert *et al*, 1992; Calvopina *et al*, 2003).

In a study by Calvopina *et al* (1998), the clearance from *Paragonimus* eggs in the sputum of patients was 100% (15/15 subjects) for praziquantel and 87.5% (14/16 subjects) for triclabendazole by 90 days post-treatment. In this trial, six patients in the triclabendazole group and another patient in the praziquantel group remained infected on day 90 post-treatment. A high parasitic load might explain these findings. It was observed that most of the patients who failed to clear of *Paragonimus* eggs had high fluke burdens (Calvopina *et al*, 2003).

Unfortunately, the fluke burden of the patients was not determined in this study. Failure to clear *Paragonimus* eggs may possibly be explained by a continuing re-exposure to the parasite, which may have resulted in reinfection after the initial treatment. Persistence of infection suggests two recommendations. For such patients, a 2-dose regimen of triclabendazole (*ie*, 10 mg/kg given on two consecutive days) may be advocated, or the administration of another course of praziquantel, using 25 mg/kg three-times daily, for three days, may be considered (Calvopina *et al*, 2003). Moreover, health education regarding transmission and changes in cooking/eating practices must be strengthened.

In this study, one patient treated with praziquantel was found to be positive for *Paragonimus* eggs on day 90, following previous negative readings. Moreover, for the triclabendazole group, one patient remained persistently infected from day 60 onwards, after being cleared on day 30, while another patient had a negative sputum finding on day 30, but was positive on day 60, then negative again on day 90 post-treatment. These findings may be explained by possible false negative readings of the sputum specimens. It is worth mentioning that patients with low fluke burden may be completely asymptomatic, and sputum examination may be negative. *Paragonimus* ova may only be found in the stool for such patients (Yokogawa, 1965). As a recommendation, the sensitivity of sputum examination for *Paragonimus* eggs can be improved with a 24-hour collection (Johnson *et al*, 1985).

As for safety and tolerability, triclabendazole had slightly lower reporting of adverse drug events compared to praziquantel. It is also worth mentioning that both treatment groups did not yield any serious adverse drug events. Previous studies also found that triclabendazole was well tolerated by patients (Ripert *et al*, 1992; Calvopina *et al*, 1998, 2003).

This study therefore concludes that triclabendazole, administered at 10 mg/kg, in a single dose, has comparable efficacy, safety, and tolerability when compared to praziquantel 25 mg/kg, given three-times daily, for three days. It can thus be recommended as an alternative drug for paragonimiasis.

Because of the low sample size, the power of this study is limited. It is recommended that a larger sample size be used in a similar study. For efficacy, the earliest time of the resolution of clinical symptoms may be monitored and compared with parasitologic cure times. More objective measurements of clinical cure, such as chest radiograph and blood work-up, may be utilized to complement the physical examination findings. A more objective means of measuring compliance with medications may also be utilized.

Pursuant to the decentralization of health delivery and management as advocated by the Health Sector Reform Agenda, the task of safeguarding the people health of the people has been relegated to the local government units (LGUs). This community-based trial was undertaken in collaboration with the provincial and municipal governments of Roxas, Zamboanga del Norte. Highly organized field work by the RHU staff resulted in satisfactory follow-up among the patients included in the study. LGUs, through the rural health units (RHUs), have a great but mostly untapped potential to initiate and spearhead surveillance and control projects. The surveillance process in this study may be used by the Roxas municipal government to jumpstart a larger surveillance and control project for paragonimiasis. Alternatively, the project may be integrated into the more established surveillance and control scheme of pulmonary tuberculosis (TB), which exists in this area and is misdiagnosed as paragonimiasis.

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REDESCRIPTION OF THE TREMATODE METACERCARIAE FROM THE MULLET (*LIZA SUBVIRIDIS*) AND HALF-BEAK (*DERMOGENYS PUSILLUS*)

Janejira Pubua and Chalobol Wongsawad

Department of Biology, Faculty of Science, Chiang Mai University, Thailand

Abstract. Metacercariae from mullet and half-beak fish were examined to identify the morphological characteristics under a light microscope. Both encysted and excysted metacercariae were described and illustrated. The results of this study showed that these two trematodes species are different. They should be furter confirmed by other scientific methods such as experimental host infection to discover adult or DNA fingerprints.

INTRODUCTION

Mullet, *Liza subviridis*, is a brackish water fish that is a popular food among residents in coastal areas. Most people eat this fish raw; therefore, they are at risk of infection with the metacercarial trematode, especially *Stellantchasmus* sp, because the mullet were reported to be the second intermediate host for *Stellantchasmus* (Seo *et al*, 1979, 1984; Sohn *et al*, 1989; Hong, 2000). A group of heterophyid metacercariae encysted in the flesh mullets were identified to be *Stellantchasmus falcatus*, which is the same species of metacercariae found in the fresh water half-beak fish (*Dermogenys pusillus*) (Chai and Sohn, 1988; Saenphet *et al*, 2001; Wongsawad *et al*, 2004). The purpose of this study was to identify the metacercariae from these two fish species and describe the morphological characteristic of the encysted and excysted metacercariae.

MATERIALS AND METHODS

Twenty mullets, *Liza subviridis*, 15-25 cm long were purchased from a local fish market in Samut Sakhon Province. In the laboratory, their muscles were artificially digested using an acid pepsin solution (1 ml conc hydrochloric acid, 1 g pepsin, and 99 ml 0.85% sodium chloride solution for 1½ hours at 37°C). The digested muscle was rinsed with 0.85% sodium chloride solution and examined for *Stellantchasmus* metacercariae (Boonchot and Wongsawad, 2005). The metacercariae were collected, counted to calculate the prevalence and intensity, and measured. Both encysted and excysted metacercariae were processed on a permanent slide, fixed in 4% formalin and stained with borax's carmine, dehydrated in an alcohol series, and mounted with Permount. These permanent slides were used to examine the morphological characteristics under a compound microscope and compared with the metacercariae from half-beak fish, *Dermogenys pusillus*. Moreover, *Stellantchasmus falcatus* metacercariae were obtained from the body cavity of the half-beak fish and processed on permanent slides, which were then used to compare with the morphological characteristics of the metacercariae from the mullets.

Correspondence: Ms Janejira Pubua, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail: janejira_pubua@yahoo.com

RESULTS

The body size and details are shown in Table 1. The body size of the metacercariae from the mullets were larger than those from half-beak fish (metacercariae from mullets were 0.15-0.21 mm long, but metacercariae from the half-beak fish were 0.05 mm long) (Fig 1A, B). The excysted metacercariae of the mullet had longer organs than those of the half-beak fish. From Table 1, there were many characteristic differences between the excysted metacercariae of the mullet and the half-beak fish (Fig 2A, B). For example, the body length, body width, esophagus, ovary length, testis (right) length and

Table 1
Measurements of excysted metacercariae from mullets in comparison with those from half-beak fish.

Items	Excysted metacercariae from mullets (mm)	Excysted metacercariae from half-beak fish (mm)
Body length	0.667-0.680	0.188-0.312[a]
Body width	0.255-0.260	0.110-0.182[a]
Oral sucker		
Length	0.040-0.045	0.025-0.042
Width	0.050-0.055	0.035-0.050
Prepharynx (length)	0.020-0.022	0.003-0.020
Pharynx		
Length	0.032-0.035	0.020-0.035
Width	0.025-0.030	0.015-0.022
Esophagus (length)	0.140-0.145	0.035-0.087[a]
Ventral sucker		
Length	0.027-0.030	0.017-0.025
Width	0.032-0.035	0.020-0.027
Seminal vesicle (expulsor)		
Length	0.067-0.070	-
Width	0.035-0.040	-
Ovary		
Length	0.035-0.040	0.017-0.025[a]
Width	0.050-0.055	0.025-0.065
Testis (right)		
Length	0.112-0.120	0.095-0.110[a]
Width	0.070-0.077	0.035-0.047[a]
Testis (left)		
Length	0.115-0.117	0.097-0.102[a]
Width	0.057-0.070	0.030–0.050[a]

[a]Distinct characteristics are different in both of mullets and half-beak fish.

A B

Fig 1- A: Encysted metacercaria from mullet (scale = 0.1 mm), B: Encysted metacercaria from half-beak fish (scale bar = 0.02 mm).

width, and testis (left) length and width, from excysted metacercariae of the mullet were distinct from excysted metacercariae of the half-beak fish. Moreover, the prevalence and intensity of metacercarial infection of the mullet and half-beak fish are shown in Table 2. The prevalence of the metacercariae from mullets was 100% and intensity was 67.8, while the prevalence and intensity of the metacercariae from the half-beak fish of the previous reported were 100% and 999.5, respectively.

DISCUSSION

The body size of excysted metacercariae,

Table 2
The prevalence and intensity of metacercarial infection from mullets in comparison from half-beak fish.

Items	Metacercarial infection from mullets	Metacercarial infection from half-beak fish (Sripalwit *et al*, 2003)
Prevalence (%)	100	100
Intensity	67.8	999.5

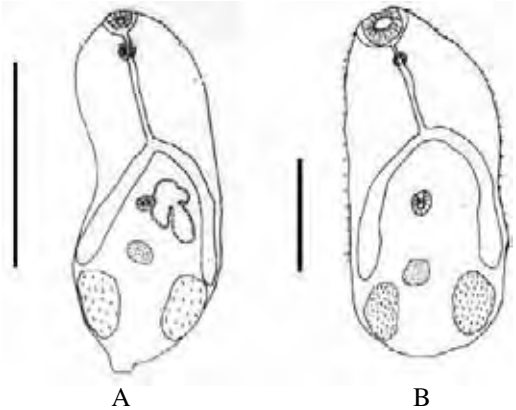

Fig 2- A: Excysted metacercaria from mullet (scale bar = 0.3 mm), B: Excysted metacercaria from half-beak fish (0.1 mm).

Fig 3- Mullet, *Liza subviridis*.

the size of the encysted metacercariae, and the esophagus length of mullets were greater than those characteristics of the half-beak fish. Besides, the intensity of the metacercarial infection of the half-beak fish was more than the metacercarial infection of the mullet. From Table 1, the size of encysted metacercariae and organs of the metacercariae from mullets were longer and wider than those from the half-beak fish were. Although the prevalences of the metacercariae of these two fish species were equal, the intensities of their metacercariae were different; the metacercarial intensity from half-beak fish was greater than the metacercarial intensity from mullets.

Several studies (Noda, 1954; Seo *et al*, 1979, 1984; Chai and Sohn, 1988) have described *Stellantchasmus falcatus* metacercariae that was collected from mullets, *Mugil cephalus*. However, the present study examined the *Stellantchasmus* metacercariae from mullets, *Liza subviridis* (Fig 3). Because of the differences between fish species, the *Stellantchasmus* metacercariae that were found in the two fish species were not similar.

The results of this study suggested that these two trematodes species are different. Further, these differences should be confirmed by other scientific methods, such as experimental host infection, to discover adult or molecular method.

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DNA FINGERPRINTS OF SOME HETEROPHYID TREMATODES FROM ADULT AND METACERCARIAL STAGES IN THAILAND

Pheravut Wongsawad and Chalobol Wongsawad

Department of Biology, Faculty of Science, Chiang Mai University, Thailand

Abstract. The DNA fingerprints of some trematodes in family Heterophyidae, *Stellantchasmus falcatus* and *Haplorchis taichui*, were investigated on adult and metacercarial stages. The other trematode, *Haplorchoides* sp was included. The molecular method, the high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) was examined with eight arbitary primers (OPA-02, OPA-08, OPA-09, OPN-02, OPN-03, OPN-09, OPX-13, and OPH-19). The bands were shown between 160-2,865 bps. The specific bands of each trematode - *S. falcatus, H. taichui*, and *Haplorchoides* sp - were 49, 56, and 42 bands respectively; and three bands were found in all trematodes. Eight arbitary primers from this study could be used for detecting the DNA fingerprints and finding the specific primer of these trematodes in the further study.

INTRODUCTION

The heterophyid trematode is the common fluke, which infects humans and causes epidemic disease in North and Northeast Thailand. The adults are able to develop in the small intestine of birds and mammals, including humans (Jongsuksuntikul *et al*, 1992; Radomyos *et al*, 1998; Waikakul, 1998). The prevalence of metacercarial stage in fishes trends to increase, resulted in the increase of high prevalence of the adult stage in humans (Radomyos *et al*, 1998). Two heterophyid trematodes, *Stellantchasmus falcatus* and *Haplorchis taichui*, were reported highly prevalent in northern Thailand, both adult (Kliks and Tantachamrun, 1974; Tantachamrun and Kliks, 1978; Radomyos *et al*, 1998) and metacercarial stages (Saenphet *et al*, 2001; Sripalwit *et al*, 2003; Wongsawad *et al*, 2004). The identification of the egg stage was uncertain regarding the morphology (Radomyos *et al*, 1998; Chuboon and Wongsawad, 2003). Mixed infection of the metacercarial stage in fish with both *Haplorchis taichui* and *Haplorchoides* sp was often found (Namue *et al*, 1998; Kumchoo *et al*, 2003; Wongsawad *et al*, 2004). The molecular amplification method, high annealing temperature-random amplified polymorphic DNA (HAT-RAPD), was used effectively to identify heterophyid trematodes, such as *Stellantchasmus falcatus* (Sripalwit *et al*, 2003), and *Haplorchis taichui* (Wongsawad *et al*, 2007). The fingerprints of heterophyid trematodes using the PCR method were used to confirm the adult stage of *Metagonimus* spp (Yu *et al*, 1997). This study aimed to describe the fingerprints of adult and metacercarial stages of *Stellantchasmus falcatus* and *Haplorchis taichui*, as well as the out-group, *Haplorchoides* sp, which could be used to identify the species with certainty.

Correspondence: Assist Prof Pheravut Wongsawad, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50202, Thailand.
E-mail pheravut@yahoo.com

MATERIALS AND METHODS

Parasitic materials

Metacercarial trematodes were collected from freshwater fishes as follows: *S. falcatus* from *Dermogenys falcatus*, and *H. taichui* and *Haplorchoides* sp from *Henicorhynchus siamensis* using 1% pepsin solution in a shaking waterbath at 370C for two hours. The adults of *S. falcatus* and *H. taichui* were prepared by oral force-feeding three-day old chicks (*Gallus gallus domesticus*) with metacercaria. Adults were collected and retained seven days postinfection from the small intestine using Baerman's apparatus. The adult of *Haplorchoides* sp was collected from the intestine of the *Hemibagrus filamentus*. All trematodes of both stages were identified to the species by observing the permanent slides under a light microscope. DNA fingerprints

The DNA fingerprints of some trematodes in the family Heterophyidae, *Stellantchasmus falcatus* and *Haplorchis taichui*, were investigated in the adult and metacercarial stages. The outgroup trematode, *Haplorchoides* sp, was also included. DNA was extracted using the DNeasy Tissue Kit (QIAGEN), 25 mg in a 1.5 ml microcentrifuge tube, and was eluted in 5 mM Tris-HCl, at pH 8.5. The molecular method by Anuntalabhochai *et al* (2000), the High Annealing Temperature-Random Amplified Polymorphic DNA (HAT-RAPD), was used with nine arbitary primers (OPA-02, OPA-08, OPA-09, OPN-02, OPN-03, OPN-09, OPX-13, OPP-15, and OPH-19). All arbitary primers were amplified DNA of all trematodes in both stages. The HAT-RAPD products were separated by 1.7 % agarose gel electrophoresis at 60 volts for 2 hours and 30 minutes, stained with 1 mg/ml of ethidium bromide for 10 minutes, and then destained in distilled water for 10 minutes (Wongsawad *et al*, 2007). The DNA bands were examined under UV transilluminator, and photographed by Kodak digital camera Gel LOGIC 100.

DNA analysis

The DNA patterns of banding of trematodes were compared with 100-bp DNA ladder plus (Fermantas) and 100-bp DNA ladder plus (BIO-RAD), and were investigated for the molecular weight using Kodak ID Image.

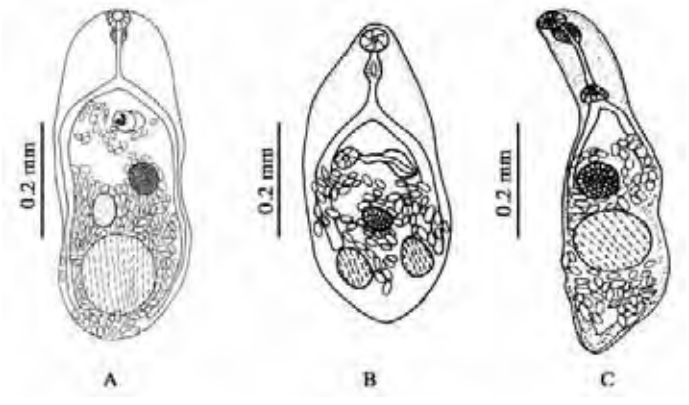

Fig 1- Trematodes: A: *Haplorchis taichui*, B: *Stellantchasmus falcatus*, C: *Haplorchoides* sp.

RESULTS

Morphological investigation

The species of trematodes were confirmed according to Pearson (1964), and Pearson and Ow-Yang (1982). The morphology and characteristics of adult worms were shown in Fig 1 (A-C). The entire body of trematodes was difficult to generally describe, but the unique characteristics of each species was observed. *S. falcatus* has a submedian ventrogenital sac with two dense minute spines, while *H. taichui* has fan-shaped acetabular spines, and *Haplorchoides* sp has long intestinal ceca at the posterior end of the body.

DNA fingerprints

Eight of the arbitary primers were the amplified DNA of all trematodes in both stages except OPP-15, which had not amplified the DNA of *S. falcatus*. The arbitary primers amplified the DNA fingerprint of each trematode in the same pattern of both stages. The results in terms of total banding number and different bands are presented in Table 1. The pattern of DNA fingerprints of both stages of the trematodes were distinguished as appear in Fig 2 (A-H). A total of 180 bands were examined as molecular weight by Kodak ID Image between 160-2,865 bps. Three bands were found in all the trematodes of the both stages by OPA-02, OPA-08, and OPX-13, with molecular weights of 580, 650, and 650 bps, respectively. The specific bands of each species were analyzed. The OPN-02 primer had the highest band number (36) and produced the highest specific band number (32). The specific bands for each trematode-*S. falcatus, H. taichui*, and *Haplorchoides* sp-were 49, 56, and 42 bands, respectively; and 3 bands were found in all trematodes.

DISCUSSION

The entire body of trematodes could be identified, with the unique characteristics of each species, but by the specialist parasitologist only. The reproductive system and unique

Table 1
The molecular weight of specific bands amplified by eight arbitary primers.

Primer	Bases sequence 5'--> 3'	Total DNA bands	Total specific bands	Molecular weight of the specific bands (bps)
OPA-02	TGCCGAGCTG	19	14	S- 1,270, 935, 875 HC- 1,300, 780, 750, 450, 190 HP - 1,330, 830, 725, 590, 130
OPA-08	GTGACGTAGG	16	14	S- 1,515, 990, 395 HC- 1,100, 970, 860, 555, 440 HP- 1,450, 1015, 875, 760, 460, 405
OPA-09	GGGTAACGCC	20	14	S- 880 , 805 , 604 , 430 , 340 , 250 HC- 960 HP- 1,080, 995, 640, 565, 407, 215
OPN-02	ACCAGGGGA	36	32	S- 1,540, 1440, 1,190, 1,110, 990, 860, 810, 782, 610, 525, 412, 365, 190 HC- 2,250, 1,465, 1,348, 1,230, 1,125, 960, 870, 815, 750, 650, 555, 435 HP- 1,088, 990, 780, 630, 405, 350, 216
OPN-03	TGCCGGCTTG	25	22	S- 1,900, 1,605, 1,290, 1,270, 1,140, 955, 510, 470 HC- 1,820, 1,410, 1,240, 1,195, 1,060, 780, 540 HP- 1,230, 820, 480, 390, 340, 245, 230
OPN-09	TGCCGGCTTG	22	21	S- 960, 900, 850, 545, 460, 370 HC- 1,380, 1,200, 990, 915, 830, 745, 560, 535, 515, 471, 395, 335 HP- 1,165, 1,005, 500
OPX-13	ACGGGAGCAA	16	12	S- 950, 670, 560, 300 HC- 1,100, 855, 760, 635, 370, 280 HP- 1,870, 390
OPH-19	CTGACCAGCC	26	20	S- 1,885, 1,235, 940, 735, 512, 250 HC- 2,865, 2,770, 2,420, 1,335, 1,145, 870, 760, 270 HP- 770, 665, 505, 487, 450, 345

S = *Stellantchasmus falcatus*; HC = *Haplorchis taichui*; HP = *Haplorchoides* sp.

characteristics organs appear distinctly in the adult stage. The mixed infections of trematodes are also difficult to identify (Namue *et al*, 1998; Wongsawad *et al*, 2004).

Eight arbitary primers from this study could be used to identify the trematode species in both adult and metacercarial stages. The protocol for detecting could be used in all species. However, adult *S. falcatus* and *H. taichui*, had to be prepared from the metacercarial stage from fish in order to confirm the species. The adult and metacercarial stages of *Haplorchoides* sp were collected from different hosts. The DNA patterns of both stages were confirmed as the same species that was introduced in the life cycle in the second intermediate host and definitive host. This method could be used accurately to detect the larval stage in the first intermediate host, freshwater snails, and the egg stage of adult worm in animals or humans. Previous

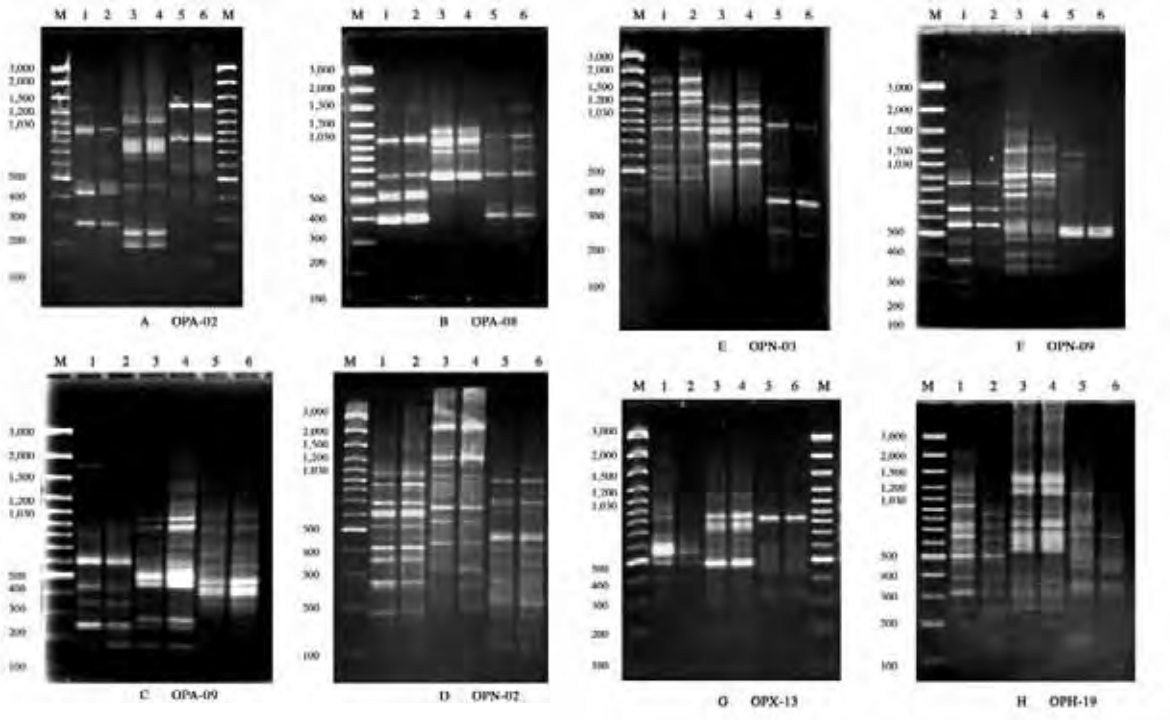

Fig 2- The fingerprints of trematode DNA by HAT-RAPD with primers: A: OPA02, B: OPA-08, C: OPA-09, D: OPN-02, E: OPN-03, F: OPN-09, G: OPX-13, H: OPH-19; (lane M: 100-bp ladder plus, lane 1: adult *S. falcatus*, lane 2: metacercaria *S. falcatus*, lane 3: adult *H. taichui*, lane 4: metacercaria *H. taichui*, lane 5: adult *Haplorchoides* sp, lane 6: metacercaria *Haplorchoides* sp).

reports have illustrated the similarity of the egg stages of heterophyid trematodes and liver fluke (Kliks and Tantachamrun, 1974; Radomyos *et al*, 1998; Kumchoo *et al*, 2003; Wongsawad et al, 2003). The DNA fingerprint of the heterophyid trematode, *Metagonimus* spp, was detected by Yu *et al* (1997) and Yang *et al* (2000), respectively. Recently, the HAT-RAPD method has been used to analyzed the DNA fingerprint of *Stellantchasmus falcatus* (Sripalwit *et al*, 2003), and the DNA quantities and qualities of some trematodes (Wongsawad *et al*, 2007). From this study, eight arbitary primers could be used to detect the DNA fingerprints of these trematodes and to find the specific primer of each trematode for further study.

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WELCOME REMARKS AND INTRODUCTION TO SYMPOSIUM ON CESTODE ZOONOSES IN ASIA AND THE PACIFIC

Akira Ito

Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan

WELCOME REMARK

We are living on the horns of a dilemma. On one hand, we like or love our own traditional local life styles, whereas on the other hand, we are challenged to adopt, or accept, or adapt the globally standardized modern life style. Therefore, we are asked to keep a balance between the two crucially different life styles. Emergent and re-emergent infectious diseases also have a similar background. All participants attending the 5th Food- and Water-Borne Parasitic Zoonoses are a rather peculiar, strange, or unique minority, because we all have enormous knowledge, experience, skills, as well as great interest and curiosity in parasitic zoonoses and simultaneously have a voluntary spirit to challenge for control of these diseases. A few months ago, 30-40 American people, mainly in California, became ill after eating flesh fried, or raw, or even live fresh water crabs (*Geothelphusa dehaani*) in so-called Americanized Japanese restaurants. None of them was of Asian origin. At that time, approximately 3,000-4,000 people were estimated to have eaten the crabs. However, local clinicians had little knowledge of such unexpected diseases, and some patient(s) were treated surgically (Bartlett, 2006). What was the disease? Yes, it is very easy for all of us to speculate or suspect what it was! It was paragonimiasis. Unfortunately, the crabs were imported to USA from Japan. This is just one example that unexpected or non-indigenous parasitic diseases may cause some outbreaks in foreign countries through importation of wild animals, as either foods or pets. We should remind ourselves that such wild animals, including such crabs, are full of pathogens. Trading of wild animals should be prohibited, or we may have emergent or re-emergent infectious diseases anywhere in the world. Both WHO and FAO have joined more closely to invite those who are actively working on these parasitic zoonoses. I would like to launch a word WAFP. Any kind of infectious disease, either emergent or re-emergent, may be caused through contaminated Water, contaminated Air, contaminated Food, or contaminated People. I have no doubt that all participants attending this Bangkok meeting will exchange mutual updated information, begin better collaborations, and create better friendships.

Correspondence: Akira Ito, Department of Parasitology, Asahikawa Medical College, Midorigaoka Higashi 2-1-1-1, Asahikawa 078-8510, Japan.
E-mail: akiraito@asahikawa-med.ac.jp

PHILOSOPHY AND STRATEGY FOR COLLABORATION IN ASIA AND THE PACIFIC

Sustainable collaborations are only available with the philosophy that local data collected by local people are of local people, and for local people first. People from developed countries, whether politicians, semi-politicians, or researchers who want to undertake any kind of cooperation, including collaboration on field surveys in developing countries, should bear this in mind. We welcome anyone who is willing to encourage local researchers through real collaboration, including transfers of knowledge, experience, and technology that is essential for doing evidence-based science. We in Asia and the Pacific are challenged by how to write scientific articles in English. However, if any one tried to publish while ignoring the original contributions by local researchers can never be acceptable. Local people recognize such work as some kind of invasion or theft of local jewels. All local data are first for local collaborators,

with encouragement. No one should be able to buy local data or publish such data as his or her original work without a real scientific contribution other than payment of some money or sweet words.

INTERNATIONAL WORKSHOP AND SYMPOSIUM SPONSORED BY MINISTRY OF EDUCATION, JAPAN (MEXT)

Based on such a philosophy and strategy, I have organized several workshops and symposia from 2000 in Asia (Table 1). The Chengdu meeting in 2000 was the first, with Chinese researchers and officials from the central and local governments in China joining together with experts from around the world. It was supported by my own research funding from the Japan Society for the Promotion of Science (JSPS) and by the Asahikawa Medical Fund (former President was Prof Yoshihiko Kubo). The late Dr Carlo Urbani from WHO Hanoi joined us just when he got the job in Hanoi. Unfortunately, he passed away due to SARS (severe acute respiratory syndromes) on 29th March 2003, just before the proceedings of the Chengdu meeting was published (Ito *et al*, 2003a). The article was dedicated to him lest we forget his great contribution. Dr Urbani and I also organized a symposium on taeniasis/cysticercosis at the 3rd Food-borne Parasitic Zoonoses (FBPZ) in Bangkok, in December 2000, (Ito and Urbani, 2001). In 2003 and 2005, I organized a symposium on "Echinococcosis" at the 4th FBPZ in Bangkok (Ito, 2004) and a symposium on "Taeniasis/Cysticercosis and Echinococcosis focused on Asia and the Pacific" in Asahikawa, Japan (Ito *et al*, 2006a). These two meetings, as well as seminars for the transfer of technology on taeniasis/cysticercosis and echinococcosis for three years during 2003-2005 were sponsored by a special fund for the promotion of science and technology from the Japanese Ministry of Education (MEXT) (Ito *et al*, 2006b). From 2003, we organized four seminars and invited 26 trainees and 14 lecturers from 14 countries. Programs of technical transfer are summarized in Table 2. All trainees prepared their own parasite, fecal, and/or serum samples from their home countries for molecular and serological analysis. Preparation of specific antigens for serology

Table 1
International workshop or symposium on cestode zoonoses organized by Asahikawa Medical College.

Year	Month/days	City, Country	Title	References
2000	Jul 16-18	Chengdu, China	Workshop on towards multilateral collaboration and cooperation for the control of echinococcosis, cysticercosis and other parasitic zoonoses in western China	Ito *et al*, 2003a
2000	Dec 6-8	Bangkok, Thailand	Symposium on Cysticercosis at the 3rd FBPZ	Ito and Urbani, 2001
2003[a]	Dec 2-4	Bangkok, Thailand	Symposium on Echinococcosis at the 4th FBPZ	Ito, 2004
2005[a]	Jul 4-8	Asahikawa, Japan	Symposium on Taeniasis/Cysticercosis and Echinococcosis focused on Asia and the Pacific	Ito *et al*, 2006b
2006[b]	Nov 28-30	Bangkok, Thailand	Symposium on Cestode Zoonoses in Asia and the Pacific at the 5th FBPZ	This issue
2007[b]	Jun 12-18	Okinawa, Japan	Symposium on Cestode Zoonoses in the Pacific at the 21st Pacific Scientific Congress	

[a]: Sponsored by a special fund for promotion of science and technology from Ministry of Education Japan (2003-2005).

[b]: Sponsored by the Asia-Africa Scientific Platform program of JSPS (2006-2008).

Table 2
The seminar programs for technical transfers useful for detection of taeniasis/cysticercosis, cystic echinococcosis (CE) and alveolar echinococcosis (AE).

Serology:

- Cysticercosis: Both ELISA and Immunoblot using native glycoproteins (GPs), either purified by preparative isoelectric focusing or by affinity chromatography using polyclonal and monoclonal antibodies against GPs and recombinant chimeric antigens.
- Cystic echinococcosis: Both ELISA and Immunoblot using purified Antigen B from native hydatid cyst fluid, recombinant Antigen B8/1.
- Alveolar echinococcosis: Both ELISA and Immunoblot using purified Em18 and recombinant Em18.

Mitochondrial DNA:

- Multiplex PCR using a single egg, a single metacestode or a proglottid of adult worm for differentiation of three human *Taenia* species and two genotypes of *Taenia solium* and *Echinococcus* species.

using their own homemade parasite materials was strongly recommended (Ito *et al*, 2006b,c).

INTRODUCTION TO THE SYMPOSIUM ON CESTODE ZOONOSES IN ASIA AND THE PACIFIC

The present symposium at the 5th FBPZ is sponsored by the Asia-Africa Scientific Platform (AASP) program with a grant from JSPS to me. This special fund is for the establishment of the research center for cestode zoonoses in Asia and Africa. Invited speakers are from Thailand (1), Indonesia (1), China (2), Japan (4), UK (1), and France (1) who are working actively based on their own original science and technology. The symposium is divided into two sessions, morning and afternoon. The former is focused on taeniasis/cysticercosis, which is chaired by Dr Malinee Anantaphruti and myself, and the latter is focused on echinococcosis and others, which is chaired by Prof Philip S Craig and myself. At the taeniasis/cysticercosis session, Dr Marcello O Sato will offer molecular tools for serology and mitochondrial DNA identification of taeniasis and cysticercosis (Sato *et al*, 2006). Dr Munehiro Okamoto re-launches the unresolved debate on Asian *Taenia,* based on mitochondrial and nuclear DNA studies (Ito *et al*, 2003b; Okamoto *et al* unpublished data). These topics give advanced tools in immunology and molecular biology for basic and epidemiological studies on taeniasis/cysticercosis. Drs Tiaoying Li (Li *et al*, 2006), Toni Wandra (Wandra *et al*, 2006), Malinee Anantaphruti (Anantaphruti *et al*, 2007), and Durga Joshi (Joshi *et al*, unpublished data) present the current situation of taeniasis and cysticercosis in China, Indonesia, Thailand, and Nepal respectively. At the echinococcosis and others session, the present situation of echinococcosis in Asia is first overviewed by Prof PS Craig (Craig *et al*, unpublished data), and then molecular tools for epidemiology of alveolar echinococcosis is introduced by Dr J-M Bart (Bart *et al*, unpublished data), and serology using Antigen B 8 kDa subunits (AgB8/1-8/5) expressed differentially through developmental stages of *E. multilocularis* and *E. granulosus* is overviewed by Dr Wulamu Mamuti (Mamuti *et al*, 2005). Dr Yamasaki describes a molecular work on paraffin embedded histopathological specimens using *Taenia* spp (Yamasaki *et al*, 2006), *Echinococcus* spp, *Diphyllobothrium* spp, and *Spirometra* spp (Yamasaki *et al*, unpublished data).

On December 1, I give a talk on "The present situation of taeniasis and cysticercosis in Asia and the Pacific" at another symposium, the Joint International Tropical Medicine and Malaria (JITMM) 2006, as a brief overview of taeniasis/cysticercosis, and summary of the symposium on taeniasis/cysticercosis on November 29.

21st PACIFIC SCIENTIFIC CONGRESS, 12-18, 2007 JUNE

In June 2007, we will organize another symposium on cestode zoonoses in the Pacific during emerging infectious disease sessions at the 21st Pacific Science Congress, in Okinawa. This symposium will also be sponsored by the AASP-JSPS with a grant to A Ito. I do expect that we in Asia and the Pacific can make our own contribution to science and technology using our own materials from our home countries. For such action, we need international collaborators who are willing to join us and work together with high appreciation of the local contribution.

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THE PRESENT SITUATION OF TAENIASIS AND CYSTICERCOSIS IN ASIA AND THE PACIFIC

Akira Ito[1], Munehiro Okamoto[2], Toni Wandra[1,3], Hariadi Wibisono[3], Malinee T Anantaphruti[4], Jitra Waikagul[4], Tiaoying Li[1,5] and Dongchuan Qiu[5]

[1]Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan; [2]Department of Laboratory Animal Science, School of Veterinary Medicine, Tottori University, Japan; [3]Directorate General, Communicable Disease Control and Environmental Health, Ministry of Health, Jakarta, Indonesia; [4]Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; [5]Sichuan Institute of Parasitic Diseases, Sichuan CDC, Chengdu, China

Abstract. *Taenia solium* cysticercosis is briefly overviewed. Atypical neurocysticercosis cases that lack specific information from clinical manifestation, neuroimaging, serology, or histopathologic examination require molecular identification of histopathologic specimens for obtaining of concrete evidence of infection with metacestodes of *T. solium*. The present situation of taeniasis and cysticercosis in Asia and the Pacific is overviewed based on our joint projects in Indonesia, China, and Thailand. Both *T. saginata* from beef and *T. asiatica* from the viscera of pigs occur sympatrically in China and in Thailand; whereas, such a sympatric occurrence is not confirmed in Indonesia, where the religious taboo about food is quite strict.

INTRODUCTION

Taeniasis in Asia and the Pacific has a unique spectrum because three species (*Taenia solium*, *T. saginata*, and *T. asiatica*) have been reported in this area (Ito *et al*, 2003a, 2004). Regarding the third species, *T. asiatica*, it remains debatable whether it is an independent species. Study that is more detailed would be necessary on mitochondrial and nuclear DNA in co-endemic areas in Asia and the Pacific before any conclusion could be made (Okamoto *et al*, unpublished data). Cysticercosis is known only from *T. solium*, although *T. asiatica* requires pigs as the intermediate host, which is similar to *T. solium* (Ito, 1992; Ito *et al*, 2003a). Recent molecular evidence strongly suggests that *T. asiatica* is very close to *T. saginata* and is a sister-species of *T. saginata* and has a far distance from *T. solium* (Hoberg *et al*, 2000, 2001; Hoberg, 2006; Nakao *et al*, 2002; Eom, 2006). Based on molecular and morphological studies (Eom, 2006), it is predicted that cysticercosis in humans, attributable to *T. asiatica,* does not occur because cysticercosis is unknown as its sister species, *T. saginata* (Ito *et al*, 2003a). There is no doubt that there are hot spots of taeniasis and cysticercosis in Asia and the Pacific (Ito *et al*, 2003a).

Correspondence: Akira Ito, Department of Parasitology, Asahikawa Medical College, Midorigaoka Higashi 2-1-1-1, Asahikawa 078-8510, Japan.
E-mail: akiraito@asahikawa-med.ac.jp

TAENIA SOLIUM CYSTICERCOSIS

Taenia solium cysticercosis is one of the most potentially lethal parasitic diseases worldwide. The main cause of late-onset epilepsy in developing countries that are endemic areas for *T. solium* is expected to be due to a cysticercus or cysticerci of *T. solium* (Ito *et al*, 2006a; Takayanagui and Odashima, 2006). The importance of clinical manifestation, neuroimaging, serology, and molecular confirmation of neurocysticercosis (NCC) has been briefly overviewed (Ito *et al*, 2006a). It is known that approximately 10% of NCC cases may show typical imaging figures, and almost all active NCC cases with multiple cysts in the brain show specific antibodies to the specific antigens, either native glycoproteins or recombinant proteins (Tsang *et al*, 1989; Ito *et al*, 1998; Chung *et al*, 1999; Sako *et al*, 2000, 2006; Hancock *et al*, 2003; Sato *et al*, 2003, 2006). Therefore, with patients who have a history of residence or visiting endemic areas of NCC, we have to be reminded of NCC. Serology to detect specific antibodies using highly specific antigens, either produced at CDC, USA, or Asahikawa

Medical College, Japan, is highly useful before surgery (reviewed by Ito *et al*. 2006a). However, if NCC cases with a solitary cyst, or even with multiple cysts, show only atypical imaging figures and no specific antibody response, we would have no critical identification of such cases. No clinical manifestation of NCC is specific or unique to NCC. Therefore, we have to consider some other diseases caused by other parasitic, bacterial, or viral infections, and malignant tumors as well as NCC. In the case of racemose form of NCC showing hydrocephalus in non-endemic areas, we rather consider toxoplasmosis, tuberculosis, and malignant brain tumors, etc. The difficulty in a confirmative diagnosis of NCC or other diseases in the brain is the lack of necropsy cases, especially in endemic developing countries. However, in some relatively rare cases, histopathological specimens may be available after brain surgery.

Histopathological examination of resected lesions often reveals the hooklets as well as the suckers and the unique morphological structures of cestodes. So far as we know, we usually conclude that such cases are metacestodes of *T. solium*. However, is it really sound without molecular confirmation? There is no real concrete evidence on the speciation if they have no hooklets. It might be due to technical loss of such structure through processing, or it might be due to some other taeniid metacestodes that rarely infect humans or are underestimated without molecular identification. Therefore, it is essential for us to identify the species based on molecular evidence. Such topics have just been overviewed (Yamasaki *et al*, 2005; Ito *et al*, 2006a,b).

THE PRESENT SITUATION OF TAENIASIS AND CYSTICERCOSIS IN ASIA AND THE PACIFIC

A long-standing puzzle has been that adult taeniid tapeworms expelled from people in Asia-Pacific seem to be *T. saginata*, the beef tapeworm, although these people eat pork rather than beef (Yokogawa, 1935; Hsieh, 1960; Huang *et al*, 1966; Ito *et al*, 2003a). This is now generally considered to be due to the third species, *T. asiatica* (Fan, 1988; Fan *et al*, 1995; Eom and Rim, 1993; Simanjuntak *et al*, 1997; Hoberg *et al*. 2000, 2001; Hoberg, 2006; Eom, 2006). *T. asiatica* has been reported from Taiwan, China, South Korea, Indonesia, Philippines, and Vietnam where local people eat pork rather than beef (Ito *et al*, 2003a, 2004). Therefore, it is now clear that some areas where we had identified *T. solium* and *T. saginata* are rather due to *T. solium* and *T. asiatica,* or due to *T. solium* and *T. asiatica* and *T. saginata*. It is necessary for us to re-examine the historical specimens identified to be *T. saginata* in Asia and the Pacific (Ito *et al*, 2003a).

Present situation in Indonesia

The majority of Indonesian people is Muslim. Therefore, *T. solium* and *T. asiatica* that are caused by ingestion of raw or under cooked pork and viscera are expected to be absent. However, in some areas or islands, where the majority is Hindu, such as in Bali, or Christians in Papua (formerly Irian Jaya) or North Sumatra, *T. solium* or *T. asiatica* are common. So far as we know, *T. solium* taeniasis/cysticercosis is recognized in Papua, but there is no other taeniid species (Simanjuntak *et al*, 1997; Wandra *et al*, 2003; Margono *et al*, 2006). In North Sumatra, *T. asiatica* is rather more common than *T. solium*. So far as we have examined, there is no *T. solium* case in Samosir Island in Lake Toba, North Sumatra (Wandra *et al*, unpublished data). In Bali, *T. solium* cysticercosis has been known to be endemic. However, it is currently rare (Sudewi *et al*, in press). Rather, *T. saginata* due to consumption of raw beef (beef *lawar*) is becoming much more common, because Balinese can eat beef, even if they are Hindu. In Bali, there is no *T. asiatica* case at all. It is expected to be due to the local eating customs. They eat uncooked minced pork with blood or beef (pork *lawar* or beef *lawar*) but do not eat uncooked viscera of pigs. This is a crucial difference between Balinese and Batak people in North Sumatra who like uncooked viscera of pigs. In North Sumatra, *T. asiatica* is common due to the consumption of uncooked or undercooked viscera of pork (*sang sang*). Therefore, there is no area in Indonesia where we can expect sympatric occurrence of the three taeniid species. It is mainly due to the barrier of the religion and differences in taste for

raw or undercooked viscera, as shown in Bali and North Sumatra (Wandra *et al*, 2006a,b).

Present situation in China

T. solium cysticercosis is one of the most common parasitic diseases in China (Ito *et al*, 2003b) because the favorite cuisine in China is pork. Both *T. solium* and *T. saginata* have commonly been reported in China (Chen *et al*, 2005). However, there are few reports demonstrating *T. asiatica* in China (Eom *et al*, 2002; Yamasaki *et al*, 2004). Most recent work in the Tibetan population in Sichuan, China has suggested that the three species are sympatrically distributed there (Li *et al*, 2006). Local people eat raw or uncooked minced pork and viscera. It is expected to be the basic risk factor for both *T. solium* and *T. asiatica*. We have to re-examine "*T. saginata*," where both *T. solium* and *T. saginata* have been reported (Eom *et al*, 2002; Yamasaki *et al*, 2004). An up-dated overview of taeniasis/cysticercosis in China is presented at the 5th FBPZ meeting in Bangkok (Li *et al* 2007).

Present situation in Thailand

So far as we know, there is no evidence of the occurrence of *T. asiatica* in Thailand (Bowles and McManus, 1994; Morakote *et al*, 2000). All specimens examined for mitochondrial DNA were confirmed to be *T. saginata*. The most recent work in Kanchanaburi, western part of Thailand, *Taenia* specimens from 24 patients were collected. They were identified morphologically to be *T. solium* and *T. saginata*. Later, the "*T. saginata*" were confirmed to be *T. saginata* (n = 7) and *T. asiatica* (n = 6) by mitochondrial DNA analysis in Asahikawa. Among these, one patient expelled three worms. Two had scoleces with armed hooklets and were expected to be *T. solium,* and the remainder had no hooklets and was expected to be "*T. saginata*." With mitochondrial DNA analysis of scoleces on glass slides, using T base-pair excision test (Yamasaki *et al*, 2004), "*T. saginata*" was confirmed not to be *T. saginata* but rather *T. asiatica*. It was the first dual infection case of *T. solium* and *T. asiatica* confirmed by molecular tools (Anantaphruti *et al*, 2007).

Present situation in other countries in Asia and the Pacific

Through the special fund from the Ministry of Education, Japan to A Ito (2003-2005), we organized 4 technical transfer seminars during the three-year period. A total of 40 researchers from 14 countries were invited to attend the seminars with full financial travel support from this special fund (Ito *et al*, 2006b). We invited others from the Philippines, Vietnam, Lao PDR, Nepal, and Mongolia in Asia, as well as from China, Indonesia, and Thailand, as summarized above, and from other countries in Asia. So far as we know, we could have confirmed *T. asiatica* and *T. solium* from the Philippines, whereas *T. saginata* and *T. solium* were confirmed from Vietnam, Lao PDR, and Nepal (Yamasaki *et al*, unpublished data). Only *T. saginata* has been confirmed in Mongolia (Myadagsuren *et al*, 2007). It is known that *T. asiatica* is distributed in Vietnam (Le *et al*, 2003). In these countries, and in China, Thailand, and Indonesia further systematic survey should be carried out for getting detailed information on the three species and background information on the life cycles.

PERSPECTIVES

Historically, there have been few cases of dual infection with *T. solium* and *T. saginata*. Such dual infection may occur but, if such cases were recorded from Asia and the Pacific, we would expect it to be due to *T. solium* and *T. asiatica* through eating raw or uncooked minced pork with viscera, which is still rather common in China, Vietnam, northern part of Thailand, and perhaps in Myanmar and some other countries. Such historical specimens of *T. saginata* may be re-examined (Ito *et al*, 2003a; Yamasaki *et al*, 2006; Anantaphruti *et al*, 2007).

There are many strategies that have been launched for the control of taeniasis/cysticercosis in humans and pigs. So far as we know, although there is no active strategy such as vaccination of pigs for control of *T. solium* cysticercosis or treatment of taeniasis solium other than sustainable education and recommendation of no free access of pigs to human feces by keeping pigs indoors and by the use of ratlines, it appears

to be true that *T. solium* cysticercosis has been eradicated or has become rare in Europe, Japan, Korea, and Bali in Indonesia. Therefore, sanitary disposal of human feces is expected to prevent infection of pigs and is the essential procedure in the control of taeniasis solium (Schantz *et al*, 1993a; Pawlowski, 2006).

CONCLUSION

Taeniasis is one of the meat- or food-borne parasitic zoonoses common in Asia and the Pacific. It is caused by eating raw or uncooked minced pork and viscera, and beef. Through eating minced pork and viscera, both *T. solium* and *T. asiatica* are expected to be more common everywhere that local people love to eat in Asia and the Pacific. Among the three human *Taenia* species, *T. solium* is the most important as a public health issue because it causes neurocysticercosis as well. The distribution of these *Taenia* species may still be controlled in general by the taboos of religions. However, unexpected outbreaks of cysticercosis may be introduced, even in Muslin or Jewish societies, through immigrants or visitors who are asymptomatic taeniasis carriers (Schantz *et al*, 1993b; Hira *et al*, 2004). Cut-off of the life cycle of these tapeworm infections should be carried out on both human and animal sides. Sustainable education of people in endemic areas and the challenge for the production of safe meat are urgent tasks.

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ASIAN *TAENIA*: SPECIES OR SUBSPECIES?

Munehiro Okamoto[1], Minoru Nakao[2], Eiko Tachi[1], Yasuhito Sako[2], Marcello O Sato[2], Hiroshi Yamasaki[2], Kazuhiro Nakaya[3] and Akira Ito[2]

[1]Department of Parasitology, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Japan; [2]Department of Parasitology, [3]Animal Laboratory for Medical Research, Asahikawa Medical College, Japan

Abstract. Asian *Taenia* is a human tapeworm found in Asian countries where people eat undercooked visceral organs of pigs contaminated with metacestodes. It was designated as a new species in Taiwan due to its unique lifecycle, which was confirmed by experimental infections; but described as a genotype, strain, or subspecies of *T. saginata* (*T. saginata asiatica*) due to molecular phylogenetic analyses. Several non-armed adult worms, which had been collected from humans in non-sympatric endemic areas, were examined. First, the mitochondrial DNA was typed by multiplex PCR and sequencing; and then a nuclear gene, elongation factor-1 alpha (EF-1 alpha) gene, was sequenced. Although intraspecific variation of EF-1 alpha gene was not detected, the sequence of EF-1 alpha gene of Asian *Taenia* was distinctly different from that of *T. saginata*. There was no phylogenic discrepancy between the mitochondrial genes and that from EF-1 alpha gene. These results suggest that the hybridization between Asian *Taenia* and *T. saginata* has not occurred as far as we examined. Recently, it was reported that there were endemic areas where sympatric distribution of *Taenia solium*, *Taenia saginata*, and *Taenia asiatica* was confirmed. In the future, we intend to examine samples from such sympatric zones.

INTRODUCTION

Asian *Taenia* is a human tapeworm found in Asian countries where people eat the undercooked visceral organs of pigs contaminated with metacestodes. It was first recognized in Taiwan, and subsequently in many other Asian countries including China, South Korea, Indonesia, Philippines, and Vietnam (Simanjuntak *et al*, 1997; Ito *et al*, 2003; Eom, 2006). The morphological features of Asian *Taenia* resemble those of *T. saginata*; therefore, it has been equated for a long time with *T. saginata*. However, the life cycle of this cestode was known to be different from classical *T. saginata* Goeze (1782) in its intermediate host as well as in the infected organs (Fan, 1988; Fan *et al*, 1988; Eom *et al*, 2002). Unlike classical *T. saginata*, which infects the skeletal muscle of cattle, Asian *Taenia* in its larval stage infects visceral organs of the pig, such as the liver, omentum, serosa, and lungs. Based on these differences, the Asian *Taenia* has been tentatively named as *T. saginata* (*taiwanensis* strain) (Cross and Murrell, 1991). The close relationship with *T. saginata* was also supported by molecular approaches (Zarlenga *et al*, 1991; Bowles and McManus, 1994). Eom and Rim (1993) described it as a new species (*T. asiatica* sp. n.), based on more detailed studies of the morphology and life cycle, which was reported by Fan (1988). However, some researchers insist that it should be regarded as a genotype, strain, or subspecies of *T. saginata* (*T. saginata asiatica*) because of the morphological similarity of the adult stage and the phylogenetically close relationship (Ito *et al*, 2003; McManus, 2006). There is considerable ongoing debate regarding the taxonomic position of Asian *Taenia* and whether it should be regarded as a genotype, strain, subspecies, or sister species of *T. saginata*.

In this report, we discussed what is known about Asian *Taenia* and considered its taxonomic position.

Correspondence: Munehiro Okamoto, Department of Laboratory Animal Science, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, 4-101 Koyamacho-Minami, Tottori 680-8553, Tottori, Japan.
E-mail: mokamoto@muses.tottori-u.ac.jp

TERMINOLOGY

In the literature concerning Asian *Taenia*, the following terms are often used:

Strain: variants within a species that have been characterized by using one or more criteria;
Subspecies: a taxonomic subdivision of a species consisting of an interbred, usually geographically isolated population;
Sister species: species derived from a common ancestral species shared by no other species; and
Sibling species: species that are extremely similar in appearance but are nonetheless reproductively isolated from one another.

Although these four terms describe relationships between closely related organisms, the meanings of the first two terms are clearly different from those of the latter two terms. The former terms imply the "same species," while the latter terms imply "distinct species."

MORPHOLOGY

Eom and Rim (1993) described the Asian *Taenia* as *T. asiatica* based on four morphological characteristics: 1) the existence of a rostellum on the scolex of the adult, 2) the posterior protuberance in gravid proglottids, 3) the large number of uterine twigs, and 4) wart-like formations on the scolex. However, Fan *et al* (1995) compared the morphological characteristics of the adults and cysticerci of both cestodes and claimed that these four characteristics were not specific for Asian *Taenia*: 1) a rostellum was observed in only 33% of the scolices, 2) posterior protuberance of the gravid proglottid was also observed in *T. saginata*, 3) uterine twigs and main branches were similar in number between Asian *Taenia* and *T. saginata*, and 4) wart-like formations were observed in the larvae of both tapeworms. Fan *et al* (1995) concluded from their findings that the Asian *Taenia* was a subspecies of *T. saginata* (*T. saginata asiatica*). The adult worm of Asian *Taenia* is very similar to that of *T. saginata*. If an adult worm from a human was provided, one could not identify, using only morphological criteria, whether it was Asian *Taenia* or *T. saginata*. However, this fact does not confirm that Asian *Taenia* is the same species as *T. saginata*. There is a possibility that the Asian *Taenia* is a sibling species of *T. saginata*. In the light of this, it is impossible to determine by morphology alone whether Asian *Taenia* is a species distinct from *T. saginata*.

HOST SPECIFICITY AND ORGANOTROPISM

As described above, the main intermediate host of Asian *Taenia* is a domestic pig, and cysticerci are usually found in the visceral organs. Conversely, the cysticerci of classical *T. saginata* are found in the muscle of cattle (beef). Therefore, it seems that host specificity of Asian *Taenia* is quite different from that of *T. saginata*. However, the metacestodes of Asian *Taenia* were also found in the liver of cattle that were experimentally infected with eggs (Fan, 1988; Fan *et al*, 1988; Eom *et al*, 2002). In addition, the cysticerci of classic *T. saginata* were also found in the liver of slaughtered cattle (Belino, 1975; van Veen, 1979), but never found in the liver of pigs.

There are several strains of *Echinococcus granulosus*, and the host specificity of some strains is quite different from that of other strains (*eg*, G1: sheep strain *vs* G4: horse strain). In the case of the genus *Taenia*, Azuma *et al* (1995) examined the infectivity of four laboratory-reared isolates of *T. taeniaeformis* in various rodents. Each of four isolates was most infective to the rodent species from which the original metacestode had been isolated in the field: the SRN and KRN isolates were most infective to the rat; the BMM isolate, to the mouse; and the ACR isolate, to the gray red-backed vole (Fig 1). Furthermore, intraspecific variation of infectivity to intermediate hosts is widely recognized among taeniid cestodes. However, based only on differences of infectivity between Asian *Taenia* and *T. saginata*, we seem to have no rationale to distinguish them as species.

GENETIC DIFFERENCE

Molecular biological studies were also applied to the debate regarding the taxonomic position of Asian *Taenia*. Bowles and McManus

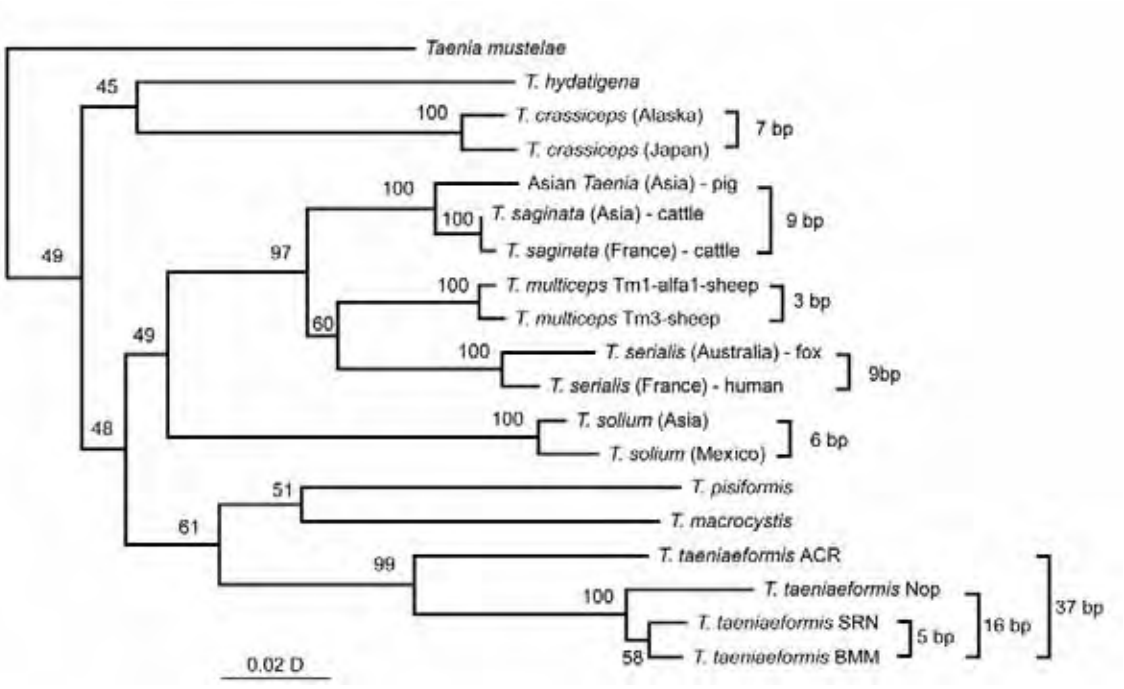

Fig 1- Phylogenetic relationships of the genus *Taenia* inferred from the mitochondrial CO1 gene sequences.

(1994) examined partial sequences of the CO1 gene from several species of the genus *Taenia*, including Asian *Taenia* and *T. saginata*, and inferred the phylogenetic relationships of taeniid cestodes. They concluded that Asian *Taenia* is a genetically distinct entity but is closely related to *T. saginata*, and its taxonomic placement as a subspecies or strain is a more appropriate than a formal designation as a new species. After their report, sequences of the same region of the CO1 gene have been reported from several species of taeniid cestodes. Then we constructed the phylogenetic tree of the genus *Taenia* from the CO1 gene sequence data (Fig 1).

DNA sequence data were aligned using the CLUSTAL W computer program (Thompson *et al*, 1994). The evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980), and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using the PHYLIP 3.63 computer program. Bootstrap values (Felsenstein, 1985) expressed in percentages out of 1,000 replicates are given near each branch. *Catenotaenia* sp from Hokkaido, Japan was used as an outgroup species to root the tree. Intermediate host species from which each isolate was isolated are as follows: *T. crassiceps* (Alaska): tundra vole (*Microtus oeconomus*), *T. crassiceps* (Japan): Japanese grass vole (*Microtus montebelli*), *T. taeniaeformis* ACR: gray red-backed vole (*Clethrionomys rufocanus bedfordiae*), *T. taeniaeformis* Nop: Japanese field mouse (*Apodemus argenteus*), *T. taeniaeformis* SRN: Norway rat (*Rattus norvegicus*), *T. taeniaeformis* BMM: house mouse (*Mus musculus*).

Because the length of the CO1 gene sequence used for phylogenetic analysis was not very long (391 bp), the bootstrap value of each node of species was not always high. Therefore, the relationships between taeniid species are obscure in this tree. However, the bootstrap value of the node that divides Asian *Taenia* and *T. saginata* is very high. In addition, nucleotide substitutions between Asian *Taenia* and *T. saginata* occur in only 9 out of the 391 nucleotide positions examined. This difference is comparable to intraspecific variations found in other *Taenia* species. These indicate that Asian *Taenia* is more closely related to *T. saginata*. When using

other DNA sequences, almost the same results are obtained. How do we evaluate these facts?

According to Eom (2006) and Hoberg (2006), recent molecular evidence strongly suggests that *T. asiatica* is a sister-species of *T. saginata*, and therefore a distinct species. McManus (2006), however, asserts that molecular studies support the opinion that the classification of Asian *Taenia* as a subspecies or strain of *T. saginata* is more appropriate than its designation as a separate species. Which stance is more reasonable? The taxonomic status, species or subspecies, could not be determined from DNA sequence data. Compared with interspecific variations among *Taenia* species, the nucleotide difference between Asian *Taenia* and *T. saginata* is very small. The nucleotide difference between two species is proportional to the time since speciation. There may be very few differences between the nucleotide sequences of the two species that have speciated more recently. Although comparison of sequence data is objective, inferring the taxonomic status from such data is quite subjective, as is doing so from the morphological or biological data.

THE BIOLOGICAL SPECIES CONCEPT

When considering the issue of species or subspecies, we must define what constitutes a species. There are several competing theories or "species concepts" (Mayden, 1997). The most widely accepted are the morphological species concept, the biological species concept, and the phylogenetic species concept. The biological species concept defines a species as a group of individuals that are actually, or potentially, interbreeding, and that are reproductively isolated from other such groups. Mayr's definition is one of the more commonly used definitions because it has an intuitive appeal to what we think species are, and because it addresses the biological reality that reproduction is at least in part a key to what species are. According to Mayr (1969), "species" are groups of populations (which are groups of individuals living together that are separated from other such groups) that can potentially interbreed or are actually interbreeding, and that can successfully produce viable, fertile offspring (without the help of human technology).

Although "subspecies" means a taxonomic subdivision of a species consisting of an interbreeding, usually geographically isolated population of organisms, a sympatric distribution of these two tapeworms is known in some endemic zones. If Asian *Taenia* is the same species as *T. saginata*, hybridization between Asian *Taenia* and *T. saginata* should occur in sympatric zones. However, no hybrids have been so far identified. In this regard, the number of isolates that have been examined from sympatric endemic areas is limited.

Then, we tried the cross breeding experiments. We can easily obtain cysticerci of both cestodes from immunodeficient, *SCID* mice (Ito *et al*, 1997; Ito and Ito, 1999). It has been demonstrated that cysticerci developed in *SCID* mice are fully developed and capable of infecting humans (Nakaya *et al*, 2006). In February 2006, we carried out experimental infections. Three volunteers ingested the cysticerci. Two persons were positive controls. One person ingested each cysticercus. However, only *T. saginata* successfully infected its host, and crossbreeding experiments have not yet succeeded. Therefore, examination of field samples is very important at this time.

How could we detect the hybridization? As described above, data from mitochondrial DNA sequences indicate that Asian *Taenia* is genetically distinct from *T. saginata*. This suggests that Asian *Taenia* has been isolated from *T. saginata* for some time. Therefore, Asian *Taenia* and *T. saginata* each should have its own type of nuclear DNA. If the hybridization did not occur, phylogeny from mtDNA would be consistent with that from any nuclear DNA. Conversely, if the hybridization has occurred in the past, there would be heterozygous individuals that carry two different alleles (one is derived from Asian *Taenia* and the other is derived from *T. saginata*) in some nuclear loci. Perhaps there are homozygous individuals who carry another type of nuclear genes that is different from that of the mitochondrial gene. However, hybrids have not yet been identified. Therefore, we usually use mtDNA for diagnosis of species (Yamasaki *et al*, 2004). In order to determine

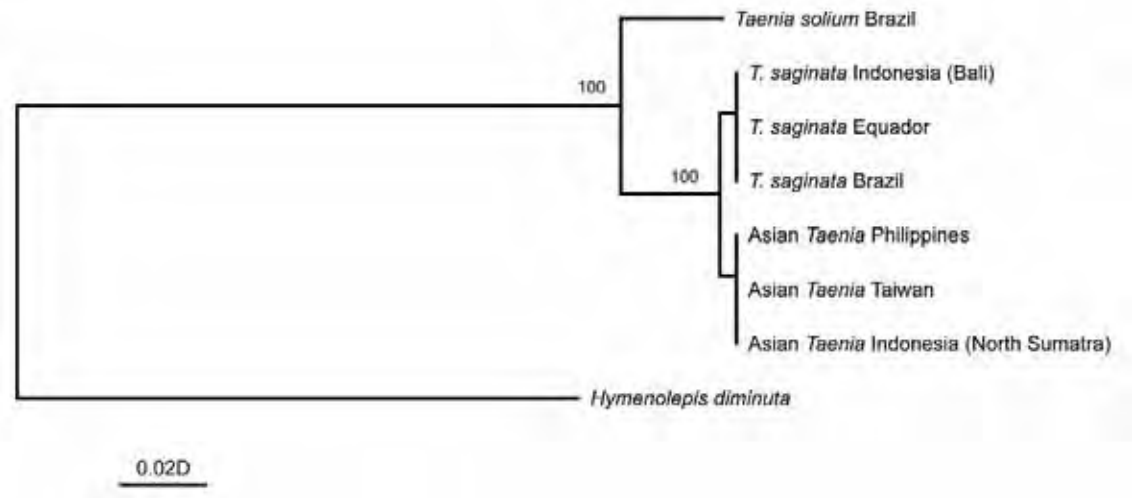

Fig 2- Phylogenetic relationships of the genus *Taenia* inferred from the nuclear EF-1 alpha gene sequences.

whether the hybridization has occurred or not, the mitochondrial DNA type should be determined first, and then nuclear DNA type might be examined. We have selected the nuclear elongation factor 1 alpha gene (EF-1 alpha gene) as a target gene. Although EF-1 alpha is a highly conserved ubiquitous protein involved in translation, it is a single gene and therefore suitable for our purpose.

Several non-armed adult worms, which had been collected from human in non-sympatric endemic areas, were examined. First, mitochondrial DNA were typed by multiplex PCR (Yamasaki *et al*, 2004) and sequenced, and then the EF-1 alpha gene was sequenced. Although intraspecific variation of EF-1 alpha gene was not detected, the sequence of the EF-1 alpha gene of Asian *Taenia* was distinctly different from that of *T. saginata*. The phylogenetic relationship of human *Taenia* that was inferred from the EF-1 alpha gene sequences is shown in Fig 2. Phylogenetic analysis used was same as Fig 1. *Tentacularia* sp (AF124799) was used as an outgroup species to root the tree. There was no discrepancy between the phylogeny from mitochondrial genes and that from EF-1 alpha gene. These results suggest that the hybridization between Asian *Taenia* and *T. saginata* has not occurred, as far as we have examined.

Recently, it was reported that there are endemic areas where sympatric distribution of *Taenia solium*, *Taenia saginata* and *Taenia asiatica* have been confirmed (Eom *et al*, 2002). In the future, we intend to examine samples from such sympatric zones.

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TAENIASIS/CYSTICERCOSIS IN CHINA

Tiaoying Li[1], Akira Ito[2], Philip S Craig[3], Xingwang Chen[1], Dongchuan Qiu[1], Xiaonong Zhou[4], Ning Xiao[1] and Jiamin Qiu[1]

[1]Institute of Parasitic Diseases, Sichuan Centers for Disease Control and Prevention, Sichuan Province, People's Republic of China; [2]Department of Parasitology, Asahikawa Medical College, Japan; [3]Biomedical Sciences Research Institute and School of Environment and Life Sciences, University of Salford, United Kingdom; [4]Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai, People's Republic of China

Abstract. This review concerning the status of taeniasis/cysticercosis in China has been compiled from various reports of studies conducted over the past 30 years, most of which have appeared in national publications in Chinese. Previous hospital reports and epidemiologic surveys have indicated that taeniasis/cysticercosis has been distributed throughout 671 counties in 29 provinces or prefectures of China. There are an estimated three million cysticercosis cases nationwide, while *Taenia asiatica* has presently been confirmed by DNA typing as occurring in Sichuan, Yunnan, and Guizhou Provinces. Although efforts to reduce transmission of taeniasis/cysticercosis have occurred in most endemic areas over the last 30 years, reports conducted over the past 15 years in southwest China have indicated that cysticercosis is emerging as a serious public health problem in this area. The most recent available information on this food-borne parasitic disease, from a Tibetan population in Sichuan, is presented in this review. In addition, recommendations for a national surveillance program for cysticercosis are discussed.

INTRODUCTION

Human taeniasis refers to food-borne infections with adult tapeworms: *Taenia solium*, *Taenia asiatica* (from pigs), or *Taenia saginata* (from bovids). Cysticercosis is a tissue infection with the larval cysticercus or metacestode stage of tapeworms, and occurs most commonly in pigs and cattle. The larval stage of *Taenia solium* can also infect humans and cause cysticercosis/neurocysticercosis, which is considered widespread in the developing countries of Latin America, Africa, and Asia (Del Brutto, 1999; Murrell, 2005). *T. solium* taeniasis/cysticercosis is particularly prevalent in rural areas and is associated with poverty and poor sanitation; where raw or undercooked pork is consumed, and scavenging pigs have access to human feces (Sarti *et al*, 1992; Pawlowski and Murrell, 2001; Burneo and Garcia, 2002). In endemic areas, neurocysticercosis is an important contributor to neurological morbidity (Garcia *et al*, 1991), and the major cause of acquired epilepsy in the world (Commission on Tropical Diseases, 1994).

It is accepted that human taeniasis and cysticercosis are present in Asia and the Asian-Pacific region (Ito *et al*, 2006). *T. solium* occurs in several Asian countries, including China, India, Indonesia, Thailand, Lao PDR, Cambodia, Nepal, Philippines, Myanmar, Vietnam, and Korea, where local people consume undercooked/raw pork (Singh *et al*, 2002). In China, the emergence of cysticercosis as a serious public health problem was recognized by the Chinese Government. Therefore, intervention measures for taeniasis/cysticercosis control have been carried out since the 1970s in some endemic areas, such as Heliongjiang, Jilin, Henan, and Fujian Provinces, where mass screening and treatment for taeniasis carriers, treatment of cysticercosis patients and pigs, enhancement of meat inspection, and population education programs were conducted. Consequently, infection of taeniasis and prevalence of cysticercosis in humans and swine in these endemic areas were reported to be

Correspondence: Tiaoying Li, Institute of Parasitic Diseases, Sichuan Centers for Disease Control and Prevention, 6 Middle School Road, Chengdu 610041, Sichuan Province, People's Republic of China.
Tel: +86 28 85589512; Fax: +86 28 85589563
E-mail: litiaoying@sina.com

greatly reduced (Table 1) (Sun *et al*, 1984; Sun, 1995; Xu *et al*, 1998). Since the open-market policy was implemented nationwide after 1989, there have been great increases in the numbers of small private butchers and slaughterhouses without strict meat inspection. Since then, more and more cases of taeniasis/cysticercosis have been recorded in provincial hospitals. For example, 4,504 cases of cysticercosis that originated from Jilin Province were identified in local hospitals during 1987-1994, while about 2,528 cysticercosis patients were reported from the Affiliated Hospital of Shandong Province during 1991-2002 (Liu *et al*, 1997; Li *et al*, 2004).

The purpose of this review is to make national authorities, scientists, and the international community aware of the emerging situation concerning human cysticercosis and other zoonotic *Taenia* infections in China. Furthermore, the most recent information available on taeniasis/cysticercosis in Tibetan populations in Sichuan Province, southwest of China, is presented.

EPIDEMIOLOGY

Previous epidemiological surveys and hospital reports on taeniasis/cysticercosis have indicated an extensive distribution of this disease in almost all the country's 29 provinces or autonomous regions; with highly endemic areas in northeast, north, central, northwest, and southwest regions (Xu *et al*, 1999; Ito *et al*, 2003; Murrell, 2005). The impact has been estimated at about 3 million cysticercosis cases and US$ 121 million of annual economic loss in pork production nationwide (Xu, 2002).

Northeast region

Taeniasis/cysticercosis was previously known to be highly endemic in Heliongjiang, Jilin, and Liaoning Provinces several decades ago. Active control intervention measures have been implemented in these areas under the government's supervision since the 1970s, which resulted in the current lower endemicity. In Heliongjiang Province, during the period of 1975 to 1980, 1,551 *Taenia* carriers were detected and treated, and 1,571 *Taenia* worms were collected in Fuyu County (Sun *et al*, 1984), while treatment of 4,211 *Taenia* cases and collection of 4,311 tapeworms during 1974 to 1991 was recorded in Tailai County (Li and Yang, 1993). Recent information from a study conducted in Harbin and Nehe Counties during 1993 to 1994 revealed that human cysticercosis seropositivity, tested by indirect hemagglutination test (IHA), ranged from 2.29% to 4.32% (Table 1) (Li *et al*, 1996; Ji *et al*, 1996). By the end of 1991, a total of 138,419 cases of taeniasis were treated and 140, 984 tapeworms (of which 17,321 were identified as *T. saginata*) were obtained from across Jilin Province. Infection of human taeniasis reduced from 0.0585% in 1983 to 0.0081% in 1993 (Table 1) (Sun, 1995). Infection of human taeniasis in Dalian District of Liaoning was reported to decrease from 0.72% in 1980 to 0.039% in 1995 (Li *et al*, 1996), while another study in this Province during 1995-1997 disclosed a taeniasis prevalence of 0.0485% (38/78,274) and a prevalence of human cysticercosis of 0.0345% (27/78,274) (Table 1) (Li *et al*, 1998).

Table 1
The status of taeniasis/cysticercosis in some endemic areas of China.

Province	Year	Taeniasis %	Cysticercosis %		Reference
			Seropositivity	Prevalence	
Heliongjiang	1993-1994	-	2.3-4.3	-	Li *et al*, 1996
Liaoning	1995-1997	0.05	-	0.03	Li *et al*, 1998
Jilin	1993	0.008			Sun, 1995
Shandong	2000	0.05	-	0.06	Liu *et al*, 2002
Qinghai	1997-1998	0.4	14.7	-	Wu *et al*, 2001
Inner Mongolia	1996-1998	0.8	10.8	0.1	Zhang *et al*, 2000

North Region

A serological survey of human cysticercosis in three regions of Shanxi, Hebei, and Inner Mongolia, conducted in 1992, indicated that 328 (0.76%) individuals were seropositive for anti-*T. solium* metacestode antibodies, and the highest seropositivity was observed to be 2.1% recorded in Inner Mongolia. In addition, 351 of 43,220 subjects (0.81%) reported epileptic seizures. Of these, 102 (29.1%) were observed to be seropositive for antibodies to *T. solium* cysticerci. Furthermore, 0.44% of individuals reported expulsion of *Taenia* proglottids, and 0.52% subjects were identified to have subcutaneous nodules (Wei *et al*, 1994). A hospital survey in Inner Mongolia reported by Zhang Bin found that 814 cysticercosis patients were recorded in a Zhelimu hospital from 1994 to 1995, with case distribution over 41 counties (Zhang *et al*, 2000). An epidemiological survey conducted at 10 study sites in 5 cities in Inner Mongolia during 1996 to 1998 found that 10.78% (441/4,092) individuals were seropositive for *T. solium* cysticerci antibody by IHA, with the range 2.72% to 26.36%. Additionally, 5 persons (0.12%) were identified as having cysticercosis and 33 (0.81%) subjects were confirmed to be *Taenia* carriers by microscopic fecal examination (Table 1) (Zhang *et al*, 2000). Ikejima *et al* (2005) also published clinical and serological data of *T. solium* cysticercosis patients in Inner Mongolia.

Northwest Region

More data are available from Qinghai Province than other provincial regions in the northwest, such as Shaanxi, Gansu, and Ningxia. An epidemiological survey performed during 1997 to 1998 in Huzhu County of Qinghai Province found that 0.39% (4/1,024) individuals were *Taenia* eggs positive by Kato-Katz. Furthermore, 14.71% (159/1,081) were seropositive for *T. solium* cysticercus antibody by ELISA, and 0.56% (6/1,081) residents reported symptoms that were considered to indicate cysticercosis (Table 1) (Wu *et al*, 2001). During 2002 to 2004, another coproparasitological study was conducted more widely in eastern Qinghai Province and recorded a taeniasis prevalence of 0.08% and 1.64% cysticercosis seroprevalence among 5,943 individuals in this area (Wu *et al*, 2005).

Central Region

Taeniasis/cysticercosis appears to be widely distributed in the central region of China, including Shandong, Henan, Anhui, and Hunan Provinces. Due to active control measures conducted in these regions, human prevalence of taeniasis/cysticercosis was greatly reduced. For example, a survey in Shandong Province during 1997-2000 indicated a *Taenia* prevalence of 0.048%, a cysticercosis prevalence of 0.057%, and a seropositivity of IgG4 against *T. solium* cysticerci of 1.91% (Table 1) (Liu *et al*, 2002), in comparison with 0.30% and 0.71% for *Taenia* infection and cysticercosis prevalence, respectively, obtained during the 1990s in the same area (Cao *et al*, 1995). A prevalence range of taeniasis of 0.04% to 1.01% was reported from Henan Province at the end of 1980s, where an incidence of human cysticercosis of 0.6% was also recorded (Zhang *et al*, 1991; Tian *et al*, 1994). In a 2001 survey performed in 2 million residents in Luohe County of Henan Province, only 6 persons were diagnosed as *Taenia* carriers and 26 individuals were confirmed to have cysticercosis, which indicated that parasite transmission had reduced compared to the status 10 years prior (Li *et al*, 2003b). Very limited data are available from Anhui Province. A survey conducted in a mining area of south Anhui indicated that 14 (0.95%) individuals were seropositive for *T. solium* cysticercus antibodies, of which 12 (0.81%) were confirmed microscopically to be *Taenia* carriers (Wang, 2002).

Southwest Region

Sichuan, Yunnan, and Guizhou Provinces are located in this region, with a total population of about 160 million, of which 30 million people belong to ethnic minorities. The western part of Sichuan Province is situated on the Tibetan Plateau, while Yunnan and Guizhou Provinces lie on the Yun-Gui Plateau, the fourth biggest Plateau in China. Previous reports indicated a very high endemicity of taeniasis/cysticercosis in this region.

A 1988-1991 mass survey covering 53,061 individuals from 28 counties of Yunnan Province, using microscopic fecal examination, indicated a widespread occurrence of taeniasis in 15 counties with an average prevalence of 0.9%, the highest prevalence of 17.4% was recorded in Lanping County, where ethnic Pumi populations reside. In addition, a taeniasis prevalence of 4.1% was recorded for the Dali area, mainly inhabited by ethnic Bai and Han Chinese (Zhang *et al*, 1994). Another village-based survey on taeniasis in ethnic Bai populations of Dali Prefecture with fecal examination found a taeniasis prevalence range of 13.2%-19.5%, and an additional 0.5% individuals were diagnosed with cysticercosis by biopsy of subcutaneous nodules (Table 2) (Fu *et al*, 1994; Fang *et al*, 1995). In a community-based survey in an ethnic Yi community of Dali Prefecture, 121 fecal samples were examined microscopically, and 34.7% were positive with *Taenia* eggs. The frequent consumption of raw pork was strongly related to the infection (Table 2) (Fang *et al*, 2002). In 2002, Du *et al* (2002) reported that 67% of 521 subjects in Lanping County (Yunnan) had expelled *Taenia* tapeworm proglottids during the previous three months, and a strong correlation between *Taenia* carriers and consumption of raw pig liver was observed, that is, 78.5% of subpopulations who consumed raw pig liver reported a history of *Taenia* proglottid expulsion, compared to 38.0% to those who didn't eat raw pig liver. The species of *Taenia* worms obtained from Lanping County were subsequently identified as *T. asiatica* by molecular DNA analysis (Zhang *et al*, 1999; Wang and Bao, 2003). In addition, both *T. solium* and *T. asiatica* were confirmed to exist in the Dali area of Yunnan Province (Wang and Bao, 2003). During the period 1991-1996, 1,086 cases of cysticercosis were recorded in the Affiliated Hospital of the Dali Institute of Schistosomiasis Prevention and Control, which originated from 12 counties of Dali Prefecture, with ethnic Bai (59.2%) and Han Chinese populations (34.8%) as the dominant patient groups (Luo *et al*, 1998).

A survey of porcine cysticercosis by postmortem inspection was conducted in 12 counties of Dali Prefecture during the period 1990-1995. The results indicated a wide distribution of this infection in all study areas, with an average prevalence of 0.87% to 3.86% recorded in pigs from Binchuan County (Table 3) (Wei *et al*, 1997).

Table 2
Infection of taeniasis (microscope) in Dali Prefecture, Yunnan Province.

Year	Locality	No examined	No positive	%	Reference
1992	Dali (ethnic Bai)	653	86	13.2	Fu *et al*, 1994
1993	Eryuan (ethnic Bai)	753	147	19.5	Fang *et al*, 1995
2000	Dali (ethnic Yi)	121	42	34.7	Fang *et al*, 2002
	Total	1,527	275	18.0	

Table 3
Infection of porcine cysticercosis (postmortem inspection) in Dali Prefecture, Yunnan Province during 1990-1995.

Locality	No examined	No positive	Infection %
Dali	489,034	2,285	0.5
Binchuan	72,236	2,791	3.9
Weishan	183,488	5,095	2.8
Eryuan	126,941	1,583	1.3
Total	871,699	11,754	1.3

Previous hospital reports from Sichuan Province indicated a distribution of taeniasis/cysticercosis that centered in the "ethnic minority" regions, which include Ganze Tibetan Prefecture, Aba Tibetan and Ethnic Qiang Prefecture, Liangshan Ethnic Yi Prefecture, and Panzhihua District, where about 4 million people belong to an ethnic minority (Liu and Lei, 1993; Ni, 1995; Li *et al*, 2003a). Results from a village-based survey in an ethnic Yi community of Xide County of Liangshan Prefecture in 1993, showed that 4.0% (62/1,542) of individuals reported a history of *Taenia* proglottid expulsion, 0.5% presented with subcutaneous nodules and 7.3% were seropositive for *T. solium* cysticercosis antibodies (Zhou *et al*, 1993). The most recent study conducted in a Tibetan population of Ganzi Prefecture indicated a very high prevalence of taeniasis (22.5%), and a significant occurrence of late-onset epilepsy (8.5% prevalence, 16.4% seropositive for *T. solium* antibodies) attributable in large part to probable neurocysticercosis caused by *T. solium* (Li *et al*, 2006). In this study, modern laboratory tests were applied, including multiplex PCR for *Taenia* species identification, a coproDNA test, and coproantigen detection by enzyme-linked immunosorbent assay (ELISA). In addition, a serological test (ELISA) using specific glycoproteins (GPs) or chimeric recombinant antigens, was used to screen for exposure to cysticercosis. The results of this study demonstrated the co-existence of all three species of human *Taenia* (*T. saginata*, *T. solium*, and *T. asiatica*) in a Tibetan population in China. The human beef tapeworm, *T. saginata* was however the dominant species causing human taeniasis in this population. A total of 30.5% of 661 persons reported proglottid expulsion (anamnesis) and 18/21 proglottids were confirmed by PCR as *T. saginata* and 3 as *T. asiatica*. In addition, 21.5% of persons were positive for *Taenia* coproantigens. Cysticerci from one local pig were also confirmed after DNA analysis as *T. solium*. A high prevalence of late-onset epilepsy (8.5%) was reported in local inhabitants, although the overall *T. solium* cysticercosis seroprevalence was 4.0%. A strong correlation was shown between the prevalence of epilepsy/convulsions in this community and seropositivity against *T. solium* cysticercosis. That is, serology was positive in 16.4% of Tibetan subjects with epilepsy, compared to a 2.0 % seropositivity for populations without epilepsy. This suggests the possibility of neurocysticercosis (NCC). *T. solium* should therefore be considered as a potential emergent public health problem in Tibetan communities in this region of Sichuan.

A previous study conducted in Zhaojue County of Liangshan Prefecture (Sichuan) randomly sampled at postmortem 30 pigs from 5 townships of which 9 were found to be infected with cysticercosis (Fu *et al* 1998). Porcine cysticercosis has now been reported from all 17 counties of Liangshan Prefecture, with high endemicity centralized in ethnic Yi communities, where the prevalence in pigs ranged from 3.3% to 10.4% with the highest at 25-30% (Zhang *et al*, 2003). Based on the data recorded in the Liangshan prefectural abattoir, 291 (0.7%) out of 40,791 pigs slaughtered in 2001 were positive with cysticerci, compared to 0.5% (137/29,331) in the year 1990.

A recent national survey of parasitic diseases in China by the Ministry of Health, indicated an average taeniasis fecal prevalence of 0.28% (983/356,629), with the highest taeniasis prevalence (21%) in the Tibet Autonomous Region (Ministry of Health, China, 2005). Based on the study of Li *et al* (2006) cited above, we expect that most of this infection in Tibet AR was due to *T. saginata*.

Only 3 human cases of cysticercosis with autochthonous infections were reported from Guizhou Province in 1980, 1992, and 2002, respectively (Lin, 1980; Fu and Liu, 1992). However, a study of porcine cysticercosis performed in 9 districts of this province in the 1990s via postmortem inspection indicated an extensive distribution of this disease in the study area, with an average prevalence of 7.6% (328/4,292), with a highest infection rate of 12.1% recorded in those pigs raised by ethnic Yi (Table 4) (Qian *et al*, 1998). We can therefore suggest that human taeniasis/cysticercosis is probably also highly endemic in these regions. In addition, the occurrence of the species of *T. asiatica* was also confirmed in Guizhou Province by DNA genotyping (Wang and Bao, 2003).

Table 4
Infection of porcine cysticercosis in different Minority owners in Guizhou Province (postmortem inspection).

Minority	Miao	Buyi	Yi	Zhuang	Hui	Shui	Dong	Gelao	Total
No. dissected	798	973	314	567	694	305	473	168	4,292
No. positive	63	86	38	21	62	14	36	8	328
% Infection	7.9	8.8	12.1	3.7	8.9	4.6	7.6	4.8	7.6

Fig 1- Map of China with the locality of the capital (Beijing) and the locality of 5 highly endemic areas. 1: Sichuan Province; 2: Yunnan Province; 3: Guizhou Province; 4: Qinghai Province; 5: Inner Mongolia.

CONCLUSION

Currently, *Taenia solium* taeniasis and cysticercosis are highly endemic in China, primarily in Yunnan, Sichuan, and Guizhou in the southwest, and in Qinghai provinces and Inner Mongolia in the northwest and northern regions (Fig 1). Several risk factors appear to be important, including a common practice of consuming raw or undercooked pork, the use of free-ranging pigs, inadequate disposal of human feces, absence of meat inspection, poor hygiene, and low socio-economic levels. The national authorities as well as local health services therefore need to give increased priority to *T. solium* taeniasis/cysticercosis control and prevention in high endemic regions of China. Active detection and treatment of *Taenia* carriers, and improved treatment of human and even swine cysticercosis should be considered. In addition, promotion of health education, sanitation, and enhancement of meat inspection also need to be improved in endemic regions. Application of specific serology for *T. solium* cysticercosis and coprotests for *Taenia* spp infection with high sensitivity/specificity (Ito *et al*, 2003) is needed to accelerate successful surveillance and control of this unique zoonotic disease in China.

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TAENIASIS/CYSTICERCOSIS IN INDONESIA, 1996-2006

Toni Wandra[1], Sri S Margono[2], Made S Gafar [3], John M Saragih[4], Putu Sutisna[5], Nyoman S Dharmawan[6], AA Raka Sudewi[7], AA Depary[8], Hemma Yulfi[8], Dewi Masyithah Darlan[8], Iriani Samad[1], Munehiro Okamoto[9], Marcello Otake Sato[10], Hiroshi Yamasaki[10], Kazuhiro Nakaya[11], Philip S Craig[12] and Akira Ito[10]

[1]Directorate General Disease Control and Environmental Health, Ministry of Health, Jakarta; [2]Department Research and Community Service, University of Indonesia, Jakarta; [3]Papua Provincial Health Office Services, Papua; [4]National Institute of Health and Development, Ministry of Health, Jakarta; [5]Department of Parasitology, Faculty of Medicine; [6]Faculty of Veterinary Medicine, [7]Department of Neurology, Faculty of Medicine, University of Udayana, Bali; [8]Department of Parasitology, University of Sumatra Utara, North Sumatra, Indonesia; [9]Department of Animal Science, School of Veterinary Medicine, University of Tottori, Tottori; [10]Department of Parasitology, [11]Animal Laboratory for Medical Research, Asahikawa Medical College, Asahikawa, Japan; [12]University of Salford, Greater Manchester, UK

Abstract. Three human taeniid species, *Taenia solium, Taenia saginata,* and *Taenia asiatica* are distributed in Indonesia. This brief review summarizes the present situation of taeniasis/cysticercosis, distribution of the three human taeniid species, and the risk factors/transmission of this parasitic disease in Bali, Papua, and North Sumatra, Indonesia.

INTRODUCTION

Taenia saginata or *Taenia asiatica* is a tapeworm parasite that causes taeniasis, the presence of adult worms in the human small intestine. *Taenia solium* can cause two distinct clinical presentations: taeniasis, and cysticercosis, the presence of larval stages in tissues. By consuming raw or undercooked beef/pork contaminated with metacestodes (cysticercus/cysticerci) of *T. saginata*, *T. asiatica*, or *T. solium*, humans get infected with the metacestodes, which become adult tapeworms within a few months. The intermediate host becomes infected by ingesting eggs or gravid segments of one of the three taeniid species released from tapeworm carriers. In humans, the ingestion of eggs of *T. solium* happens through contaminated food, vegetable, water, etc. It is also due to autoinfection, directly through anal-oral route, or by internal autoinfection, reflux of the proglottid or eggs from the intestine into the stomach (Subianto *et al*, 1978; Bakta, 1987; Sanchez, 2003). Cysticercosis can affect many anatomical areas, but it becomes more prominent in the central nervous system (CNS), causing neurocysticercosis (NCC). NCC is the most common parasitic disease of the CNS and one of the most important causes of epilepsy (Sanchez, 2003; Takayanagui and Odashima, 2006).

Taeniasis/cysticercosis is still a very important health problem in certain areas of Indonesia. The disease in this country is caused by three cestodes, that is, *T. solium, T. saginata*, and *T. asiatica* (Margono *et al*, 2006; Suroso *et al*, 2006; Wandra *et al*, 2006a). These three cestodes were reported from three known endemic provinces for taeniasis/cysticercosis: Bali (*T. solium* and *T. saginata*), Papua (*T. solium*), and North Sumatra (*T. asiatica*).

Historically, *T. saginata* taeniasis in Indonesia was reported in East Java in 1867 (Oemijati, 1977), and *T. solium* taeniasis in East Kalimantan in 1940 (Bonne, 1940, cited in Suroso *et al*, 2006). Cases with taeniasis and/or cysticercosis have also been reported sporadically from several other provinces (Simanjuntak *et al*, 1997; Suroso *et al*, 2006).

Correspondence: Toni Wandra, Directorate General Disease Control and Environmental Health, Ministry of Health, Indonesia, Jalan Percetakan Negara 29, Kotak Pos 223 Jakarta 10560, Indonesia.
Tel: 62-21-4247608 #119; Fax: 62-21-4200949
E-mail: twandra@yahoo.com

GEOGRAPHICAL DISTRIBUTION

Bali

A recent survey for human taeniasis and cysticercosis in four villages in four districts of Bali (Gianyar, Badung, Denpasar, Karang Asem Districts) in 2002-2005, indicated that among 540 local people, the prevalence rates of *T. saginata* taeniasis were found to range from 1.1% in Badung in 2004 and in Karang Asem in 2006, to 27.5% in Gianyar (Ketewel village) in 2004. The prevalence rates of *T. saginata* taeniasis increased dramatically in Gianyar, including in 2002 (25.6%) and 2005 (23.8%), compared to previous surveys in 1977 (2.1%) and in 1999 (1.3%), respectively (Simanjuntak *et al*, 1977; Sutisna *et al*, 2000). It is possibly due to the increase in the number of families who consume raw beef (beef *lawar*) (Wandra *et al*, 2006a). However, when 48 taeniasis patients in Gianyar, who expelled tapeworm after treatment with praziquantel in 2002-2005, were re-examined in 2003-2006, there were no cases of re-infections with *T. saginata*. All of them reported that they stopped eating beef *lawar* after they recognized that they harbored tapeworms. However, a few people could not stop eating beef *lawar*, and, after a few months, they expelled tapeworms again.

In another village of Gianyar (Pagesangan) in 2006, only 2 (3.6%) of *T. saginata* taeniasis patients among 56 local people were found. Based on mitochondrial DNA analysis, all 66 tapeworms expelled from 66 worm carriers during 2002-2006 were confirmed to be *T. saginata*. There is no evidence for the existence of *T. asiatica* in humans in Bali (Wandra *et al*, 2006a; Wandra *et al*, unpublished data). The source of infection with *T. saginata* taeniasis was related to the consumption of a local raw-beef dish (beef *lawar*) with inadequate meat and food (*lawar*) inspection. The risk factors for *T. saginata* taeniasis are age, gender, level of education, consumption of beef *lawar*, and source of *lawar* (Wandra *et al*, 2006a).

By contrast, *T. solium* taeniasis/cysticercosis is now rather rare in Bali, probably due to improvements in sanitation and pig husbandry. Among 596 local people in five villages in 4 districts of Bali in 2002-2006, there was no indication of *T. solium* taeniasis, history of epileptic seizures (suspected neurocysticercosis), and subcutaneous nodules (suspected subcutaneous cysticercosis) except for 2 seropositive out of 451 serum samples, including 0.8% (1/125) in Gianyar in 2002, and 2.8% (1/36) in Karang Asem in 2006, respectively (Wandra *et al*, 2006a, Wandra *et al*, unpublished data).

Papua

An epidemiological study on taeniasis/cysticercosis was carried out in 11 sub-districts in 5 districts of Papua (Jayawijaya, Merauke, Manokwari, Paniai, and Nabire) for ten years (1996-2005). A total of 1,474 persons were diagnosed by anamnesis (questionnaire) and physical examinations; 146 stools samples for detection of *Taenia* spp by copro-ELISA test. The serum samples of human (1,444), pigs (272) and dogs (125) were available for detection of antibodies against *T. solium* cysticercosis.

A total prevalence of 13.0% (19/146) for taeniasis was found in Jayawijaya, including 14/88 (15.9%) in 1999, and 5/58 (8.6%) in 2001. The seroprevalence of cysticercosis in humans by sub-district in Papua was detected to range from 0.0% (0/60) in a non-endemic area, Merauke Sub-District, Merauke in 1998, to 45.8% (44/96) in a highly endemic area, Assologaima Sub-District, Jayawijaya in 1996 (Wandra *et al*, 2000, 2003; Ito *et al*, 2003, 2004; Wandra *et al*, unpublished data).

The seroprevalence of cysticercosis in pigs and dogs in Jayawijaya ranged from 8.5% to 70.4% (1998-1999), and from 4.9% to 33.3% (2000-2002), respectively. Necropsy of 4 seropositive dogs found cysticerci of *T. solium* in the brain and heart (4 all) and in the muscle (2) as well. Mitochondrial DNA (mtDNA) analysis of proglottids and cysts obtained either from human, pigs, or dogs and metacestodes grown in NOD/shi-*scid* mice were confirmed to be *T. solium* Asian genotypes (Wandra *et al*, 2000; Ito *et al*, 2002, 2003, 2004). There were no *T. saginata* or *T. asiatica* human cases in Papua.

Bivariate analysis of available data for age, sex, levels of education, and the habit of hand washing before eating in a group with seropositive cysticercosis (n = 102-158) and a

control in a group with seronegative cysticercosis (n = 355-576) in Jayawijaya showed that the factors associated with cysticercosis for local people were age ($p < 0.01$), levels of education ($p < 0.01$), and washing hand before eating ($p < 0.05$). It is suggested that the 18 years or older group, low level of education, and the habit of not washing hands before eating were the important factors associated with cysticercosis, particularly in Jajawijaya, Papua (Wandra *et al*, unpublished data).

A study in four Sub-districts of Jayawijaya (Assologaima, Wamena Kota, Kurulu, and Hubikosi) among 506 families in 1996-2005 showed that only 17% (86/506) defecated in a toilet, 6.3% (32/506) in the backyard, 1.8% (9/506) in the river, 64.6% (327/506) in the garden, and 10.3% (52/506) in the forest (Wandra *et al*, unpublished data).

The number of contaminated districts in Papua has increased from one district (Pania) in the 1970s (Margono *et al*, 2006) to 4 districts (Paniai, Jayawijaya, Manokwari and Nabire) in the past 10 years, due to the local people in Papua moving from one district to another district, sometimes bringing their pigs with them, so that the disease appears to have spread from endemic areas to other districts, particularly where the ethnic groups such as Akari, Dani, Lani, and Yale are predominant, as related to their life style and socio-cultural characteristics (Suroso *et al*, 2006; Wandra *et al*, unpublished data).

There was no evidence that Merauke had already been contaminated, because a woman showing a high antibody titer in 1997 was a transmigrant from another province of Indonesia (Wandra *et al*, 2000, 2003; Ito *et al*, 2003, 2004). There is no data available on taeniasis/cysticercosis from other districts of Papua.

North Sumatra

In Samosir Island in Lake Toba, North Sumatra the prevalence rates of taeniasis during 1972-1990 were reported to range from 1.9% to 20.7% (Kosin *et al*, 1972; Cross *et al*, 1976; Koeshardjono *et al*, 1987; Kosman *et al*, 1990 cited in Depary, 2003). Repeated epidemiological surveys of taeniasis/cysticercosis during 2003-2006 on 240 local people showed that 6/240 (2.5%) were infected with *T. asiatica*, including 2/58 (3.4%) in 2003 and 4/182 (2.2%) in 2005 (Wandra *et al*, 2006b).

Mitochondrial DNA analysis of proglottid samples isolated from patients showed that all 6 expelled tapeworms were confirmed to be *T. asiatica*. There is no evidence for the existence of *T. solium* or *T. saginata* in Samosir Island. The main risk factor of *T. asiatica* taeniasis for the people is due to preparation of a local traditional dish (*sang-sang*) at home, in local restaurants, and during traditional or religious celebrations. While cutting pork into small pieces, the people sometimes try to eat the uncooked viscera (liver). This is completely different from the Bali people who do not like the taste of uncooked viscera (Wandra *et al*, 2006b).

CONCLUSION

The recent survey for human taeniasis and cysticercosis in Bali indicated that the increase in the number of cases of *T. saginata* taeniasis might be due to the increasing number of families who consume raw beef (beef *lawar*). *T. solium* taeniasis/cysticercosis is now rather rare, probably due to the improvement in sanitation and pig husbandry. Both *T. solium* and *T. saginata* are distributed in Bali. There is no evidence for the existence of *T. asiatica* in humans in Bali, probably due to the local people liking uncooked meat with blood (pork *lawar*), but not liking uncooked viscera. *T. solium* is distributed in Papua. The population aged 18 years or older group, low level of education, and the habit of not washing hands before eating were the important factors associated with cysticercosis, particularly in Jajawijaya. The number of contaminated districts with taeniasis/cysticercosis has increased. Mitochondrial DNA (mtDNA) analysis of parasite was confirmed to be *T. solium* Asian genotypes. In Samosir Island, North Sumatra, where *T. asiatica* taeniasis is still found, it was possibly related to the consumption of the uncooked viscera of local pigs. Considering the differences in religions, cultures, socio-economic characteristics, and levels of education, control programs of taeniasis/cysticercosis should be adapted to the specific local area and be evidence based.

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PRESENT SITUATION OF PORCINE TAENIASIS AND HUMAN CYSTICERCOSIS IN NEPAL

Durga Datt Joshi[1], Padam Raj Bista[1], Akira Ito[2] and Hiroshi Yamasaki[2]

[1]National Zoonoses and Food Hygiene Research Center (NZFHRC), Chagal, Kathmandu, Nepal;
[2]Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan

Abstract. Human and porcine taeniasis/cysticercosis is reportedly one of the major zoonotic diseases in Nepal. Out of 250 pig carcass examined, 34 (13.6%) carcass were positive for taenia cyst. Out of 724 patients who presented with seizure at Kathmandu Model Hospital, 61% of all seizures and 72% of focal seizures were due to NCC. Forty-six cases of NCC were studied at Shree Birendra Hospital, 20 (43.5%) were from Eastern Development Region of Nepal. Data collected from 543 patients at Patan Hospital, Lalitpur were analyzed; out of which, 40 (7%) were diagnosed to have neurocysticercosis, based on clinical profile and neuroimaging. Fifty-four cases of seizure disorders were treated at Om Hospital and Research Center in Kathmandu; out of which, 24 (43.7%) patients were diagnosed as neurocysticercosis. Risk factors include the pig production system, the food culture, inadequate regulatory mechanisms, and low priority in the control program, and are the factors associated with the high prevalence of infection. Rapid expansion of small-scale pig producers and processors has led to a significant increase in cysticercosis in pigs and humans.

INTRODUCTION

WHO estimated that 50 million persons, predominantly from developing countries, are infected with taeniasis, and 50,000 people die of the disease each year. Taeniasis refers to a human infection with the adult tapeworms: *Taenia solium* and *Taenia saginata*. The disease in human and pigs is an ancient parasitic disease that has been rooted in developing countries and is now emerging as a major health problem of global dimensions (Sciutto *et al*, 2000). The infection is also present in India, Pakistan, northern China, Thailand, and Nepal (Schantz *et al*, 1992). *Taenia* cysts were observed for the first time in pig meat slaughtered in Kangeswari, Kathmandu, Nepal (Joshi, 1973, 1991).

Taeniasis refers to a human infection caused by the adult tapeworm of *Taenia solium* and *Taenia saginata*. The infective stage of *T. solium* (*Cysticercus cellulosae*) develops in the pig, while that of *T. saginata* (*Cysticercus bovis*) develops in buffalo and cattle. The adult stages of *T. solium* and *T. saginata* are obligatory intestinal parasites for man. An infection with the larval stage of *T. solium* is called cysticercosis. The infection frequently occurs in populations living in poor sanitary conditions, and people infected with *T. solium* can initiate the spread of proglottids into an endemic environment. Cysticercosis is, therefore, a communicable infectious disease among humans residing in poor and unhygienic communities.

Human and porcine taeniasis/cysticercosis are reported to be among the major zoonotic diseases in Nepal (Poudyal, 1998; Gaihre, 2000; Thapa, 2000; Joshi *et al*, 2001a, 2003, 2005). Particular ethnic groups, which could comprise up to 25% of the population of Nepal, are pig farmers and pork consumers with very low hygienic and sanitation practices, and with no control of pig husbandry and slaughtering. Epilepsy cases in Nepal are increasing, with studies showing that up to 7.3 per 1,000 population may suffer from epilepsy, and almost 50% of the cases are due to neurocysticercosis (Rajbhandari, 2003). Joshi *et al* (2001a) showed that the seroprevalence by ELISA and prevalence by lingual palpation was 23.5% (204 pig sera) and 32.5% (419 pig tongues), respectively.

Correspondence: Durga Datt Joshi, National Zoonoses and Food Hygiene Research Center (NZFHRC), Chagal, Kathmandu, Nepal.
E-mail: ddjoshi@healthnet.org.np

Background information on pig slaughtering and marketing

There are no modern slaughtering places or slaughterhouses, and no one undertake

antemortem or postmortem examinations of the animals in Nepal. Thus, slaughtered animals are transported to the pork meat shops by bicycles, rickshaws, or taxis in a very unsanitary way, without meat inspection. The total pig population is about 935,075 in the country. Of which, 53% are reared in the Eastern Development Region; and, the Far-western Development Region represents the lowest pig population in of the country at 5% (Table 1).

Nepal, especially with people of different ethnic, religious, and food habits are equally concerned with human taeniasis, as some of the ethnic groups (Tharu) in the plains (*terai*) region keep pigs (which are considered to be the reservoirs for *Taenia solium*) as their cash crop/property that could be sold in times of need, usually during festivals.

Risk factors

The following risk factors are associated with porcine taeniasis and human cysticercosis in Nepal:

1. Pigs are reared by the group/community of low economic status, low level of household sanitation, low level of personal hygiene, and low level of education.

2. Free-range pig rearing is being carried out by allowing pigs to scavenge and eat human feces, the cost of subsistence raising of pigs in unsanitary conditions, households lacking latrines as well as out-door human defecation near or in pig rearing areas, deliberate use of human feces as pig feed, connecting pigpens to human latrines, indiscriminate defecation in the public areas, and use of sewage effluents, sludge, or night soil as fertilizer in vegetable crop fields.

3. Food habits and preparation of fast foods for human population is changing resulting in people having different habits of eating, human carriers involved in pig rearing, lack of meat inspection, unregulated animal traders and meat sellers selling infected pigs or meat, cultural preferences for eating raw or improperly cooked meat, risky local traditions and customs, frequent pork consumption, and low national priority for the control of pig diseases.

4. Prevalence rate in Nepal: reliable data on prevalence and epidemiological information is lacking; however, the presence of taeniid adults in certain ethnic group people is estimated to be 10-50%, while the porcine cysticercosis rates are estimated to be 14-32%.

Risk factors, production systems, the food culture, inadequate regulatory mechanism, and low priority in the control programs are the associated factors of high prevalence of the infection. Rapid expansion of small-scale pig producers and processors has led to significant increases in cysticercosis in pigs and humans (Ratala, 2006).

Meat consumption patterns in a rural farm community study is shown in Fig 1, that is, 60% people eat pork meat, and 35% mixed buffalo and pork meat. Overall, 68.2% of respondents consumed cooked pork; whereas, boiled pork was

Table 1
Pig population by development region.

Development region	Pig population	Pig population (%)	Pork production (mt)	Pork production (%)
Eastern	495,230	53.0	7,556	49.1
Central	157,371	16.8	3,413	22.2
Western	108,449	11.6	1,879	12.2
Mid-western	126,172	13.5	1,934	12.6
Far-western	47,853	5.1	607	3.9
Total	935,075	100.0	15,389	100.0

Source: Department of Livestock Services, 2005.

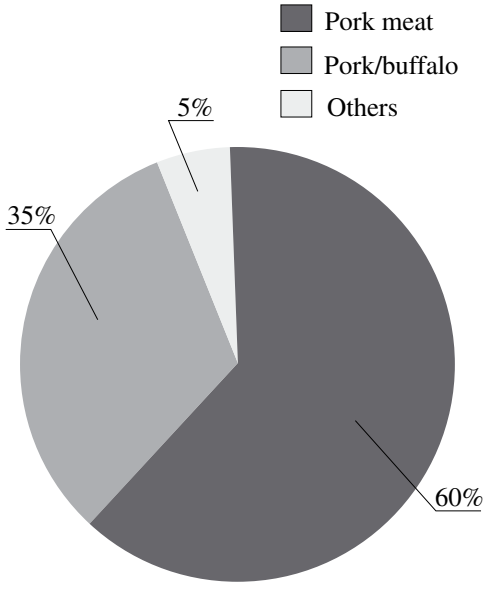

Fig 1- Meat consumption patterns in rural farm community.

less consumed by 4.3%, and only 8.3% consumed raw pork (Sharma *et al*, 2006). Pig grazing in the study area showed that about 73% pigs are raised in open fields, and only 27% in shelters. This causes risk factors in disease transmission to human and pig, thereby, completing the lifecycle of *Taenia solium* parasites (Joshi *et al*, 2006b).

CYSTICERCOSIS

Porcine cysticercosis

Two detailed studies were carried out in Nepal, one in Sunsari, Syangja, and another in Kathmandu valley on taeniasis in pig population during the years 2001-2005. Joshi *et al* (2001a) showed that the seroprevalence by ELISA and prevalence by lingual palpation were 23.5% (204 pig sera) and 32.5% (419 pig tongues), respectively. Validation of lingual examination with serology and DNA-PCR techniques was also studied. (Joshi and Wallingham, 2006) carried out a study in 2005 where the prevalence of lingual palpation (examination) of porcine cysticercosis was 10.5% (21/200), seroprevalence by ELISA method was 22.5% (45/200), and prevalence of *T. solium* cysticercus in the postmortem carcass organs examined was 20.5% (41/200).

The DNA analysis of cysticerci by multiplex PCR method determined them to be the *T. solium* Asian genotype. These study results showed that, out of 45 seropositive pigs, only 41 were cysticerci positive of which 21 were lingual examination positive pigs (Joshi *et al*, 2006c).

In another study a total of one hundred ten buffaloes and thirty swine were examined for the presence of cysticercosis at the various abattoirs of Rampur and Narayangath bazaar, at Chitwan. Two buffaloes (1.9%) and two swine (6.7%) were found to be infected with cysticercosis. In buffaloes, cysticerci were recovered from the liver and lungs; whereas, in case of swine it was obtained from the muscles of diaphragm and the neck (Rana *et al*, 2006).

Concerning the consumption of pork, 68.2% of the respondents consumed cooked pork, 4.3% of them consumed boiled pork, while 8.3% of the people consumed raw pork (Sharma *et al*, 2006).

Pig carcass examined in Sunsari and Kathmandu Valley. Out of 250 pig carcasses examined, 34 (13.6%) pig carcasses had *Taenia* cyst positive in Sunsari and Kathmaìÿu valley (Poudyal, 1998).

Human cysticercosis

Family health parasitic survey, Chitwan. A total of 183 stool samples were examined in the Mushar community of Chitwan. The prevalence rate of intestinal parasites was found to be 77.1%; out of which, females had higher prevalence (*ie*, 79.2%) than those of males (*ie*, 74.4%), of which the prevalence rate of *Taenia* sp was 1.6%. In this community, there was no toilet; that is, all of them defecated in the open field (forest, bank of river, etc). Out of 183 people interviewed, 107 (58.5%) of Mushars did not use a toilet but defecated in an open field. Only 76 (41.5%) people out of 183 interviewed used a toilet.

Family health parasitic survey, KMC. Out of 211 persons, stool samples taken in ward 19 (Joshi *et al*, 2006a), 132 (62.1%) were infected by different species of parasites, of which *Taenia solium* constituted 1.4%.

Epidemiological study, Department of Health Services. In one epidemiological study (Bista, 2006), it was found that higher prevalence

was seen in females (61%) as compared to males (39%).

TU teaching hospital. Sixty-six cases of neurocysticercosis (NCC) were seen at the neurology service of TU Teaching Hospital (Agrawal, 2006). The numbers of males and females were almost equal (36/30). Of these 66 cases, 77.2% presented with seizures of one or the other type. CT scans showed single ring-enhancing lesions in 42 cases (63.6%) and multiple ring enhancing lesions in the remaining 24 cases (36.3%). Maximum lesions were seen in parietal region 63.6%, followed by frontal (13%), and temporal and occipital (9% each). A total of 681 stool samples from hospital and clinics were examined (Sherchand, 2006). Tapeworm (*T. solium*, *T. Saginata,* and *H. nana*) infections were found in 52 (7.6%) cases; in which, 36 (69.2%) were infected with *H. nana*, and 16 (30.8%) with *T. solium* or with *T. saginata*, in 2005. Although people of all ages were infected, most of the cases, 37 (71.1%), were 19-50 years old; the 29-40 year-old age group was the most infected (25%).

Kathmandu Model Hospital. Two studies were carried out in Kathmandu Model Hospital during August 2000 to May 2001 (Neopane, 2006; Panta, 2006). Enzyme-linked immunoelectro-transfer blot (EITB) and radio imaging techniques were used to diagnose neurocysticercosis (NCC). It was found that 724 patients presented with seizures; out of which, 61% of all seizures and 72% of focal seizures were due to NCC. The mean age of presentation was 13 years, and both sexes were equally affected. Seventy-one percent of these patients had stage II lesions (ring-enhancing lesion), and the remaining patients had stage III lesions. Sixty-nine percent of them had a solitary lesion; whereas, 31% had multiple lesions (Panta, 2006).

Shree Birendra Hospital, Chhanuni, Kathmandu. Forty-six cases of NCC were studied at Shree Birendra Hospital, Chhauni (Neopane, 2006), of which 20 (43.5%) were from the Eastern Development Region of Nepal. This indicates that more people of this area rear pigs and eat pork, which could be infected with the *Taenia solium* parasite. Out of 46 cases, 54% were males, while 46% were females (Fig 2).

Patan Hospital, Lalitpur. Another study (Chaudhary 2006) was carried out as a retrospective analysis of data of patients admitted to Patan Hospital in Lalitpur, Nepal with the

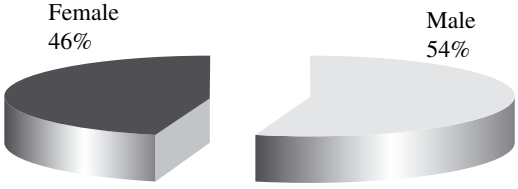

Fig 2- Sex-wise distribution of seizure cases.

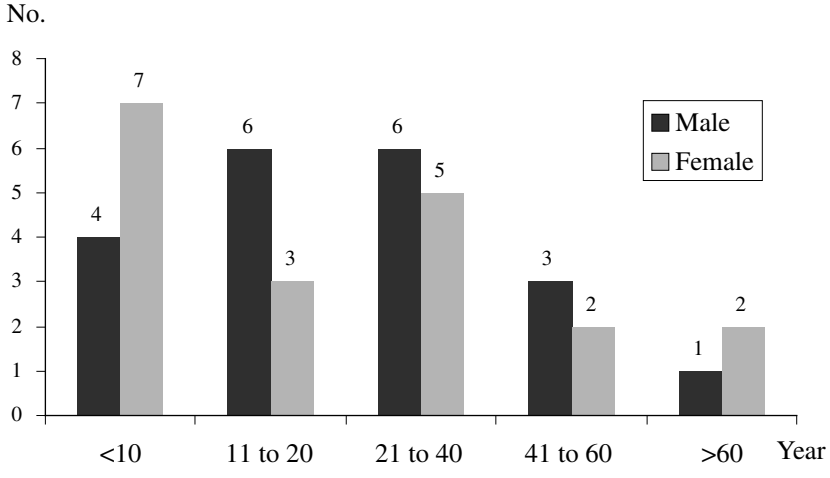

Fig 3- Age and sex distribution of seizure patients at Patan Hospital, Lalitpur.

initial presentation as seizure. A total of 543 patients' data were analyzed out of which 40 (7%) were diagnosed to have neurocysticercosis, based on clinical profile and neuroimaging. Age and gender distributions of seizure patients are shown in Fig 3. Patients' ages ranged from 2-75 years.

Om Hospital, Chabahil, Kathmandu. One of the retrospective studies (Sharma 2006a) included 24 cases of neurocysticercosis (out of 55 cases of seizure disorder) who attended at neurosurgical OPD, Om Hospital, Chabahil, with the history of seizures from 15 October to 15 November 2005. Over a period of one month 54 cases of seizure disorders were treated at a neurosurgical OPD, out of them 24 (43.7%) patients were diagnosed as neurocysticercosis. 23 (41.9%) patients had no intracranial lesions and labelled as idiopathic. Causes of seizures in remaining 15% were infection, tumors, congenital cysts and infarction, respectively.

RECOMMENDATIONS

Southeast Asian countries, including Nepal, need to address important issues and risk factors of porcine taeniasis and human cysticercosis. Based on these issues, a long-term disease control plan has to be formulated and implemented by member countries with the support from WHO and other donor agencies: identifying the groups most at risk, with good prevention programs, and identifying means to increase access to diagnosis for epilepsy for the rural poor; with evidence-based treatment for NCC (Tun and Margarita, 2006). The following recommendations were made based on this review:

Ensure that pigs are protected from ingesting feed and water contaminated with human feces.

Adopt occupational health safety measures.

Strict bio-security measures should be adopted.

Keep closed herds.

Notifiable diseases should be reported to the authority concerned.

Proper herd health record keeping.

Sewage treatment is an important factor.

Clean-up an infected pig prior to slaughter.

Improve sanitation and hygiene.

Proper handling of meat and by-products.

Proper cooking of meat.

Treatment of human carrier and cysticercotic pigs.

Identify the high-risk production pockets and rural pig breeding areas and target them.

Health education for all the stakeholders.

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CYSTICERCOSIS AND TAENIASIS IN THAILAND

Malinee T Anantaphruti[1], Jitra Waikagul[1], Hiroshi Yamasaki[2] and Akira Ito[2]

[1]Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand;
[2]Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan

Abstract. Taeniasis is a common food-borne parasitic zoonosis in Thailand. Infection rates by stool examination vary, depending on the place and time of examination, and have been reported as 0.2-7.0%. Nationwide data indicate that most cases occur in the north and northeast (approximately 1.2%). By molecular analysis, *T. asiatica* infection was first discovered in Thailand in 25% (6/24) of taeniasis cases. The identifications were based on either multiplex PCR, base excision sequence scanning thymine-base (BESS T-base) analysis, and DNA sequencing of PCR products using *cytochrome coxidase* subunit 1 and *cytochrome b* genes. DNA sequencing showed that the *T. solium* in these patients were the Asian genotype. By molecular identification, a dual infection of *T. solium* and *T. asiatica* was first found in Thailand, which was also the first in Asia. The usefulness of molecular analysis for identifying human taeniid cestode infections is stressed. Cysticercosis, a potentially fatal chronic disease in humans, is caused by *C. cellulosae* developed in humans by ingestion of *T. solium* eggs. From 1947 to 2004, approximately 500 cases of human cysticercosis have been reported in Thailand, while the actual number of cases is speculated to be several times more.

INTRODUCTION

Human taeniasis is still common and a relatively serious public health problem worldwide. It is caused by either *Taenia saginata* (the beef tapeworm) or *Taenia solium* (the pig tapeworm) in the intestine. In Asia, a third of *Taenia* infections is caused by *Taenia asiatica* as has been reported in many countries (Ito *et al*, 2003, 2004). The larval stage of *T. saginata*, *Cysticercus bovis*, is found in the muscles of cattle; whereas *Cysticercus cellulosae* of *T. solium* and *Cysticercus viscerotropica* of *T. asiatica* (Eom and Rim, 1993; Eom, 2006) are found in the muscles and in the viscera, respectively, of pigs. Humans become infected with these *Taenia* species in the intestine by ingesting uncooked or poorly cooked beef, pork, or pork viscera that are contaminated with cysticerci. Eggs excreted into the environment from the adult worms of *T. solium* from tapeworm carriers can develop into *C. cellulosae*, which can cause cysticercosis, a fatal disease in humans as well as in pigs and even in dogs (Ito *et al*, 2003, 2004). Therefore, taeniasis solium is an important food-borne taeniid zoonosis in many developing countries, including Thailand.

Correspondence: Malinee T Anantaphruti, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.
Fax: 66 (0) 2643-5600
E-mail: tmmtr@mahidol.ac.th

HUMAN CYSTICERCOSIS

Cysticercosis, caused by the *C. cellulosae* of *T. solium*, is expected to be one of the most potentially lethal helminthic infections in humans and an important public health problem worldwide. When eggs that are released from *T. solium* carriers are ingested by humans, pigs, or dogs, the hatched oncospheres develop into cysticerci in many tissues and organs and cause various types of cysticercosis. Neurocysticercosis of the central nervous system (CNS) is the most serious in humans because a single cysticercus or multiple cysticerci develop in the CNS, mostly in the brain (Ito *et al*, 2006; Takayanagui and Odashima, 2006). The most common symptom is epileptic seizure. Another relatively serious type is ophthalmic cysticercosis, which often causes a high degree of visual impairment. An asymptomatic type is subcutaneous or muscle cysticercosis. This review is based on reports of approximately 500 cysticercosis cases, mainly published in Thailand.

Distribution and sex

Most cases of cysticercosis were reported

in hospitals in Bangkok, especially in medical school hospitals without any record of patient domicile. Residential analyses of patients were found in two reports. In 25 patient records from Rajvithi Hospital in 1997, patients were mainly from the northern provinces (44%); the remainder were from the central (32%), northeastern (20%), and southern regions (4%) (Techathuvanan, 1997). Among 98 cases reported from Prasat Neurological Hospital, more cases were from the north (27.6%), Bangkok (25.5%) and the central region (25.5%), followed by the northeast (11.2%) and east (10.2%) (Jitsukon and Towanabut, 1989). There were more cases of cysticercosis reported from Hospitals in the northern provinces than the northeastern provinces (Menakanit, 1963; Tantajumroon and Thitasut, 1966; Chotmongkol, 1988, 2001). The existing residential data summarized in this review showed most cases (68 cases) were from the central region, including the eastern provinces and Bangkok, followed by the northern (58 cases) and northeastern regions (32 cases), and rarely from the south (2 cases). The disease is found more often in males than females, at a ratio of 1.9: 1 among about 400 reported cases

Neuro- and soft tissue-cysticercosis

Neurocysticercosis (NCC) in humans was first mentioned in Thailand in 1947 in a textbook for medical students at Siriraj Hospital. The first case was diagnosed in 1934 in an autopsied female, who resided in Tak Province, showing hundreds of *C. cellulosae* in the cerebral cortex. The second case was also an autopsied, a male from Ubon Ratchathani Province in 1939 with a large number of cysticerci in the grey matter of the brain (Daengsvang and Tansurat, 1947). Four males with epileptic convulsions were reported from the same hospital in 1954; many subcutaneous nodules were found in all these cases, and pathological examinations of sectioned nodules revealed cysticercosis (Viranuvatti and Toochinda, 1954). In 1962, four NCC cases confirmed by autopsy were reported (Chitanond and Indavasu, 1962). These early cases were from government hospitals in Bangkok Metropolis, and the diagnosis was mainly based on pathological biopsy specimens and post-mortem samples.

The introduction of computed tomography (CT) and magnetic resonance imaging (MRI) has increased the number of diagnosed cysticercosis patients in the country. Between 1979-1988 and 1981-1986, 98 and 132 cases, respectively, were diagnosed as NCC at Prasat Neurological and Siriraj Hospitals (Bhoopat *et al*, 1989; Jitsukon and Towanabut, 1989). A five-year retrospective study of 25 cases in Rajvithi Hospital during 1993-1997 showed that 60% (n = 15) in CNS, 24% (n = 6) in soft tissues, and 16% (n = 4) in the eye (Techathuvanan, 1997).

Soft-tissue cysticercosis (STCC) involves nodules in subcutaneous tissues (subcutaneous cysticercosis, SCC) and muscle. As STCC does not in general cause serious manifestations, STCC cases have not been frequently reported. Diagnosis was based on pathological examination of surgical specimens. Daengsvang and Tansurat (1947) recorded the first three cases having subcutaneous nodules at Siriraj Hospital. A number of cases of STCC co-existed with NCC cases. Seven out of eight neurologically symptomatic patients admitted to the Neurological Section at Phramongkutklao Hospital were confirmed to have subcutaneous nodules (Punyadassaniya, 1972). The same author conducted a retrospective study of patient records from the Pathology Section: 70 SCC cases (48 males, 22 females) were recorded during 1955-1972. Chitchang (1966) reported 20 SCC patients (18 males and 2 females, aged 13-54 years) in 1961-1965. The sites commonly affected were the extremities. Neurologic symptoms were demonstrated in 5 of these 20 SCC cases. SCC cases from the two above reports were from the same source; thus, the number of cases might overlap. CT-based diagnosis of 132 NCC cases at Siriraj Hospital showed that 13 patients had subcutaneous nodules or characteristic muscular calcification (Bhoopat *et al*, 1989). Similarly, the in-patient hospital records at Prasat Neurological Hospital showed that 5% of 98 NCC cases had subcutaneous nodules. It was interesting that 23% and 58% of the cases showed X-ray calcification on thigh and chest, respectively (Jitsukon and Towanabut, 1989).

At Chiang Mai University Hospital in the north in 1962-1966, 13 cases of cysticercosis

were found by pathology: 10 were STCC and 3 others were NCC (one patient had subcutaneous nodules over the entire body). Cysticercus was confirmed by biopsy of a 1-cm nodule (Tantajumroon and Thitasut, 1966). At Khon Kaen University Hospital in the northeast, 2 of 12 NCC cases showed soft tissue cyst calcification (Chotmongkol, 1988).

From 1947 to 2000, approximately 300 cases of NCC and 100 cases of STCC were recorded. The reports of STCC cases that appear in the literature might be lower than the actual situation. Patients with subcutaneous nodules might not be concerned about themselves, and the physicians might misdiagnose the nodules as sebaceous cysts, lymphadenitis, tumors, or other skin lesions (Chitchang, 1966).

Racemose cysticercosis

Abnormal growth of *Cysticercus* appears as multiple cysts attached to the central stem by stalks, like a bunch of grapes, producing racemose cysticercosis. The cystic larva, *Cysticercus racemosus*, contains no scolex. The first recorded case, a 36-year-old male from Kamphaeng Phet, a northern province, was observed to have a sparganum-like object involving the base of the brain and spinal subarachnoid space in 1969 (Pradatsundarasar, 1980). In 1971, a cyst-like parasite object was found in the subarachnoid space of the brain of a female Bangkok resident and was diagnosed as a sparganum-like tapeworm (Pradatsundarasar *et al*, 1971). Histological sections of these two cases were later re-identified as *C. racemosus* (Pradatsundarasar, 1980; Chayapum *et al*, 1993). Two more cases were reported in 1980 in the ventricles of the brain of Thai males (Patharangkura *et al*, 1980). In the south of Thailand where taeniasis was rarely found, racemose cysticercosis in the basal subarachnoid space of the brain and the thoracic subarachnoid space was reported in a male from Yala Province (Chayapum *et al*, 1993). Recently, racemose NCC in the subarachnoid space at the cistern of the brain through the lumbar cistern was found in a patient from Phitsanulok, a northern province. The patient presented with paraparesis and bilateral hearing loss (Jarupant *et al*, 2004). The disease caused by *C. racemosus* is quite severe and potentially fatal, because relapse occurs frequently. Until 2004, 6 cases were reported as racemose cysticercosis in Thailand.

Ocular cysticercosis

Ocular cysticercosis (OCC) has been reported occasionally, compared with NCC and STCC. In 1956, almost 10 years after the first record of cysticercosis in Thailand, OCC cases in 2 females who presented with painful and dimmed visual acuity were first reported in the literature (Kanjanarun, 1956a,b). In Chiang Mai, a 20-year-old male had a cyst-like mass in the posterior chamber of the eye (Khamboonruang and Mahasuwan, 1971). In 1997, 1 case of cysticercosis in the vitreous and 3 cases of subretinal cysticercosis were diagnosed from 25 cysticercosis cases admitted at Rajvithi Hospital (Techathuvanan, 1997). A live cyst and a 9.0 mm cyst were successfully removed from the subretina of a 25- and a 36-year-old male, respectively, from northeast Thailand (Kittiponghansa *et al*, 1988; Lerdvitayasakul and Lawtiantong, 1991). Until 1997, approximately 10 OCC cases were reported in the literature.

Cysticercosis of other organs

Cysticercosis was also reported in other organs of the body. An autopsy of a 65-year-old male conducted at Siriraj Hospital in 1947 revealed a 0.5 cm cyst at the myocardium of the left ventricle. The patient had no particular heart symptom (Daengsvang and Tansurat, 1947). An autopsy of a 40-year-old male who had died of NCC showed several cysts in the heart and lungs. The cyst was confirmed as *C. cellulosae* by pathological examination (Saengsingkaeo and Bunnag, 1965). Two cysticerci were found in the thyroid gland of a 28-year-old female who died of heavy NCC with >1,500 cysts in the brain (Leelachaikul and Chuahirun, 1977).

Cysticercosis co-existing with taeniasis

Cysticercosis co-existing with taeniasis is not really uncommon. Chotmongkol (1992) reported that stool examinations of three NCC patients revealed one with *Taenia* eggs. Four percent of 98 NCC cases registered at Prasat Neurological Hospital had proglottids in the stool (Jitsukon

and Towanabut, 1989). Of 25 cysticercosis cases diagnosed at Rajvithi Hospital, one patient had a history of expulsion of *Taenia* proglottids in the feces, while another had *Taenia* eggs in the feces (Techathuvanan, 1997).

TAENIASIS

Taeniasis is a common food-borne parasitic zoonosis in Thailand. The disease is known to be caused by the two species, *T. saginata* and *T. solium*.

Prevalence and distribution

In the north and northeast of Thailand, people customarily consume raw or undercooked meat; therefore, taeniasis is relatively common among the residents there. Reported occurrences were generally based on findings of eggs in stool specimens. The latest nationwide stool survey, conducted in 2001 by the Ministry of Public Health with 17,025 residents of any age, showed the highest *Taenia* infection rate was 1.25% in the north, followed by 1.17% in northeast (Ministry of Public Health, 2001). In an early nationwide survey in 1957, the infection was highest in the northeast, with 3.4% prevalence (Vajrasthira and Harinasuta, 1957). Several nationwide surveys later showed infection was higher in the north and northeast, between 1-2%, and lower in the central region (<1%), and rare in the south (Table 1) (Vajrasthira and Harinasuta, 1957; Preusaraj *et al*, 1982; Jongsuksantigul *et al*, 1992; Jiradit *et al*, 1997; Ministry of Public Health, 2001).

Individual reports in some locations showed variable prevalence. A high prevalence (5.9%) was found among 1,450 hilltribe people residing in 6 upper northern provinces (Mae Hong Son, Chiang Mai, Chiang Rai, Lampang, Lamphun, Phayao), and 1.9% prevalence among 2,540 hilltribe people along the Thai-Lao PDR border of Nan Province (Wijit and Kraewsan, 2001; Maipanich *et al*, 2004). In the northeast, the infection rate was 7.0% among 438 stool samples from Udon Thani Province, in 1967 (Chularerk *et al*, 1967). Two decades later, in 1987, it was still high, at 5.9% among 202 residents of Kalasin (Ektaseng *et al*, 1987). In a survey in 1996, the prevalence rate was 3.3% in 5,125 residents from 7 provinces of the region (Nakhon Phanom, Maha Sarakham, Yasothon, Roi Et, Ubon Ratchathani, Loei, and Chaiyaphum), (Sithithaworn, unpublished data). The prevalence was rather low in Khon Kaen Province, at 0.2% in 395 stool samples from villagers (Rhongbutsri and Kitvatanachai, 2002).

Table 1
Chronological nationwide prevalence (%) of *Taenia* infection, by regions, Thailand.

Regions	Years				
	1957[a]	1982[b]	1992[c]	1997[d]	2001[e]
North	1.2	0.94	2.33	1.8	1.25
	(99/8,389)	(80/8,485)	(284/12,146)		(48/3,841)
Northeast	3.4	1.13	1.17	1.5	1.17
	(6,531/192,499)	(165/14,582)	(149/12,705)		(50/4,287)
Central	0.2	0.66	0.23	0.2	0.16
	(45/21,478)	(89/3,548)	(33/13,924)		(9/5,613)
South	0	0.06	0	0	0
	(0/41,337)	(4/6,724)	(0/6,388)		(0/3,284)
Total	2.5	0.78	1.03	0.9	0.63
	(6675/263,703)	(338/43,339)	(466/45,163)		(107/17,025)

Number in parenthesis are No. positive/Total

[a]Vajrasthira and Harinasuta, 1957; [b]Preuksaraj *et al*, 1982; [c]Jongsuksantikul *et al*, 1992; [d]Jiradit *et al*, 1997; [e]Ministry of Public Health, 2001.

In the central region, infection rates were relatively low if compared with the north and northeast. In the west-central, along the Thai-Myanmar border, a prevalence of 2.8% was found among 286 Thai troops working in Ratchaburi (Maneeboonyang *et al*, 2004) and 0.7% among 761 residents of Kanchanaburi (Anantaphruti *et al*, 2004). In and around Bangkok Metropolis, of 189 adults, 25% were positive for intestinal parasitoses, and 2% of these were infected with *Taenia* sp (Pitisuttithum *et al*, 1990).

Higher prevalence figures were shown in an investigation of worm purging following drug administration; 102 (14.9%) taeniasis cases were found among 681 residents who received praziquantel in 16 northeastern provinces. The infection rate was higher in males than in females (Radomyos *et al*, 1994). A similar investigation was performed in 16 northern provinces, where 21 (4.9%) of 431 residents were infected with *Taenia* sp (Radomyos *et al*, 1998).

About 40-50 taeniasis cases are admitted to the Outpatient Clinic of the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Bangkok each year (Hospital for Tropical Diseases, 2002, 2003, 2005). This figure was similar to previous records for the period 1977-1978, where 39 patients who discharged segments of *Taenia* sp attended the same Hospital (Charoenlarp *et al*, 1981).

Taenia species

T. saginata infection was more common than *T. solium*. The scolices evacuated by 34 patients and the proglottids expelled from another 23 patients were all identified as *T. saginata* (Manmontri, 1949; Chularerk *et al*, 1967; Charoenlarp *et al*, 1989). *T. solium* was found in 3/35 *Taenia*-infected patients; whereas, all others were identified as *T. saginata* by the morphological characteristics of the scolex, except 2 cases with proglottids without scolex (Chirasiri, 1963; Charoenlarp *et al*, 1981). Molecular analysis of 37 samples of proglottids from taeniasis patients in Chiang Mai showed that they all were *T. saginata* (Bowles and McManus, 1994; Morakote *et al*, 2000). *T. solium* infection seems to be rare in Thailand, but cysticercosis cases were quite often reported, as mentioned above. Anantaphruti *et al* (2007) reported a high prevalence of *T. solium* infection in a remote area in Kanchanaburi, a west-central province. Sixteen persons of 24 taeniasis episodes expelled scolices with or without proglottids. Based on morphological characteristics of the scolex, 31% (5/16) of cases were confirmed to be *T. solium*. Consequently, the expelled proglottids/scolex from these patients were also identified as *T. solium* Asian genotype based on molecular analysis by either BESS-T analysis (base excision sequence scanning thymine-base), multiplex PCR, or DNA sequencing (methods described by Yamasaki *et al*, 2002, 2004, 2006). Eleven cases of *T. solium* infection from 24 taeniasis patients (46%) were found in this study area. Moreover, by these molecular identification methods, it was the first evidence of *T. asiatica* from Thailand; both *T. asiatica* and *T. saginata* are sympatrically occurring in 6 and 7 patients, respectively (Anantaphruti *et al*, 2007).

Worm burden and mixed infection

Each taeniasis patient generally harbored only one *Taenia* sp worm. Among the scolices expelled from 58 *T. saginata* cases, the maximum number of scolices was 3 in 1 patient and 2 in 10 patients. The remaining 47 patients harbored a single scolex each (Viranuwatti, 1952; Chirasiri, 1963; Charoenlarp *et al*, 1981, 1989). Based on the proglottids evacuated, Chaneyayothin (1971) reported 5 *T. saginata* worms expelled from one patient after treatment with a Thai herbal drug. Unfortunately, no scolices were expelled for numbering confirmation. More worms were recorded in *T. solium* infections. The maximum number of scolices found was 6 in one patient. Three scolices were found from 3/5 cases who purged scolices (Anantaphruti *et al*, 2007).

Mixed infections of two species of *Taenia* have been reported. Chirasiri (1963) reported that a patient evacuated a scolex of *T. solium* together with proglottids identified as *T. saginata*. Unfortunately, no scolex was found for specific confirmation of *T. saginata* in this case. In 2002, two scolices of *T. solium* together with one scolex of *T. saginata* were expelled from a patient in Kanchanaburi Province. The worms were identified as *T. solium* Asian genotype and *T. asiatica* confirmed by molecular

analysis. This dual infection of *T. solium* and *T. asiatica* was the first case found in Asia. It concluded that molecular analysis is highly useful for identification of human taeniid cestodes (Anantaphruti *et al*, 2007).

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USEFULNESS OF IMMUNOLOGICAL AND MOLECULAR TOOLS: PROGRAMS TOWARDS CONTROL AND ERADICATION OF CYSTICERCOSIS IN ENDEMIC AREAS

Marcello Otake Sato[1,2], Yasuhito Sako[1], Minoru Nakao[1], Toni Wandra[1,3], Hiroshi Yamasaki[1], Kazuhiro Nakaya[1,4] and Akira Ito[1]

[1]Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan; [2]Laboratório de Parasitologia, Escola de Medicina Veterinária e Zootecnia, Universidade Federal do Tocantins, Araguaína-TO, Brazil; [3]Directorate General Disease Control and Environmental Health, Ministry of Health, Jakarta, Indonesia; [4]Animal Laboratory for Medical Research, Asahikawa Medical College, Asahikawa, Japan

Abstract. Neurocysticercosis (NCC) in the human brain is caused by *Taenia solium* and is one of the most serious parasitic zoonoses. Programs for the control of the taeniasis/cysticercosis complex are focused on elimination of the parasite in both humans and animals. The accuracy and the specificity or sensitivity of the detection tests for real cysticercosis and taeniasis cases are important for the control and eradication of taeniasis/cysticercosis in endemic and non-endemic areas. We discuss the advantages and disadvantages of immunological and molecular tools currently available for diagnosis of cysticercosis and taeniasis. ELISA and immunoblot were used with native and recombinant antigens for the detection of human NCC cases and animal cysticercosis cases. Genotyping of *T. solium* and two other human *Taenia, T. saginata*, and *T. asiatica* and copro-DNA tests were used for detection of worm carriers. These tools, available not only for identification of patients or carriers but also for epidemiological studies, are briefly reviewed.

INTRODUCTION

Three species of human *Taenia* are recognized: *T. solium*, *T. saginata*, and *T. asiatica*. It is possible to delimit areas of occurrence and endemicity of each species worldwide (Schantz, 2002). All three species are important public health problems in endemic areas as foodborne zoonoses. Cysticercosis/taeniasis is caused by the "pork tapeworm," *T. solium*, and is an international emergent and re-emergent zoonosis. It is the major etiological agent of seizures in endemic areas constituting a major public health problem for most of the developing world (Garcia *et al*, 1991, 2005; Tsang *et al*, 1995; Schantz, 2006). Neurocysticercosis, caused by *T. solium*, is the main cause of seizures in endemic areas (Garcia *et al*, 1991, 2005), where it is a well-known problem. Now, it is also an important issue in travel medicine programs as the disease is recrudescing in non-endemic countries.

Correspondence: Akira Ito, Department of Parasitology, Asahikawa Medical College, Midorigaoka Higashi 2-1-1-1, Asahikawa 078-8510, Japan.
E-mail: akiraito@asahikawa-med.ac.jp

The methods used for detection of cysticercosis and taeniasis are performed using direct and indirect techniques used for finding evidence of the parasite from different sources. Recently, we have performed serology using *T. solium* glycoproteins purified from cyst fluid by preparative isoelectric focusing electrophoresis (IEFE), affinity chromatography and recombinant antigens (Ag1V1/Ag2) using ELISA, and immunoblots for detection of antibodies (Ito *et al*, 1998; Sako *et al*, 2000; Sato *et al*, 2006a). Our routine techniques also include amplification of traces of mitochondrial DNA, searching for direct evidence of the worm by PCR, Multiplex PCR, PCR-RFLP, BESS-T, and DNA sequencing from several material sources making differential diagnosis and clear up any doubt regarding the causative agent of a suspected case (Yamasaki *et al*, 2002, 2004a, 2005, 2006). These techniques are applied in different situations depending on the purpose of the test, whether it is either clinical differential diagnosis or a part of a control/eradication epidemiological program; in other words, the usefulness of one or other diagnostic test depends on the area (endemic/non-endemic) and/or the suspected cases.

Generally, the programs for the control of taeniasis/cysticercosis complex are focused on elimination of the parasite in humans and animals through the use of chemotherapy or elimination of infected hosts in developing and developed countries (Schantz, 2006; Pawlowski *et al*, 2005; Pawlowski, 2006), with the aim of either control and eradication of the parasite or prevention of the parasite entrance in a non-endemic area. This article will review and discuss our experience with different diagnostic methods and their role on taeniasis/cysticercosis control programs.

Geographical distribution of the human *Taenia*

The presence of the adult worm is reported mainly in under-developed countries, and has a direct relationship with the lack of both sanitary conditions and proper meat inspection for human consumption (Schantz, 2006; Ito *et al*, 2003a). These two main characteristics of endemic areas maintain the life cycle of the parasite. An important geographical peculiarity is the co-existence of three species of human *Taenia* in the same area, as we reported in Thailand (Anantaphruti, 2007). In some cases, the endemic areas have natural qualities that can attract migrants, susceptible hosts, who are potentially exposed to the infective agents for taeniasis and/or cysticercosis (Sato *et al*, 2006b; Wandra *et al*, 2006) and possibly becoming worm carriers to non-endemic areas, which results in the introduction of an exotic disease. Even if taeniasis is not an important public health problem in a specific area with a well established system of sewage treatment, the lack of both basic hygiene practices and detection of taeniasis patients can put at risk the people living around a worm carrier, because the parasite is releasing viable eggs that can cause cysticercosis (Schantz *et al*, 1992).

By observing the geographical distribution of *T. solium* it is possible to distinguish two scenarios: 1) developed countries with reported cysticercosis cases in humans, but not in animals, and with no reported taeniasis cases or very rare cases of taeniasis (there are imported exotic disease in humans or a recrudescent disease); and 2) underdeveloped countries with taeniasis (possibility of three species) and cysticercosis cases in humans and animals, with the risk of exporting taeniasis and cysticercosis cases to humans and likelihood to be an endemic area.

Genotypes of *T. solium, T. saginata,* and *T. asiatica*

Full sequencing of the mitochondrial DNA of *T. solium* from worldwide samples allowed us to observe regional genetic differences in the parasite. Intraspecific pairwise divergences between the two genotypes range from 0.9-1.3% for the *Cox1* gene and from 1.6-2.1% for the *Cytb* gene. Divergences of American and African samples (0 ± 0.4% in *Cox1* and 0 ± 0.6% in *Cytb*) were also observed. *T. solium* African/American and Asian genotypes were determined (Nakao *et al*, 2002). Concerning other human taeniid tapeworms, *T. saginata* and *T. asiatica*, pairwise divergences between these closely related species were observed, reaching 4.7% for *Cox1* gene and 4.0% for *Cytb* gene (Nakao *et al*, 2002). Genotypic differences were also observed in our study of the antigens for diagnosis of cysticercosis. Nucleotide substitutions in the first intron of *Ag2* allow differentiating African/American and Asian genotypes of *T. solium*, which constitute an alternative marker for differentiation of *T. solium* genotypes (Sato *et al*, 2006a). The use of these genotypic differences as markers allowed us to determine the original area of isolates and subsequent geographic classification. This is remarkable information for the travel medicine specialty.

DIAGNOSING CYSTICERCOSIS

Human cysticercosis is usually diagnosed after the late onset of neurological symptoms, as seizures. A patient's history of seizures will support the diagnosis of cysticercosis, but it is not reported as a standard clinical sign for cysticercosis, which makes the clinical diagnosis difficult. Seizures and neurological symptoms appear generally five years after the exposure to the infective agent; however, with high variability that depends on the number, location, size, and viability or stage of degeneration of the cysts (Garcia *et al*, 2005; Takayanagui and Odashima, 2006). Clinical diagnosis is usually done after the

onset of symptoms, meaning that the evolution of the disease in the host is already completed. In suspected cases, the metacestodes may be visible by neuroimaging exams as computed tomography (CT) or magnetic resonance imaging (MRI). In these cases, perilesional edemas around calcified lesions are usually present in and directly related with seizures and other focal neurological manifestations (Garcia *et al*, 2005).

In pigs, cysticercosis usually has no symptoms; the clinical diagnosis is based on inspection of areas of predilection of the parasite as tongue and eyes. The tongue inspection method only detects heavily infected pigs. Usually this method has low sensitivity and specificity (Sato *et al*, 2003, 2006b). For this reason, an early detection of human cysticercosis may decrease the number of the clinical form of the disease, making possible a safe, accurate, and supervised treatment of the disease. In the case of swine cysticercosis, its early and accurate detection leading to the proper treatment of truly infected pigs will improve the profit rate for the farmers who at the same time will provide safer pork products for the consumers.

Antigens and serology

The antigens used in the cysticercosis serodiagnosis are purified glycoproteins (GPs) with molecular weights ranging between 8 kDa and 50 kDa (Tsang *et al*, 1989). GPs used for cysticercosis diagnosis are a 120 kDa protein complex in *T. solium* cyst fluid (Lee *et al*, 2005). It is comprised of six subunits varying in size from 14-38 kDa with 14 or 18 kDa backbone proteins; it generates a highly specific antibody response by the hosts. The synthesis of those GPs using isoelectric focusing electrophoresis (IEFE) is described by Ito *et al* (1998); resulting in highly specific tests for the detection of antibodies against GPs. IEFE purified antigens could be used with similar specificity and sensitivity in ELISA and immunoblot for the detection of, not only infected persons, but also infected pigs and even infected dogs (Ito *et al*, 2002b; Sato *et al*, 2003, 2006a,b; Wandra *et al*, 2003, 2006). The 8 kDa backbone protein of the GP antigens belongs to a family of proteins that is closely related to that of the antigens for *T. solium* (Sako *et al*, 2000, 2006; Hancock *et al*, 2003). These proteins usually show from 0-3 N-glycosylation sites (Sako *et al*, 2000; Sato *et al*, 2006a). Glycoforms with different masses or a different number of N-linked oligosaccharides are the putative cause of the different banding pattern of GPs from Asian, African, or American geographical origin.

A crucial characteristic to point out is that, although there are some antigenic differences from region to region, all the antigens have shown

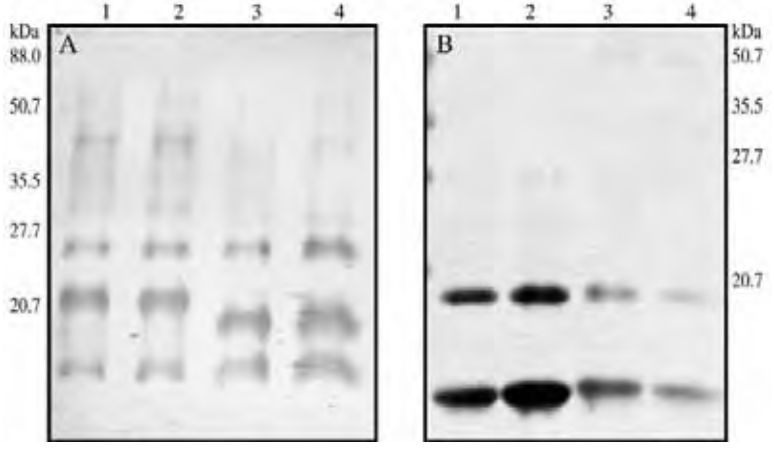

Fig 1- Immunoblot of *T. solium* glycoproteins purified by monoclonal (4F10) affinity. Panels A and B correspond to 4F10 monoclonal affinity purified glycoproteins before (A) and after treatment with N-Glycosidase (B) using 4F10 monoclonal antibodies as a probe. The lanes are GPs from 1- Africa (Tanzania), 2- America (Brazil), 3- Asia (China) and 4- Asia (Indonesia).

the same immunoblot banding pattern after deglycosylation (Fig 1). This indicates that the GP protein backbone is similar to the antigens from different geographical origins, and the protein backbones, not the carbohydrate components of the antigen, are sufficient for serodiagnosis purposes (Sato *et al*, 2006b). Sako *et al* (2000) described four different antigens deriving from a native Asian antigen and synthesized a chimeric recombinant (RecT) antigen for the detection of infected humans and animals by both ELISA and immunoblot (Ito *et al*, 2002b; Sato *et al*, 2003, 2006a,b; Wandra, 2003). Serological tests using African, American, or Asian GPs and recombinant antigens showed a correlation higher than 92%, which indicates that both RecTs and GPs (African, American, or Asian) are suitable for serology in humans and swine worldwide. In conclusion, glycosylation causes more bands to show in the immunoblot tests; however, this feature does not affect significantly the sensitivity of the test in immunoblots or ELISAs then RecTs are recommended for diagnosis of cysticercosis.

Practical examples of the usefulness of the serology

In a study with experimentally infected pigs, we showed the limit of detection for the ELISA tests as 2.5 cysts per animal, at least 1 month after the infection. The field-infected animals are usually carrying more than 2.5 cysts. Thus, it is possible to detect the total number of infected animals (Sato *et al*, 2003). We also showed that serology methods in swine are more useful than the tongue inspection method, detecting accurately more *T. solium* cyst infected pigs. Our results found that approximately 70% of PTI (positive at tongue inspection) pigs and 30% NTI (negative at tongue inspection) pigs positives by serology using GPs or RecTs (Ag1V1/Ag2) (Sato *et al*, 2003). Regarding the detection of cysticercosis in humans, there is a debate about the better source of material for detecting NCC. In spite of the actual recommendation that the cerebrospinal fluid (CSF) should be used for the detection of NCC (Del Brutto *et al*, 2001), our results with a blind test of serum samples and encephalic fluid showed 100% of correlation (Takayanagui *et al*, unpublished data), which indicates that serology is suitable for antibody detection for cysticercosis, especially NCC. Another interesting example concerns highly suspected cases by MRI and CT-Scan showing negative results using GPs and Ag1V1/Ag2 ELISA and immunoblot. According to our experience, these cases may be confirmed after surgical excision of the lesion to be a cystic type of neoplasia (Ito *et al*, 2006a). The serology using either GPs or Ag1V1/Ag2 ELISA was also used for detecting asymptomatic patients in northeast Brazil in a newly described endemic area (Sato *et al*, 2006b), and had been used for seroepidemiology in Indonesia and China with high accuracy and sensitivity (Wandra *et al*, 2003; Margono *et al*, 2003, 2006; Ikejima *et al*, 2005; Ito *et al*, 2006b).

Molecular tools for diagnosis of cysticercosis

The observation of parasitic material is the most important step for diagnosis in parasitology. However, classical identification is sometimes not possible due to the impossibility of getting the whole parasite, or even a morphologic characteristic of the sample (Ito *et al*, 2002a, c, 2006a; Yamasaki *et al*, 2004b). Characterization of the full sequence of mitochondrial DNA of *T. solium* worldwide allowed us to design useful tools for the diagnosis of parasitic material, even if all the morphological characteristics are lost. These techniques do not distinguish between the stages of the parasite; consequently, they can be applied to identify either adults or metacestodes (Ito *et al*, 2002a,c, 2006a; Yamasaki *et al*, 2002, 2004a, 2005, 2006). The analysis of the nucleotide sequences of full human *Taenia* spp mDNA genome makes possible the determination of the genes suitable for use as diagnostic tools. Observing the alignment sequence entropy graphic of the sequence data, some genes (*eg*, *cox1*, *cob*) have substitutions between species conserved within the genotypes. These data were used to design strategies to discriminate *T. saginata*, *T. asiatica*, *T. solium* and also Asian and African/American genotypes of *T. solium*.

With the use of the obtained sequence information, it was possible to determine restriction enzyme patterns and then design a PCR-RFLP (Yamasaki *et al*, 2002), which in turn made it

possible to differentiate the genus *Taenia* and even genotypes of *T. solium* (Nakao *et al*, 2002). In addition, this method is very useful because it does not require sequencing; however, it is time consuming because of the incubation. Despite its usefulness, this technique is not suitable to perform at the field level because of the need for proper storage and preservation of the enzymes, which is essential for getting accurate results. Studying the sequence data of the cytochrome oxidase *c* subunit 1 (*cox1*) gene, it was possible to design a set of genotype-specific primers that could be combined to produce a multiplex PCR to distinguish Asian, African, and American genotypes of *T. solium*, *T. saginata*, and *T. asiatica* in only one reaction batch. This tool allowed us to diagnose human *Taenia* species from the most diverse origins: eggs, fresh worms, worms fixed in alcohol, biopsies, pathology slides, stools, and other sources of DNA (Ito *et al*, 2002a, c, 2006a; Yamasaki *et al*, 2004b, 2005, 2006). Diagnosis is made by observing bands with specific molecular sizes in agarose gel, without sequencing. This is an extremely useful tool because sequencing facilities are not present in most part of laboratories in the under-developed world.

USEFULNESS OF THE TECHNOLOGIES IN ENDEMIC AND NON-ENDEMIC AREAS

The major difference between cysticercosis and taeniasis control programs in endemic and non-endemic areas is the objectives of the programs. While programs in endemic areas are designed to stop or minimize transmission and spreading of the disease complex, in non-endemic areas it is done for differential diagnosis and confirmation of the disease in exotic cases. For both programs, the reliability of the tests is a *sine qua non* for the accomplishment of the proposed objectives. For endemic areas, it is ideal to detect all the potential human and animal parasite-carriers through programs of mass detection involving field surveys.

ELISA performed in serum samples is the most indicated method for the triage and screening of a population from a study area because the sensitivity and specificity of the antigens, GPs or recombinants, are very high

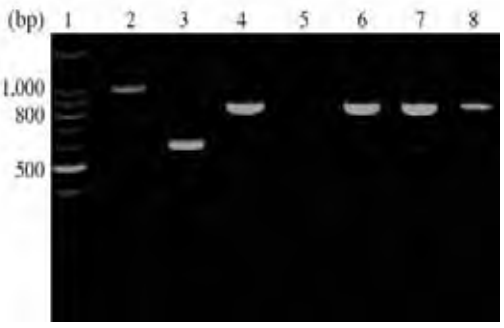

Fig 2- Multiplex PCR technique in a field survey in Indonesia (Bali). Lane 1 is molecular marker 100bp. Lanes 2, 3, 4, and 5 are positive controls for *T. solium*, *T. asiatica*, *T. saginata*, and a negative control, respectively. Lanes 6 and 7 are samples extracted from segments of an expelled worm. Lane 8 copro-PCR with a sample extracted from fecal sample of a suspected taeniasis case.

(Sato *et al*, 2003, 2006b; Margono *et al*, 2003, 2006; Wandra *et al*, 2003), detecting more accurately the true positives. It is a highly useful tool to determine the ways to conduct or establish control programs, to be done in an area under a control program for improving results or when it is the first contact in a new area (Sato *et al*, 2006b). Multiplex PCR technique has already proved it has the ability to be applied in the field in a survey in Indonesia (Bali) (Fig 2), where we could detect worm carriers accurately using DNA extracted from feces and specimen of a suspected taeniasis case with successful results.

CONCLUSION

The use of serology and molecular techniques in endemic areas is a palpable reality. The reliability of the methods for application in the field or in laboratories in developing countries with limited facilities has already been proved, with successful results. These tools have real contributions for the improvement of the taeniasis/cysticercosis control and eradication programs worldwide.

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MITOCHONDRIAL DNA DIAGNOSIS FOR CESTODE ZOONOSES: APPLICATION TO FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE SPECIMENS

Hiroshi Yamasaki[1], Kazuhiro Nakaya[2], Minoru Nakao[1], Yasuhito Sako[1] and Akira Ito[1]

[1]Department of Parasitology, [2]Animal Laboratory for Medical Research, Asahikawa Medical College, Japan

Abstract. A PCR-based technique was applied for definitive diagnosis using formalin-fixed, paraffin-embedded sections (FPES) from patients with cestode zoonoses, such as cysticercosis, alveolar echinococcosis, sparganosis, and diphyllobothriasis. DNA samples extracted from thin sections were used for PCR-amplification of mitochondrial cytochrome *c* oxidase subunit 1 gene. Although DNA were fragmented because of formalin fixation, smaller sizes of the gene fragments were stably amplified in all samples examined, and causative cestode species were identified by DNA sequencing. This study demonstrated that mitochondrial DNA analysis using FPES is a powerful tool, not only for routine and retrospective diagnosis, but also for genetic polymorphism analysis of the cestode species.

INTRODUCTION

Diagnosis of cestode zoonoses such as cysticercosis, echinococcosis, and sparganosis in humans is performed based on clinical manifestations, imaging examination, serology, and/or histopathology. Although the histopathology provides highly definitive evidences for diagnosis of cestodiasis, it is occasionally difficult to specify causative parasites due to the degeneration and/or calcification of the lesions, or artifacts in preparation. Recently, we reported two *Taenia solium* cysticercosis cases where histopathological findings were not confirmatory, but PCR-based mitochondrial DNA analysis using formalin-fixed paraffin-embedded sections (FPES) was highly useful for definitive diagnosis (Yamasaki *et al*, 2005, 2006a). Therefore, in order to diagnose such cases or to identify the cestode species found in the histopathological specimens, the authors have applied the PCR-based techniques for other parasitic diseases. In this study, the importance of molecular analysis using FPES in clinical diagnosis and parasitological studies was described with selected cestode zoonosis cases. In place of formalin, widely used as a tissues fixative, alternative fixatives that are currently used for molecular analysis are also introduced.

Correspondence: Dr Hiroshi Yamasaki, Department of Parasitology, Asahikawa Medical College, Asahikawa 078-8510, Japan.
E-mail: hyamasak@asahikawa-med.ac.jp

MATERIALS AND METHODS

Formalin-fixed paraffin-embedded sections examined

Twenty samples from 19 patients with cysticercosis, alveolar echinococcosis (AE), sparganosis, and diphyllobothriasis were examined (Table 1). The tissues and plerocercoids resected from patients, and broad tapeworms expelled from diphyllobothriid patients were fixed with formalin and processed into FPES. Most FPES were prepared within the previous five years, but three samples from AE patients (cases 1-3) were archival specimens prepared 13-28 years ago. In sparganosis cases, formalin-fixed (case 1) and frozen plerocercoids (case 2) were used as DNA sources. For molecular analysis, unstained sections (5 sections with 10-μm thickness) were used; however, HE- (cases 2 and 3 in AE) and PAS-stained sections (case 6 in AE) attached onto the slide glasses were used.

DNA diagnosis

DNA samples were prepared from FPES using either DEXPAT (TaKaRa Shuzo, Japan) or DNA Isolator PS kit (Wako Pure Chemicals, Japan). The amplification of a target gene, a mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*), was performed with the protocol (94ºC, 1 minute; 58ºC, 30 seconds; 72ºC, 30 seconds; 35 cycles) (Yamasaki *et al*, 2004; 2005). The PCR-amplified products were run on 2-3% agarose gels or 10% polyacrylamide

gel; the nucleotide sequences of the amplicons were analyzed by direct DNA sequencing. The samples for sequencing were prepared using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (ABI PRISM, USA) and DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer.

RESULTS

Although the resected lesions and cestode parasites had been fixed with formalin prior to molecular analysis, the smaller sizes of *cox1* gene fragments were successfully amplified. Two different kits used for extraction of DNA from FPES provided similar results for amplification of target genes.

Cysticercosis

We have experienced seven cysticercosis cases confirmed by mitochondrial DNA analysis of the biopsied tissues. In Cases 1 and 3, the long sizes of 1.8-kb and 984-bp *cox1* were successfully amplified (Yamasaki *et al*, 2004, 2006a); however, smaller sizes (224-410 bp) of *cox1* fragments were stably amplified in other cases. In Case 4 with numerous cysts in the thigh and gluteal regions, for instance, resected cysts were extremely calcified and cysticercosis was not confirmed histopathologically (Fig 1-A). However, the 224-bp *cox1* fragments were amplified in FPES prepared from three cystic lesions (Fig 1-B, lanes 1-3), and it was revealed the lesions were derived from an Asian genotype of *T. solium* by DNA sequence analysis (Tsuda *et al*, 2007, in press). The patient was thought to have been exposed to *T. solium* approximately 60 years ago in Okinawa, where it was once an endemic area for *T. solium* cysticercosis. In Case 6 (Matsuoka *et al*, 2007), the patient presented with repeated epileptic seizures and was diagnosed as cysticercosis by histopathology (Fig 1-C). Because the patient had repeated travels during 1999-2005 to Korea, Bali (Indonesia), and Mexico where cysticercosis is still endemic, it was considered that the patient was exposed to *T. solium* in Mexico because of the patient's travel history in 2005. In order to presume the locality where the patient was infected, molecular analysis using FPES was performed. Subsequently, 224- and 720-bp *cox1*

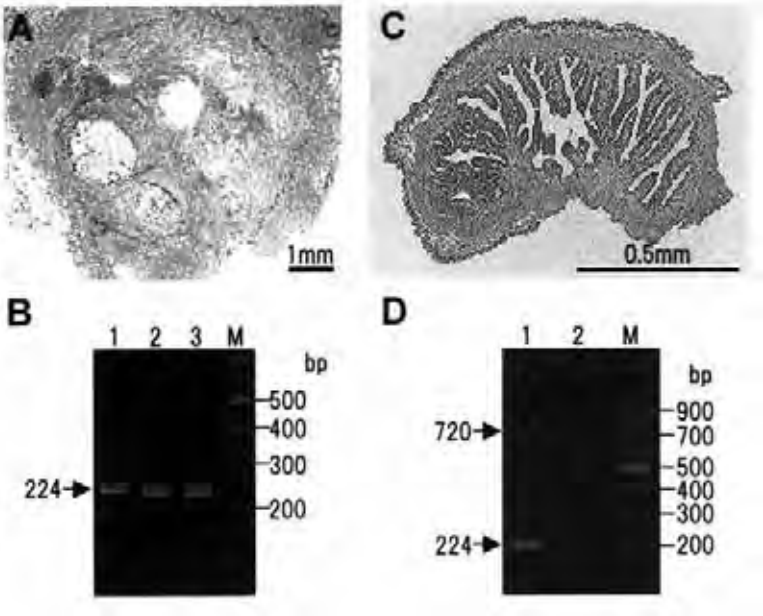

Fig 1- Cysticercosis. A and B, histopathological findings of biopsied lesion and 224-bp *cox1* gene fragments amplified from 3 cystic lesions (lanes 1-3) in case 4. C and D, a taeniid cysticercus showing characteristic labyrinth-like structure (from Matsuoka *et al*, 2007) and 224-bp and 720-bp (faint) *cox1* amplified by PCR (lanes 1 and 2) in case 6.

fragments were amplified by PCR (Fig 1-D), and the causative agent was identified as the Asian genotype of *T. solium* based on a nucleotide at position 723 of *cox1* (data not shown). With more detailed genetic polymorphism analysis of geographical isolates, it would be possible to specify the locality where the patient was exposed to *T. solium*.

Alveolar echinococcosis

In AE cases caused by *Echinococcus multilocularis* metacestodes, six cases were from Hokkaido, northern Japan, where AE is still endemic, and Cases 2 and 3 were from Rebun Island, a previously endemic area in Hokkaido. Case 1 was an interesting case from a remote area on Honshu, the main island of Japan, and was originally described as an autochthonous infection in 1979 (Yoshimura *et al*, 1979). According to Yoshimura *et al* (1979), the patient never went outside of Honshu and was diagnosed as AE by autopsy (Fig 3-A). Therefore, to confirm whether *E. multilocularis* from Case 1 is identical to *E. multilocularis* isolates from Hokkaido, PCR-based sequence analysis was performed using archival FPES samples. As a result, only smaller sizes (99-123 bp) of *cox1* fragments were amplified (Fig 2-B). The 220-bp *cox1* was not amplified, even in nested PCR (data not shown). For determining the complete sequence of *cox1* from Case 1, overlapped *cox1* fragments with molecular sizes of 100~131-bp were amplified by PCR using 16 primer sets (data not shown). Subsequently, it was proven *E. multilocularis cox1* from the Case 1 was identical to those of the isolates from Hokkaido. It is still undetermined how the patient got infected with *E. multilocularis* in the remote area.

Conversely, in AE Cases 2, 3, and 6, HE-(Cases 2 and 3) and PAS-stained sections (Case 6) attached onto the slide-glasses were used as DNA sources. In these cases, DNA Isolator PS kit was utilized. In Cases 2 and 3, 220-bp *cox1* fragments were amplified (data not shown). In Case 6, in addition to 220-bp *cox1*, 410-bp *cox1* fragment was amplified (Fig 2-D, lanes 1-2), however the amplification of longer 825-bp *cox1* fragment was not successful (Fig 2-D, lane 3). These results demonstrate that HE- and PAS-stained sections are also useful materials for retrospective studies

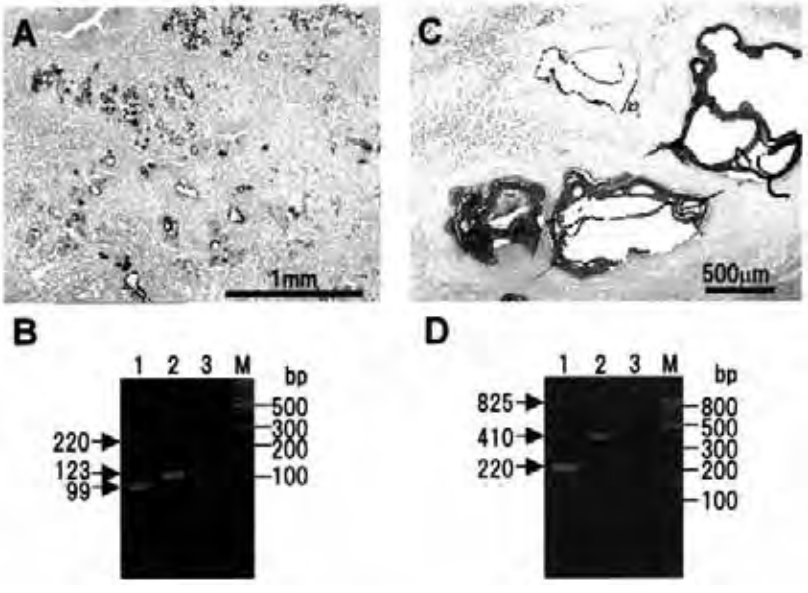

Fig 2- Alveolar echinococcosis. A and B, typical histological finding showing AE (PAS stain) and smaller sizes of *cox1* amplified by PCR from the archival FPES (lanes 1 and 2) in case 1. C and D, PAS-stained histopathology and PCR-amplified 224- and 410-bp *cox1* fragments (lanes 1 and 2) in case 6. No PCR products with molecular sizes of 220- and 825-bp *cox1* fragments were amplified in case 1 (lane 3 in B) and case 6 (lane 3 in D), respectively.

because many histopathological specimens are stained with HE and/or PAS.

Sparganosis and diphyllobothriasis

Sparganosis caused by the infection of larval *Spirometra* plerocercoid is sporadically found in Japan. The diagnosis is easy if the plerocercoid is surgically removed from patients presenting creeping eruption. In Japan, the *Spirometra* species causing sparganosis is considered *Spirometra erinaceieuropaei*. However, whether the causative plerocercoid is really *S. erinaceieuropaei* is difficult to determine based on the morphology. To identify the plerocercoid species accurately or examine genetic polymorphism among the plerocercoids, molecular analysis was performed. Fig 3-A shows a formalin-fixed plerocercoid used for DNA extraction from Case 1(Yoshikawa *et al*, 2002). The target *cox1* with molecular sizes of 445~480-bp *cox1* fragments were successfully amplified from the plerocercoid (lanes 1 and 2 in Fig 3-B, Case 1) as well as other two cases, and sequence analysis of the PCR products revealed the *Spirometra* plerocercoids were all identified as *S. erinaceieuropaei*, although a genetic divergence (1.1-2.4%) was observed.

Diphyllobothriasis cases caused by the infection of the adult broad tapeworm, *Diphyllobothrium* spp, are frequently found in regions where people eat raw salmon and trout that cross the oceans. *Diphyllobothrium latum* that is distributed in North Europe has been considered a cestode species causing diphyllobothriasis in humans for over a century. However, the species was described as *Diphyllobothrium nihonkaiense*, based on the morphology (Yamane *et al*, 1986), and it has been demonstrated that *D. nihonkaiense* is a distinct species from *D. latum* at the mitochondrial genome levels (Nakao *et al*, 2007). *D. nihonkaiense* is currently recognized as a cestode species causing diphyllobothriasis in Japan. According to Yamane *et al* (1986), one distinct morphology for differentiating *D. nihonkaiense* from *D. latum* is the position of cirrus sac: it is situated obliquely against the anterior-posterior axis in *D. nihonkaiense*, whereas it is situated horizontally in *D. latum*. However, the morphological criterion is not absolute for the differentiation of the species because the position of segments to be examined or preparation of sagittal sections. We also rarely find diphyllobothriasis caused by the marine *Diphyllobothrium* cestodes, other than

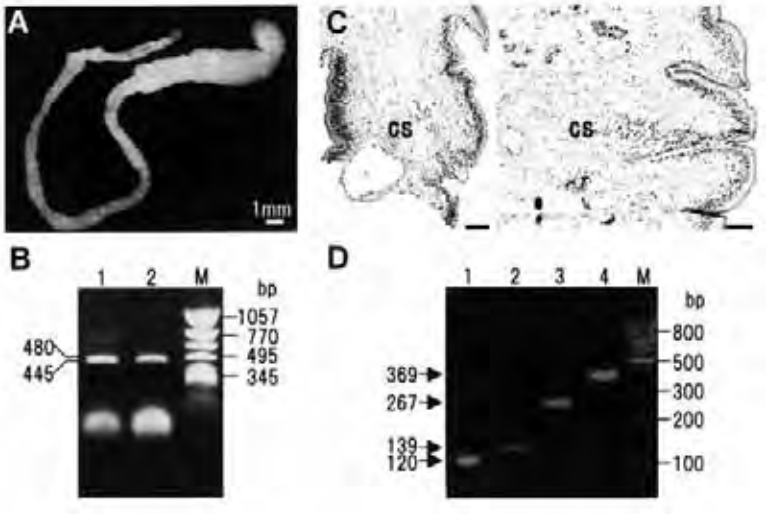

Fig 3- Sparganosis and diphyllobothriasis. A and B, a formalin-fixed plerocercoid used as DNA source (from Yoshikawa *et al*, 2003) and PCR-amplified 445-480-bp *cox1* fragments (lanes 1-2) in case 1. C, sagittal sections showing different positions of cirrus sac (cs) of 2 diphyllobothriid tapeworms expelled from case 1 (left) and case 2 (right). D, PCR-amplified 120-369-bp *cox1* fragments (lanes 1-4) in case 1. Bars in C = 100 μm.

D. nihonkaiense, and it may be necessary to identify the cestode species at molecular level in parasitology. Fig 3-C shows sagittal sections of diphyllobothriid cestodes expelled from two Japanese children (Cases 1 and 2). The cestode (left) can be identified as *D. nihonkaiense* from the cirrus sac situated obliquely, but the other one (right) shows morphology resembling *D. latum*. In molecular analysis using FPES of the segments, 120~396-bp *cox1* fragments were amplified in a diphyllobothriid tapeworm (Fig 3-D, lanes 1-4 from Case 1) as well as other two tapeworms, and all diphyllobothriid tapeworms were identified as *D. nihonkaiense*.

DISCUSSION

In the present paper, the authors stressed the importance of molecular-pathological diagnosis using FPES in clinical diagnosis and parasitology with selected cestode zoonosis cases. In echinococcosis and sparganosis and diphyllobothriasis, because serology and histopathology and/or morphology provide reliable diagnostic results, molecular diagnosis may be not necessary for clinical diagnosis. However, the serological and histopathological diagnosis of cysticercosis is occasionally not confirmatory. Cysticercosis cases with a solitary cyst and calcified lesions are frequently seronegative, although the cysticercosis with multiple cysts (Case 5, Table 1) is serologically positive as pointed by Sako *et al* (2000) and Sako and Ito (2001). Histopathological findings are also not absolute in cases where the cystic lesions are degenerated and/or calcified (Yamasaki *et al*, 2004, 2005; Tsuda *et al*, 2007; Ishikawa *et al*, 2007). Furthermore, the causative *Taenia* species is exclusively *T. solium* in cysticercosis in humans; however, there have been reported cysticercosis cases caused by zoonotic *Taenia* species other than *T. solium*, such as *Taenia crassiceps* in AIDS patients or by unusual racemose-type *T. solium* in HIV-positive individuals (reviewed by Yamasaki *et a*l, 2006b, c). Having considered these situations, molecular analysis using FPES is highly informative and useful, not only for identification of the *Taenia* species in cysticercosis (Yamasaki *et al*, 2004, 2005, 2006b, c; Ito *et al*, 2006), but also for genetic polymorphism analysis of causative cestode species causing echinococcosis, sparganosis and diphyllobothriasis.

Although it is known that fixation in formalin of tissues and processing of tissues to paraffin wax degrades DNA molecules because of cross-linking caused by formalin, it is possible to extract DNA of up to 200 bp from the archival FPES (Bianchi *et al*, 1991). In practice, it seems to be difficult to solubilize formalin-fixed tissues directly as compared with thin sections. In our experience, 1.8-kb, 984-bp or 720-bp *cox1* were able to amplify in some cysticercosis cases (Yamasaki *et al*, 2006a; Matsuoka *et al*, 2007); however, smaller 224-bp *cox1* fragments are more stably amplified in most cysticercosis cases (Yamasaki *et al*, 2005, 2006a; Matsuoka *et al*, 2007; Tsuda *et al*, 2007; Ishikawa *et al*, 2007). In AE cases, only 100-131-bp *cox1* fragments were amplified in the archival specimen as shown in Fig 2. In recently prepared specimens, 220-410-bp *cox1* fragments were amplified; however, the amplification of longer 825 bp-*cox1* fragment was not successful. In sparganosis and diphyllobothriasis cases, 445-480-bp and 120-396-bp *cox1* fragments were amplified, respectively, from FPES although amplification of larger sizes of *cox1* fragments were not performed in these zoonosis cases. The sizes of the PCR products could be due to the quality of formalin used in pathology departments and the times taken to fix samples.

In this study, the target genes were successfully amplified when HE- and PAS-stained sections from AE patient were used. This implies that stained specimens are available for routine and retrospective studies. The PCR-based diagnosis was also useful for differentiating AE from disease similar to AE having tortuous laminated layers (Yamasaki *et al*, unpublished data). In our department, although the FPES is now used for diagnosing parasitic diseases and genotyping of the parasites, it is highly interested in demonstrating parasite-specific DNA by in situ PCR using FPES, in particular, in the obsolete cysticercosis case where the structure of cysticercus is extremely degenerated. The PCR-based diagnosis using FPES is highly beneficial for providing definitive information, not only

Table 1
Demographic data on cestode zoonosis cases examined in this study.

Case #	Patient	Lesion	Serology	Histopathology	Year	Reference
Cysticercosis						
1	53/F, Japanese	Brain (solitary)	(-)	Confirmed	2002	Yamasaki *et al*, 2004
2	9/F, Filipino	Brain (solitary)	(-)	Not confirmed	2003	Yamasaki *et al*, 2005
3	83/M, Japanese	Muscles (calcified)	(-)	Not confirmed	2003	Yamasaki *et al*, 2006
4	87/M, Japanese	Muscles (calcified)	(-)	Not confirmed	2005	Tsuda *et al*, 2007
5	28/F, Indian	Brain (multiple)	(+)	Not confirmed	2005	This study
6	24/F, Japanese	Brain (solitary)	(-)	Confirmed	2005	Matsuoka *et al*, 2007
7	38/F, Japanese	Brain (solitary)	(-)	Not confirmed	2005	Ishikawa *et al*, 2007
Alveolar echinococcosis						
1	62/F, Japanese	Liver	Not done	Confirmed	1978	Yoshimura *et al*, 1979
2	73/F, Japanese	Liver	Not done	Confirmed	1982	This study
3	62/F, Japanese	Liver	Not done	Confirmed	1992	This study
4	?/F, Japanese	Liver	Not done	Confirmed	1993	This study
5	48/F, Japanese	Liver, bone	(+)	Confirmed	2004	This study
6	64/F, Japanese	Liver	(+)	Confirmed	2006	This study
7	47/F, Japanese	Liver	(+)	Confirmed	2006	This study
Sparganosis						
1	64/M, Japanese	Brain	(+)	Confirmed	2002	Yoshikawa *et al*, 2002
2	67/F, Japanese	Abdominal skin	(+)	Confirmed	2005	This study
3	58/F, Japanese	Orbit, forehead	(+)	Confirmed	2006	This study
Diphyllobothriasis						
1	4/M, Japanese	Intestine	Not done	Confirmed	2006	This study
2	3/F, Japanese	Intestine	Not done	Confirmed	2006	This study

in clinical parasitology, but also for genetic polymorphism analysis among the causative cestode species. Such technologies are currently applied for various diseases, for instance, parasitic diseases (Muller *et al*, 2003; Yamasaki *et al*, 2004, 2005, 2006a; Boer *et al*, 2006; Rivasi *et al*, 2006), tuberculosis (Hofman *et al*, 2003; Schewe *et al*, 2005), Lyme borreliosis (Chou *et al*, 2006), virus-related diseases (Ikegaya *et al*, 2005; Bryan *et al*, 2006) and cancers (Paik *et al*, 2005; Yatabe *et al*, 2006).

Fixation of tissues with formalin results in well-preserved morphology, but it leads to fragmentation of DNA and RNA to a high degree, which substantially constricts the spectrum of applicable molecular analysis (Bianchi *et al*, 1991; Vollmer *et al*, 2006). As an alternative fixative, a versatile methacarn has been introduced for genomic DNA analysis in microdissected paraffin-embedded tissue specimens (Uneyama *et al*, 2002). By using the fixative, extensive portions of DNA of up to 2.8 kb could be amplified by nested PCR using DNA extracted from a 1 x 1-mm area of cerebral tissues. On the other hand, a novel HOPE (Hepes-Glutamic acid buffer-mediated Organic solvent Protection Effect)-fixative (DCS Innovative Diagnostik Systeme, Germany) has also been currently utilized for PCR-based analysis (Goldmann *et al*, 2002; Sen Gupta *et al*, 2003), *in situ* hybridization (Umland *et al*, 2003; Vollmer *et al*, 2006), immunohistochemistry (Olert *et al*, 2001; Umland *et al*, 2003; Vincek *et al*, 2003), Western blot (Uhlig *et al*, 2004) and tissue microarray analysis (Goldmann *et al*, 2004, 2005; Vollmer *et al*, 2006). The most remarkable feature of the HOPE-fixative is the extremely low degradation of nucleic acids (Olert *et al*,

2001; Wiedorn *et al*, 2002; Vincek *et al*, 2003); it allows us to preserve and extract high molecular weight DNA and RNA of > 20 kb and proteins in combination with excellent morphological results comparable to formalin-fixed tissues. In AE, it would be highly interesting to investigate gene-expression profiling between echinococcal foci and non-echinococcal tissues using a DNA microarray prepared from paraffin-embedded sections fixed with HOPE.

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EVALUATION OF 8-kDa SUBUNITS OF ANTIGEN B FROM *ECHINOCOCCUS MULTILOCULARIS* FOR SERODIAGNOSIS OF ECHINOCOCCOSIS

Wulamu Mamuti[1,2], Yasuhito Sako[1], Marshall Lightowlers[3], David Jenkins[4], Jinfeng Kang[2] and Akira Ito[1]

[1]Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan; [2]Department of Parasitology, Xinjiang Medical University, Urumqi, China; [3]Veterinary Clinical Center, University of Melbourne; [4]Australian Hydatid Control and Epidemiology Program, Australia

Abstract. Antigen B (AgB) is a thermostable polymeric lipoprotein of 160 kDa and an important diagnostic antigen for serodiagnosis of human cystic echinococcosis due to its promising sensitivity and specificity. Now it has been proven that this protein is encoded by a gene family. Our recent studies demonstrated the existence of homologues of these genes in *E. multilocularis*. Five different cDNAs and corresponding genomic DNA clones encoding homologues of *EgAgB* 8-kDa subunits were identified as EmAgB8/1, EmAgB8/2, EmAgB8/3, EmAgB8/4, and EmAgB8/5. These genes appeared to have been expressed in a developmentally regulated manner in the parasite life cycle. Our current work focused on serological evaluation of recombinant proteins of EmAgB 8-kDa subunits for the immunodiagnosis of human echinococcosis and dogs infected with *Echinococcus*. Western blot analysis indicated that rEmAgB8/1 is the most useful antigen for serodiagnosis of human cystic echinococcosis with highest sensitivity and specificity (81.1%) among the five subunits of EmAgB; rEmAgB8/3 can be used as a candidate antigen for establishment of coproantigen test for diagnosis of dogs infected with *Echinococcus*.

INTRODUCTION

Cystic echinococcosis (CE) and alveolar echinococcosis (AE), caused by the larval stage of *Echinococcus granulosus* and *Echinococcus multilocularis*, respectively, are the clinically and epidemiologically most important forms of human echinococcosis (Craig *et al*, 1996; Schantz *et al*, 1999; Pawlowski *et al*, 2001; Ito *et al*, 2003). The metacestode of *E. granulosus* usually develops in patients into a fluid-filled unilocular cyst with relatively thick cyst walls and an additional fibrous outer layer, originating from the host. By contrast, the metacestode of *E. multilocularis* exhibits a multivesicular, tumor-like infiltrating structure that has a poorly defined barrier between parasite and host tissue, usually containing a semisolid matrix rather than fluid (Siles-Lucas and Gottstein, 2001).

Correspondence: Wulamu Mamuti, Department of Parasitology, Xinjiang Medical University, Xinyi Road, No 8, Urumqi 830054, Xinjiang China
Tel: 86-991-4362316; Fax: 86-991-4323628.
E-mail: mwulamu@hotmail.com

Patients infected with either parasite are exposed to a variety of parasite-derived antigenic molecules that may evoke host-immune responses. Antigen B (AgB) was described initially from *E. granulosus* hydatid cyst fluid (Oriol *et al*, 1971); it is a polymeric lipoprotein of 160 kDa. In SDS-PAGE, the EgAgB disassociates to produce several subunits with molecular sizes of 8, 16, 24, and 32 kDa; the higher-sized subunits are composed of the 8-kDa subunit component (Lightowlers *et al*, 1989; González *et al*, 1996). Furthermore, the 8-kDa subunit band itself was also found to be comprised of a protein family (EgAgB, 8-kDa monomers) that is encoded by a multigene family (Fernández *et al*, 1996; Chemale *et al*, 2001; Arend *et al*, 2004). The AgB is a highly immunogenic major component of hydatid cyst fluid; it is a most sensitive and specific parasite antigen among other *Echinococcal* antigens in the serodiagnosis of human CE. Serological studies utilizing EgAgB revealed cross-reactions with antibodies in the sera of patients with AE infections (Lightowlers *et al*, 1989; Maddison *et al*, 1989; Ito *et al*, 1999; Mamuti *et al*, 2002).

This suggests that a molecule closely related

to EgAgB was also expressed in the metacestode of *E. multilocularis*. Further studies using molecular and serological tools confirmed the existence of AgB in the metacestode of *E. multilocularis*. A partial DNA homologous to the EgAgB8/1 (EmAgB8/1) was isolated from metacestode of *E. multilocularis* (Frosch *et al*, 1994), and its full-length cDNA was isolated and characterized by Mamuti *et al* (2004). Serological studies revealed that the recombinant protein encoded by EmAgB8/1 (rEmAgB8/1) demonstrated almost the same serodiagnostic value as that of the recombinant protein encoded by EgAgB8/1 (rEgAgB8/1). Recently, four additional cDNAs that encode other 8-kDa subunit monomers of EmAgB were isolated from vesicles, protoscoleces, and immature adult worms of *E. multilocularis*. These genes are expressed in a regulated manner at different developmental stages of the parasite (Mamuti *et al*, 2006). However, the serodiagnostic values of the recombinant proteins of other EmAgB 8-kDa subunit monomers are still unknown. This study aimed to evaluate the serodiagnostic properties of the remaining 8-kDa subunit monomers of EmAgB and to select the most sensitive and specific recombinant protein for serodiagnosis of human echinococcosis and dogs infected with *E. granulosus* adult worms.

MATERIALS AND METHODS

Serum samples

Human serum samples of CE and AE were collected from the following groups of patients after obtaining appropriate ethics approvals and patients' informed consent (Table 1). Thirty serum samples were taken from patients with CE confirmed by surgery in China and 60 serum samples from CE patients confirmed by serology using Antigen 5, with or without surgery, in Australia (Lightowlers *et al*, 1989, 1995). These were followed by WB and ELISA with antigen B in hydatid cyst fluid of *E. granulosus* at Asahikawa Medical College (AMC), Japan (Mamuti *et al*, 2002). Fifty serum samples were taken from AE patients diagnosed by surgery at Hokkaido University Hospital and by Em18-serology and at AMC, Japan; and 23 serum samples were taken from AE patients diagnosed by surgery plus serology in France using a commercially available kit (Liance *et al*, 2000; Ito *et al*, 2002). These serum samples were obtained from patients who had been confirmed as CE or AE, and who do not necessarily represent samples taken from patients prior to their commencement of treatment, by either surgery and/or chemotherapy. Fifteen serum samples were collected in Australia from *E. granulosus* infected dogs, confirmed by necropsy after obtaining appropriate ethics approval.

Recombinant antigens and Western blots

The recombinant proteins corresponding to each 8-kDa subunit monomer of EmAgB were expressed in a bacterial expression system and purified as described in a previous report (Mamuti *et al*, 2004, 2006, 2007). SDS-PAGE and Western blots were carried out as described by Ito *et al* (1999), with some modifications. In brief, approximately 2 mg of each recombinant protein that corresponded to a related 8-kDa subunit monomer of EmAgB was separated on a two-dimensional 4-20% polyacrylamide gradient gel (6 cm wide, Tefco Co, Nagano, Japan) and transferred electrophoretically onto a PVDF membrane. Each membrane was cut into fifty strips and probed with diluted human sera (1:50). Bound antibodies were detected using rec-Protein G-peroxidase conjugate (Zymed Laboratories, USA) at 1:1,000 dilution, and 4-chloro-1-naphtol (Nacalai Tesque Inc, Japan) as substrate at a final concentration of 0.05%.

RESULTS

Echinococcosis in humans

Recombinant proteins corresponding to each 8-kDa subunit monomer of EmAgB were successfully expressed and purified from the bacterial lysate. The purified recombinant proteins were applied for subsequent serological tests in Western blots after removal of fusion partners. The results obtained in Western blots are summarized in Table 1. The sensitivity of these recombinant proteins to detect IgG antibodies in serum samples from CE and AE varied with each other. The representative data

are shown in Fig 1. The rEmAgB8/1 showed the highest sensitivity (81.1%) to detect IgG antibodies in serum samples from CE patients than that (41.1%) from AE patients. These results are in agreement with our previous study and the possible reason underlining such phenomenon was discussed in our previous report (Mamuti *et al*, 2004). However, all other recombinant antigens revealed lower reactivities with these serum samples from both CE and AE. Especially, the rEmAgB8/3 showed lowest reactivity with CE serum samples rather than other recombinant antigens. This may be due to the lower immunogenicity of the EmAgB8/3;

Table 1
Summary of serological results obtained in Western blots.

Serum sample sources	No. of samples tested	No. of serum sample positive (%) with indicated antigens			
		rEmAgB8/1	rEmAgB8/2	rEmAgB8/3	rEmAgB8/4
CE patients from:					
Australia	60	48 (80.0)	26 (43.3)	5 (8.3)	29 (48.3)
China	30	25 (83.3)	20 (66.7)	5 (16.7)	16 (53.3)
AE patients from:					
Japan	50	19 (38.0)	7 (14.0)	9 (18.0)	11 (22.0)
France	23	11 (47.8)	7 (30.4)	6 (26.1)	5 (21.7)
Dogs infected with *E. granulosus* from: Australia	15	ND	ND	1 (6.7)	ND

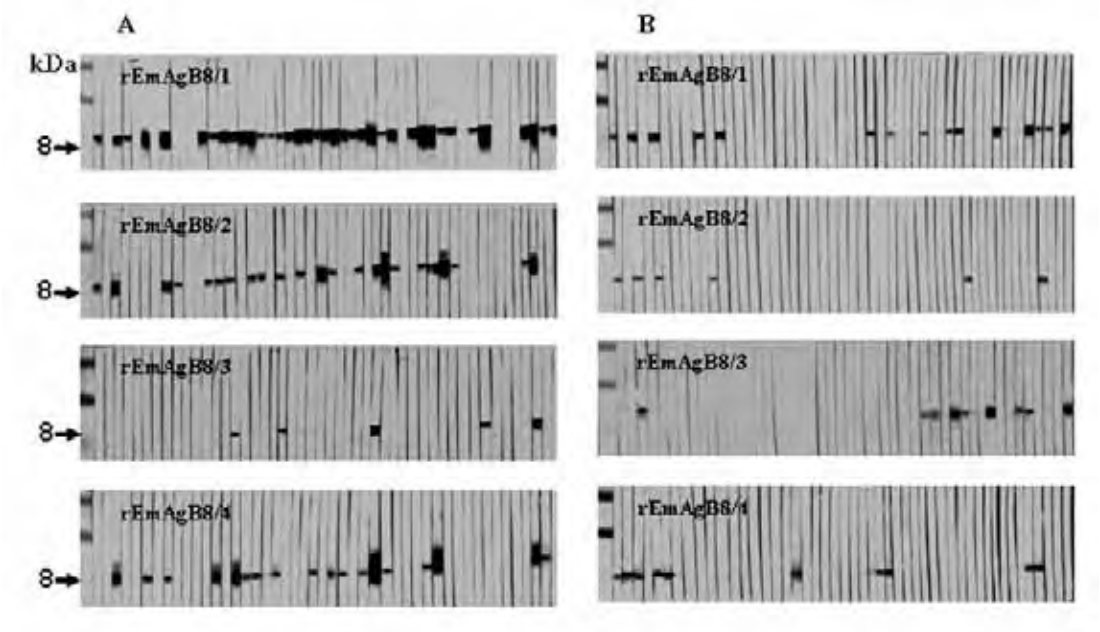

Fig 1- Comparative analysis of recombinant proteins of EmAgB 8-kDA subunit monomers by Western blots. Representative data are shown. The rows 1-4 are the membranes which were transferred with rEmAgB8/1-rEmAgB8/4, respectively. The membrane then probed with serum samples from CE patients (column A) or AE patients (column B). The numbers on the left are molecular sizes (in kilodaltons) of the reacted antigens with IgG antibodies in patients' sera.

even it was expressed in a considerable amount in metacestode stage of the parasite (Mamuti *et al*, 2006). Comparative analysis revealed that rEmAgB8/2, rEmAgB8/4, or rEmAgB8/3 could only be detected by the CE serum sample, which could detect rEmAgB8/1 (Fig 1, column A). However, this was not evident when these antigens were probed with serum samples from AE patients. In these cases, some serum samples were positive with rEmAgB8/3 or rEmAgB8/4, but not positive with the EmAgB8/1. This may be associated with distinctive post-translation, polymerization process, and immunogenic mechanism of the antigen B 8-kDa subunits in these two species.

Echinococcosis in dogs

Additional Western blots were carried out with 15 serum samples from dogs infected with *E. granulosus* adult worm using rEmAgB8/3, because this antigen was expressed in a greater amount in adult worms (Mamuti *et al*, 2004). We found that this recombinant antigen was only detected by one of these 15 AE serum samples (Fig 2). The low reactivity of this antigen with infected dog serum samples may due to the locality of the adult worm; the *E. granulosus* is an intestinal parasite of dog, which may not be able to produce strong serum IgG antibodies to detect the parasite antigen. However, it could be applicable for the establishment of a coproantigen test for the diagnosis of dogs infected with *Echinococcus*, as it is expressed in a greater amount in adult worms of the parasite.

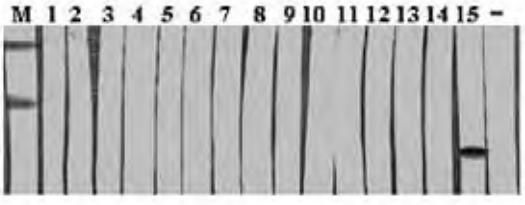

Fig 2- Western blot result of dog serum samples reacted with rEmAgB8/3. Lanes 1-15 were probed with serum samples from 15 dogs infected with *E. granulosus*. The last lane (-) was probed with a serum sample obtained from a parasite free dog as negative control. Lane M, protein maker.

DISCUSSION

The smallest subunit (8-12 kDa) of native AgB in hydatid cyst fluid is recognized to be strongly immunogenic in patients with echinococcal infections (Leggatt et al, 1992; Maddison *et al*, 1989). In a previous study, we have observed that the sensitivity of recombinant EmAgB8/1 is similar to that of EgAgB8/1 to detect the specific total IgG antibodies in serum samples from patient with AE and CE (Mamuti *et al*, 2004). The sensitivity of other EmAgB 8-kDa subunit monomers that we have described here are also not improved for detection of IgG antibodies in serum sample from patients with AE, although they were detected in parasite vesicles and protoscoleces. This may be because the AgB in patients with CE initially accumulated in the fluid of unilocular cysts of metacestode, after being secreted and aggregated into bigger molecules contact with the host immune system. In contrast, the AgB in patients with AE have less chance to accumulate and aggregate into bigger molecules before interacting with the host immune system due to the tumor-like infiltrating structure of the metacestode with no limiting host-tissue barrier. Usually parasite antigens with a bigger molecule size are more immunogenic than the smaller ones to evoke host immune responses. This may be one of the reasons why AgB is more sensitive to detect specific IgG antibodies in serum samples from CE patients than in from AE patients. Rott *et al* (2000) reported that the recombinant protein of EgAB8/2 showed no cross-reactions with serum samples from AE patients in their experiment. This may be due to an insufficient number of serum samples from AE patients. We found the rEmAgB8/2 showed positive reactions with about 20% (14/73) of serum samples from AE patients. In addition, our previous study showed that EmAgB8/2 has more than 94% homology at protein level to that of EgAgB8/2 (Mamuti *et al*, 2006). In conclusion, first, rEmAgB8/1 is the most promising recombinant antigen for serodiagnosis of human echinococcosis among the other 8-kDa subunit monomers of EmAgB. Second, rEgAgB8/3 can be used as a candidate antigen for establishment of a coproantigen test

for diagnosis of dogs infected with *E. granulosus* and *E. multiloculais*.

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PARASITIC INFECTION: A RECURRING PHENOMENON IN MALAYSIA

V Nissapatorn[1], YAL Lim[1], I Jamaiah[1], M Rohela[1] and A Khairul Anuar[2]

[1]Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur; [2]College of Medical and Allied Health Sciences (MAHSA), Kuala Lumpur, Malaysia

Abstract. A total of 255 patients including 179 (70.2%) of non-HIV and 76 (29.8%) HIV-infected patients were recruited in this descriptive study. The subjects was significantly found to be male Chinese (157; 61.6% ***vs*** 74; 47.1%) followed by female Malays (98; 38.4% ***vs*** 35; 35.7%) ($p<0.05$). The majority of subjects (124; 48.6%) were in the age group of 21-39 years, however, no statistical difference was found between the various age groups ($p>0.05$). Overall seroprevalence of latent *Toxoplasma* infection was 82/183 (44.8%) being; 3 (3.7%) positive for IgM, 74 (90.2%) for IgG, and 5 (6.1%) for IgG and IgM antibodies. The prevalence was more relatively found in the Chinese (28; 15.3%) and Malays (27; 14.8%) than others ($p<0.05$). While, 23/76 (30.3%) of HIV-positive patients were shown *Toxoplasma* seropositivity. The majority of these subjects (138/181; 76.2%) were significantly asymptomatic ($p = 0.000$), while the others were clinically evident cases of toxoplasmosis. Of this, 37 patients were included in differential diagnosis relating to ocular diseases and only 4 patients were confirmed as having ocular toxoplasmosis. Toxoplasmic encephalitis (TE) was based on presumptive diagnosis, particularly found in 5 patients with AIDS. Seventeen patients were clinically diagnosed as having malaria being; 8 for *P. vivax*, 4 for *P. falciparum*, 3 for *P. malariae*, and 2 for mixed infections. All cases resolved satisfactorily after treatment with antimalarial drugs. Other important emerging parasitic diseases were also detected in these patients including amebiasis (2), blastocystosis (1), cryptosporidiosis (1), filariasis (1), and giardiasis (2) during the time of this study.

INTRODUCTION

Parasitic infections have historically been some of the most common causes of human disease; which pose economic, health, and social problems to the people living in developing countries, including Malaysia. The impact of parasitic infections on human lives has been a topic of great interest in the field of tropical medicine. We therefore conducted this study to determine the prevalence of parasitic infections and the incidence of clinical cases of these diseases from patients admitted to the University of Malaya Medical Center (UMMC), Kuala Lumpur. This data will further enhance the existing baseline information and the implementation of the standard strategies in terms of prevention and proper management.

Correspondence: Dr Veeranoot Nissapatorn, Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: (603) 7967-6618 ; Fax: (603) 7967-4754
E-mail: nissapat@hotmail.com

MATERIALS AND METHODS

Patients

This retrospective and descriptive study was carried out at the Department of Parasitology, University of Malaya Medical Center (UMMC), Kuala Lumpur, Malaysia. This institution is the oldest university tertiary referral hospital, with 863 beds. It serves as a focus for public services and as a teaching center. Data from a total of 255 patients, including 179 (70.2%) of non-HIV and 76 (29.8%) of HIV-positive patients, were collected and recorded, including laboratory results from blood, serum, and stool samples, in the standardized data collection sheet during May 2004 to February 2006. All positive cases were further investigated, based on the patients' demographic profiles, clinical presentations, relevant laboratory data, and their treatment outcome, from the medical record office, University of Malaya Medical Center. The patients' information was included in the same questionnaire.

Diagnosis of diseases

Various investigations were carefully reviewed according to our study's objectives. The standard

criteria for diagnosing these parasitic infections are as follows:

1. Diagnosis of giardiasis was made by stool examination (concentration technique) for the present of *Giardia* trophozoites or cysts.

2. Diagnosis of cryptosporidiosis was made by the stool examination for the presence of *Cryptosporidium* oocysts and confirmed by modified Ziehl-Neelsen staining techniques.

3. Diagnosis of malaria was made by microscopic identification of peripheral blood smear to detect the presence of *Plasmodium* spp. Also, NOW® MALARIA, USA was serologically used for the antigen detection of *Plasmodium* spp.

4. Diagnosis of seropositivity for *Toxoplasma* infection was via detection of anti-*Toxoplasma* IgG, IgM, or both by either one of the standard ELISA commercial kits (Trinity Biotech, Bray, Ireland; Veda-lab, Alencon Cedex, France) in accordance with the manufacturer's instruction.

5. Diagnosis of seropositive for amobiasis was detected by serologic tests such as IHA or Cellognost® Amoebasis, USA. For filarial infections, peripheral blood smear was used to detect the presence of filarial worms. Few of serological diagnostic kits were available for the antigen detection of Brugia spp and *Wuchereria bancrofti* (NOW® ICT FILARIASIS, USA), also for the antibodies detection of Brugia spp (*BRUGIArapid*™, Malaysia) and *Wuchereria bancrofti* (WB Malaysia). Positive results of these parasitic infections were identified by experienced technicians and confirmed by clinical consultants.

6. Clinical toxoplasmosis was detected in the different two groups of patient, and the diagnoses were determined for a) ocular toxoplasmosis, by clinical presenting signs and symptoms, opthalmoscopic examination, serodiagnosis of *Toxoplasma* infection, and responses to anti-*Toxoplasma* therapy, and b) congenital encephalitis was diagnosed by anti-HIV-positive status, CD4 <200 cells/mm3 (excluded from other of immunocompromised), neurological signs and symptoms, seroevidence of anti-*Toxoplasma* antibodies, and response to anti-*Toxoplasma* therapy.

Statistical analysis

All the findings obtained were entered, edited, and analyzed using statistical software, SPSS version 10 (SPSS Inc, Chicago, USA). The data with quantitative variables were expressed as median and range; whereas, qualitative variables were expressed as frequency and percentage. Statistical analyses were estimated using either the chi-square test or Student's *t* test, where appropriate. A p-value of <0.05 was regarded as statistically significant.

RESULTS

The demographic profiles of the study subjects during May 2004 to February 2006 (21 months) are presented in Table 1. A total of 255 patients, including 179 (70.2%) of HIV-negative and 76 (29.8%) HIV-positive patients were recruited into this retrospective and descriptive study. The age range was from 1 to 81, with a mean of 38.4 ± (SD) 15.31 years. The male:female sex ratio was 1.6:1. The study subjects were significantly found to be male Chinese (157; 61.6% *vs* 74; 47.1%), followed by female Malays (98; 38.4% *vs* 35; 35.7%) ($p < 0.05$). The majority of patients (124; 48.6%) were in the 21-39 year-old age group; however, no statistical difference was found between the various age groups. Interestingly, HIV transmission was found more significantly in males compared with females (56; 35.7% *vs* 20; 20.4%) ($p < 0.05$).

Toxoplasma seropositive and negative status was detected in 181/255 (71.0%) subjects. The overall seroprevalence of *Toxoplasma* infection was 82/181 (45.3%); including 3 (3.7%) positive for IgM, 74 (90.2%) for IgG, and 5 (6.1%) for both IgG and IgM antibodies (Fig 1). The age range was from 1 to 81 years, with a mean of 40 ± (SD) 14.73 years. The sex ratio (M:F) was 1.6:1. The higher prevalent rates were found in males (52; 28.4%) and equally distributed in the subjects between the age group of 20 to 39 and 40 to 59 years (34; 18.8%). However, no statistical difference was found between these associations. Interestingly, the prevalence was significantly related to local racial origins, particularly in Chinese (28; 15.5%) and Malays (27; 14.9%), when compared to others ($p = 0.000$). Twenty-three (30.3%) of 76

Table 1
Demographic profiles of 255 study subjects attended at the University of Malaya Medical Centre (UMMC) during May 2004 to February 2006.

Variable	No. subjects (255)		p-value
	Male n = 157 (%)	Female n = 98 (%)	
Age range = 1-81 years			
Mean = 38.4 (±SD) 15.3 years			
M:F = 1.6:1			
Age group			0.182
≤ 20	11 (7.0)	12 (12.2)	
21-39	75 (47.8)	48 (49.0)	
40-59	50 (32.0)	32 (32.7)	
≥ 60	21 (13.4)	6 (6.1)	
Race			0.023
Malay	35 (22.3)	35 (35.7)	
Chinese	74 (47.1)	29 (29.6)	
Indian	24 (15.3)	20 (20.4)	
Other	24 (15.3)	14 (14.3)	
Country of origin			0.627
Malaysia	131 (83.4)	84 (85.7)	
Outsider	26 (16.6)	14 (14.3)	
HIV status			0.010
Positive	56 (35.7)	20 (20.4)	
Negative or unknown	101 (64.3)	78 (79.6)	
Toxoplasma status			0.879
Positive	52 (33.1)	30 (30.6)	
Negative	61 (38.9)	38 (38.8)	
Unknown	44 (28.0)	30 (30.6)	
Case of toxoplasmosis			0.000
Asymptomatic	153 (97.5)	92 (63.9)	
Ocular toxoplasmosis	2 (1.3)	2 (2.0)	
Toxoplasmic encephalitis	2 (1.3)	4 (4.1)	

HIV-positive patients showed seropositivity for latent *Toxoplasma* infection. The majority of these subjects (138/181; 76.2%) were significantly asymptomatic (p = 0.000), while the others were clinically evident cases of toxoplasmosis. From 37 patients who were included in differential diagnosis related to ocular diseases, 33 of them were suspected as having ocular toxoplasmosis and the other 4 patients were confirmed cases. The life-threatening condition of toxoplasmosis was more commonly found in immunocompromised patients, particularly that involving the brain. From this study, the designation of toxoplasmic encephalitis (TE) was based on presumptive diagnosis, including 1 case in a patient with lymphoma, and 5 cases in AIDS patients.

Concerning malaria, 18 cases were found to be positive for peripheral blood smear (17) or serological (1) detection for malarial infections, including 8 with *P. vivax*, 4 with *P. falciparum*, 3 with *P. malariae*, and 2 with mixed infections. Of these cases, 17 patients were clinically diagnosed as having malaria. Twelve cases were found among locals, including Chinese (6), Malays (5), and an Indian (1). The other five cases were from foreign workers where 4 cases and 1 case came

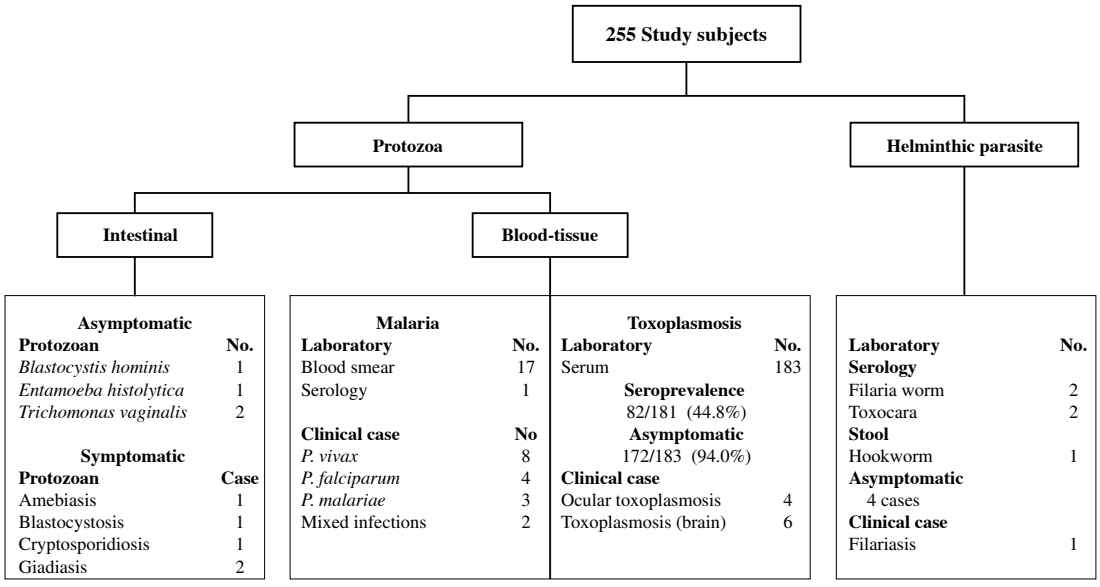

Fig 1- The prevalence and clinically evident cases of parasitic diseases from 255 study subjects during May 2004 to February 2006.

from India and Tanzania, respectively. Malaria occurred more commonly in males (14) and in the younger age group (10) between 20 to 39 years. All cases resolved satisfactorily after treatment with antimalarial drugs.

Regarding helminthic infections, three cases had a seropositive titer for filarial infection, and one case was confirmed clinically as having filariasis. Three cases were asymptomatic with hookworm (1) and *Toxocara* (2) infections. Concerning intestinal protozoan parasites, the majority of positive laboratory investigations were asymptomatic, including 3 for *Blastocystis*, 2 for *Trichomonas*, and 1 for *Entamoeba* infections. Among the symptomatic cases, there was 1 each for amebiasis, blastocystosis, and cryptosporidiosis: in addition, 2 cases were diagnosed for giardiasis. Overall, there was no report of outbreaks, drug resistance, or death relating to any of these parasitic infections during the time of this study.

DISCUSSION

Parasitic diseases have been found in both immunocompetent and compromised hosts in any given geographical distribution. The clinical impacts have been recognized as some of the most common infectious, opportunistic, endemic, and killer diseases in the regional or global arenas.

Overall, the incidence of parasitic diseases was comparatively lower than a previous study conducted in the same setting (Nissapatorn *et al*, 2005). Malaria was the most commonly occurring parasitic disease, and *P. vivax* and *P. falciparum* were the more frequent causes found in this study. Malaria continues to present a significant challenge in tropical countries where over 2 millions people die each year due to this disease. No drug resistance was identified among the affected patients; however, chloroquine resistant falciparum malaria has been reported as early as in 1963, 1995, and 1998 (Jamaiah *et al*, 2005). The continuing expansion of resistance of *P. falciparum* to antimalarial drugs in common use results in the increasing need for different drug combinations (Socheat *et al*, 2003). This is causing great concern, as this species is highly prevalent in tropical Africa, the Amazon, and Southeast Asia (Le Bras *et al*, 2006). Promising results from recent vaccine trials carried out in malaria-naïve and -endemic populations have provided important insights into what will be required of a successful vaccine (Ballou, 2005).

We hope that with this upcoming solution could certainly ease the overwhelming burden relating to malaria in term of control program management in developing countries.

Toxoplasmosis has been listed as a priority among parasitic infections reported in Malaysia. Although this silent parasitic infection has long been afflicting this country, the seroprevalence of latent *Toxoplasma* infection remains high. Toxoplasmosis represents a clinical problem that focuses the attentions of several specialties due to the multiorgan pathology it induces and difficulties in evaluating the activity of the morbid process (Kociecka, 2001). Due to its medical importance, this finding indicated that toxoplasmosis needs a high index of suspicion in suspected cases of ocular disease, particularly in anterior or posterior uveitis. There was no case found in this study of reactivated toxoplasmosis diagnosed after post surgery. Interestingly, one study found that reactivation of ocular toxoplasmosis might develop after LASIK surgery (Barbara *et al*, 2005). Therefore, this infectious disease should be included in differential diagnosis in both medical and surgical involvements with unknown etiological origin and clinical presentations consistent with toxoplasmosis.

Toxoplasmic encephalitis (TE) is based on presumptive diagnosis, and more commonly found in AIDS patents compared with other groups of immunocompromised patients, as indicated in our study as well as others. Neuropathological conditions associated with AIDS are still present in approximately 70-90% of patients and can be the result of HIV itself or of opportunistic infections (Del Valle and Pina-Oviedo, 2006). The majority of patients had secondary reactivation of latent *Toxoplasma* infection that predominantly involved the brain rather than other organs, as also shown in this study. Nevertheless, one study demonstrated that ocular or orbital disease might be the first manifestation of life-threatening systemic toxoplasmosis in HIV-positive patients (Lee *et al*, 2006). However, universal access to HAART has definitely changed both the mortality and morbidity of AIDS (Silva and Araujo, 2005) and has remarkably reduced the incidence of AIDS defining illnesses including TE (Podlasin *et al*, 2006). Due to the benefits of HAART, we support the proposal whereby HIV-infected adult patients who receive effective HAART therapy, primary and secondary prophylaxis against TE can be safely discontinued after the CD4+ T-cell count has increased to more than 200 cells/mm3 for more than 3 months (Miro *et al*, 2006). Nevertheless, it would depend on each case, where there is a need to evaluate the patients' accessibility, availability and affordability of HAART. Then the use of HAART would serve to treat AIDS-related TE fully without the need for chemoprophylaxis. The combination of fansidar and clindamycin is commonly use in treating TE in our hospitals. So far, there has not been any other superior regimen identified for the treatment of TE. The choice of therapy will often be determined by the availability of a therapy such as TMP-SMX, which appears to be an effective alternative therapy for TE in resource poor settings where pyrimethamine and sulfadiazine are not available (Dedicoat and Livesley, 2006). TE is still being seen as one of the most common opportunistic infections in patients with AIDS, particularly in this region.

Gastrointestinal protozoan parasites pose a basic public health concern because of high prevalence rates, particularly in the developing countries. The problem might be more serious because of asymptomatic intestinal parasitic infections (Nuchprayoon *et al*, 2002), as also might be the case in this study. Our data showed 5 cases of symptomatic amebiasis, giardiasis, and cryptosporidiosis in these patients. Of these cases, only cryptosporidiosis was diagnosed in an AIDS patient who presented with chronic diarrhea. Persistent diarrhea and weight loss are the predominant features of cryptosporidiosis (Moolasart *et al*, 1995), and it causes severe and chronic life-threatening gastroenteritis in immunocompromized patients (Zardi *et al*, 2005). Therefore, a high index of suspicion is appropriate for this opportunistic pathogen in HIV-positive patients who have these specific presentations.

Cryptosporidiosis has been one of the most important parasites that is considered by the Centers for Disease Control and Prevention (CDC), Atlanta, as an AIDS-defining illness. In

Malaysia, reports of cryptosporidiosis in patients with AIDS has been very limited (Kamel *et al*, 1994; Nissapatorn *et al*, 2003; Lim *et al*, 2005), and not much attention is given, particularly in clinical practice. The possible explanations are that the routine but specific diagnostic methods have not been established for the laboratory; and, despite the efficacy of passive immunotherapy or some chemotherapeutic agents (*eg*, paromomycin and nitaxozanide), no significant benefits have been demonstrated (Zardi *et al*, 2005). With the introduction of antiretroviral drugs, one study indicated that the increasing immunity due to these drugs might be an important factor in the prevention of opportunistic protozoan infections among HIV-infected patents (Wiwanitkit and Srisupanant, 2006). An increased inhibitory effect was demonstrated when aminoglycoside paromomycin was combined with the protease inhibitors (Hommer *et al*, 2003), and it could be considered as alternative for the development of new drugs that might prove effective against this enigmatic parasite and other related opportunistic parasitic infections (Pozio, 2004). Further studies are required to elucidate the clinical affect of this pathogen, particularly among non-receiving HAART patients, the majority of whom are living in this region.

B. hominis is a cosmopolitan, unicellular, polymorphic protozoan parasite. This organism is one of the most common intestinal parasites found in the large intestine of humans, and the fecal-oral route is the mode of transmission. *B. hominis* has been found in both symptomatic and asymptomatic individuals. This organism however remains controversial and is currently the subject of extensive debate regarding its role as a human pathogen (Nigro *et al*, 2003). In our study, 3 out of 4 positive individuals were asymptomatic. The varying prevalence of *B. hominis* infection from different geographical distributions was comparatively high, up to 46% (Yakoob *et al*, 2004a,b). Further epidemiological surveillance in different groups of the population is required, therefore, to verify the actual prevalence of *B. hominis* infection in this country. The clinical significance of *B. hominis* has been reported worldwide and has certainly gained much attention. In our study, blastocystosis was diagnosed in a patient with symptomatic diarrhea. The role of this organism as a potential intestinal pathogen relating to travelers (Jelinek *et al*, 1997); irritable bowel syndrome (IBS) (Ashford and Atkinson, 1992; Hussain *et al*, 1997); immunosuppressed patients including cancer, hematological malignancy, and HIV/AIDS (Devara *et al*, 1998; Tasova *et al*, 2000; Lebbad *et al*, 2001); hypoalbuminemia and anasarca (Nassir *et al*, 2004); and chronic urticaria (Gupta and Parsi, 2006) has been consistently reported in different settings. In addition, relapse in IBS patients was found to be associated with *B. hominis* (Mylonaki *et al*, 2004). Supporting this clinical aspect, there is a possibility that a subgroup of *B. hominis* could be pathogenic in some patients (Tungtrongchitr *et al*, 2004). With the advance of molecular studies, we should be able to identify this unusual characteristic of *B. hominis* in the near future.

Concerning treatment, metronidazole is considered a standard drug of choice for treating this organism, as was shown in our study. Nevertheless, previous studies have reported that *B. hominis* cysts were resistant, not only to choline (Zaki *et al*, 1996), but also to metronidazole (Zaman and Zaki, 1996; Haresh *et al*, 1999). Interestingly, trimethoprim-sulfamethoxazole was found to be highly effective against this organism (Ok *et al*, 1999) and in some individuals with severe *Blastocystis* infection (Moghaddam *et al*, 2005). In addition, *B. hominis* isolates showed greater *in vitro* susceptibility with furazolidone compared with metronidazole and complete resistance with ciprofloxacin (Yakoob *et al*, 2004a). A recent study suggested that nitazoxanide could be effective in treating *B. hominis* infection in some patients (Rossignol *et al*, 2005). Overall, we recommend further study to elucidate the role of *B. hominis* still; in case of the absence of other causes, *B. hominis* should be considered as a pathogen (Carbajal *et al*, 1997). Also, more clinical drug trial studies, including herbal extracts (Sawangjareon and Sawangjareon, 2005), are required to verify the efficacy of treatments against *B. hominis*.

In this study, only one case was found having clinically evidence of filariasis caused by *Brugia malayi*. Filariasis has been one

of the most important parasitic infections in Malaysia, where only *Wuchereria bancrofti* and *Brugia malayi* are reported to cause human disease. Brugian filariasis accounts for about 13 million cases of the estimated 119 million cases worldwide of active infection and chronic disease (Jamail *et al*, 2005). Almost half of the cases of brugian filariasis are confined to Southeast Asia countries; while India and China account for the other half of the global burden (Michael *et al*, 1996). Improvement in diagnostic techniques for lymphatic filariasis in this country has been remarkably successful and well received. An earlier study showed that monoclonal antibodies have been used to detect circulating antigens and parasite-specific antibodies in filariasis (Abdullah *et al*, 1993). Subsequently, PCR-ELISA has been developed to detect *B. malayi* infection in an area of low endemicity in Malaysia, where more infections were detected, and where it was more reproducible compared to Southern hybridization (Rahmah *et al*, 1998a). Due to its high specificity and sensitivity, serological detection (IgG4 ELISA) would therefore be very useful as a tool in diagnosis if compared to thick blood smear examination (Rahmah *et al*, 1998b, 2001; Lim *et al*, 2001). A further study found that this ELISA format would also be able to demonstrate the decline in IgG4 titer post-treatment (Noordin *et al*, 2003). A rapid dipstick test (Brugia Rapid) has been used to detect specific anti-filarial antibody and has also been tested among suspected cases in our study. The Brugia Rapid is a promising diagnostic tool, not only for detecting *B. malayi*, but also *B. timori* infections; it would be especially useful for the brugian filariasis elimination program in terms of screening and diagnosis, both in Malaysia and related endemic countries worldwide (Rahmah *et al*, 2001, 2003a,b; Noordin *et al*, 2003; Supali *et al*, 2004).

In conclusion, the diversity of parasitic infections is still very much alive in both asymptomatic and symptomatic conditions; which poses a basic public health problem in this tropical rainforest country, Malaysia. Malaria is still the most common parasitic disease; it mostly occurs among locals with a history of traveling to endemic areas. Toxoplasmosis remains one of the most important parasitic diseases, and more efforts in terms of diagnosis and management are needed to curb the primary infection and its consequences of clinical disease. Filariasis is generally well controlled in this country; however, filariasis control programs need to be constantly enforced, particularly in certain endemic areas in this region. Intestinal protozoan parasitic infections share their modes of transmission and pose a significant public health problem. Therefore, more efforts are needed to examine the incidence of symptomatic cases, particularly emerging parasitic infections like cryptosporidiosis or even those with an uncertain pathogenic role, such as that of blastocystosis. Overall, we recommend further studies to be carried out as similar epidemiological and clinical surveillance to monitor closely the trend of parasitic infections periodically.

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DIVERSITY OF HELMINTHS FOUND IN CHANNID FISHES FROM BUNG BORAPHET

Monruedee Chaiyapo[1], Chalobol Wongsawad[1] and Pheravut Wongsawad[1]

[1]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

Abstract. Thirty-five channid fish, including *Channa lucius*, *Channa micropeltes*, and *Channa striata*, were collected from Bung Boraphet, Nakhon Sawan Province during April 2006 and investigated for helminthic infection. There were seven species of helminths : three species of cestodes (*Senga* sp, *Senga chiangmaiensis*, and larval stage in order Proteocephala), two species of trematodes (*Acanthostomum* sp and *Clinostomum* sp), a species of Acanthocephala (*Pallisentis nagpurensis*), and a species of nematode. The prevalence (%) of helminths in each species was 100, 20, 76, 20, 20, 100, and 20, respectively. The highest intensity of cestode larva in Order Proteocephala of *Channa striata* was 75.2%.

INTRODUCTION

Bung Boraphet is the largest freshwater swamp in Thailand. Its substantial size supports an abundance of diverse animals. Local residents benefit from the natural resources of this swamp, including remedies, clothing, building materials, and food (Kor-anantakul *et al*, 2000). Fish provides occupation and high protein food for people who live there. Channid fish are the favorites of people because of their delicious flesh and versatility for cooking. If these fish have been prepared with unhygienic cooking methods, there is the possibility of getting parasitic infection from the infected fish. Investigations of helminthic infection in these fish provide essential information for people to use for control and protection from parasites. Previously, there were only a few studies of parasitic infection of channid fish from provinces in the northern and central parts of Thailand (Sirikanchana, 1983, 1988; Wongsawad *et al*, 2004). To fulfill the essential need for knowledge, this study aimed to examine the diversity of helminths found in channid fish from Bung Boraphet, Nakhon Sawan Province.

MATERIALS AND METHODS

Nets and hooks were used to collect channid fish from Bung Boraphet, Nakhon Sawan Province during April 2006. Thirty-five individual channid fish included 5 *Channa lucius*, 5 *Channa micropeltes*, and 25 *Channa striata*. Each fish was photographed, and the essential data were collected, including total length, body weight, and other morphological characteristics for fish species identification. Each fish was dissected and examined in all parts (fins, gills, scales, and visceral organs) under a stereo microscope. The helminths were removed, counted, fixed in 4% formalin, stained with hematoxylin or borax carmine, dehydrated in alcohol series, and mounted with Permount. Species of worms were identified by morphological examination, as prescribed (Yamaguti, 1958, 1959, 1961, 1963; Schmidt, 1986; Sirikanchana, 1988: Wongsawad *et al*, 2004).

RESULTS

Thirty-five channid fish from Bung Boraphet were examined for helminthic infection. Species of helminths, prevalence (%), intensity of infection, and site of infection are shown in Table 1. There were seven species of helminths: three species of cestodes (*Senga* sp, *Senga chiangmaiensis*, and larval stage of Order Proteocephala), two species of trematodes (*Acanthostomum* sp and *Clinostomum* sp), a species of Acanthocephala *Pallisentis nagpurensis*, and a species of nematode. The prevalence rates of helminths in each species were 100, 20, 76, 20, 20, 100, and 20, respectively. *Senga* sp in *Channa micropeltes* (100%) and *Pallisentis nagpurensis* in *Channa*

Correspondence: Monruedee Chaiyapo, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50202, Thailand.
E-mail : chaiyapo@chiangmai.ac.th

Table 1
Species, prevalence, intensity, and site of helminths in channid fish from Bung Boraphet, Nakorn Sawan Province.

Species of fish	Species of helminths	Prevalence (%)	Intensity of infection	Site of infection				
				Sc	St	In	Li	Sp
Channa micropeltes	Cestoda:							
	Senga sp	100	63.8		+	+	+	
Channa lucius	**Acanthocephala:**							
	Pallisentis nagpurensis	100	67			+	+	
	Cestoda:							
	Senga chiangmaiensis							
	Nematoda: Unknown	20	0.6			+	+	
	Trematoda:							
	Acanthostomum sp	20	0.2			+		
	Clinostomum sp							
		20	0.2	+				
		20	0.2				+	
Channa striata	**Acanthocephala:**							
	Pallisentis nagpurensis	52	6.9		+	+	+	
	Cestoda:							
	Order Proteocephala	76	75.2			+	+	+

Key: Sc = scale, St = stomach, In = intestine, Li = liver, Sp = spleen

lucius (100%) had the highest prevalence of helminths. The highest intensity of infection in each species of fish was 63.8 in *Senga* sp, from *Channa micropeltes*; 67 in *Pallisentis nagpurensis*, from *Channa lucius*; and 75.2 in larval stage of cestode, from Order Proteocephala of *Channa striata*. *Channa lucius* had the highest species diversity of helminths, including five species. The highest dispersion of helminths was three sites: recorded in *Senga* sp from stomach, intestine, and liver; *Pallisentis nagpurensis* from stomach, intestine, and liver; and cestode larva cystacanth from intestine, liver, and spleen. The specific dispersion of helminths was only one site, recorded in *Acanthostomum* sp from scale, *Clinostomum* sp from liver, and nematode from intestine.

DISCUSSION

From this survey, 7 species of helminths were recovered in channid fishes. We observed that the posterior part of scolex in *Senga* sp (Fig 1) from *Channa micropeltes* is broader than *Senga malayana* (Sirikanchana, 1988), and their hook size is bigger than *Senga chiangmaiensis* (Wongsawad *et al*, 2004). From this study, *Senga chiangmaiensis* (Fig 1a,b) was found in *Channa lucius*. A previous report (Wongsawad *et al*, 2004) found this cestode in four species of fish: *Lepidocephalichthys burmanicus, Mystacoleucus marginatus, Mastacembelus armatus*, and *Monopterus albus*. This implied that *Senga chiangmaiensis* has low specificity of hosts. An immature cyst of cestode order Proteocephala was newly recorded in *Channa striata*. Sirikanchana (1983) found the immature cyst in a different order of cestode in the same species of fish. In this investigation, Acanthocephala *Pallisentis nagpurensis* from *Channa striata* and *Channa lucius* was the same species of parasite that was found in *Ophicephalus striatus* from Sing Buri (Sirikanchana, 1983) and Bangkok (Sirikanchana, 1988). *Acanthostomum* sp was recovered from *Channa lucius* in this study, whereas it was formerly found in *Channa gachua* in Mae Sa

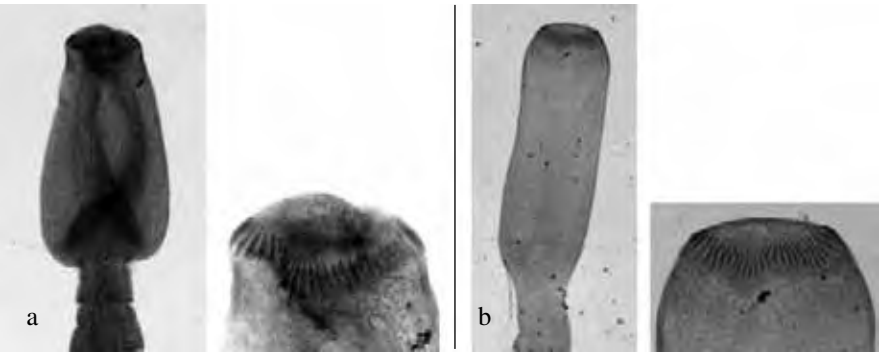

Fig 1- Scoleces and hooks of cestodes: a) *Senga* sp; b) *Senga chiangmaiensis*.

(Wongsawad *et al*, 2004). The metacercaria of *Clinostomum* sp was found in *Trichogaster microlepis* from Bung Boraphet (Yooyen *et al*, 2005), while we found the adult stage of this species from another host, *Channa lucius*, in this study. We have concluded that *Channa lucius* received the metacercarial stage of *Clinostomum* from *Trichogaster microlepis* to explain the cycle of this parasite from intermediate host to definitive host.

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ECTOPARASITES ON MURID RODENTS CAUGHT IN MTS. PALAY-PALAY/MATAAS NA GULOD NATIONAL PARK, LUZON ISLAND, PHILIPPINES

Hazel Anne V Luyon and Cristina C Salibay

Biological Sciences Department, College of Science, De La Salle University-Dasmariñas, Dasmariñas, Cavite, Philippines

Abstract. Rodents in lowland, midland and upland areas of the forest of Mts. Palay-palay/Mataas na Gulod National Park (MPMGNP), Luzon Island, Philippines were trapped using a live capture method. All rodents trapped belonged to genus *Rattus* namely *R. norvegicus* (35.9%), *R. everetti* (27.8%), *R. tanezumi* (20.5%) and *R. argentiventer* (15.8%). Ectoparasites were recovered from the rodents through scraping of host's skin, hair and nails, and collection of dead ectoparasites after insecticide dusting. Among the ectoparasites identified based on their morphological characteristics were *Polyplax spinulosa* (68.0%), *Chirodiscoides caviae* (13.2%), *Laelaps nuttali* (11.5%), *Ornithonyssius bacoti* (4.5%) and *Xenopsyllia cheopis* (2.6%). The infestation rate of the parasite varied based on the rat species, with *P. spinulosa* parasitizing all the rodents caught, and *R. norvegicus* having the highest infestation rate. *Chirodiscoides caviae* parasitized predominantly *R. norvegicus*, while *L. nuttali* was found mainly on *R. tanezumi*. *Ornithonyssius bacoti* was found on both *R. tanezumi* and *R. norvegicus*, while *X. cheopis* was only recovered on *R. argentiventer*. Ectoparasite infestation was also influenced by the gender of the host, with male rats (71.43%) manifesting a significantly higher ($p<0.05$) infestation rate than female rats (28.57%). All recovered ectoparasites were common parasites of rats. Infested rats near human habitations in the area warrant possible rodent-borne diseases among the residents thus, an investigation of the occurrence of rodent-borne diseases among the dwellers may provide epidemiologic pattern related to such diseases.

INTRODUCTION

Parasitism of vertebrates, including rats, by terrestrial arthropods has been most widespread in lairs, nests and other host habitations (Harwood and James, 1979). The diversity and infestation variations in ectoparasites among wild rodents may indicate the prevalence of representative parasites on their hosts and may reflect their host specificity required for their survival and proliferation (Soliman *et al*, 2001b). They also noted that environmental conditions, such as season, topography and vegetation can affect rodent hosts and their ectoparasites (Soliman *et al*, 2001a).

Pestiferous species of rats and mice in the Philippines are known to cause damage to property and crops (Sanchez *et al*, 1985; Salibay and Claveria, 2005). Human interaction with rats potentially exposes the former to the risk of contracting zoonotic diseases caused by viruses, bacteria, protozoa, or invertebrates (Sanchez *et al*, 1985; Harkness and Wagner; 1989; Stoffolano and Romoser, 1998). One of the areas where murid rodents thrive is tropical forests (Heaney and Regalado, 1998).

The presence of humans as disturbance gradients in natural forests is associated with the presence of wild rodents in natural habitats (Sanchez *et al*, 1985; Heaney *et al*, 1999). Arthropologic activities have greatly altered the surrounding environment for habitation, such as forests. The incidence of rodent-borne diseases transmittable to humans has increased and becomes unavoidable because of the close association between rodents and man (Harwood and James, 1979; Sanchez *et al*, 1985). In addition, environmental manipulation, such as agricultural conversion, industrialization, use of pesticides for crop control and management, established in the host's habitat may increase arthropod populations. This is because destruction of the natural habitats of hosts results in limiting their

Correspondence: Cristina C Salibay, Biological Sciences Department, College of Science, De La Salle University-Dasmariñas, Dasmariñas, Cavite, Philippines.
Tel: 063-046-4164531, Fex: 063-046-4164524
E-mail: hvluyon@mail.dasma.dlsu.edu.ph

spatial dimension, hence hosts become congested in a "smaller" habitat, giving way to easier transmission of ectoparasites from one host to another.

Taking into account the role of rodents as carriers/reservoir hosts of disease-carrying agents (Harkness and Wagner, 1989) and their close proximity to human habitations (Gratz, 1988; Nowack, 1991; Salibay and Claveria, 2005), especially in rural areas (Soliman *et al*, 2001a,b), and as inhabitants of forests (Nava *et al*, 2003), this study was conducted to determine the rodent host preference of ectoparasites in terms of host species and gender collected in habitats at different area elevations.

MATERIALS AND METHODS

The study site

Mts. Palay-palay/Mataas na Gulod National Park (MPMGNP) is a 4,000-hectare mountain range situated within the municipality of Ternate and Maragondon in Cavite, and Nasugbu, Batangas, provinces in Southern Luzon, Philippines (DENR, 1992). The range includes three main peaks which were considered as the forest habitats in this study, Palaypalay, Mataas na Gulod, and Pico de Loro, with elevations of 595 meters above sea level (masl), 622 masl and 648 masl, respectively. The collection sites at the MPMGNP were determined using stratified random sampling with reference to the forest trail. Each habitat was divided into low elevation (LE) (0-207 masl), middle elevation (ME) (208-414 masl), and high elevation (HE) (415 and above). The sampling site was approximately 1 km from human habitation, agricultural, and infrastructural sites. Likewise, within the selected habitat, the point of reference was 15 meters from a human trail.

Host capture

The technique for capture of wild rodents was adopted from the guidelines and works of PCAARD (1985); Sanchez *et al* (1985); Walker (1994); Heaney *et al* (1999); Soliman *et al* (2001b); ASM ACUC Guidelines (2003); and Salibay (2004) with slight modifications. The survey of rodents along the study site used the elevational transect method. This method was set in trails along an elevational transect at elevation areas of the three habitats. The study also adopted the 100 meter-transect line live capture method. In each sampling station, 20 spring door wire traps with food bait, which included bananas, grilled coconut meat, dried fish and earthworms, were deployed with an interval of 5 meters for each collection site. The sites were further subdivided into subplots to avoid collection of rats at exactly the same plot per collection site. The traps were set before dusk and checked in the early morning. In the absence of a rodent, traps with fresh bait were left in place for two to three days, then transferred to another location. The captured rats were transported to the DLSU-D Natural History Laboratory for processing.

Host euthanasia and preservation (ASM ACUC Guidelines, 2003)

Since some ectoparasites leave the body of the host shortly after death, the captured rodents were transferred from the cages to closed containers before the euthanization process to ensure the collection of the ectoparasites present on the body of the euthanized hosts. Under open-air field conditions or well-ventilated areas, chloroform was appropriated for euthanization since it also kills ectoparasites. Collected rodents were processed with formalin and preserved in glass jars with 70-80% ethanol. After the experiment, the rodents were disposed of properly.

Rodent identification

The rodents were identified using several references (Sanchez *et al*, 1985; Heaney *et al*, 1999; Salibay, 2004). Individual rodent autopsy for host identification was performed assessing for morphological differences according to physical characteristics and external measurements, which included weight and head, hind foot, tail and body lengths. The physical characteristics and morphometrics of the murid rodents recorded for identification were *R. norvegicus* and *R. tanezumi* (Sanchez *et al*, 1985; Salibay, 2004), *R. everetti* (Heaney *et al*, 1999) and *R. argentiventer* (Sanchez *et al*, 1985). Authentication of pre-identified species was done at the Mammalogy Section, Zoology Division of the National Museum, Manila, Philippines.

Ectoparasite collection

The ectoparasite collection followed the protocol of Soliman *et al* (2001b), and Walker (1994) using the host search method. The ectoparasites were rendered inactive on the body of the host using chloroform. The individual rodents were dry-combed with a toothbrush in a postero-anterior direction to collect parasites. The snout, ears, limbs, and axillary regions of rodents were combed. Wet-combing in a postero-anterior (PA) direction was followed by placing the dead rodent immersed in collecting tray with 70% ethanol. The parasites were then filtered through filter paper and transferred into a glass vial (3 cc) by forcefully flushing them with 70% ethanol using a pipette.

Fleas and lice were collected by parting the hair and picking up the lice with forceps or using a fine comb. Dusting the host with an insecticide and collecting the dead parasites as they fell onto a collecting tray was done. Mites were collected by skin scraping using as improvised plastic scraper with thin, flat edges, or a scalpel held at 90o to the skin. The scraper was wetted with oil or glycerol making a temporary mount on a microscope slide for examination. Spots of blood were drawn from epidermis to check for sarcoptic mites. Affected host skin with dermatitis was incised over nodules to check for demodectic mange mites.

Ectoparasite preservation

The preservation and identification of ectoparasites was performed following the methods of Walker (1994) and Soulsby (1982). The specimens collected were submerged in 75% ethanol and additional water for liquid preservation. Glycerol was added as one-tenth part of the total volume of ethanol using 5 to 25 ml thick-walled, wide-mouth glass containers. Dry and wet-combed collections were processed for temporary mounting, and preservation (permanent mounting) on glass slides. Temporary mounting was used for counting the ectoparasites and permanent mounting was used for photomicroscopy and identification.

For temporary mounting, fresh specimens were mounted in glycerol directly on a microscope slide, a cover slip was placed, and the specimen was directly examined under a dissecting microscope. For permanent mounting, the specimens were fixed by immersion in glacial acetic acid with formalin for 5 hours. After fixation, specimens were dehydrated by soaking them in 10% potassium hydroxide solution for one day, then washed with water for a few minutes, followed by a 30 minute-soaking in 10% acetic acid and washed with water before starting the dehydration procedure with ethanol. The specimens were dehydrated by soaking in several mixtures of ethanol and water from 75% to 95% ethanol at one hour for each mixture and were cleared by soaking the specimen with xylene. The specimen was transferred from the xylene to a glass slide, a drop of Canada balsam was placed on the slide and a cover slip was placed.

Ectoparasite identification

Mounted specimens were examined microscopically using dissecting and binocular microscopes to study their morphological characteristics for identification. The identity of the ectoparasites was established using identification guides by Walker (1994), and the works of Harwood and James (1979), and Salazar and Cabrera (1969).

Ectoparasite counting

The visual examination or indirect counting method adopted from the work of Clayton and Walther (1997) was used in this study. Counting of ectoparasites was done using a counter while ectoparasites were rendered inactive in a Petri dish or on a dissecting microscope.

Data gathering and analysis

Infestation rate of recovered ectoparasites from their rat hosts was based on Soliman *et al* (2001b) using the formula:

$$\frac{N1}{N2} \times 100$$

Where N1 = Number of ectoparasite recovered from a particular individual host species
N2 = Total number of ectoparasites recovered

Ectoparasite burden per rat host is calculated as:

$$\frac{R1 \times 100}{R2}$$

Where R1 = Number of individual rat infested according to gender

R2 = Total number of rats infested

Statistical analysis

Influence of the elevation to the number of caught rodents was determined using *t*-test and regression analysis model. The rodent species collected in terms of forest area habitats of the MPMGNP was tested using univariate and two-way ANOVA at $p \leq 0.05$. Ectoparasites per rat hosts were tested using two-way ANOVA at $p \leq 0.05$. Chi-square (χ^2 test) was applied to compare the ectoparasite burden on male and female host groups.

RESULTS

Murine rodent species collected

Four species of rodents, all from genus *Rattus* were collected from MPMGNP, namely: *R. norvegicus* (Norway rat), *R. everetti* (common Philippine forest rat), *R. tanezumi* (Oriental house rat) and *R. argentiventer* (ricefield rat). Of the species collected, *R. norvegicus* (39.3%), was significantly ($p \leq 0.05$) the most prevalent, followed by *R. everetti* (28.6%), *R. tanezumi* (21.4%) and *R. argentiventer* (10.7%). The number of wild rats caught in terms of elevation was significantly ($p \leq 0.05$) the highest at low elevation (55.4%), followed by middle (28.6%), then high (16.1%) elevations (Fig 1). As the elevation increased, the frequency of rats decreased.

Ectoparasites of rodents

The number and species of ectoparasites recovered from the rats are presented in Table 1. The results indicate all rats collected were parasitized by ectoparasites, with *R. norvegicus* having the highest number of parasites recovered (35.9%). This was followed by *R. everetti* (27.8%), *R. tanezumi* (20.5%), and *R. argentiventer* (15.8%). Although *R. tanezumi* did not show a high infestation rate compared to *R. norvegicus* or *R. everetti*, it harbored four species of parasites. *Rattus everetti* and *R. argentiventer* had the least number of types of ectoparasites recovered.

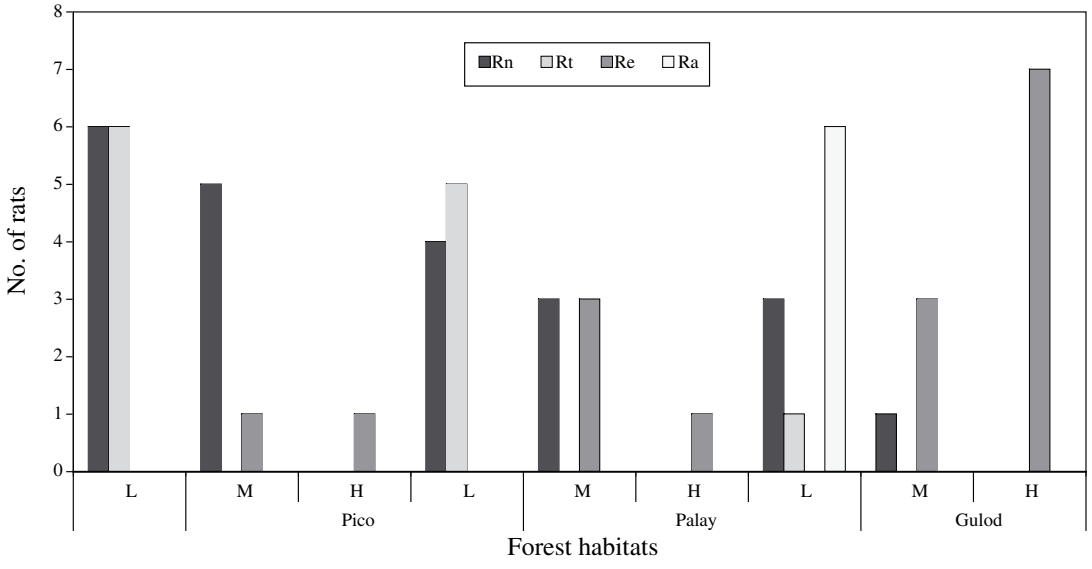

Fig 1- The number of rats collected at different elevations in forest habitats. R.n. (*R. norvegicus)*; R.t. (*R. tanezumi)*; R.e. (*R. everetti*); R.a. (*R. argentiventer*).

Table 1
Summary of ectoparasite infestation rates (IR) on murine rodents.

Rattus species (total number collected)	Number of Ectoparasite present (%IR)						Total per rodent species	Infestation rate (%)
	X. cheopis	*P. spinulosa*	*C. caviae*	*L. nuttali*	*O. bacoti*	*Thrips* sp		
R. norvegicus (22)	0 (0)	116 (24.8)	26 (5.6)	18 (3.8)	7 (1.5)	1 (0.2)	168	35.9
R. tanezumi (12)	0 (0)	50 (10.7)	12 (2.5)	20 (4.3)	14 (3)	0 (0)	96	20.5
R. everetti (16)	0 (0)	90 (19.2)	24 (5.1)	16 (3.4)	0 (0.)	0 (0)	130	27.8
R. argentiventer (6)	12 (2.6)	62 (13.2)	0 (0)	0 (0)	0 (0)	0 (0)	74	15.8
Total per ectoparasite (%)	12 (2.6)	318 (68.0)	62 (13. 2)	54 (11.5)	21 (4.5)	1 (0.2)	468	

Two species of ectoparasites of the class Insecta and three species of the class Arachnida were collected from the rodents (Fig 2). In the class insecta, the spined rat louse, *Polyplax spinulosa* (68.0%), dominated the infestation of all rodents. However, the Oriental rat flea, *Xenopsylla cheopis* (2.6%) was recovered only on *R. argentiventer*, and with a minimal infestation rate. Of the arachnids recovered, the scab mite, *Chirodiscoides caviae* constituted 13.2% of the collected rats, with the highest infestation rate being in *R. everetti*, followed by *R. tanezumi* and *R. norvegicus*, while none were found on *R. argentiventer*. The common rat mite, *Laelaps nuttali* had on 11.5% infestation rate, which was recovered from the three hosts: most frequently in *R. tanezumi*, followed by *R. norvegicus* then *R. everetti*. The tropical rat mite, *Ornithonyssus bacoti* (4.5%) infested both *R. tanezumi* and *R. norvegicus*.

The presence of *Thrips* sp, a plant-sap sucking insect, which belongs to *Thysanoptera*, found on *R. norvegicus*, was found least commonly in this study.

Ectoparasite infestation relative to host gender was significantly higher ($p<0.05$) in male rats than in females (Table 2). This may be due to the fact that male rats are bigger in size and are more active so that they have high chances of being infested.

DISCUSSION

The present study found two ectoparasites from the class Insecta and 3 from the class Arachnida, all of which are ectoparasites on *Rattus* species (Harwood and James, 1979; Eduardo and Mercado, 1981; Gratz, 1988). Similar to earlier surveys (Salazar, 1977; Durden and Page, 1991; Soliman *et al*, 2001a,b) of ectoparasites on commensal murid rats, these are known to be found on *Rattus* spp, and are not classified as being host specific (Salazar, 1977; Soulsby, 1982; Walker, 1994). This was evident with *P. spinulosa* found in all rat species collected, which may be indicative of rat-to-rat transmission within and among the different species of hosts. The findings of this study are similar to those in studies by Durden and Page (1991) and Soliman *et al* (2001b) of the presence of mites on rodents. They collected *L. echidnina*, *L. nuttali* and *O. bacoti*; *X. cheopis*; and *Hoplopleura pacifica* and *P. spinulosa* from *R.r.palelae*, *R. argentiventer*, *R. exulans* and *M.m. castaneus*, in Sulawesi Utara, Indonesia (Durden and Page, 1991), and *R. norvegicus* and *R. rattus* from rural Egypt (Soliman *et al*, 2001b). Both studies indicate that *L. nuttali*, *O. bacoti*, *X. cheopis* and *P. spinulosa* were associated with murid rodents, especially *Rattus* species.

Of the *Rattus* species, *R. argentiventer* is

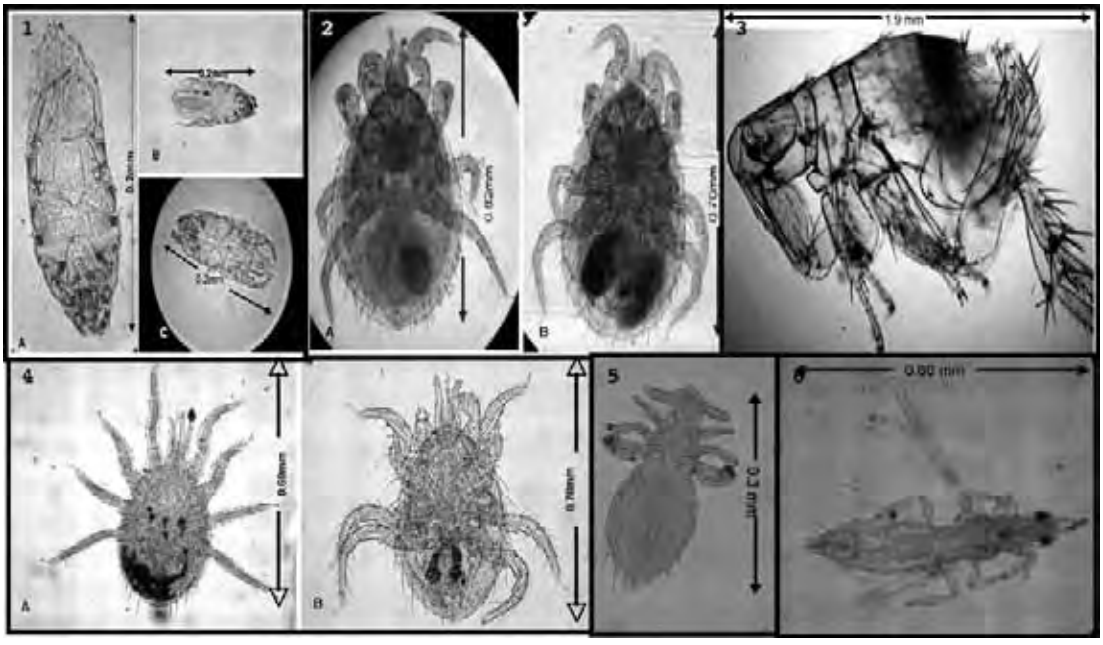

Fig 2- Recovered ectoparasites from wild *Rattus* spp. 1. *Chirodiscoides caviae* adult, 1A and 1C. Male;1B. Female. 2. *Laelaps nuttali* adult, 2A. Male; 2B. Female. 3. *Xenopsyllia cheopis*, adult. 4. *Ornithonyssius bacoti*, 4A. Nymph 4B. Adult. 5. *Polyplax spinulosa*, adult. 6. *Thrips* sp, adult.

Table 2
Ectoparasite burden relative to host gender.

Host	Number of ectoparasites on rodents (%)	
	Male	Female
R. norvegicus	12 (21.4)	10 (17.9)
R. tanezumi	8 (14.3)	4 (7.1)
R. everetti	16 (28.6)	0
R. argentiventer	4 (7.1)	2 (3.6)
Total	40 (71.4)	16 (28.6)

considered important in the maintenance of campestral or sylvatic plague caused by fleas associated with field rodents. This may cause such disease in rural areas even if there is no outbreak of plague cases (Harwood and James, 1979; Liat *et al*, 1980). In the present study, the presence of *X. cheopis* solely in *R. argentiventer* may indicate a close association between the flea and this rodent species.

A study done by Gratz (1988) proved that the chances of encountering *Rattus* species varried depending on the rat's habitat preference. *Rattus norvegicus* has a broad habitat, while *R. tanezumi* thrives in trees or on vine-covered fences and landscaped residential or industrial sites, as well as in the vegetation of riverbanks and streams. Their preferences may be influenced by their food sources. This preference may lessen the chances of encountering other rat species in natural forests, such as *R. argentiventer*, which prefers agricultural areas, thereby influencing the transmission of ectoparasites within the same species or among different species.

According to Harwood and James (1979), the majority of siphonapterans, such as *X. cheopis*, are known to leave their host and may transfer to hosts of the same or different species. However, in the present study, it was recovered only from *R. argentiventer*, which may indicate this rodent may not be as mobile as other *Rattus* species.

Notably, *O. bacoti*, which parasitized *R. tanezumi* and *R. norvegicus*, has a strong association with non-native rats in suburban areas of Manila, Philippines (Salazar, 1977). The present study also shows that *O. bacoti* were recovered more often from *R. tanezumi*

and *R. norvegicus* than from *R. everetti* and *R. argentiventer*, which are rodents of forest and agricultural areas, respectively. Non-native murid rats may be more adaptable to disturbed areas (Sanchez *et al*, 1985; Heaney *et al*, 1999; Soliman *et al*, 2001a), and tend to be more susceptible to ectoparasitism compared to native species. In addition to becoming more adaptable, Soliman *et al* (2001b) found a higher infestation rate with ectoparasites with greater body size of the host. This finding is in congruence with the results of the present study, where *R. norvegicus* and *R. everetti* had significantly higher infestation rates and interestingly, were larger rats than *R. tanezumi* and *R. argentiventer*. The high infestation rate of parasite in *R. norvegicus* may be attributable to the greater number of such species in the habitat; thus, changes in rat-to-rat transmission with the ectoparasite is also high within the species (Salazar, 1977; Harwood and James, 1979; Sanchez *et al*,1985; Gratz, 1988).

Relative to host gender, the highest percentage of ectoparasites recovered was from male rats. This may be attributable to the fact that male rats are more active and can travel significantly farther than females; their broad diet makes them adaptive to travel longer distances (Sanchez *et al*, 1985; Heaney *et al*, 1999; Soliman *et al*, 2001b), making them more susceptible to ectoparasitic infestation. The age of the commensal rat hosts, as well as the species of the ectoparasite, are also important factors that influence the infestation rate (Soliman *et al*, 2001b); however, such factors were not considered in this study.

Environmental modification, such as forest clearing and subsequent conversion into agricultural land may influence the presence of pestiferous vectors, such as *R. norvegicus* and *R. tanezumi*, as well as their ectoparasites, as was reported by Harwood and James (1979); Sanchez *et al* (1985). The high infestation rate of ectoparasite species in *Rattus norvegicus* and *R. tanezumi* can be attributed to these species preference for more congested areas where houses and other settlements are located (Sanchez *et al*, 1985) compared to *R. everetti*, which dwells only in areas near its habitat with less disturbance caused by the presence of human settlements, and *R. argentiventer*, which thrives in agricultural areas (Sanchez *et al*, 1985; Heaney *et al*, 1999). This results in a greater chance for a physical encounter among rat species in congested areas, and the transfer of ectoparasites from one host to another, as in the case of *R. tanezumi* and *R. norvegicus* in this study.

In natural forest conditions, rats in the wild are considered to be cleaner (Heaney and Regalado, 1998) compared to those that are found in urban areas (Salazar, 1977). This is because rat species dwelling in forests consume fruits or crops, while those dwelling in urban areas are found in poorly sanitized areas and in nearby garbage dumpsites. Salazar (1977) also noted rat species caught in urban areas had poorer hygiene. In this study, some rats recovered in the forest areas were of the same species as those urban dwellers, indicative of the infiltration of urban rats in forest areas especially at lower elevations.

In conclusion, although, the degree of infestation of ectoparasite varies among the host species, the elevation and habitat where the species were collected did not show direct influence as to the infestation rate of the ectoparasites.

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ECTOPARASITE SPECIES FOUND ON DOMESTIC DOGS FROM PATTAYA DISTRICT, CHON BURI PROVINCE, THAILAND

Chadaporn Nuchjangreed and Wararat Somprasong

Department of Medical Science, Faculty of Science, Burapha University, Chon Buri, Thailand

Abstract. The prevalences of ectoparasites and their densities were determined. A total of 460 ectoparasites were collected from 83 untreated household dogs in Pattaya District, Chon Buri Province. Four hundred and six ticks were found and classified into two species: *Rhipicephalus sanguineus* (n = 356, 77.4%), and *Boophilus microplus* (n = 50, 10.9%). All fleas found on the studied dogs were *Ctenocephalides canis* (n = 54, 11.7%). Sixty-seven dogs were infested with ticks and the prevalence rate of tick-harboring domestic dogs was 80.7%. Only 21 dogs were infested with fleas, resulting in a prevalence rate of 26.5%. The densities of *R. sanguineus, B. microplus* and *Ct. canis* infestations in the dogs were 5.4, 2.6, and 2.6, respectively. The stages of *R. sanguineus* were larvae (0.6%), nymphs (1.7%), adult males (49.4%) and adult females (48.3%). The stages of *B. microplus* were nymphs (16%) and adult females (84%). For *Ct. canis*, only adult stages (5.7% males and 94.3% females) were found. The results indicate that *R. sanguineus* is the most prominent species of ectoparasite found on household dogs, followed by *Ct. canis*. *B. microplus*, which is commonly found on cattle, was also found on dogs. The fleas seemed to be less of a problem than ticks, and flea larvae were not discovered on domestic dogs in this area.

INTRODUCTION

Ectoparasites, such as ticks and fleas, live on domestic dogs. They can cause severe dermatitis and may act as vectors for pathogenic agents, resulting in serious diseases not only in dogs, but also in humans. Hard ticks, *Rhipicephalus sanguineus*, are the vectors of a wide range of important diseases worldwide, including viruses, bacteria, and protozoa. These include: rocky mountain spotted fever (spotted fever group, SFG), boutonneus fever, african tick fever, russian tick typhus, Q fever, encephalitis, tularemia, relapsing fever and Lyme disease (Service, 1996). More recently, ticks have been implicated as vectors of additional diseases in North America, including anaplasmosis, babesiosis, and ehrlichiosis. Ticks are also involved in tick paralysis, the condition caused by a toxin or toxins found in the saliva of ticks. *Ctenocephalides canis*, *Ctenocephalides felis felis*, *Pulex irritans* and *Echidnophaga gallinacea* (from poultry) are fleas usually detected in dogs. Their presence is generally associated with skin disorders (dermatitis), pruritus, severe itching and allergic reactions in infested hosts. They may also cause pest problems in contaminated environments. They also act as vectors of pathogenic agents, such as rickettsia disease (murine typhus), bacterial disease (plague) and viral disease (myxomatosis)(Koutinas *et al*, 1995). Dog fleas and cat fleas are identified as intermediate hosts for dog tapeworms (*Dipylidium caninum*) and dwarf tapeworms (*Hymenolepis nana*)(Kettle, 1984).

In urban or suburban areas, people traditionally raise dogs as pets. Thus, pet owners may contact disease from ectoparasites via their dogs. One way to prevent disease is to treat pets as family members. Health check-ups protect pets from infestation by ectoparasites. One potential and effective way to prevent diseases is to provide a good environment for the pet. Thus, a knowledge of types of species, density and prevalence of ectoparasites is needed to effectively control them.

Several surveys have pointed out the importance of ectoparasites in small animals. However, there are differences in respect to their frequency and geographical locations (Chesney, 1995; Koutinas et al, 1995; Nithikathkul et al, 2002, 2005; Shimada et al, 2003). The

Correspondence: Chadaporn Nuchjangreed, Department of Medical Science, Faculty of Science, Burapha University, 169 Longhard Bangsaen Road, Saensook Sub-District, Mueang District, Chon Buri Province, 20131, Thailand.
Tel +66 (038) 745900 ext 3163
Fax +66 (038) 393497
E-mail: Nuchchadaporn@hotmail.com

presence of *P. irritans*, *C. canis* and *C. felis* has been described in dogs and cats in Chile (Alcaino *et al*, 2002). In Thailand, Ixodid ticks on domestic animals in Samut Prakan Province and ectoparasites on domestic dogs (*Canis lupus familiaris*) were studied in Mueang District, Khon Kaen (Nithikathkul *et al*, 2002, 2005). Surveys of flea species from dogs were conducted in Nakhon Chaisi District, Nakhon Pathom Province (Nateeworanart *et al*, 2005). However, information regarding ectoparasites on domestic dogs in Chon Buri Province have not yet been reported.

The purpose of this study was to identify ectoparasites found on domestic dogs in Pattaya District, Chon Buri Province, Thailand. The species, the percentage, density and prevalence of ectoparasites collected from selected domestic dogs at three veterinary clinics located in Pattaya District were studied.

MATERIALS AND METHODS

Dogs of various breeds, sexes and ages were examined for ectoparasites at three veterinary clinics in Pattaya District between January and March 2004. All dogs presenting for a clinical veterinary consultation on one regular working day per week, per practice, were examined. Dogs were examined regardless of prior therapeutic or prophylactic insecticide treatment.

Ectoparasites were collected by hand from the ears, tails, necks and legs of dogs over a 15-minute period. Captured specimens were counted and placed small plastic containers. They were then fixed in 70% alcohol in a vial (with the labeled host and time data *eg*, details about breed of dogs, date and time and address of household where the dogs live). Captured specimens were preserved in 70% alcohol until identification was finished. The ectoparasites were kept in 10% KOH (clearing agent) 2-3 day before identification. Species determination was based on microscopic examination; the nomenclature of Peus (1938) was used for identifying specimens. The stage and species of ectoparasites were identified.

The percentage, density and prevalence of infestation were calculated using the following formula: Percentage of ectoparasites = (number of specific ectoparasites/total number of ectoparasites) x 100; Density of infestation = number of specific ectoparasites/number of dogs with their corresponding specific ectoparasite infestion; Prevalence of infestation = number of existing cases/total population at a specific point in time; Estimated prevalence of infestation = number of animals infested with an ectoparasite species/number of dogs sampled) x 100.

RESULTS

A total of 460 ectoparasites (406 ticks, 54 fleas) were collected from 83 untreated household dogs in Pattaya District, Chon Buri Province. By taxonomy, 406 ticks were classified into 2 species of ectoparasites: *Rhipicephalus sanguineus* (77.4%, n=356) and *Boophilus microplus* (10.9%, n=50). The most common tick was *R. sanguineus* and the least common tick was *B. microplus*. A total of 54 fleas were found, all of them *Ctenocephalides canis* (11.7%, n=54).

The prevalence of ectoparasites and their density were determined in 83 untreated dogs. Sixty-seven dogs were infested by ticks. The overall prevalence rate for ticks on domestic dogs was 80.7%. *R. sanguineus* was found in 79.5% (n=66) and *B. microplus* in 22.9% (n=19). Only 21 dogs were infested by fleas, with a prevalence rate of 26.5%. The densities of *R. sanguineus, B. microplus* and *Ct. canis* infestations in dogs were 5.4, 2.6, and 2.6, respectively (Table 1).

The stages of *R. sanguineus* included larvae (0.6%), nymphs (1.7%), adult males (49.4%) and adult females (48.3%). The stages of *B. microplus* were nymphs (16%) and adult females (84%). For *R. sanguineus*, all stages of ticks were found. However, only nymphs and adult females of *B. microplus* were found. The only species of fleas found in domestic dogs from Pattaya District was *Ct. canis*. Adult males (5.7% and and females (94.3%) *Ct. canis* were found (Table 2). Flea larvae were not discovered on any of the studied domestic dogs.

DISCUSSION

In household dogs, *R. sanguineus* was the

Table 1
Species, percentage, estimated prevalence and density of ectoparasites found on domestic dogs in Pattaya District.

Species	Percentage of all ectoparasites (%)	Estimated prevalence of ectoparasites (%)	Density of ectoparasites
Rhipicephalus sanguineus	77.4	79.5	5.39
Boophilus microplus	10.9	22.9	2.63
Ctenocephalides canis	11.7	26.5	2.57

Percentage of ectoparasites = (number of specific ectoparasites/total number of ectoparasites) x 100; Density of infestation = number of specific ectoparasites/number of dogs with corresponding ectoparasite infestion; Estimated prevalence of infestation = number of animal infested by an ectoparasite species/number of dogs sampled) x 100.

Table 2
Stages of ectoparasites found on domestic dogs in Pattaya District.

Species/ Stage	Adult male (%)	Adult female (%)	Nymph (%)	Larvae (%)
R. sanguineus	49.4	48.3	1.7	0.6
B. microplus	-	84.0	16.0	-
Ct. canis	5.7	94.3	-	-

most common species, followed by *Ct. canis* and *B. microplus*. These findings are similar to those found by other investigators (Guglielmone *et al*, 1991; Michael *et al*, 1996). *Haemaphysalis longicornis*, *H. flava* and *I. ovatus* were more common in dogs living in rural areas than urban or suburban areas. Infestation with *R. sanguineus* in dogs was associated with being in an enclosed yard while exposure to woodlands was associated with *H. flava* and *I. ovatus* infestation (Shimada *et al*, 2003). All stages of *R. sanguineus* were found. However, only nymphs and adult females of *B. microplus* were found. *B. microplus* is commonly found on cattle (Kettle, 1984). We postulated the development of transportation in Pattaya District has altered the animal life. Cattle and dogs live in the same area. Over time, *B. microplus* may have adapted themselves to live on dogs.

Nithikathkul *et al* (2002) reported only two species of Ixodid ticks, *R. sanguineus* and *B. microplus* were found. *R. sanguineus* was the most common species in *Canis lupus familiaris* and *B. microplus* was the most common species in cattle (*Bos indicus* and *Bos taurus*, cross-bred) in Bang Phli District. The density of *R. sanguineus* was highest in the winter; the density of *B. microplus* was highest in the summer. The number of ticks depended on the geographic location, animal host and season. The high percentage of *R. sanguineus* may be due to the temperature in the winter is optimum for these ticks; temperature affects the growth, development, reproduction and survival of *R. sanguineus*. In contrast, temperature and/or moisture may not affect the growth and reproduction of *B. microplus* (Nithikathkul *et al*, 2002).

Fleas seemed to be less of a problem for dogs. *Ct. canis* was the only flea species found. *Ct. felis* and flea larvae were not discovered on domestic dogs in this area. These findings are not consistent with the findings of the polyxenous nature of fleas studied by other investigators (Chesney, 1995; Clark, 1999; Visser *et al*, 2001; Alcaino *et al*, 2002). *Ct. canis* tends to be a predominant species in rural locations and *C. felis* is more common in urban areas (Alcaino *et al*, 2002; Beck *et al*,

2006). Koutinas *et al* (1995) identified dog fleas collected from 129 dogs and 38 cats. *Ct. canis* was the most common species found on dogs (71.3%). The prevalence on cats was substantially lower (5.3%). *Ct. felis* was found on 97.4% of cats and 40.3% of dogs surveyed from various parts of northern Greece. Among the less prevalent flea species, *Pulex irritans* (0.8%) and *Xenopsylla cheopis* (0.8%) were detected on dogs. There were mixed infestations in 13.2% of dogs and 2.6% of cats in the Greek study. Flea-associated dermatoses were observed in 26 dogs (20.2%) and 4 cats (10.5%). Flea-allergic dermatitis, with its typical manifestations, was found in 10 dogs (38.5%) possessing skin lesions. Of 4 flea-allergic cats, three presented with miliary dermatitis and one the symmetrical hypotrichosis (Koutinas *et al*, 1995).

Alcaino *et al* (2002) studied flea species on dogs in three cities of Chile. The only species of fleas found on dogs were *Ctenocephalides felis felis* (41.8%), *Ctenocephalides canis* (39.4%) and *Pulex irritans* (18.8%). The three species were found in three cities, and differences regarding their frequencies were detected ($p<0.05$). *Ct. felis* was the predominant species found on dogs in Santiago and Concepcion. However in Osorno, the most southern city, the predominant species was *Ct. canis* (78.7%). Osorno is much more rural than Santiago, which is the capital city, and according to other authors, *Ct. canis* is a predominant species in rural areas, while *Ct. felis* is more common in urban locations (Beresford-Jones, 1981). Similar findings were seen in England (Edward, 1969), and Denmark (Kristensen *et al*, 1978). In contrast, Beck *et al* (2006) studied the epidemiology and population dynamics of flea infestations in dogs and cats; the preliminary results did not indicate a relationship between climatic conditions and flea infestation rates. Similarly, no differences in infestations rates were detected between urban and rural areas.

From the results of this study, environmental conditions are likely to change ectoparasite behavior, growth, development, survival rates and reproduction. Season and geographical area appeared to affect ectoparasite prevalence and density in domestic animals. These differences may be due to temperature and/or moisture variations each season which affect the growth and reproduction of ectoparasites (Nithikathkul *et al*, 2002). The development of transportation and relocation of animals possibly affects the density, prevalence and epidemiology of ectoparasite infestations in domestic dogs in Pattaya District. However, there were differences in the spectrum of ectoparasite species related to geographical areas (rural versus urban; mountains versus plains and/or waterfront), seasonal occurrence, and the host susceptibility to these ectoparasites (Harman *et al*, 1987, Scheidt, 1988; Cruz-Vazquez *et al*, 2001).

In conclusion, data of the current study show *R. sanguineus*, had the highest percentage, density and prevalence of tick species. *Ct. canis* was the most prominent of flea species on domestic dogs in Pattaya, in agreement with Thailand literature on ectoparasites (Nithikathkul *et al*, 2002, 2005). The worldwide distribution of ticks and fleas, their role as vectors for a variety of pathogens, their involvement in flea allergy dermatitis, and their general public health impact demonstrate the need for effective ectoparasite control. Further investigations are needed for a better understanding of ectoparasite infestations (species, percentages, densities and prevalences of ectoparasites), the geographical patterns of occurrence and distribution, and seasonal climatic factors that influence development in other animals such as cats, rodents, stray dogs, pigs, and cattle.

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THE POTENTIAL FOR *AEDES ALBOPICTUS* (SKUSE) (DIPTERA: CULICIDAE) TO BE A COMPETENT VECTOR FOR CANINE HEARTWORM, *DIROFILARIA IMMITIS* (LEIDY)

Sonthaya Tiawsirisup and Morakot Kaewthamasorn

Division of Parasitology, Department of Veterinary Pathology,
Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

Abstract. The susceptibility of *Aedes albopictus* (Skuse) to canine heartworm, *Dirofilaria immitis* (Leidy), was determined and compared between Thai (TH) and US (US) strains of this mosquito species to assess the likelihood of its potential to be a competent vector for *D. immitis*. There were 6 individual experiments with 1,053 mosquitoes in this study. *Ae. albopictus* were allowed to feed on *D. immitis* infected dogs with different levels of microfilaremia, which were 663 ± 79 (mean ± SE), 1,410 ± 93, 2,463 ± 208, 3,490 ± 211, 5,000 ± 257 and 7,480 ± 551 microfilariae (mf)/ml of blood. Infection rates with the TH strain infective stage larvae (L3) of *D. immitis* were 5, 40, 13 and 14% after taking a blood meal with 663, 2,463, 3,490 and 5,000 mf/ml, respectively, as determined on day 14 post-blood feeding (PBF). Infection rates with the US strain with L3 of *D. immitis* were 8, 20, 25, 18 and 35% after taking a blood meal with 663, 2,463, 3,490, 5,000 and 7,480 mf/ml, respectively, as determined on day 14 PBF. The vector efficiency index (VEI) is defined as the average number of L3 multiplied by 100 and divided by the average number of ingested mf. VEI of TH strain were 3.2, 8.5, 20 and 2.4 after taking a blood meal with 663, 2,463, 3,490 and 5,000 mf/ml, respectively, as determined on day 14 PBF. VEI of US strain were 4.2, 13.5, 58.3 and 5.7 after taking a blood meal with 663, 2,463, 3,490 and 5,000 mf/ml, respectively, as determined on day 14 PBF. This study indicates that both TH and US strains of *Ae. albopictus* were competent vectors for *D. immitis*, however, field studies need to be carried out to determine the possible role of *Ae. albopictus* in the transmission cycle of *D. immitis* in field conditions.

INTRODUCTION

Filariasis, a disease caused by the filarial nematode, is important in tropical countries, including Thailand. *Brugia malayi* and *Wulchereria bancrofti* are filarial nematodes that cause filariasis or elephantiasis in humans. Important filarial nematodes in dogs are *Dirofilaria immitis* and *Brugia pahangi*, the adults of which are found in the heart and lymph nodes of an infected dog, respectively. *D. immitis* causes hematological and serum chemistry changes (Niwetpathomwat *et al*, 2006) and serious illness in dogs, since adults of this nematode reside in the right ventricle and sometimes in the pulmonary artery. *D. immitis* also infects other mammals, including humans. This nematode, however, cannot complete its life cycle in humans but may cause pulmonary nodules and granulomas and subcutaneous nodules in infected humans (Levinson *et al*, 1979; Tsung and Liu, 2003; Oshiro *et al*, 2004).

The mosquito is a biological vector for this nematode, which facilitates the development of microfilaria to an infective larval stage. Infective larvae are transmitted during the feeding process of the infected mosquito. Different mosquito species have different abilities to be vectors of *D. immitis* because of their anatomy. A study by Tiawsirisup *et al* (2005) found that Thai strains of *Aedes aegypti* and *Culex quinquefasciatus* may serve as biological vectors for *D. immitis* in laboratory conditions. However, there have been no studies of the vector competency of *Ae. albopictus* in Thailand.

Ae. albopictus (Skuse), the Asian tiger mosquito, is a flood water mosquito, considered a competent vector for many pathogens in both field and laboratory conditions. Previous studies have shown its potency as a biological vector of

Correspondence: Sonthaya Tiawsirisup, Division of Parasitology, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Henri-Dunant Road, Pathumwan, Bangkok 10330, Thailand.
Tel: +66(0)2218-9664 Fax: +66(0)2252-1704
E-mail: sonthaya.t@chula.ac.th

various arboviruses, such as chikungunya virus, dengue virus and West Nile virus (Tiawsirisup *et al*, 2004, 2005). This mosquito is widely distributed in America, Africa, Europe, Australia, and Asia, including Thailand (Chareonviriyaphap *et al*, 2003). It was first introduced into Europe in 1979 and the United States in 1985 (Sprenger and Wuithiranyagool, 1986). Used tire shipments between countries were considered the main pathway that brought *Ae. albopictus* from Asian countries to others (Reiter and Sprenger, 1987).

Its original habitat is tropical forest. Larvae and pupae are found in natural containers, including bamboo stumps, coconut shells, tree holes and rock holes, however, they are also found in artificial containers. It primarily feeds on humans however it also feeds on animals, including cattle, swine, dogs, cats, rats, and chickens (Ponlawat and Harrington, 2005). Mixed blood meals may be detected from this mosquito; since it is a multiple-host feeder, it may serve as a bridge vector that carries some pathogens from infected animals to humans.

This study investigated whether *Ae. albopictus* in Thailand may be a competent vector for *D. immitis*, and whether there is any difference in vector competency between the Thai (TH) and US (US) strains of *Ae. albopictus*.

MATERIALS AND METHODS

Mosquito specimens

TH and US strains of *Aedes albopictus* raised for more than 10 generations, were used for this study. The TH strain of *Ae. albopictus* was kindly provided by Dr Padet Siriyasatien, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Thailand and the US strain of *Ae. albopictus* was kindly provided by Dr Wayne A Rowley, Department of Entomolgy, Iowa State University, USA. All mosquitoes were maintained in controlled environmental conditions (28 ± 2ºC and 80 ± 10% RH) at the Division of Parasitology, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

Ae. albopictus was selected for this study because this mosquito is widely distributed in Thailand. The TH and US strains were selected for this study to examine whether there were any variations in vector competency between the mosquito strains.

Experimental animals

Two mixed-breed-local dogs naturally infected with *D. immitis* were used in this study as sources of infected blood meal for the mosquitoes. They were kept in the laboratory animal facilities, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

Microfilaria counting

Infected dog blood. One milliliter of blood was collected from the cephalic vein during mosquito feeding. A three-line-smear was made from 20 µl of blood on a glass slide, allowed to air dry, hemolized in distilled water, fixed in absolute methanol, and stained with 10% Giemsa. The stained slide was then examined for microfilariae under a light microscope.

Blood fed mosquitoes. Mosquitoes were randomly selected after a blood meal. Mosquitoes were individually dissected and the blood meal was removed from the midgut, mixed with distilled water and smeared on a glass slide. The slide was fixed in absolute methanol, stained with 10% Giemsa. The stained slide was then examined and microfilariae counted under a light microscope.

Vector competence of *Ae. albopictus* for *Dirofilaria immitis*

Three- to 5-day-old mosquitoes were used in this study. The mosquitoes were deprived of sucrose for 24 - 48 hours before feeding on the infected dog. The dog was sedated with 2 mg/kg body weight of Xylazine HCl and anesthetized with 10 mg/kg body weight of pentobarbital sodium. The dog was then placed on a mosquito cage where the mosquitoes were allowed to feed for 30 minutes. A group of 50 blood-fed mosquitoes were transferred into plastic cups and maintained in the mosquito laboratory. Mosquitoes were randomly selected from mosquito cups each tested day. The wings and legs were removed and the mosquitoes were dissected. Each mosquito organ was examined for *D. immitis* larvae under a light microscope.

Data analysis

The vector competentcy of *Ae. albopictus* for *D. immitis* in this study was evaluated. The infection rate, defined as the number of blood-fed mosquitoes that had infective stage or third stage larvae (L3) in their body multiplied by 100 and divided by the number of tested mosquitoes. Vector efficiency index (VEI) is defined as the average number of L3 developed in the mosquito multiplied by 100 and divided by the average number of ingested microfilariae.

Infection rates per strain were compared for each microfilaria level and between each tested day. Infection rates were also compared between strains on each tested day at the same microfilaria level. Pairwise Fisher's exact test was used for comparison. Observed differences were considered significant at $p < 0.05$.

RESULTS

There were 6 experiments with different microfilaria (mf) levels in this study. TH and US strains of *Aedes albopictus* were allowed to feed on *Dirofilaria immitis* infected dogs at 663 ± 79, 1,410 ± 93, 2,463 ± 208, 3,490 ± 211, 5,000 ± 257 and 7,480 ± 551 mf/ml of blood. Blood-fed mosquitoes were dissected and examined for third stage larvae (L3) on days 7-18 post-blood feeding (PBF) and defined as being infected mosquitoes if they had L3. The infection rates and vector efficiency index (VEI) are shown in Tables 1 and 2, respectively. Infection rates for the US strain were determined for days 7 and 14 PBF and they were 0 and 35%, respectively ($p < 0.001$). The other evaluations were performed on day 14 PBF and thereafter.

After taking a blood meal with 663 ± 79 mf/ml, infection rates in the TH strain were 5, 8, and 10% and infection rates in the US strain were 8, 10, and 13% on days 14, 15, and 16 PBF, respectively. The ranges of VEI for these mosquitoes were 3.2-6.5 and 4.2-12.5 for TH and US strains, respectively. There were no significant differences in infection rates between the two strains tested.

After taking the blood meal with 1,410 ± 93 mf/ml, infection rates with the TH strain were 3, 13, and 7% and infection rates with the US strain were 10, 3, and 23% on days 16, 17, and 18 PBF, respectively. The ranges of VEI for these mosquitoes were 6.7-13.3 and 2.5-41.7 for the TH and US strains, respectively. There were no significant differences in infection rates between the two strains tested.

After taking a blood meal with 2,463 ± 208 mf/ml, infection rates with the TH strain were 40 and 33% and infection rates with the US strain were 20 and 12% on days 14 and 15 PBF, respectively. Ranges for VEI of these mosquitoes were 8.5-13.4 and 10.8-13.5 for the TH and US strains, respectively. There was a significant difference between the infection rates for the TH and US strains on day 15 PBF at this mf level ($p=0.0391$).

After taking a blood meal with 3,490 ± 211 mf/ml, infection rates for the TH strain were 13 and 41% and infection rates of the US strain were 25 and 28% on days 14 and 15 PBF, respectively. The ranges of VEI for these mosquitoes were 20-46.7 and 45.8-58.3 for the TH and US strains, respectively. There were no significant differences in the infection rates between the strains tested at this mf level.

After taking a blood meal with 5,000 ± 257 mf/ml, infection rates for the TH strain were 14, 14, and 0% and infection rates for the US strain were 18, 16, and 25% on days 14, 15, and 16 PBF, respectively. The ranges of VEI for these mosquitoes were 0-3.2 and 2.9-6.7 for the TH and US strains, respectively. There was a significant difference between the infection rates for the TH and US strains on day 16 PBF at this mf level ($p=0.001$).

Infection rate comparisons within strain for tested days at each mf level showed significant differences in infection rates between days 14 and 15 PBF ($p=0.0227$) and between days 15 and 16 PBF ($p=0.0219$) for the TH strain after taking a blood meal with 3,490 ± 211 mf/ml and significant differences in infection rates between days 15 and 16 PBF ($p=0.0159$) and days 14 and 16 PBF ($p=0.0159$) with the TH strain after taking a blood meal with 5,000 ± 257 mf/ml.

Infection rate comparisons within the TH strain for mf levels on day 14 PBF showed significant differences in the infection rates in mosquitoes that took a blood meal with 663 and

Table 1
Infection rates in Thai (TH) and US (US) strains of *Aedes albopictus* with *Dirofilaria immitis* infective stage larvae determined on days 7-18 post-blood feed (PBF).

Blood meal microfilariae per ml (Mean ± SE)	Mosquito strain	Day (PBF)	No. mosquitoes tested	Infection rate (95% confidence interval)
663 ± 79	TH	14	40	5 (1, 17)
	US	14	40	8 (3, 20)
	TH	15	40	8 (3, 20)
	US	15	40	10 (4, 23)
	TH	16	40	10 (4, 23)
	US	16	30	13 (5, 30)
1,410 ± 93	TH	16	30	3 (1, 17)
	US	16	30	10 (3, 26)
	TH	17	30	13 (5, 30)
	US	17	30	3 (1, 17)
	TH	18	30	7 (2, 21)
	US	18	30	23 (12, 41)
2,463 ± 208	TH	14	20	40 (22, 61)
	US	14	20	20 (8, 42)
	TH	15	52	33 (22, 46)
	US	15	34	12 (5, 27)
3,490 ± 211	TH	14	30	13 (5, 30)
	US	14	20	25 (11, 47)
	TH	15	32	41 (26, 58)
	US	15	43	28 (17, 43)
	TH	16	32	13 (5, 18)
5,000 ± 257	TH	14	50	14 (7, 26)
	US	14	50	18 (10, 31)
	TH	15	50	14 (7, 26)
	US	15	50	16 (8, 29)
	TH	16	40	0
	US	16	40	25 (14, 40)
7,480 ± 551	US	7	40	0
	US	14	40	35 (22, 50)

2,463 mf/ml, at 2,463 and 3,490 mf/ml and at 2,463 and 5,000 mf/ml.

Infection rate comparisons in the US strain among the mf levels of on day 14 PBF showed a significant difference in the infection rates between the mosquitoes that took a blood meal with 663 and 7,480 mf/ml.

DISCUSSION

Canine heartworm, *Dirofilaria immitis*, is a common filarial nematode in dogs in Thailand (Choochote *et al*, 1987; Niwetpathomwat *et al*, 2006). The life cycle of this nematode involves infected dogs and a mosquito vector. The risk for

Table 2
Vector efficiency index (VEI) for Thai (TH) and US (US) strains of *Aedes albopictus* for *Dirofilaria immitis* determined on days 14-18 post-blood feed (PBF).

Blood meal microfilariae (mf) per ml (Mean ± SE)	Mosquito strain	No. mf in mosquito midgut (Mean ± SE)	No. L3 in mosquito (Mean ± SE)	Day (PBF)	VEI[a]
663 ± 79	TH	3.1 ± 0.4	0.1 ± 0.1	14	3.2
	US	2.4 ± 0.4	0.1 ± 0.1	14	4.2
	TH	3.1 ± 0.4	0.2 ± 0.1	15	6.5
	US	2.4 ± 0.4	0.2 ± 0.1	15	8.3
	TH	3.1 ± 0.4	0.2 ± 0.1	16	6.5
	US	2.4 ± 0.4	0.3 ± 0.2	16	12.5
1,410 ± 93	TH	1.5 ± 0.2	0.1 ± 0.1	16	6.7
	US	1.2 ± 0.2	0.1 ± 0.1	16	8.3
	TH	1.5 ± 0.2	0.2 ± 0.1	17	13.3
	US	1.2 ± 0.2	0.03 ± 0.03	17	2.5
	TH	1.5 ± 0.2	0.1 ± 0.1	18	6.7
	US	1.2 ± 0.2	0.5 ± 0.2	18	41.7
2,463 ± 208	TH	8.2 ± 1.8	0.7 ± 0.2	14	8.5
	US	3.7 ± 0.7	0.5 ± 0.3	14	13.5
	TH	8.2 ± 1.8	1.1 ± 0.3	15	13.4
	US	3.7 ± 0.7	0.4 ± 0.2	15	10.8
3,490 ± 211	TH	3.0 ± 0.6	0.6 ± 0.4	14	20.0
	US	2.4 ± 0.4	1.4 ± 0.7	14	58.3
	TH	3.0 ± 0.6	1.4 ± 0.5	15	46.7
	US	2.4 ± 0.4	1.1 ± 0.5	15	45.8
	TH	3.0 ± 0.6	0.4 ± 0.2	16	13.3
5,000 ± 257	TH	12.5 ± 1.8	0.3 ± 0.1	14	2.4
	US	10.5 ± 2.2	0.6 ± 0.3	14	5.7
	TH	12.5 ± 1.8	0.4 ± 0.2	15	3.2
	US	10.5 ± 2.2	0.3 ± 0.2	15	2.9
	TH	12.5 ± 1.8	0	16	0
	US	10.5 ± 2.2	0.7 ± 0.2	16	6.7

[a] VEI defined as the average number of L3 developed in the mosquito multiplied by 100, divided by the average number of ingested microfilariae.

infection with *D. immitis* depends on the level of mircrofilaremia in the infected dog and mosquito. To determine a vector in nature, many criteria have to be considered, including the number of mosquitoes in that area, the number of infected dogs, blood feeding preference, frequency of mosquitoes, and the ability of the mosquito to facilitate the development of the infective larval stage (L3).

Aedes albopictus was selected for this study because it is found in rural and urban areas in Thailand. They are an important vector, similar to *Ae. aegypti*. Both infection rate and vector efficiency index (VEI) were used in this study to

indicate the vector competency of this mosquito in laboratory conditions. We found no L3 in blood-fed mosquitoes examined on day 7 PBF. This is similar to the findings of Nayar and Knight (1999) who found the development of microfilaria (mf) to L3 in the Malpighian tubules of the mosquito happened on days 14-17 PBF.

The potential for mosquitoes to be infected with *D. immitis* is higher when mf levels in the blood meal are higher (Tiawsirisup and Nithiuthai, 2006). The development of *D. immitis* mf to L3 in the mosquito takes place in the mosquito's Malpighian tubules. Large concentrations of microfilariae cause injury to the Malpighian tubules which can cause mosquito mortality, particularly during the first week PBF (Apperson *et al*, 1989; Nayar and Knight, 1999).

The VEIs in the TH strain of *Ae. albopictus* were 3.2, 8.5, 20 and 2.4 and in the US strains of *Ae. albopictus* were 4.2, 13.5, 58.3 and 5.7 after taking blood meals with 633, 2,463, 3,490 and 5,000 mf/ml, respectively, when tested on day 14 PBF. This shows there is a correlation between VEI and microfilaria level in the blood meal except when the microfilaria level in the blood meal is higher than 5,000 mf/ml, which may cause mosquito mortality.

There are many levels of susceptibility of *Ae. albopictus* to the development of *D. immitis* mf to L3. A previously study of *Ae. albopictus* in the United States found the development of mf to L3 could not occur (Apperson *et al*, 1989). The development of *D. immitis* larva in some strains of *Ae. albopictus* was arrested at the end of the first larval stage in the Malpighian tubules, and was an expression of refractoriness, *ie* the infection rate of *Ae. albopictus* with L3 will be less than what it was supposed to be. The susceptibility of *Ae. albopictus* to *D.immitis* infection also has a genetic basis (Nayar and Knight, 1999). This study showed the US strain of *Ae. albopictus* used in this study is a competent vector for *D. immitis*. The US strain in this study was collected from the state of Missouri, USA. The infection rate with L3 was 28% and the VEI was 45.8 after a blood meal with 3,490 ± 211 mf/ml, tested on day 15 PBF.

This study tested the susceptibility of some strains of *Ae. albopictus* from Thailand and the United States to *D. immitis* in laboratory conditions. Field studies need to be performed to assess the role of this mosquito in the transmission of *D. immitis* in nature. The susceptibility of this mosquito in nature may be different due to inbreeding in a restricted environment. Inbreeding can result in large numbers either susceptible or resistant (Nayar and Knight, 1999). *Ae. albopictus* has been found to be a natural vector for *D. immitis* in some countries, such as Japan and Italy (Konishi, 1989; Cancrini *et al*, 2003).

Infection of the mosquito with *D. immitis* can also cause an elevated dissemination rate of some viruses in the mosquito, such as chikungunya virus, because the mf of *D. immitis* cause a hole during penetration through the midgut epithelial layer. When the midgut of the mosquito is punctured immediately after the mosquito ingested a virus, a higher dissemination rate is observed for that mosquito (Zytoon *et al*, 1993a). Transovarial transmission of the virus in the mosquito has also been observed in laboratory conditions (Zytoon *et al,* 1993b). Co-infection with arbovirus and microfilaria of any filarial nematode are more likely to increase the infectivity of the mosquito with the virus.

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SEASONAL ABUNDANCE AND BITING ACTIVITY OF *ANOPHELES ACONITUS* (DIPTERA: CULICIDAE) IN CHIANG MAI, NORTHERN THAILAND

Anuluck Junkum[1], Benjawan Pitasawat[1], Benjawan Tuetun[1], Atiporn Saeung[1], Eumporn Rattanachanpichai[1], Narissara Jariyapan[1], Narumon Komalamisra[2], Motoyoshi Mogi[3], Udom Chaithong[1] and Wej Choochote[1]

[1]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand;
[2]Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand;
[3]Division of Parasitology, Department of Microbiology, Saga Medical School, Japan

Abstract. The seasonal abundance and nocturnal biting activity of *Anopheles aconitus*, a secondary vector of malaria in Thailand, were investigated from January 2005 to February 2006. Seasonal changes in abundance were mainly influenced by monthly rainfall, with a major peak occurring from August to November. This species preferred to bite animals rather than humans, and favored biting humans outdoors rather than indoors. The biting activity was highest at dusk and/or in the evening, but was low throughout the remaining night-hours. A unimodal pattern of biting, with a peak occurring at 06:00-08:00 PM was seen in *An. aconitus*. The overall parous rate was 51.19% (35.85-75.00%). During peak abundance (August to November), a parous rate ranging from 42.86 to 61.57 was observed. Of 1,198 dissected specimen stomachs, 0.17% (2/1,198) were found to be infected with oocysts. No sporozoites were detected in any of the 1,198 specimen salivary glands.

INTRODUCTION

Anopheles (*Cellia*) *aconitus* has been incriminated as a natural vector of *Plasmodium vivax* in central Thailand (Gould *et al*, 1967; Scanlon *et al*, 1968). It has also been reported as a vector for malaria in other countries, such as Indonesia (Kirnowardoyo, 1985; Kirnowardoyo and Supalin, 1986), Bangladesh (Maheswary *et al*, 1992) and Malaysia (Rahman *et al*, 1993). Recently, three karyotypic forms of *Anopheles aconitus*, Form A (X_1, X_2, Y_1), Form B (X_1, X_2, Y_2) and Form C (X_1, X_2, Y_3), have been reported sympatrically from Mae Taeng District, Chiang Mai Province, northern Thailand (Baimai *et al*, 1996). Subsequently, the latter two karyotypic forms were confirmed as efficient laboratory vectors for both *P. falciparum* and *P. vivax* (Junkum *et al*, 2005a). Based on the results of ovarian nurse cell polytene chromosome and isoenzyme investigations, and hybridization experiments, DNA sequences of ITS1, ITS2, D3, COI and COII regions of *An. aconitus*, Forms B and C are considered as conspecific cytological races in the Thai population (Jariyapan *et al*, 2005; Junkum *et al*, 2005b). Even though the above results indicate an extensive population genetic study of *An. aconitus*, very little concerning the biological and/or ecological aspects is known for strains found in northern Thailand. In view of its important role as a natural vector for *P. vivax*, and the lack of biological and/or ecological information, a more thorough study of *An. aconitus* is needed. Therefore, the seasonal abundance and nocturnal biting activity of *An. aconitus* in Mae Taeng District, Chiang Mai Province, northern Thailand are described below.

Correspondence: Wej Choochote, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.
Tel: 66 (053) 945342; Fax: 66 (053) 217114
E-mail: wchoocho@mail.med.cmu.ac.th

MATERIALS AND METHODS

Study site

The study site was Ban Pang Mai Daeng, Mae Taeng District, Chiang Mai Province, northern Thailand (endemic for malaria, where Baimai *et al* (1996) determined three karyotypic forms of *An. aconitus*) was chosen as the study area (Fig 1).

Fig 1- Map of Thailand showing Chiang Mai Province (CM), where mosquito collections were performed at Mae Taeng District. Chiang Mai is situated at a latitude of 18° 47′N and longitude of 98° 59′E in northern Thailand, approximately 581 km away from the capital city, Bangkok (BK), central Thailand.

Specimen collection

Collections were carried out twice a month (two consecutive nights) from 06:00 PM to 06:00 AM, from January 2005 to February 2006. For human bait, two persons at each site [one inside a house (indoors) and one outside a house (outdoors)] sat and exposed their legs as bait. One catcher at each site caught all anopheline mosquitoes attempting to bite using an aspirator. For animal bait, one calf was tethered approximately 10 m from the human bait, and all anopheline mosquitoes attempting to land and bite the calf were collected using a battery-powered aspirator. The collection time for each hour was divided into 50 minutes for catching and 10 minutes resting. Adults caught hourly were preserved in separate cups, supplied with 5% sucrose solution and transported to the insectary. The species were identified using the morphological keys of Harrison (1980). The stomach and salivary glands for all identified *An. aconitus* specimens were dissected for oocyst and sporozoite investigations, and 2 ovaries for parity identification (Detinova, 1962). Climatic data, *ie*, temperature and relative humidity, were measured every 10 minutes from 06:00 PM to 06:00 AM using a handy-typed digital thermometer and hygrometer, whereas rainfall data were obtained from the Northern Meteorological Center. The year was divided into three seasons following the Thai Meteorological Department, which bases its records on rainfall and temperature values: hot season (mid February to mid May), rainy season (mid May to mid October), and dry-cool season (mid October to mid February).

RESULTS

The numbers of captured *An. aconitus* females on human and water-buffalo bait are shown in Table 1. In total, 1,334 adult *An. aconitus* females were captured indoor from humans (8), outdoor on humans (165) and on water-buffalo bait (1,161), (Table 1).

The mosquitoes were more abundant during the rainy season (August) and the dry-cool season (November), with peaks obtained during the transitional month of October in the late rainy season and beginning of the dry-cool season from both human bait positioned outdoors (24.50/night) and water-buffalo bait (150.50/night) (Fig 2). Statistical analysis revealed a positive association between the monthly number of mosquitoes and rainfall ($r = 0.96$, $p < 0.05$); but no significant relationship between monthly number and temperature ($r = -0.02$, $p > 0.05$), or monthly number and relative humidity ($r = 0.55$, $p > 0.05$) on human bait collection was found. Similar results were also seen with water-buffalo bait collections, *ie*, the monthly number was positively associated with rainfall ($r = 0.58$, $p < 0.05$), but it did not correlate with temperature ($r = -0.07$, $p > 0.05$) or humidity ($r = 0.55$, $p > 0.05$).

The nocturnal biting of *An. aconitus* on human bait outdoors and water-buffalo bait from 06:00 AM - 06:00 PM during a major peak

Table 1
Number of *An. aconitus* females collected on human and water-buffalo bait from January 2005 to February 2006; collections were performed from 06:00 AM - 06:00 PM for two consecutive nights each month.

Months	Human bait		Water-buffalo bait	Total no.	Parous rates (no.)	Mean temperature in °C (range)	Mean % humidity (range)	Mean rainfall in mm
	Indoors	Outdoors	Outdoors					
Jan 05	0	3	17	20	40 (8/20)	22.56 (17.10-29.43)	68.27 (51.67-78.00)	0.0
Feb 05	0	1	11	12	41.67 (5/12)	25.58 (20.47-29.07)	49.15 (28.33-64.33)	4.9
Mar 05	0	1	5	6	50 (3/6)	27.76 (23.97-31.40)	45.56 (38.00-51.00)	24.7
Apr 05	0	0	4	4	75 (3/4)	28.16 (25.73-31.90)	61.01 (44.33-70.00)	57.2
May 05	0	2	9	11	60 (6/10)	28.45 (25.93-31.37)	62.71 (45.33-76.00)	104.7
Jun 05	0	2	12	14	42.86 (6/14)	27.52 (26.10-29.87)	76.47 (59.33-86.67)	193.5
Jul 05	1	3	25	29	37.04 (10/27)	25.14 (23.07-27.53)	90.31 (79.33-96.67)	179.1
Aug 05	1	22	136	159	56.69 (72/127)	26.13 (24.80-27.80)	85.01 (74.00-90.00)	155.2
Sep 05	3	41	294	338	42.86 (129/301)	26.93 (24.10-29.80)	79.08 (70.00-88.67)	436.3
Oct 05	1	49	301	351	56.10 (161/287)	25.78 (24.47-27.13)	77.78 (70.00-83.00)	192.0
Nov 05	0	32	241	273	61.57 (141/229)	23.92 (19.63-26.50)	80.72 (72.00-87.67)	22.8
Dec 05	2	7	53	62	35.85 (19/53)	20.79 (19.47-23.07)	66.06 (49.67-80.00)	27.9
Jan 06	0	2	29	31	42.86 (12/28)	22.13 (16.97-25.17)	61.28 (43.00-73.67)	0.0
Feb 06	0	0	24	24	38.10 (8/21)	24.65 (23.30-26.37)	59.13 (45.00-70.33)	0.0
Total	8	165	1,161	1,334	51.19 (583/1,139)	-	-	-

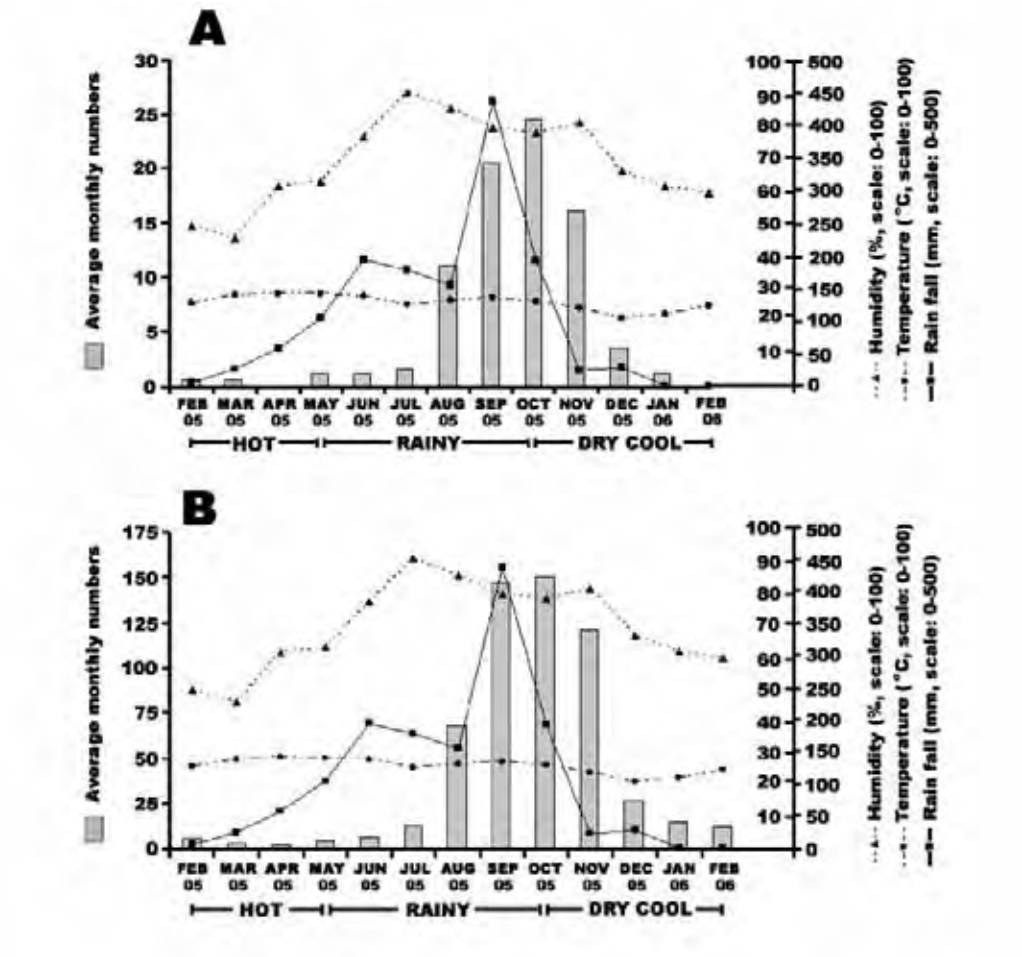

Fig 2- Seasonal changes in abundance of *An. aconitus*; A: biting on human bait positioned outdoors; B: biting on water-buffalo bait.

of abundance (total number of females caught over two consecutive nights was greater than 20 in humans and greater than 50 in water-buffalo bait) in August, September, October, November and December (Table 2, Figs 3 and 4).

A unimodal pattern, with a peak at dusk and/or in the evening (06:00 - 08:00 PM) was seen on both humans and water-buffalo bait, although the temperature gradually decreased and humidity gradually increased during the night, but this did not appear to be a main cause of bites in the evening, since both temperature and humidity were high enough for mosquito activity. Nevertheless, variations in hourly peak from month to month were plainly observed (Fig 4 A, B, C).

Dissections stomach and salivary glands for oocysts and sporozoites in 146 females caught from human bait yielded zero positive samples, whereas 2 stomachs (one caught in October, and the other in November) from a total of 1,052 females dissecter [0.19% (2/1,052) oocyst rate] captured from water-buffalo bait were positive for 2 and 5 immature oocysts, respectively.

Parity was determined for 1,139 females. Overall, 51.19% (35.85-75.00%) of the mosquitoes were parous. Based on the higher number of caught females during August to November compared to the other months, the high parous rates were 56.69, 42.86, 56.10 and 61.57% in August (rainy season), September (rainy season), October (transitional month of the

Table 2
Average nightly number of *An. aconitus* bites on humans positioned outdoors and water-buffalo bait collected from 06:00-08:00 PM in each representative month for evaluation of abundance.

Collection time	Rainy season						Dry-cool season			
	Aug 05		Sep 05		Oct 05		Nov 05		Dec 05	
	H	B	H	B	H	B	H	B	H	B
06:00-07:00 PM	3.5	23	8.5	40.5	12	63	14	96	2.5	12
07:00-08:00 PM	4.5	34.5	10.5	59.5	7.5	29.5	1.5	8.5	1	5.5
08:00-09:00 PM	2.5	6	1	17.5	4	7.5	0.5	3.5	0	4.5
09:00-10:00 PM	0.5	2	0	1.5	1	3.5	0	2.5	0	1.5
10:00-11:00 PM	0	1	0.5	2.5	0	4	0	0	0	2
11:00-12:00 PM	0	0	0	0.5	0	4.5	0	0	0	0.5
00:00-01:00 AM	0	0	0	1.5	0	0	0	0	0	0
01:00-02:00 AM	0	0	0	3.5	0	5.5	0	0	0	0
02:00-03:00 AM	0	0.5	0	5	0	3.5	0	1	0	0
03:00-04:00 AM	0	0	0	5.5	0	11	0	1	0	0
04:00-05:00 AM	0	1	0	4.5	0	8	0	2.5	0	0.5
05:00-0:600 AM	0	0	0	5	0	10.5	0	5.5	0	0
Biting activity pattern	U (07:00-08:00 PM)	U (07:00-08:00 PM)	U (07:00-08:00 PM)	U (07:00-08:00 PM)	U (06:00-07:00 PM)	U (06:00-07:00 PM)	U (06:00-07:00 PM)	U (06:00-07:00 PM)	U (06:00-07:00 PM)	U (06:00-07:00 PM)

H: human bait; B: water-buffalo bait, U: unimodal; ( ): major peak time

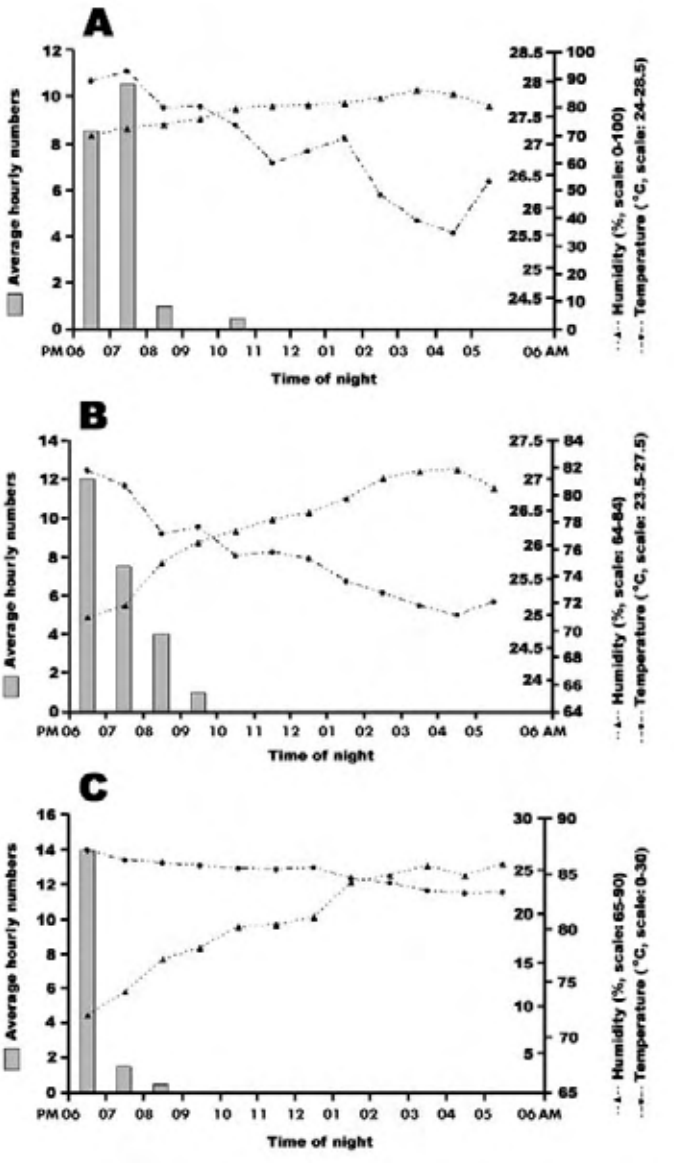

Fig 3- Variations in hourly biting activities of *An. aconitus* on human bait positioned outdoors from 06:00 PM – 06:00 AM. (A) September 2005, (B) October 2005, and (C) November 2005.

rainy season and dry-cool season), and November (dry-cool season), respectively (Table 1). The parous rate of 42.86% in September differed significantly from the other months ($\chi^2 = 20.97$, $p < 0.05$).

DISCUSSION

Previous studies of the seasonal abundance and nocturnal biting activity of adult *An. aconitus* from various types of geographical locations indicated the variations from one location to another, and these factors were presumably influenced by climatic conditions and types of bait (Chow *et al*, 1960; Reid, 1968; Harrison, 1980; Rahman *et al*, 1993). In the present study conducted in the rice plain of Mae Taeng District, Chiang Mai Province, northern Thailand, where

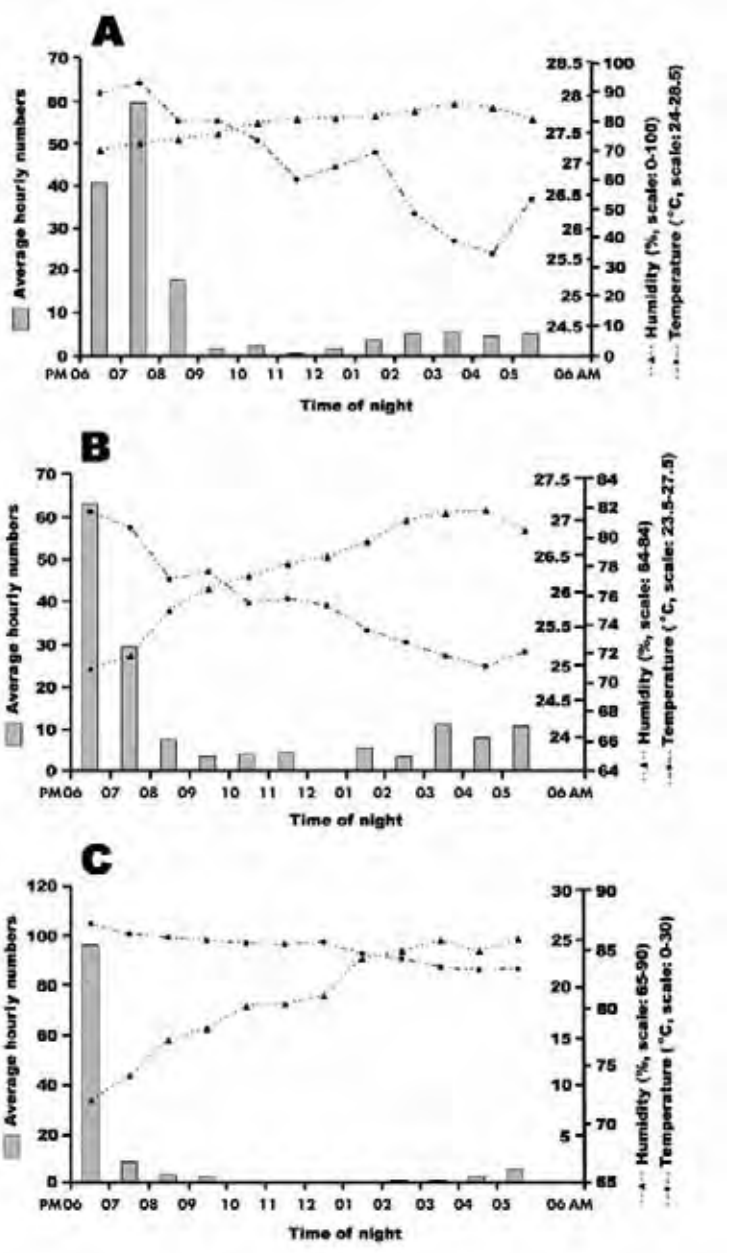

Fig 4- Variations in hourly biting activities of *An. aconitus* on water-buffalo bait from 08:00 PM-06:00 AM. (A) September 2005, (B) October 2005, and (C) November 2005.

a high volume of rainfall occurs between June to October (peaking in September), the population densities of *An. aconitus* obtained from both human and water-buffalo bait showed a greater abundance during the rainy season (August) to dry-cool season (November) (peaking in October). Additionally, seasonal changes in monthly number had a positive association with rainfall, but no correlation with monthly temperature or relative humidity. These results are in contrast to those found by Rahman *et al* (1993), who carried out during a seasonality study of *An. aconitus* in an endemic village, Kampong Bongor, located near the Malaysia-

Thailand border (longitude 101° 11′ N, latitude 5° 30′ N). Although the monthly rainfall was similar to the present study, the population densities demonstrated were more abundant from March to May (peaking in May), when the rainfall was extremely low, indicating no correlation between monthly number and rainfall, and presumably, other factor(s) were involved.

The results of nocturnal biting activity exhibited that *An. aconitus* bit both humans and animals, with the animal biting densities significantly higher throughout the seasons of occurrence. Additionally, the *An. aconitus* bit humans outdoors approximately 20.63 times more often than indoors, and bit animals about 6.71 times more often than humans. Our study is generally in agreement with one in Pathum Thani Province, in the central plain of Thailand (Harrison, 1980). At this location, *An. aconitus* bit humans outdoors approximately 8.34 times more often than indoors, and bit animals about 8.10 times more often than humans. The results of biting patterns on both humans and animals revealed that *An. aconitus* had a unique unimodal pattern that peaked at dusk and/or in the evening (06:00-08:00 PM) throughout the rainy to dry-cool seasons. This situation increases the frequency of man-vector contacts, since these time periods are coincidental with various activities near human households.

Parous rates on a monthly basis were used to evaluate mortality and daily survival rates in mosquitoes (Samarawickrema, 1967; Upatham *et al*, 1988). In our study, the average parous rate was 51.2% (583/1,139), in comparison to 21.0% (12/57) in Pathum Thani Province, where *An. aconitus* was incriminated (positive for both oocysts and sporozoites on dissection) as a vector for *P. vivax* (Gould *et al*, 1967), and 65.7% (44/67) in Buri Ram Province (Harrison, 1980). The result of a very low oocyst rate (0.2% (2/1,052)), and no sporozoites from dissected salivary glands of *An. aconitus* during the 14-month study period, may indicate the effect of the antimalaria program in the area (Division of Malaria, 2005). Efficient laboratory vectors for *P. falciparum* and *P. vivax* (Junkum *et al*, 2005a) have a longer survival rate, as determined by a high parous rate, and the outdoor biting behavior at dusk, may increase the chances of transmitting malaria to man, particularly during outbreaks and/or re-emerging periods.

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RETROSPECTIVE STUDY OF DENGUE FEVER (DF) AND DENGUE HEMORRHAGIC FEVER (DHF) PATIENTS AT UNIVERSITY MALAYA MEDICAL CENTER, KUALA LUMPUR, MALAYSIA IN THE YEAR 2005

I Jamaiah, M Rohela, V Nissapatorn, FT Hiew, A Mohammad Halizam, H Noor Liani and AR Siti Khairunnisaak

Department of Parasitology, Faculty of Medicine,
University of Malaya, Kuala Lumpur, Malaysia

Abstract. The aim of this retrospective study was to determine the number of dengue fever (DF) and dengue hemorrhagic fever (DHF) cases admitted to University Malaya Medical Center (UMMC) in the year 2005 together with their clinical presentations and epidemiology. The data for 2005 was collected from the medical records department of UMMC. A total of 1,279 cases were admitted in 2005 with DF (81%, n=1,040) and DHF (19%, n=239). January had the greatest number of cases of DF and DHF (22%, n=281) and April had the least (4%, n=49). The greatest number of DF cases (38%, n=392) were seen in the 20-29 year age group, while the greatest number of DHF cases (32%, n=76) were in the 10-19 year age group. In regard to race, the greatest number of cases were seen in Malays (48%, DF; 49%, DHF). Males were more commonly infected than females. The majority of patients infected were students (30%, n=385). All patients with DF and DHF presented with fever. The symptoms reported included nausea and vomiting, joint pain, gum bleeding and dehydration. The mean value hemoglobin, white blood cell count and platelet count were 14.4 g/dl, 4 $\times 10^3$/dl, 75 $\times 10^6$/dl, respectively for DF and 15.1 g/dl, 4 $\times 10^3$/dl, 52 $\times 10^6$/dl respectively for DHF. The majority of patients were treated with hydration therapy. There were three deaths reported, caused by dengue shock syndrome (DSS).

INTRODUCTION

Dengue is the most common mosquito-borne viral disease of humans that in recent years has become a major international public health concern. The World Health Organization (WHO) estimates that more than 2.5 billion people are at risk for dengue infection, and 50 million cases occur annually with 22,000 deaths (WHO, 2002). The dengue virus is an enveloped single stranded RNA virus of the family Flaviridae. There are four serotypes which share genetic and antigenic features, but infection with one serotype does not provide long-term protection against other serotypes. The principal vector is the day biting *Aedes aegypti*, which typically breeds in clean stagnant water in a wide variety of sites, including man-made containers in domestic and peri-domestic urban areas (Kumarasamy, 2006).

Correspondence: I Jamaiah, Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
E-mail: jamaiah@ummc.edu.my

DF was first reported in Malaysia in 1902 and DHF in 1962. Since then, epidemics of dengue cases have been reported regularly. The first major Malaysian epidemic of DHF with severe manifestations occurred in 1973, with 969 reported cases and 54 deaths (Wallace *et al*, 1980). DHF, though endemic in the sixties, emerged as a major public health problem in Malaysia from 1973 onwards (Shekhar and Huat, 1992, 1993). Until the end of September 2005, 29,196 cases of dengue had been reported with 76 deaths. The increasing trend in the incidence of dengue infection is a cause for concern (Kumarasamy, 2006).

DF is characterized by fever which lasts from 5 to 7 days with two or more of the following symptoms: headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, or leukopenia.

DHF is defined as an acute febrile illness with hemorrhagic manifestations (shown by a positive tourniquet test, petechiae, ecchymoses or purpura, or bleeding from the mucosa, gastrointestinal tract, injection sites, or other locations), a platelet

count <100,000/mm^3 and objective evidence of plasma leakage due to increased vascular permeability, shown by either a fluctuation in hematocrit ≥20% during the course of illness and recovery or clinical signs of plasma leakage, such as pleural effusion, ascites, or hypoproteinemia (WHO, 1997).

The main objective of this retrospective study was to determine the number of DF and DHF cases admitted to the UMMC for the year 2005 and note their clinical presentations and epidemiology.

MATERIALS AND METHODS

The case records of all patients admitted with DF and DHF to the UMMC in the year 2005 were reviewed in detail. The data collected included date of admission, age, sex, race, nationality, address, occupation, clinical presentations, laboratory investigation and treatment. Analysis was done using SPSS 11 and Microsoft Excel 2003.

RESULTS

A total of 1,279 cases were reported at UMMC for the year 2005. Eighty-one percent (n=1,040) of reported cases was DF and 19% (n=239) were DHF, giving a DF to DHF ratio of 4:1.

The greatest number of reported cases of DF were in the age group of 20-29 years (38%, 329 cases) and of DHF were in the age group of 10-19 (32%, 76 cases) (Fig 1).

Fig 2 shows the total number of DF and DHF cases by month. The greatest number of cases were reported in January (22%, n=281); 22%

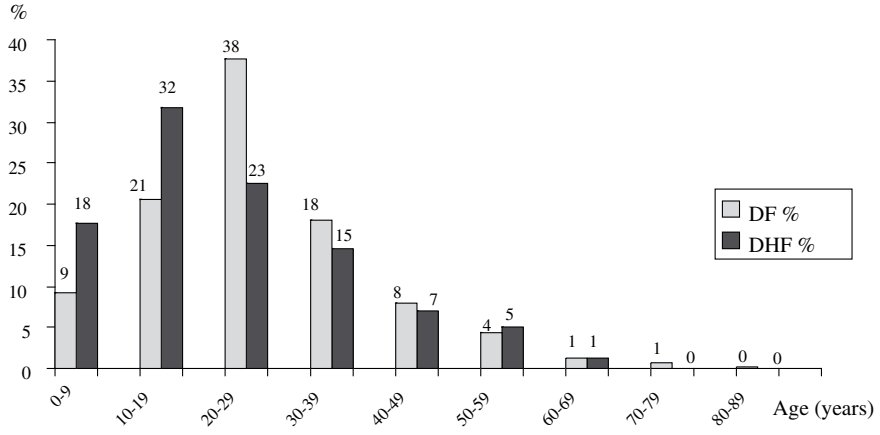

Fig 1- Age distribution of DF and DHF.

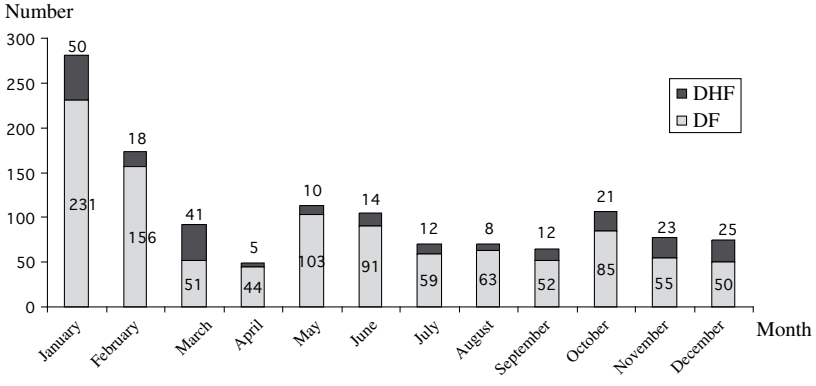

Fig 2- Number of DF and DHF cases by month.

Table 1
Socio-demographic characteristics cases with of DF and DHF.

Variables		DF		DHF		Total		p-value
		n	%	n	%	n	%	
Race	Malay	497	48	118	49	615	48	0.813
	Chinese	231	22	49	21	280	22	
	Indian	184	18	46	19	230	18	
	Others	128	12	26	11	154	12	
Sex	Male	614	59	148	62	762	60	0.412
	Female	426	41	91	38	517	40	
Nationality	Malaysian	919	88	213	89	1,132	89	0.741
	Non-Malaysian	121	12	26	11	147	11	
Dengue serology result	Positive	779	75	204	85	983	77	0.002[a]
	Negative	121	12	13	6	134	10	
	Unknown	140	13	22	9	162	13	

[a] Chi-square test is significant at α=0.05 level

Table 2
Occupations among DF and DHF patients.

Occupation	No.	%
Student	385	30.1
Factory worker	54	4.2
Housewife	46	3.6
Teacher	22	1.7
Soldier	20	1.6
Restaurant worker	18	1.4
Businessman/woman	18	1.4
Maid	17	1.3
Contractor	17	1.3
Technician	14	1.1
Clerk	14	1.1
Pensioner	13	1.0
General worker	13	1.0
Salesman	12	0.9

(n=231) of the total were DF cases and 21% (n=50) were DHF cases. The fewest number of dengue cases was reported in April (4%, n=49) with 4% (n=44) of the total were DF cases and 2% (n=5) were DHF cases.

Table 1 shows the majority of DF and DHF cases were reported among ethnic Malays, males and Malaysian citizens. The dengue serology test was positive in 77% (n=983). In 13%, the results of the dengue serology test were unavailable (unknown).

All patients with DF and DHF reported fever. Nausea and vomiting, joint pain, gum bleeding and dehydration were significantly associated with DF and DHF (p-value 0.004, 0.043, 0.003, and 0.001, respectively) (Table 3).

Hydration therapy was the commonest treatment. Paracetamol was prescribed to all patients. Platelet transfusions were given to DHF patients when needed. Three patients died due to complications of DHF.

Table 4 shows that the mean (standard deviation) hemoglobin, white blood cell count and platelet count were 14.4 ± 1.8 g/dl, 4.19 ± 2.87 × 10^3/dl, 75.53 ± 50.83 × 10^6/dl, respectivelyfor DF and 15.1 ± 2.0 g/dl, 4.42 ± 2.70 × 10^3/dl, 51.86 ± 41.01×10^6/dl for DHF, respectively. However, data were missing for platelet count on 2 cases and for hemoglobin and white blood cell count on 3 cases.

DISCUSSION

The ratio of DF:DHF cases for the year 2005 at UMMC was 4:1. This is lower than that reported by the Ministry of Health (MOH), Malaysia. The ratio of DF:DHF for years 1996

Table 3
Symptoms of DF and DHF.

Symptoms		DHF		DF		Total		p-value
		n	% within case	n	% within case	n	% within case	
Fever	yes	239	100.0	1,040	100.0	1,279	100.0	.[a]
	no	0	0.0	0	0.0	0	0.0	
Myalgia	yes	117	49.0	442	42.5	559	43.7	0.710
	no	122	51.0	598	57.5	720	56.3	
Headache	yes	113	47.3	434	41.7	547	42.8	0.128
	no	126	52.7	606	58.3	732	57.2	
Nausea and vomiting	yes	183	76.6	696	66.9	879	68.7	0.004[b]
	no	56	23.4	344	33.1	400	31.3	
Petechia	yes	62	25.9	200	19.2	262	20.5	0.260
	no	177	74.1	840	80.8	1,017	79.5	
Rash	yes	80	33.5	353	33.9	433	33.9	0.940
	no	159	66.5	687	66.4	846	66.1	
Cough	yes	48	20.1	199	19.1	247	19.3	0.717
	no	191	79.9	841	80.9	1,032	80.7	
Abdominal pain	yes	101	42.3	272	26.2	373	29.2	0.000[b]
	no	138	57.7	768	73.8	906	70.8	
Joint pain	yes	62	25.9	207	19.9	269	21.0	0.043[b]
	no	177	74.1	833	80.1	1,010	79.0	
Gum bleeding	yes	61	25.5	176	16.9	237	18.5	0.003[b]
	no	178	74.5	864	83.1	1,042	81.5	
Dehydration	yes	94	39.3	297	28.6	391	30.6	0.001[b]
	no	145	60.7	743	71.4	888	69.4	

[a] No statistics were computed because fever was a constant.

[b] Chi-square test is significant at α=0.05 level

Table 4
Mean hemoglobin, white blood cell count and platelet count.

Cases		Hemoglobin (g/dl)	WBC ($x10^3$/dl)	Platelet count ($x10^6$/dl)
DF	Mean	14.4	4.19	75.53
	n	1,037	1,037	1,038
	Std deviation	1.8	2.87	50.83
DHF	Mean	15.1	4.42	51.86
	n	239	239	239
	Std deviation	2.0	2.70	41.01
Total	Mean	14.5	4.23	71.10
	n	1,276	1,276	1,277
	Std deviation	1.8	2.84	49.99

and 1997 were 26:1 and 23:1, respectively (MOH, 1996 and 1998). This may be because the area covered in this study was small, mostly Selangor and Kuala Lumpur, compared to MOH which covered all the states of Malaysia.

Our study showed the 20-29 years age group had the greatest number of cases of DF (38%, n=329) and the 10-19 years age group had the greatest number of cases of DHF (32%, n=76). The MOH for Malaysia also reported similar findings. They found the 15-29 year old age group had the highest incidence rate for DF (MOH, 2000). Fang *et al* (1984) reported that most cases were over age 15 years. These studies are different from the epidemics of the 1970s and 1980s, where the majority of cases were children below age 15 years old (Wallace *et al*, 1980; Hussin *et al*, 2005). DHF is primarily a disease of children under 15 years in hyperendemic areas (Halstead *et al*, 2001).

January had the greatest number of DF and DHF cases with 22% (n=281) while the least was in April at 4% (n=49) (Fig 2). Hussin *et al* (2005) also reported similar findings with the greatest incidence in January and the least in May. Shekhar and Huat (1992-1993) reported a peak incidence in August for DHF cases. Wallace *et al* (1980) reported cases occurred mainly from May to September. Fang *et al* (1984) reported the majority of cases occurred from July to October. The MOH reported the high incidence of dengue was probably the result of an increase in breeding places at construction sites (MOH, 2000).

The cases reported to UMMC showed Malays as the most commonly affected ethnic group, followed by Chinese, Indians and others. They were the most common ethnic group admitted. Hussin *et al* (2005) and Jamaiah *et al* (2005) also reported similar findings. This differs from the MOH report that showed Chinese as the majority of cases with dengue (43%), followed by Malays (39%), and Indians (6.3%) (MOH, 1996). Shekhar and Huat (1992-1993) reported male Chinese as the commonest group affected with both DF and DHF. Wallace *et al* (1980) and Fang *et al* (1984) also reported that most cases occurred among Chinese.

The majority of DF and DHF patients were students (30%, n= 385), followed by factory workers (4%, n=54) and housewives (3.6%, n=46) (Table 2). This is to be expected as most cases occurred in teenagers.

All patients (DF and DHF) in this study had fever. Hussin *et al* (2005) and Jamaiah *et al* (2005) also reported fever as the commonest symptom. However, according to the WHO, the clinical features of DF vary according to the age of the patient. Infants and young children may have a non-specific febrile illness with rash. Older children and adults may have either a mild febrile syndrome or the classical incapacitating disease, abrupt onset high fever, severe headache, pain behind the eyes, muscle and joint pains, and rash. DHF is a potentially deadly disease that is characterized by high fever, hemorrhagic phenomena and circulatory failure. The illness commonly begins with a sudden rise in temperature accompanied by facial flush and other non-specific constitutional symptoms of dengue fever. The fever usually continues for two to seven days and can be as high as 40-41°C, possibly with febrile convulsions and hemorrhagic phenomena (WHO, 2002). Petechial skin rash, epistaxis and gum bleeding were seen most commonly in mild and moderately severe cases of DHF (George and Duraisamy, 1981). Wallace *et al* (1980) reported hemorrhagic manifestations were observed in 67% of cases of DHF in General Hospital Kuala Lumpur, Malaysia.

In this study, there were three deaths. They died due to dengue shock syndrome (DSS). Mortality can be as high as 10-20% (over 40% if shock occurs) without early appropriate treatment, but it is as low as 0.2% in hospitals with staff experienced with the disease. Warning signs that dengue shock syndrome is impending include sustained abdominal pain, persistent vomiting, change in the level of consciousness (irritability or somnolence), a sudden change from fever to hypothermia, and a sudden decrease in platelet count (Innis, 1995; Rigau-Perez, 1998).

The mean value for hemoglobin, white blood cell count and platelet count were 14.4 g/dl, 4 $\times 10^3$/dl, 75 $\times 10^6$/dl, respectively for DF and 15.1 g/dl, 4 $\times 10^3$/dl, 52 $\times 10^6$/dl for, respectively DHF (Table 4). The mean platelet count for both DF and DHF were low compared to the normal value. Thrombocytopenia (<100,000/ml) constitutes one

of the most common clinical findings in the course of dengue disease (Halstead, 1982; Ramirez-Ronda and Garcia, 1994). Platelet levels tended to decline from a higher value on admission to lower levels over subsequent days, with the lowest being on day 6 of the fever (Ibrahim and Cheong, 1995). The mechanisms involved in the thrombocytopenia associated with dengue disease are not well known, however, it may be due to alterations in megakaryocytopoiesis and platelet production. This possibility is based on analysis of bone marrow biopsies from dengue patients, which demonstrated a decrease in marrow cellularity, including megakaryocytes (Nelson and Bierman , 1964; Nakao *et al*, 1989). These alterations could be due to the ability of dengue virus to infect human hematopoietic cells and impair progenitor cell growth (Nakao, 1989; Murgue *et al*, 1997).

Primary prevention of dengue mainly resides in eliminating or reducing the mosquito vector. Public spraying for mosquitoes is the most important aspect of vector control. Application of larvicides, such as Abate®, to standing water is more effective in long term control of mosquitoes. Initiatives to eradicate pools of standing water (such as in flowerpots) have proven useful in controlling mosquito borne diseases. Promising new techniques have been recently reported from Oxford University on rendering *Aedes* mosquito pest sterile (Wikipedia, 2006).

Personal prevention consists of the use of mosquito nets, repellents, cover exposed skin, use DEET-impregnated bednets, and avoiding endemic areas. This is also important for malaria prevention (Wikipedia, 2006). The control of dengue outbreaks requires a multi-pronged effort by various government agencies (Poovaneswari and Lam, 1992).

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TUBERCULOSIS IN MALAYSIA: A CONTINUING SURGE

V Nissapatorn[1], YAL Lim[1], I Jamaiah[1], H Man Chin[1], MZ Ilyana[1], MZ Nonaziah[1], A Siti Hasifah[1] and I Kuppusamy[2]

[1]Department of Parasitology, University of Malaya Medical Center, Kuala Lumpur; [2]Assunta Hospital, Petaling Jaya, Selangor, Malaysia

Abstract. The substantial influx of foreign-born persons including immigrant population into the community becomes one of the postulated reasons to be elucidated relating to this so-called "a disease without border" in Malaysia. A total of 425 TB patients, including Malaysians and foreigners, were treated at the Institute of Respiratory Medicine from May to December 2003. TB was found relatively more often in foreign laborers and Malaysians with nonspecific occupations. Tuberculin skin test (TST) was used to screen for latent TB infection and a higher positive rate of TST was found in foreign compared to local patients. Chest X-ray findings showed a higher rate of abnormalities consistent with PTB, found in the majority of both groups. Lymph node biopsy and sputum culture were used significantly to detect the presence of *M. tuberculosis* and confirm the diagnosis of TB. EHRZ+B6 was significantly the most commonly used anti-tubercular drug regimen, found in both local and foreign patients. Foreign patients were more significantly associated with non-compliance to anti-tubercular therapy. Hepatitis was one of the most common adverse drug reactions found in local patients. The presence of a greater number of illegal migrants, who are highly mobile within the country as well as across borders, and who do not undergo any health screening, further complicates the national tuberculosis control program in the future.

INTRODUCTION

Tuberculosis (TB) is one of the world's most important infectious causes of morbidity and mortality among adults. Between 8 to 9 million people develop the disease, and approximately 2 million die from TB each year (Pai *et al*, 2006). TB is mainly concentrated in developing countries, and global control efforts have been hampered by the limitations of diagnostic, prophylactic, and therapeutic tools (Freire, 2006). This resurgent disease has become a significant worldwide public health problem and is largely related to immigration from countries with a high prevalence of TB; infection with HIV; social problems, such as poverty, homelessness, and drug abuse; and dismantling of TB services (Raviglione and O'Brien, 2005). In Malaysia, about 10% of TB notified cases have been discovered among the immigrant population, particularly those from high TB burden neighboring countries (Iyawoo, 2004). Immigrants contributed more than 24% of the newly detected TB cases in Sabah, East Malaysia (Dony *et al*, 2004). The substantial influx of foreign-born persons including the immigrant population into the community has become one of the reasons why TB could be called "a disease without borders" in Malaysia. We conducted this study in order to compare the course of tuberculosis in terms of epidemiology, clinical manifestations, investigation, and treatment outcomes between Malaysian (local) and foreign-born patients. This study certainly provided a picture of the current situation of TB in these patients, particularly in the latter group. It could also be useful for the host country to establish preventive measures and regional collaboration to curb the future incidence of TB among foreign-born persons.

Correspondence: Dr Veeranoot Nissapatorn, Department of Parasitology, University of Malaya Medical Center, 50603 Kuala Lumpur, Malaysia.
Tel: 603-7967-6618; Fax: 603-7967-4754
E-mail: nissapat@hotmail.com

MATERIALS AND METHODS

A total of 425 patients including 297 (69.9%) Malaysian (local) and 128 (30.1%) foreigners who were confirmed as non-HIV infected patients and registered for tuberculosis treatment at the Institute of Respiratory Medicine (IRM) from May to December, 2003 were included in this

study. This institute is a tertiary level national referral center for respiratory diseases, situated in Kuala Lumpur, Malaysia. Any person with a respiratory problem can attend this center without a physician's referral. The majority of notified TB cases in Kuala Lumpur each year are treated there. The data was retrospectively reviewed from each patient's medical record. Socio-demographic profiles, clinical presentations, investigation results, treatment, patient compliance with therapy, and outcomes of therapy response were included in a standardized data collection sheet. A country like Malaysia still attracts more foreigners in each year to come in for different purposes, such as working and/or studying. These foreign patients were mainly from its neighboring countries: Indonesia (89), Myanmar (16), Philippines (3), Thailand (1), and Vietnam (2). Others were from Nepal (5), Bangladesh (4), India (3), Pakistan (2), and 1 each from Africa and Japan. Of these, 21 (17 Indonesian and 4 Burmese) patients resided in this country more than 10 years, while the remaining (107) had less than 10 years (range of 1 month to 9 years) residency. The group was comprised of job seekers or laborers (78; 63%), non-laborers (30; 24.2%), and others, such as housewives and students (16; 13%). The case definitions of tuberculosis were obtained from the World Health Organization (WHO, 2002).

Statistical analysis

The data were analyzed employing the statistical software, SPSS version 11 (SPSS Inc, Chicago, Ill, USA). The data with quantitative variables were described as mean and range, while qualitative variables were described as frequency and percentage. Statistical analysis was estimated using chi-square test where appropriate. A p-value of <0.05 was regarded as statistically significant.

RESULTS

Table 1 shows the demographic profiles of 425 TB patients comprising Malaysian (297, 70%) and foreigners (128, 30%) who attended the Institute of Respiratory Medicine (IRM) from May to December 2003. Overall, the incidence of tuberculosis in this study was high in both local (270; 90.9%) and foreign (119; 93%) patients. The age range was found to be older in local than in foreign patients. Both groups had the significantly highest percentage in the age group of 21-40 years, and the occurrence of TB slowly declined in the locals when compared to that of foreign patients, which decreased markedly over the years ($p = 0.000$). TB occurred more commonly in married-and-male than unmarried-and-female patients, in both groups. However, no statistical difference was found between both groups ($p > 0.05$). TB was found relatively more often in foreign laborers and Malaysian with nonspecific occupations ($p = 0.000$). A significant one-third of these patients, including 121 (40.7%) Malaysian and 12 (9.4%) foreigners, were given BCG vaccination ($p = 0.000$). Smoking was the most common predisposing factor for the occurrence of TB, found in both groups ($p > 0.05$). Both groups (39, 13.1% Malaysian *vs* 13, 10.2% foreigner) showed similar results with a previous history of contacting TB ($p > 0.05$). Intravenous drug use was more significantly found in national (49, 16.8%) than foreign (3, 2.3%) patients ($p = 0.000$).

Clinical manifestations and investigation of the 425 TB paients are presented in Table 2. A significant majority of patients in both groups had fever (Malaysian, 55% *vs* foreigner, 67.2%), loss of appetite (Malaysian, 55.2% *vs* foreigner, 67.2%), weight (Malaysian, 56.2% *vs* foreigner, 72.7%), and hemoptysis (Malaysian, 16.8% *vs* foreigner, 33.6%) ($p < 0.05$). Cough (Malaysian, 73.1% *vs* foreigner, 79%) and sputum (Malaysian, 54% *vs* foreigner, 53.1%) were also more common in these patients. However, no statistical difference was found between these two groups ($p > 0.05$). Lymphadenopathy occurred more commonly in local (59, 20%) than foreign (17, 13.3%) patients, and the cervical lymph nodes were the most common location found in both groups ($p > 0.05$). Tuberculin skin test (TST) or Mantoux test was used to screen for latent TB infection, and an optimum cut-off point ≥ 10 mm ($p = 0.07$) when compared to ≥ 5 ($p = 0.112$) or ≥ 15 ($p = 0.407$) mm was considered to be a better correlation. A higher positive rate of TST was found in foreign (32, 25%) than local (56, 19%) patients. A similarly positive ESR was shown in

Table 1
Demographic profile of 425 TB patients attended at the Institute of Respiratory Medicine (IRM) during May-December 2003.

Variable	No of patients (%)		p-value
	Malaysian (297)	Foreigner (128)	
	Aged range = 15 to 93	with a median of 39 years	
	Aged range = 15-93 years	Aged range = 18-75 years	
	Median = 44 years	Median = 30 years	
Age group			0.000
≤ 20	11 (3.7)	8 6.3)	
21-40	118 (39.7)	92 (72)	
41-60	98 (33)	23 (18)	
≥ 61	70 (23.6)	5 (4)	
Sex			0.291
Male	208 (70)	83 (64.8)	
Female	89 (30)	45 (35.2)	
Marital status			0.046
Single	102 (34.3)	57 (44.5)	
Married	195 (65.7)	71 (55.5)	
Present address			0.018
Kuala Lumpur	158 (53.2)	84 (65.6)	
Outsider	139 (46.8)	44 (34.4)	
Occupation			0.000
Laborer	46 (15.5)	78 (61.2)	
Non-laborer	47 (15.8)	30 (23.4)	
Others[a]	204 (68.7)	20 (15.6)	
BCG vaccination status			0.000
Yes	121 (40.7)	12 (9.4)	
No or unknown	176 (59.3)	116 (90.6)	
Risk factors			0.427
Yes	114 (39.4)	40 (31.3)	
Smoking	92 (31)	34 (26.6)	
Alcohol consumption	2 (0.7)	0	
Smoking and drinking	20 (6.7)	6 (4.7)	
No	183 (61.6)	88 (68.8)	
Case category			0.193
New case	270 (90.9)	119 (93)	
Old case (previous TB history)	27 (9.1)	9 (7)	
With completed treatment	10 (3.4)	1 (0.8)	
With uncompleted treatment	17 (5.7)	8 (6.3)	
History of contact with TB			0.391
Yes	39 (13.1)	13 (10.2)	
No	258 (86.9)	115 (89.8)	
Intravenous drug use (IDU)			0.000
Yes	49 (16.8)	3 (2.3)	
No/unknown			
	248 (83.2)	125 (97.7)	

[a]Others included housewife, students retired persons

the majority of local (269, 90.6%) and foreign (118, 92.2%) patients (p >0.05). Interestingly, HCV infection rates were comparatively higher in local (42, 14%) than foreign (3, 2.3%) patients (p = 0.001). Chest X-ray findings showed a higher rate of abnormalities consistent with PTB found in both groups (p = 0.014). Lymph node biopsy (p = 0.004) and sputum culture (p = 0.003) were used significantly to detect the presence of *M. tuberculosis* and confirm the diagnosis of TB.

Table 3 shows the diagnosis and treatment outcomes of the 425 TB patients. Pulmonary (lung) was the most common location (Malaysian, 58.6% *vs* foreigner, 75%), followed by extrapulmonary (Malaysian, 27.6% *vs* foreigner, 16.4%), and pulmonary with disseminated TB (Malaysian, 13.8% *vs* foreigner, 8.6%), which was significant in both groups (p = 0.005). Lymph node and lung with lymph node were the most common forms of extrapulmonary, and pulmonary with disseminated TB, respectively. EHRZ+B6 was significantly the most commonly used anti-tubercular drug regimen, found in both local (192, 64.7%) and foreign (116, 90.6%) patients (p = 0.000). A higher cure rate of ≥6 months anti-tubercular drug regimen was significantly found in both groups (Malaysian, 25.6% *vs* foreigner, 29%), compared to other regimens (p=0.004). A longer duration (≥9 and 12 months) of treatment was more commonly found in local than foreign patients. Non-compliance to therapy was more significantly found among foreign (29%) than among local (19%) patients. There were 22 patients (20 Malaysian *vs* 2 foreigner) who developed adverse drug reactions. Of these, there was hepatitis (4 patients), visual impairment (5), liver impairment (6), skin reaction-rash (4), thrombocytopenia (2), and hearing deficit (1) found in Malaysian patients; while visual impairment (1) and skin reaction-rash (1) were among the adverse reactions recorded in foreign patients. Only 1 patient was recorded having antituberculous drug (rifampicin) resistance. Overall, no MDR-TB or death was reported in these patients during the time of this study.

DISCUSSION

From our study, the incidence of TB was comparatively higher in foreign patients in this country. This is not surprising as this has also become increasingly apparent in many countries such as Australia, Canada, the Netherlands, and the United State (Rieder *et al*, 1994; Raviglione *et al* 1995; Wells *et al*, 1999). Tuberculosis was significantly more common in older locals. This could be explained because the majority of foreign patients stay temporarily in this country for the improved income or standard of living, as clearly shown in this study. The reactivation of primary infection due to lowered body immunity with age or other co-diseases such as diabetes mellitus or disease requiring the use of steroids would be alternative explanations for the local patients (Venugopalan, 2004). Moreover, a higher rate of TB cases was found in men than women in both groups. This finding is supported by most of the previous studies worldwide and this could also be explained by the higher mobility of the male group due to work requirements (Venugopalan, 2004; WHO, 2004a). Nevertheless, women progress from infection to active TB faster than men do, but the reported incidence of pulmonary TB among women is nearly always lower than for men (WHO, 2004b).

Approximately, one-third of these patients (more in local than foreign patients) were given BCG vaccination. This suggests that BCG vaccination is still compulsory, and public health awareness should be more consistently implemented to promote the national vaccination program in any given population, particularly in limited resource settings. However, the development of a vaccine better than BCG is also encouraged for the developing countries where the risk of TB infection remains high (Shimao, 2005). We observed that IDUs was more likely found among local TB patients. This supports with the fact that IDUs is not only the most common route of HIV transmission, particularly in the younger age group in Malaysia, but also a significant risk factor in contributing to the transmission of TB/HIV co-infection as reported worldwide. Therefore, the higher authorities should take more serious steps to tackle this problem, since these two diseases are very contagious and pose a major public health concern in this region.

Looking at the clinical diagnosis, our findings

Table 2
Clinical manifestations and investigation results of 425 TB patients.

Variable	No of patients (%)		p-value
	Malaysian (297)	Foreigner (128)	
Clinical manifestations			
Fever	163 (55)	86 (67.2)	0.018
Loss of appetite	164 (55.2)	86 (67.2)	0.021
Loss of weight	167 (56.2)	93 (72.7)	0.001
Cough	217 (73.1)	101 (79)	0.203
Sputum	160 (54)	68 (53.1)	0.887
Hemoptysis	50 (16.8)	43 (33.6)	0.000
Dysnea	39 (13.1)	21 (16.4)	0.374
Others	63 (21.2)	23 (18)	
Lymphadenopathy	59 (20)	17 (13.3)	0.104
Site of lymph node involvement			0.554
Cervical	42 (14.1)	10 (7.8)	
Other	17 (5.7)	7 (5.5)	
Investigation results			
Mantoux test			0.07
Positive ≥ 10 mm	56 (19)	32 (25)	
Negative	41 (13.8)	9 (7)	
Unknown	200 (67.3)	87 (68)	
ESR			0.381
Normal	10 (3.4)	6 (4.7)	
Positive ≥ 10 mm/1st hour	269 (90.6)	118 (92.2)	
Unknown	18 (6.1)	4 (3.1)	
HBV infection			0.753
Positive	14 (4.7)	4 (3.1)	
Negative	102 (34.3)	44 (34.3)	
Unknown	181 (61)	80 (62.5)	
HCV infection			0.001
Positive	42 (14)	3 (2.3)	
Negative	71 (24)	35 (27.3)	
Unknown	184 (62)	90 (70.3)	
CXR finding			0.014
Normal	47 (15.8)	9 (7)	
Positive for PTB	176 (59.3)	95 (74.2)	
Positive for PTB and disseminated	43 (14.5)	11 (8.6)	
Positive for ETB	31 (10.4)	13 (10.2)	
Lymph node biopsy			0.004
Yes	46 (15.5)	7 (5.5)	
No/Unknown	251 (84.5)	121 (54.5)	
Sputum smear			0.173
Positive	102 (34.3)	56 (43.8)	
Negative	191 (64.3)	70 (54.7)	
Unknown	4 (1.4)	2 (1.6)	
Sputum culture for MTB			0.003
Positive	125 (42.1)	77 (60.2)	
Negative	156 (52.5)	47 (36.7)	
Unknown	16 (5.4)	4 (3.1)	

Table 3
Diagnosis and treatment outcomes of 425 TB patients.

Variable	No of patients (%)		p-value
	Malaysian (297)	Foreigner (128)	
Site of organ involvement			0.246
Lung	174 (58.6)	96 (75)	
Lung and LN	25 (8.4)	9 (7)	
Lung and other (pleura, spine, larynx and miliary)	16 (5.4)	2 (1.6)	
Lymph node (LN) and/or others	39 (13.1)	9 (7)	
Miliary and/or others	20 (6.7)	5 (3.8)	
Other (pleura, bone, GIT, GUT, etc)	23 (7.7)	7 (5.5)	
Type of tuberculosis			0.005
Pulmonary	174 (58.6)	96 (75)	
Pulmonary and extrapulmonary	41 (13.8)	11 (8.6)	
Extrapulmonary	82 (27.6)	21 (16.4)	
Anti-tubercular drugs regimen			0.000
EHRZ+B6	192 (64.7)	116 (90.6)	
SHRZ+B6	85 (28.6)	9 (7)	
Other	20 (6.7)	3 (2.3)	
Treatment outcome			0.004
≥ 6 months	76 (25.6)	37 (29)	
≥ 9 months	69 (23.2)	12 (9.4)	
≥ 12 months	13 (4.4)	1 (0.8)	
On going treatment			
< 6 months	55 (18.5)	30 (23.4)	
≥ 6 months	28 (9.4)	11 (8.6)	
Loss to follow-up	56 (19)	37 (29)	
Transfer out	15 (5.1)	10 (7.8)	

showed that both groups displayed similar clinical features, which also depend upon organs involvement relating to TB disease. Pulmonary TB patients were significantly found as having clinical manifestations such as fever, loss of appetite and weight, and hemoptysis, which is in agreement with an earlier study (Chowell *et al*, 2005). This suggests that TB should be considered one of differential diagnosis for patients with persistent fever or unresolved in management of other diseases in these cases. TST was used as one of the routine investigations for the diagnosis of latent TB infection and showed a higher positive rate among foreign compared with local patients. A TST reading of 10 mm had a higher sensitivity than 15 mm (Tan *et al*, 2002) as a cut-off point in TB diagnosis (Loh *et al*, 2005), as also shown in this study. However, interpretation of TST might be difficult, particularly in the Malaysian settings of multi-ethnicity and high BCG coverage (Loh *et al*, 2005). This test is of limited value in the diagnosis of active tuberculosis because of its low sensitivity and specificity (Raviglione and O'Brien, 2005); it often gives false results (Kunst, 2006) and is therefore not applicable for the diagnosis of latent TB infection. Interferon-gamma assays are newly available tests to detect latent TB infection and might allow targeting chemoprophylaxis to reduce the burden of active TB. However, this assay is currently not routinely used (Kunst, 2006). Radiographic images from chest X-ray finding are one of the important

investigations used, particularly in diagnosing PTB. Pulmonary tuberculosis is unlikely in the absence of any radiographic abnormality (Raviglione and O'Brien, 2005). At the same time, sputum examination, particularly from sputum culture, is used not only as a significantly confirmatory method in detecting the presence of *M. tuberculosis* but also for screening of both primary drug susceptibility and resistance, which could prevent further escalation of the existing multi-drug resistant TB.

Concerning TB treatment and its outcome, a higher overall cure rate was found in local compared with foreign patients. Compliance is one of the potential factors to increase the cure rate in TB patients and the type of treatment, gender, occupation, history of contacting TB, perception in health status, attitude, knowledge, and social support were found to be significantly contributing factors (Lertmaharit *et al*, 2005). With the presently available anti-tuberculosis drugs, a six months regimen is comparatively effective in treating PTB, found in both groups of patients. In addition, the regimen of more than 9 months duration was given more in PTB-disseminated and ETB patients when compared to only PTB cases. This therefore indicates that compliance and sites of TB involvement determine the duration of treatment regimen used in these patients. A revolutionary concept for TB drug development and leveraging the potential of the existing drugs pipeline (Freire, 2006) is the future trend for the treatment of TB. In addition, a potent new drug, which has no cross-resistance with existing TB drugs, is targeted to make another great contribution to global TB control (Shimao, 2005).

Non-compliance to therapy was more likely found in foreign compared with local patients. A significant reason in this finding was occupation, with a majority of these patients being laborers (24/37) who might not understand the disease, particularly long-term treatment that requires strict cooperation throughout the course of treatment. To circumvent this problem, their employers should take the initiative to provide job security and especially medical services to prevent the spread of tuberculosis infection within their respective communities.

Adverse drug reactions were found at a higher rate among local than among foreign patients. Hepatitis was one of the most common side effects in patients with the age of more than 45 years (3/4), and there was evidence of relapse TB found in one of these patients. Supporting this finding, one study showed that the risk of hepatitis increased from 2.6% to 4.1% as age exceeded 49 years, and, if patients at risk of both hepatitis and relapse were to receive standard treatment, daily dosing was preferable (Chang *et al*, 2006). This suggests that observation of these side effects should be consistently taken into serious consideration, and communication between medical personnel and patients would be mandatory to prevent future drug resistance. So far, only one local patient developed drug resistance, while there was no report among foreign patients. However, one study showed that the risk of drug resistance was the highest for younger TB patients and among foreign-born patients from Vietnam and the Philippines (Moniruzzaman *et al*, 2006). This assists clinicians in prescribing and tailoring more appropriate anti-tuberculosis regimens for immigrants (Moniruzzaman *et al*, 2006). However, there was no registered case of multi-drug resistant tuberculosis (MDR-TB), in either local and foreign (Nissapatorn *et al*, 2005) patients found in this study. MDR-TB is not yet a serious problem in Malaysia as evidenced by the Drug Resistance Surveillance completed in 1996/1997 with WHO collaboration, and MDR-TB prevalence was found to be 0.1% in Malaysia (Iyawoo, 2004). MDR-TB, however, is the subject of great interest and has consistently gained attention worldwide. The monthly monitoring of sputum culture for AFB in the initial 6 months of treatment helps greatly to predict treatment outcomes (Yew *et al*, 2000). Moreover, one study suggested that regimens with at least four sensitive drugs are mandatory for the successful treatment of MDR-TB and fluoroquinolones are needed in the majority of cases to ensure the success of the four-drug regimen, because of frequent drug resistance or toxicity to other anti-tuberculosis drugs (Shigetoh, 2001). Overall, most patients with multi-drug-resistant tuberculosis can be cured with the use of appropriate, intensive

treatment regimens (Tahaoglu *et al*, 2001), and drug selection must rely on treatment history, results of susceptibility testing and an evaluation of the patient's adherence (API Consensus Expert Committee, 2006).

In conclusion, tuberculosis is still highly prevalent and found in a given population in Malaysia. Age group certainly contributes to the occurrence of TB. BCG vaccination and TST are still compulsory in developing countries where the incidence of TB remains high. Clinical presentations have shown similarity between both groups and depended upon the sites of TB involvement. A higher cure rate of anti-tuberculosis therapy of ≥6-month duration was found in both groups of TB patients. Medical examinations should be a strictly mandatory prerequisite in order to obtain a work permit among foreigners before they enter the country. Moreover, the presence of a substantial number of illegal migrants, who are highly mobile within the country as well as across borders, and who do not undergo any health screening, further complicates the national tuberculosis control program in the future.

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THE DETECTION OF ANTIBODY RESPONSE DURING IMMUNIZATION WITH *HELICOBACTER PYLORI* IN RABBITS BY INDIRECT IMMUNOFLUORESCENT ASSAY (IFA)

Achariya Sailasuta [1] , Anuchai Niwetpathomwat [2] Somporn Techagnamsuwan [1], Tanittha Chatsuwan [3] , Galayanee Doungchawee[4] and Pranom Puchadapirom [4]

[1]Department of Pathology, [2]Department of Veterinary Medicine, Faculty of Veterinary Science, [3]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok; [4]Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand

Abstract. Detection of antibodies against *Helicobacter pylori* by indirect immunofluorescent assay (IFA) in rabbits has been developed as a non-invasive screening method. This study aimed to detect the antibody response by IFA after immunization with *Helicobacter pylori* in rabbits. Six healthy New Zealand white female rabbits were immunized subcutaneously with *Helicobacter pylori*, 4 times, on days 0, 14, 28 and 42, were used for IFA evaluation. Blood samples were then collected from each rabbit on day 0 and weekly, for twelve weeks. *H. pylori* were coated on slides and fixed with absolute methanol and air dried. The coated slides were incubated with sample rabbit sera for 50 minutes at room temperature and washed with PBS. The slides were incubated with goat anti-rabbit IgG antibody conjugated with FITC for 50 minutes at room temperature. The slides were evaluated by fluorescent microscopy. The rabbit sera had evaluated IFA titers starting from week 4 with a peak at week 8. Antibodies were constantly detected until the end of observation (week 12). The means $\log_2$ *H. pylori* antibody titers were 10.50 ± 1.32, 11.83 ± 1.065, 13.33 ± 0.19, 14.00 ± 0.23, 14.83 ± 0.37, 15.00 ± 0, 15.00 ± 0, 15.00 ± 0, and 14.83 ± 0.90 (mean ± SE, n = 6), respectively. Given the ability to detect *H. pylori* antibody in rabbits using IFA, the results of this experiment may be useful for evaluating the epidemiology and diagnosis of *H. pylori* infection in veterinary public health studies.

INTRODUCTION

Helicobacter pylori is a common bacterial infection in humans that is responsible for a variety of gastroduodenal pathologies, including peptic and gastric ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma (Eck *et al*, 1997; Forman, 1998; Kabir, 2003; Suerbaum and Michetti, 2002). *H. pylori* infection can be diagnosed by tests requiring upper gastrointestinal endoscopy for the retrieval of a gastric biopsy specimen (microbiological culture, histological examination, and rapid urease tests) (Graham and Qureshi, 2001). During recent years, noninvasive diagnostic tests for *H. pylori* infection have gained in significance (Vaira and Vakil, 2001). Immunodiagnosis of *H. pylori* infection is attractive in comparison to other noninvasive diagnostic methods for the investigation of upper gastrointestinal symptoms (Newell and Stacet, 1993; Attallah *et al*, 2004). It has been suggested that animals are a reservoir of *H. pylori*, which may be of importance in human infection, and the role of *Helicobacter* spp in gastrointestinal diseases in dogs and cats is uncertained (Vaira *et al*, 1992). It has been known for years that gastric helicobacter-like organisms (HLO) are commonly present in the stomach of dogs but the relationship between these organisms and gastric diseases has never been resolved (Henry *et al*, 1987; Geyer *et al*, 1993; Hermanns *et al*, 1995; Eaton *et al*, 1996; Happonen *et al*, 1996; Yamazaki *et al*, 1998). Invasive *Helicobacter* spp infection diagnosis by histopathology and PCR have been investigated in necropsied dogs (Sailasuta *et al*, 2005). Indirect immunofluorescent assay (IFA) is easy to perform, has a high sensitivity and is inexpensive (Chan *et al*, 2003). It has been wildely used to screen for various infectious diseases, such as leptospirosis (Appassakij

Correspondence: Dr Achariya Sailasuta, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.
Tel:66 (0) 2218-9616; Fax: 66 (0) 2252-0779
E-mail: achariya.sa@chula.ac.th

et al, 1995; Pradutkanchana *et al*, 2003) as well as autoimmune diseases, such as bullous pemphigoid (Chan *et al*, 2003). A *Helicobacter pylori* antibody detection in rabbit serum samples by indirect immunofluorescent antibody assay has been developed (Sailasuta *et al*, 2006). This study aimed to detect antibody response by IFA in rabbits after immunization with *H. pylori* evaluate serodiagnosis as a screening technique for *H.pylori* infection in domestic animals.

MATERIALS AND METHODS

Preparation of *H. pylori* cell lysate

The *H. pylori* specimens were kindly obtained from the Department of Microbiology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The bacterial cells were harvested, washed three times in phosphate-buffered saline (PBS; pH 7.2), and disrupted by a sonicator three times at 4 ˚C for 15 seconds each time (modified from Attallah *et al*, 2004). After centrifugation at 600*g* for 10 minutes at 4 ˚C, the protein content of the supernatant solution was determined with the use of bovine serum albumin as a standard (Lowry *et al*, 1951). The supernatant was split into aliquots and stored at -20 ˚C until used.

Production of anti-*H. pylori* antibody

A group of six healthy New Zealand female rabbits were immunized subcutaneously and intramuscularly at four different injection sites: both scapula regions of the fore limbs and the thigh muscle of hind limbs. Five hundred microliters of *H. pylori* cell lysate was diluted (by volume) with Freund's complete adjuvant and 0.5 ml was used for each injection site. Immunization took place 4 times, on days 0, 14, 28 and 42. The blood samples were then collected from all rabbits at week 0 and weekly for twelve weeks. The serum samples were then separated and stored at -20 ˚C until tested.

Indirect immunofluorescence assay

For IFA, xylane coated-slides were fixed with *H. pylori* at 108 cells/ml in cold methanol (-10 ˚C) for 15 minutes and air dried. The slides were reacted with 5 µl per well of rabbit sera at dilution factors of 2, 4, 8, and 16, for 30 minutes at room temperature. The slides were washed twice with 0.15 M phosphate-buffered saline (PBS, pH 7.2) for 30 minutes, stained with a 1:50 dilution of goat anti-rabbit immunoglobulin conjugated with FITC (Dako®, Denmark) for 50 minutes at room temperature. The slides were then washed twice in PBS for 5 minutes, mounted with buffer glycerol at pH 8.0 and observed under a fluorescent microscope. The detection of apple-green color in the spiral organism was scored as 2+, 3+, or 4+ in positive cells and 0 in negative cells. When the serum sample was positive, it was then rediluted for detection of the titer. The polyclonal anti-*H.pylori* antibody (Dako®, Denmark) and

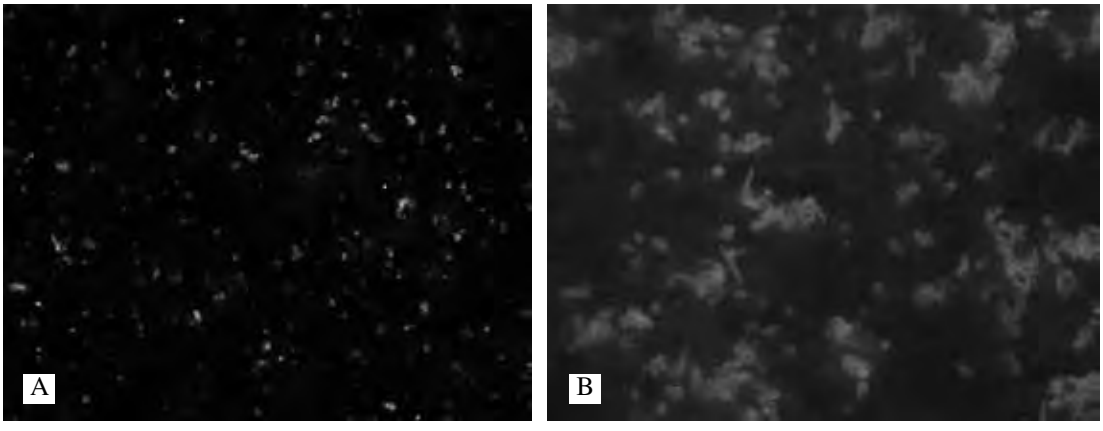

Fig 1- A: *H. pylori* under dark field microcopy (x400). B: Indirect immunofluorescence assay (IFA); *H. pylori* were positive in rabbit serum at 8 weeks after immunization, fluorescein-labeled antibody to rabbit IgG, Fluorescent microscope (x400).

Table 1
Geometric antibody titers against *H. pylori* in rabbits sera using IFA (n=6).

No	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6	Wk7	Wk8	Wk9	Wk10	Wk11	Wk12
Rabbit 1	0	0	0	13.00	13.00	13.00	14.00	15.00	15.00	15.00	15.00	15.00
Rabbit 2	0	0	0	7.00	13.00	13.00	14.00	14.00	15.00	15.00	15.00	15.00
Rabbit 3	0	0	0	13.00	13.00	14.00	14.00	15.00	15.00	15.00	15.00	15.00
Rabbit 4	0	0	0	5.00	6.00	13.00	15.00	15.00	15.00	15.00	15.00	16.00
Rabbit 5	0	0	0	13.00	13.00	14.00	14.00	15.00	15.00	15.00	15.00	13.00
Rabbit 6	0	0	0	12.00	13.00	13.00	13.00	15.00	15.00	15.00	15.00	15.00
Average	0	0	0	10.50	11.83	13.33	14.00	14.83	15.00	15.00	15.00	14.83
SD	0	0	0	3.25	2.61	0.47	0.58	0.37	0.00	0.00	0.00	0.90
SE	0	0	0	1.3281	1.065	0.1925	0.2357	0.1521	0	0	0	0.3664

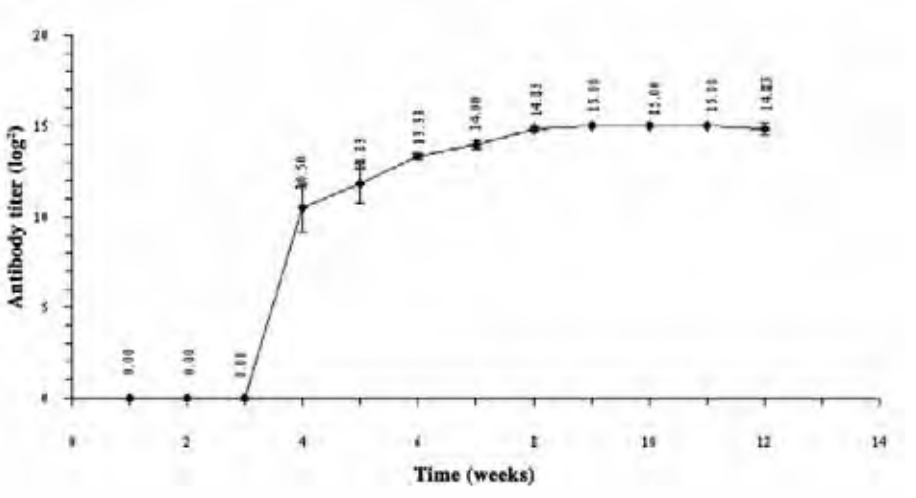

Fig 2- Mean of the $\log_2$ *H. pylori* antibody titer in rabbit sera determined by IFA during 12 weeks of observation. Serum samples collected from rabbits each week. The rabbits were immunized at days 0, 14, 28 and 42.

normal rabbit serum were used for positive and negative controls, respectively. The geometrical mean titers were used for evaluation of the titers each week in the six rabbits.

RESULTS

Reactivity of the developed anti-*H. pylori* antibodies by IFA

H. pylori lysate was observed on the fixed slide under a dark field microscope, and spiral and coccoid shapes were noted (Fig 1A). The serum samples that were collected weekly for 12 weeks. The results for the 72 serum samples examined are shown in Table 1. The means for the log2 of the *H. pylori* antibody titers were 10.50 ± 1.32, 11.83 ± 1.065, 13.33 ± 0.19, 14.00 ± 0.23, 14.83 ± 0.37, 15.00 ± 0, 15.00 ± 0, 15.00 ± 0, and 14.83 ± 0.90 (mean ± SE, n = 6), respectively. The *H. pylori* antibody titers in the rabbit sera was positive by IFA starting from week 4. The peak of the antibody titer was demonstrated at week 8 and was constantly detected until the end of observation (week 12) (Fig 2). Sera collected from the rabbits, immunized with the *H. pylori* lysate, at the week 8th had fluorescein-labeled antibody to rabbit Ig under fluorescent microscope (Fig 1B).

DISCUSSION

The IFA has been used in diagnostic laboratories successfully for 2 decades and the potential utility of IFA for the detection and quantification of leptospirosis antibody has been well documented (Appassakij *et al*, 1995; Pradutkanchana *et al*, 2003). Similarly, the capability of IFA for the detection of *H. pylori* infection in serum as a non-invasive method has been reported (Attallah *et al*, 2004). A panel of 72 serum samples showed a classical IgG immune response to *H. pylori* (Anderson *et al*, 1986; Boonpucknavig and Doungchawee, 1997). In this experiment, the sera were then examined for IFA, and the specificity of any positive reactions was checked against the control well. IFA does give some false-positive results due to unwanted positive protein in the serum (Boonpucknavig and Doungchawee, 1997). It has been reported that *H. pylori* in humans crossreacts with several antigens from *P. aeruginosa*, *H. influenzae* and *C. jejuni* (Johansen *et al*, 1995). Thus, the high antibody titers in the experiment rabbits should be reconsidered depending on the prevalence of infection. The antibody response in all six rabbits in this study were synchronized. The sera obtained from this study can be used as *H. pylori* antiserum for in-house diagnosis. The IFA described appears to be sufficiently sensitive and specific to be useful for the detection of *H. pylori* antibody. This method is an alternative, is easy-to-use, and can be applied as a non-invasive test for the detection of *H. pylori* infection in domestic animals as a benefit for veterinary public health.

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HISTOPATHOLOGICAL AND HEMATOLOGICAL EVALUATION OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*) EXPOSED TO A TOXIC CYANOBACTERIUM (*MICROCYSTIS AERUGINOSA*)

Prannapus Fahprathanchai[1], Kanokporn Saenphet[1], Kriangsak Meng-umphan[2], Yuwadee Peerapornpisal[1], Salika Aritajat[1], Supap Saenphet[1] and Paiwan Sudwan[1,3]

[1]Department of Biology, Faculty of Science,
[3]Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand;
[2]Faculty of Fisheries Technology and Aquatic Resources, Mae Jo University, Chiang Mai, Thailand

Abstract. Sub-chronic exposure of Nile tilapia (*Oreochromis niloticus*) to the toxicity of *Microcystis aeruginosa*, a toxic cyanobacterium, was investigated with emphasis on the hepatic histopathology and hematological effects. Histopathology revealed abnormalities of the fish livers exposed to *M. aeruginosa* in their diet showing hemorrhage, congestion, vacuolization, leukocyte infiltration, pyknotic cells and irregular arrangements of hepatocytes. The degree of tissue damage was found to be concentration dependent. An alteration in lymphocyte numbers was also detected in the fish exposed to *M. aeruginosa*.

INTRODUCTION

Microcystis spp are cyanobacteria which grow in marine, brackish and fresh water (Fleming and Stephen, 2001). Besides being a source of nutrition for aquatic life, many of them are capable of producing toxins. *Microcystis aeruginosa* is a toxic species which produces microcystins (Carmichael, 1994), a hepatotoxin and tumor promotor (Fischer *et al*, 2000). Pathologic lesions of the liver have been reported in rats exposed to sublethal doses of microcystin-LR (Guzman and Solter, 2002) and exposure to high levels of this toxin can lead to hepatocyte necrosis and hemorrhage, with severe cases resulting in death (Bhattacharya *et al*, 2002). Since blooming of toxic cyanobacteria has been detected in many ponds and water reservoirs in Thailand (Prommana *et al*, 2001; Peerapornpisal *et al*, 2002), a risk assessment to those aquatic organisms is needed. The aim of this study was to investigate the hepatotoxicity *M. aeruginosa* had on Nile tilapia (*Oreochromis niloticus*). The hematological effects were also investigated.

Correspondence: K Saenphet, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail: K_Saenphet@yahoo.com

MATERIALS AND METHODS

M. aeruginosa

Colonies of cyanobacteria dominated by *M. aeruginosa* forming blooms on the surface of Huay Yuak reservoir, Mueang District, Chiang Mai, Thailand were collected during April-June, 2005. *M. aeruginosa* was identified and isolated for fish diet preparation.

Animals

The Nile tilapia (*O. niloticus*) used in the experiment weighed 10.07±0.03 g. They were obtained from the Faculty of Fisheries Technology and Aquatic Resources, Mae Jo University. During a 7-day-acclimatization period, the fish were fed twice daily with a commercial fish diet. The water temperature range was 25-27ºC and the pH was 7.0-7.2. Chlorine residual was below detection limits.

Experimental design

The fish were randomized into 3 groups (20 each). Two experimental groups were fed a standard fish diet mixed with *M. aeruginosa* at proportions of 25% and 50%, respectively. The control fish received only the standard fish diet. The dialy quantity of food was 3% of the body weight for each group. Three replicates per group were conducted. At the end of the 60-day

experimental period, the fish were anesthetized with MS222 (tricaine methansulphonate) for histological and hematological investigation. Blood was collected from the caudal vein with a heparinized capillary tube. Hematocrits (PCV) and differential white blood cells were determined using standard methods (Dacie and Lewis, 1984). Immediately after each fish was bled, the liver was removed and fixed in Bouin's solution for histopathological examination using a routine histological technique with hematoxyline and eosin for staining (Brancroft and Cook, 1994).

Statistics

Means and standard deviations were calculated. The significances of the differences were analyzed with the Student's *t* -test

RESULTS

Fig 1 shows there were no histological change in the livers of the control fish. In contrast, the fish that received *M. aeruginosa* at 25% and 50% proportions of their daily diet showed severe liver damage and the degree of hepatic tissue damage increased in a concentration-dependent manner. The histopathological changes found in the treated fish livers included irregular arrangements of hepatocytes and dilation of the central

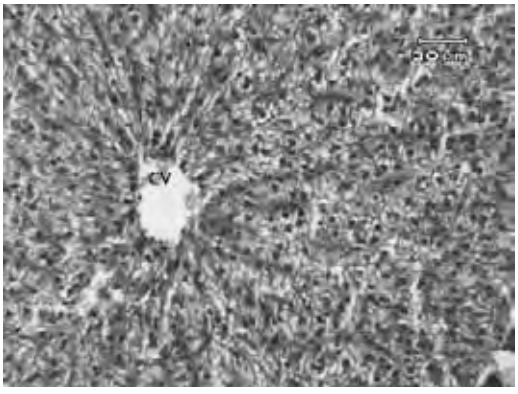

Fig 1- H & E stained section of the liver of a control fish (*O. niloticus*) showing the normal central vein (CV) and regular arrangement of the hepatic sinusoids.

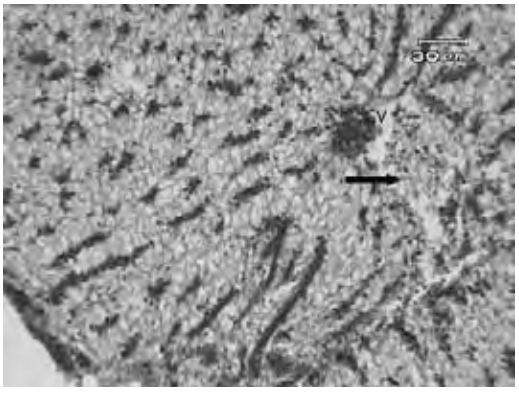

Fig 2- H & E stained section of the liver of *O. niloticus* exposed to *M. aeruginosa* showing dilation of the central vein (CV) and blood congestion (arrow). Notice the irregular arrangement of the hepatic sinusoids.

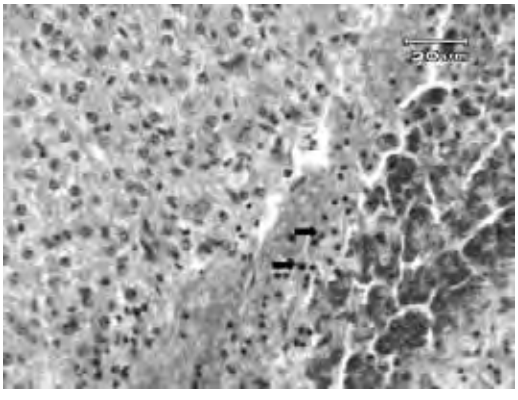

Fig 3- Leukocyte infiltration detected in hepatic tissue of *O. niloticus* exposed to *M. aeruginosa* (arrow) (H & E stain).

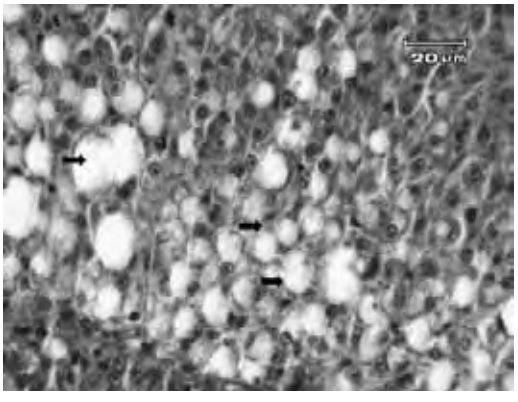

Fig 4- Numerous vacuolated cells (arrows) distributed in the hepatic tissue of *O. niloticus* exposed to *M. aeruginosa* (H & E stain).

Table 1
Defferential white blood cell counts in *O. niloticus* exposed to 25% and 50% *M. aeruginosa* for 60/days compared to controls.

Groups	White blood cells (Cells/ml^3)				
	Monocytes	Lymphocytes	Neutrophils	Eosinophils	Basophils
Control	12,968.9 ± 7,074.0[a]	76,929.4 ± 8,946.7[a]	26,232.6 ± 1,128.8[a]	884.2 ± 1,148.3[a]	294.7 ± 589.5[a]
25% *M. aeruginosa*	26,340.7 ± 14,207.8[a]	92,568.6 ± 20,937.7[a]	30,354.5 ± 1,229.0[a]	501.2 ± 777.3[a]	501.2 ± 772.3[a]
50% *M. aeruginosa*	26,935.2 ± 11,163.2[a]	48,312.4 ± 12,485.1[a]	48,882.2 ± 3,270.7[a]	3,277.9 ± 3,277.1[a]	855.1 ± 1,282.6[a]

[a] = significant differences at $p \leq 0.05$

vein accompanied by blood congestion (Fig 2). Leukocyte infiltration and vacuolization were also detected in the hepatic tissues of the treated fish (Figs 3 and 4). The results of the hematological examinations are presented in Table 1. A significant decrease in lymphocyte counts was found in the fish receiving *M. aeruginosa*, compared to controls. The PCV results in the subjects did not differ from those in the control fish (data not shown).

DISCUSSION

Along with worldwide water eutrophication, cyanotoxins are of environmental concern and fish mortality is associated with *M. aeruginosa* blooms. The histopathological finding in *O. niloticus* treated with *M. aeruginosa* indicate that cyanobacteria produce potent hepatotoxins which alter the architecture of hepatocytes and may impair their function. The congested blood vessels indicated clear evidence of hemorrhages. Although the PCV values in the treated fish were not different from those of controls, the decrease in lymphocyte number and leukocyte infiltration in hepatic tissue also supports the evidence of tissue inflammation. Histopathological and hematological alterations in *O. niloticus* exposed to *M. aeruginosa* may lead to the loss of fish homeostasis and place them under stress. The elevation of gluthatione (GSH) and malodialdehyde (MDA), important biomarkers of oxidative stress, was detected in silver carp (*Hypophthalmichthys molitrix* Val.) grown in *M. aeruginosa* water blooms (Bláha *et al*, 2004). The vacuolization of hepatocytes may also indicate an imbalance in the rate of synthesis of substance(s) in the cells and release into the systemic circulation. Inhibition of protein phosphatase in the liver of rainbow trout exposed to microcystin-LR has also been reported previously (Fischer *et al*, 2000). Since fish serve as a protein source for humans, the accumulation of hepatotoxins from *M. aeruginosa* in their organs may lead to serious problems for fish consumers.

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SPERM DENSITY AND ULTRASTRUCTURE OF SERTOLI CELLS IN MALE RATS TREATED WITH *KAEMPFERIA PARVIFLORA* WALL. EX BAKER EXTRACT

Paiwan Sudwan[1,2], Kanokporn Saenphet[1], Supap Saenphet[1] and Chalobol Wongsawad[1]

[1]Department of Biology, Faculty of Science; [2]Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

Abstract. The purpose of this study was to determine the effects of *Kaempferia parviflora* Wall. Ex Baker on the sperm density and ultrastructure of Sertoli cells in male albino rats. Three treated groups were orally administered with the rhizome extract of *K. parviflora* at various doses (60, 120, and 240 mg/kg bw/day) for 60 days. A control group received distilled water of 1 ml/day. At the end of the treatment period, the epididymal sperm density was determined and small pieces of testis were processed for ultrastructure study of Sertoli cells under the transmission electron microscope (TEM). The results showed that male rats treated with *K. parviflora* extract at the dose of 60 mg/kg bw/day had significantly higher sperm density than that of the control group. Electron micrographs demonstrated that all *K. parviflora* treated groups had prominent granules in the Sertoli cells, which secreted their substances to spermatogonia in the basal compartment of the seminiferous epithelium. Male rats treated with *K. parviflora* extract at the dose of 60 mg/kg bw/day also had highly condensed lysosome in the basal part of Sertoli cells. It was concluded that the ethanolic extract of *K. parviflora* involves sperm density and ultrastructure of Sertoli cells in male rats.

INTRODUCTION

Kaempferia parviflora Wall. ex Baker is a traditional herb of Thailand, known in Thai as *Krachaidum*. It belongs to the Zingiberaceae family. According to Thai traditional medicine, their rhizomes are believed to have sexual enhancing properties (Churdboonchart, 2000; Wutythamawech, 2000). This plant is sometimes referred to as "Thai ginseng" and has long been used among Thai men for sexual enhancement (Wutythamawech, 2000; Chaophaya Aphibhubejhr Hospital, 2006). They become a popular product and have been promoted commercially as *Krachai dum* wine, tea, and supplementary food, as well as being fermented in honey and white whisky. *K. parviflora* tea showed positive effects on the seminal vesicle and spermatogenesis at the dose of 60 and 120 mg/kg for 30 days (Jitjaingam *et al*, 2005) and the light microscope showed this plant extract increased granules of Sertoli cells and decreased serum estradiol levels (Sudwan and Saenphet, 2006). However, reports of the ethanolic extract of *K. parviflora* at 60, 120, and 240 mg/kg BW for 60 days showed a morphological change in the liver of all treated groups and reduced the time in the first 10 minutes of rat courtship behavior (Sudwan *et al*, 2006). Therefore, the effects of the ethanolic extract of *K. parviflora* on sperm density and ultrastructure of Sertoli cell need to determine. The aim of this research was to investigate the effects of ethanolic extract of *K. parviflora* rhizomes on sperm density and ultrastructure of Sertoli cell in male albino rats.

Correspondence: Paiwan Sudwan, Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200 Thailand.
Tel: 66-053-945312, Fax: 66-053-945304
E-mail: pasudwan@mail.med.cmu.ac.th

MATERIALS AND METHODS

Plant and preparation

The rhizomes of *K. parviflora* were collected from Loei Province, Thailand, and authenticated at the Botany Section, Queen Sirikit Botanic Garden, Ministry of Natural Resources and Environment, Mae Rim, Chiang Mai (voucher specimen no. 06-051717). The rhizomes were sliced, dried at 60 ˚C, ground to a fine powder, extracted with 50% ethanol in a soxhlet apparatus, and evaporated by rotary evaporation. Three

doses of the extract (60, 120, and 240 mg/kg bw) were prepared by dissolving in distilled water to the desired concentrations.

Animal and treatment

Twenty-eight adult male Wistar rats were used. The rats were purchased from the National Laboratory Animal Center, Thailand. They were housed under standard animal housing conditions at the Department of Biology, Faculty of Science, Chiang Mai University with free access to food and tap water. The animals were randomly assigned into four groups of seven rats each. Rats of the three treatment groups were orally administered with 60, 120, and 240 mg/kg bw (1 ml) of the crude extract for 60 days. The fourth group served as controls and was treated with 1 ml of the distilled water in a similar manner.

Sperm density

At the end of the treatment period, all rats were anesthetized, sacrificed, and examined for sperm density and the ultrastructure of Sertoli cells. The right cauda epididymis was cut into small pieces, and homogenized in 10 ml of 0.9% NaCl, and the sperm number was estimated in duplicate using an improved Neubauer hemocytometer. The testes were processed for histological study under the transmission electron microscope.

Ultrastructural study of Sertoli cells

Pieces of right testis were cut in 1 x 1 x 1 mm^3 and fixed in glutaraldehyde plus paraformaldehyde for conventional transmission electron microscopic procedure (Bazzola and Russell, 1998). The plastic blocks were sectioned at 500-900 nanometers, contrasted with uranyl acetate and lead citrate, and examined under the transmission electron microscopes with the cooperation of the Electron Microscopy Research and Service Center, Faculty of Science and the Medical Science Research Equipment Center, Faculty of Medicine, Chiang Mai University.

Statistical analysis

Sperm density was expressed as mean ± SE. Statistical evaluations were made using one-way ANOVA, followed by least significant difference (LSD) multiple comparisons test. Significance was set at $p \leq 0.05$.

RESULTS

The results showed that male rats treated with *K. parviflora* extract at the dose of 60 mg/kg bw/day had significantly higher sperm density than that of the control group (Fig 1). Transmission electron micrographs demonstrated that there were spermatogonia resting on the basal layer of the seminiferous epithelium in all groups (Fig 2A, 3A, 4A, 5A). During the developmental process, the cells of the spermatogenic series were supported by Sertoli cells. The Sertoli cells were bound to one another and within the tubule they divided into basal and adluminal compartments (Fig 2A, 3A, 5A). The cytoplasm

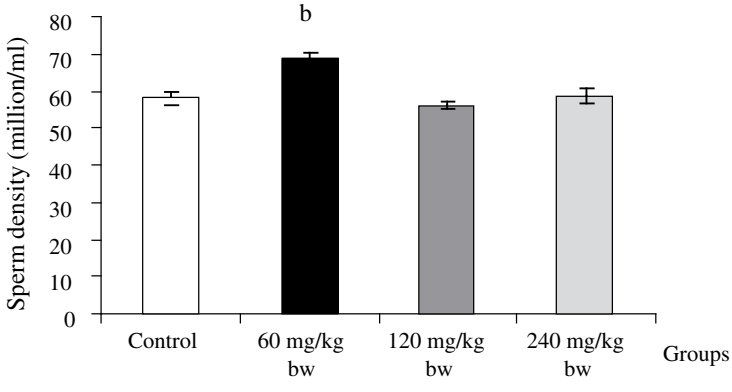

Fig 1- Sperm density in the control and treated groups for 60 days (n = 7). The values are expressed as mean ± SE. [b]Significant difference ($p < 0.5$) from control.

of the Sertoli cells of the control animals showed evidence of normal composition (Fig 2B), while all *K. parviflora* treated groups had prominent granules in their cytoplasm (Fig 3A, 3C, 4A-B, 5A). Sertoli cells secreted their substances to spermatogonia in the basal compartment of the seminiferous epithelium. Male rats treated with *K. parviflora* extract at the dose of 60 mg/kg bw/day also had highly condensed lysosome in the basal part of Sertoli cells (Fig 3B). These lysosomes contained dense particulate material and amorphous granular material. However, electron micrographs presented the normal morphology of spermatozoa in the control and treated groups (Fig 2C-D, 3D, 5B-C).

DISCUSSION

In this experiment, *K. parviflora* was given to male rats to study the sperm density and ultrastructure of Sertoli cells. Male rats treated with *K. parviflora* had more secretory granules in the cytoplasm of Sertoli cells compared with those of the control group. Electron micrographs demonstrated some granules were dropping into the intercellular spaces, which were situated beneath the cytoplasmic bridge of the Sertoli cells. These spaces supported and enclosed the spermatogonia as in a previous study using light microscope (Sudwan and Saenphet, 2006). In this manner, they were postulated to act as nutritional and metabolic supports for the developing spermatogenic cells (Fawcett, 1994; Burkitt, *et al*, 1998; Fawcett and Jensh, 2002).

Administration of *K. parviflora* extract at the dose of 60 mg/kg bw/day for 60 days in male rats produced significantly higher sperm density compared with that of the control group. It could then be assumed that the plant extract might have had an androgenic effect on increased sperm density (Watcho *et al,* 2004). Additionally, Sudwan and Saenphet (2006) observed that this plant had an effect on other steroid hormones, such as decreasing serum estradiol levels in male rats after a treatment period. Because estradiol is an androgen-antagonist hormone, it is possible that it plays a role in the improvement of the response of Sertoli cells and consequently gives a benefit to spermatogenesis.

There was an increase in the spermatogenesis in the male rats treated with *K. parviflora* extract at the dose of 60 mg/kg bw/day group. Thus, this group had more residual cytoplasm of the spermatid, which is ingested by lysosome in the Sertoli cells. The lysosome enzymes of the Sertoli cells had a role to degrade the residual bodies in the seminiferous tubule (Fawcett, 1994; Fawcett and Jensh, 2002). The ultrastructure micrographs also had plenty of lysosome and irregularly shaped conglomerates in the basal part of Sertoli cells.

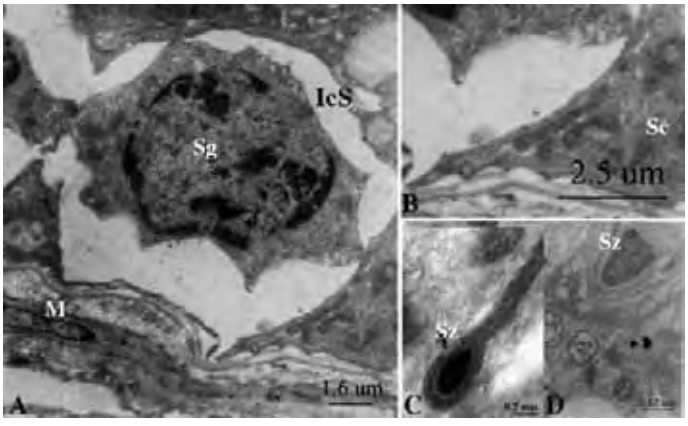

Fig 2- Electron micrographs of seminiferous tubule in the control male rats: A) Spermatogonia (Sg) resting on the basal layer of the seminiferous epithelium beneath which is a slender smooth muscle like (myoid) cells (M). It placed into basal compartment beneath the cytoplasmic bridge of Sertoli cells (Se). 4,000× (IcS = intercellular space). B) Sertoli cell cytoplasmic organelles. 4,000×. C & D) Normal morphology of spermatozoa (Sz). 6,000× and 16,000×, respectively.

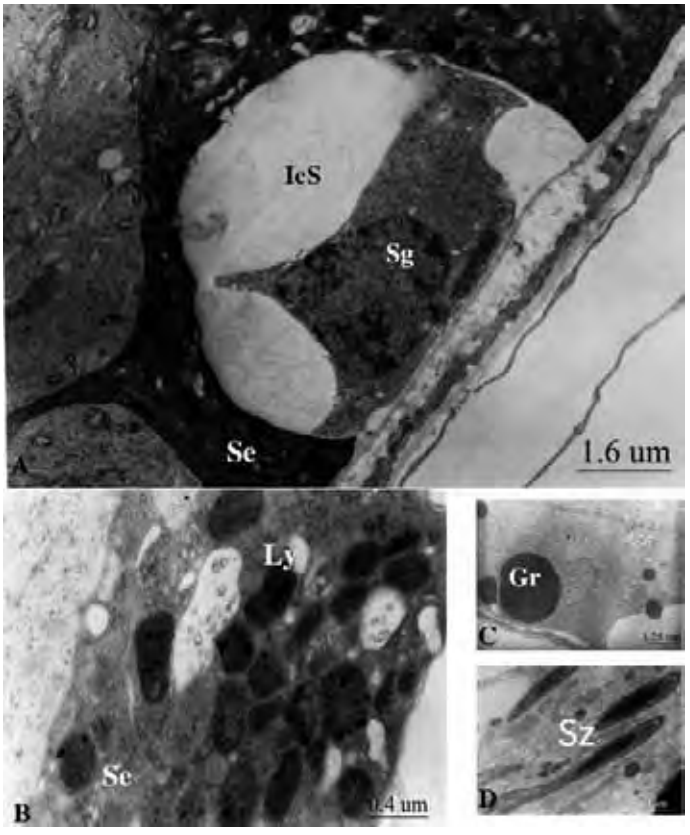

Fig 3- Electron micrographs of seminiferous tubule in the male rats treated with *K. parviflora* extract at the dose of 60 mg/kg bw/day: A) Spermatogonia (Sg) resting on the basement membrane of the seminiferous epithelium. The Sertoli cells (Se) are bound to one another and within the tubule divided into basal and adluminal compartments. 6,300×. B) Highly condensed lysosome (Ly) in the basal part of Sertoli cells shown at higher magnification. They contained electron dense particulate material and amorphous granular material. 25,000×. C) Prominent granules (Gr) in the cytoplasm of Sertoli cells. The Sertoli cells secreted their substances into the intercellular space (IcS) in the basal compartment of the seminiferous epithelium to spermatogonia. 8,000×. D) The normal morphology of spermatozoa (Sz). 8,000×.

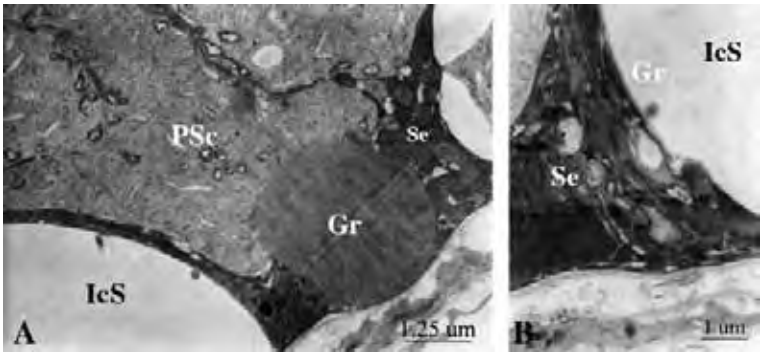

Fig 4- Electron micrographs of seminiferous tubule in the male rats treated with *K. parviflora* extract at the dose of 120 g/kg bw/day.; A) Sertoli cells (Se) resting upon the basement membrane of seminiferous tubule and their prominent granules (Gr); PSc = Primary spermatocyte. 8,000×. B) Sertoli cells having secreted their substances into the intercellular space (IcS). 10,000×.

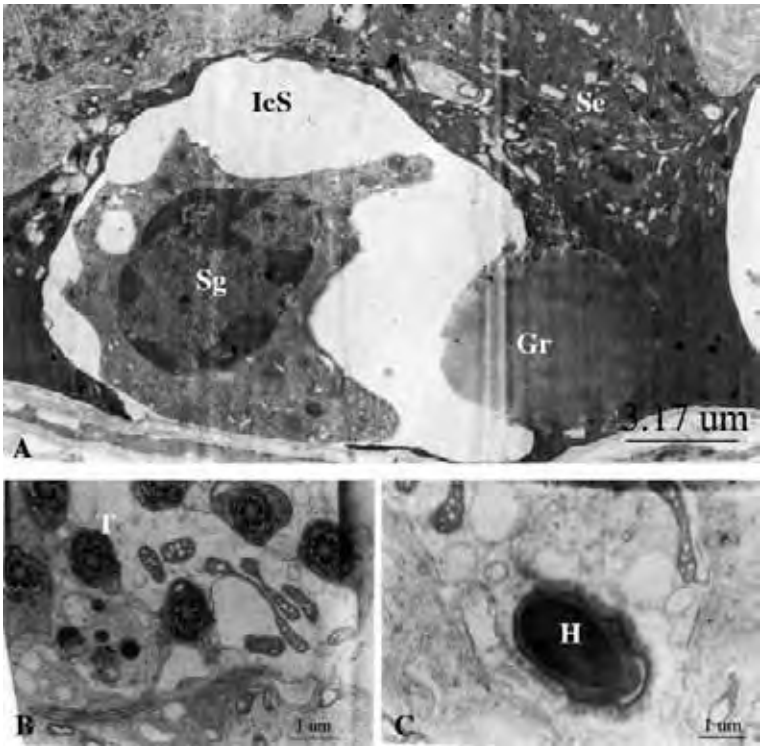

Fig 5- Electron micrographs of seminiferous tubule in the male rats treated with *K. parviflora* extract at the dose of 240 g/kg bw/day. A) Spermatogonia (Sg) resting upon the basement membrane of the seminiferous epithelium. The Sertoli cells (Se) are bound to one another and within the tubule divided into basal and adluminal compartments as the control group. One Sertoli cell has prominent granules (Gr) in its cytoplasm, which are adjacent to the intercellular space (IcS). 3,150×. B) Normal morphology of tail (T), and C) head (H) of spermatozoa (Sz). 10,000×.

This evidence demonstrated that *K. parviflora* extract induced secretory granules, which affected an increase of spermatogenesis and lead to increased amounts of lysosomes. Thus, the Sertoli cells played an important role in germ cell maturation and were highly susceptible to extraneous promotion or damage to the cytoplasmic organelles or inclusions (McCall and Eroschenko, 1988; Kuromaru *et al*, 1989; Lohiya *et al*, 2002; Sudwan and Saenphet, 2006). In conclusion, the ethanolic extract of *K. parviflora* involved in spermatogenesis, possibly mediated by Sertoli cells, leads to an increase of sperm density and secretory granules in Sertoli cells.

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