RESEARCH COMMUNICATION

Carcinogenic Human Liver Fluke: Current Status of *Opisthorchis viverrini* Metacercariae in Nakhon Ratchasima, Thailand

Natthawut Kaewpitoon[1,2]*, Soraya J Kaewpitoon[1,2], Naporn Ueng-arporn[1,2], Ratana Rujirakul[1], Seekaow Churproong[2], Likit Matrakool[2], Suprakrit Auiwatanagul[2], Banchob Sripa[3]

Abstract

Background: *Opisthorchis viverrini* infection is a serious public-health problem in Southeast Asia. It is associated with a number of hepatobiliary diseases and the evidence strongly indicates that liver fluke infection is the etiology of cholangiocarcinoma. Objectives: This study aimed to investigate *Opisthorchis viverrini* metacercarial infection in cyprinoid fish collected from 32 districts of Nakhon Ratchasima province, Northeastern Thailand during one year period from February 2010 to February 2011. Methods: A cross-sectional study was conducted, data being collected with pepsin-HCl digestion and stereomicroscope, respectively. Analysis was performed using SPSS Windows Version 12.0. Results: A total of 640 Cyprinidae family fish including 5 species were collected from different study sites, and investigated for *O. viverrini* metacercariae. The infection rate was 12.3% (79/640), predominantly in *Cyclocheilichthys armatus, C. repasson, Puntioplites proctzysron, Hampala macrolepitota* and *Hampala dispar*, respectively. The prevalence of *O. viverrini* metaceria in Nakhon Ratchasima area was 78.1%, predominantly in Sida and KiaKham Thale So. Conclusion: This findings stress that natural fish species in rural communities are still a source of *O viverrini* infection and put local people at risk, therefore public awareness and prevention campaigns are urgently required.

Keywords: Carcinogenic human liver fluke - metacercariae - Thailand

Asian Pacific J Cancer Prev, **13**, 1235-1240

Introduction

Opisthorchis viverrini, fish-borne trematode infection causes a serious public-health problem in Southeast Asia mainly Thailand, Cambodia, and Laos PDR. In Thailand, opisthorchiasis is a major public health problem in the Northeastern and Northern region, and it is estimated that 5.5 million people are infected with the causative parasite (Sripa et al., 2010). Human have been infected by ingesting undercooked fish containing infective metacercariae, this is very common in the northeastern and northern region particularly in rural areas (Sadun, 1955; Sithithaworn et al., 1997; Jongsuksuntigul & Imsomboon, 2003).

The infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis (Harinasuta et al., 1984). The experimental and epidemiological evidences strongly indicate that the liver fluke infection in the etiology of cholangiocarcinoma (CCA); the bile duct cancer (Thamavit et al., 1978; IARC, 1994). Multi-factorial etiology of CCA, mechanical damage, parasite secretions, and immunopathology may enhance cholangiocarcinogenesis (Flavell, 1981; Sripa et a., 2007).

The infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis (Harinasuta et al., 1984). The pathogenesis of *O viverrini*-mediated hepatobiliary changes may be due to mechanical irritation caused by the liver fluke suckers and/or its metabolic products (Bhamarapravati et al., 1978; Sriamporn et al., 2004). However, several authors suggest that immunopathological process may contribute to the hepatobiliary inflamation and damage (Tansurat, 1971; Viranuvatti & Stitnimankarn, 1972; Bhamarapravati et al., 1978; Pairojkul et al., 1991). The experimental and epidemiological evidence strongly implicate the liver fluke infection in the etiology of cholangiocarcinoma (CCA); the bile duct cancer (Thamavit et al., 1978; IARC, 1994). Multi-factorial etiology of CCA, mechanical damage,

*[1]Parasitic Disease Research Unit, Suranaree University of Technology, [2]Institute of Medicine, Suranaree University of Technology, Muang, Nakornrachasima, [3]Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand *For correspondence: natthawut.k@sut.ac.th*

parasite secretions, and immunopathology may enhance cholangiocarcinogenesis (Sripa et a., 2007; Flavell, 1981).

Many pathological features in the hamster model reveal evidence of such mechanism, e.g. periportal infiltration of lymphocytes and macrophages. Acute damage may be induced by parasite factors, but the progressive changes are consistent with immunopathologic mechanisms (Pairojkul et al., 1991). During liver fluke infection, inflammation, periductal fibrosis, and proliferative responses, including epithelial hyperplasia, goblet cell metaplasia, and adenomatous hyperplasia, may represent predisposing lesions that enhance susceptibility of DNA to carcinogens (Kim, 1984; Flavell & Flavell, 1986). Several *N*-nitroso compounds and their precursors occur at low levels in fermented food, such as preserved mud fish paste, pla ra, a condiment that is a ubiquitous component of the cuisine of Northeastern Thailand and Laos (Sripa et al., 2007). Some authors hypothesised that *N*-nitroso compounds are a primary carcinogen leading to CCA in humans in Northeastern Thailand (Migasena et al., 1980; Srisraluang & Boriboon, 1988). Jinawath et al showed the selective up-regulation of genes involved in xenobiotic metabolism in Thai patients with CCA, implying that these genes may be involved in the detoxification of possible carcinogens, such as nitrosamines (Jinawath et al., 2006). In hamster experimentally infected with *O viverrini*, CCA was induced by exposure to sub-carcinogenic doses of nitrosamine (Thamavit et al., 1978).

Liver fluke infection can induce the endogenous nitrosation both in humans and experimental animals. Infected hamster showed nitric oxide synthase (NOS) expression by immune effector cells in the inflamed areas and increased endogenous nitrosation of thiazolidine-4-carboxylic acid (thioproline) (Srianujata et al., 1987). In human studies, infected cases showed a higher endogenous nitrosation than uninfected cases (Srivatanakul et al., 1991; Satarug et al., 1998). Infected cases had increased levels of plasma and urinary nitrate and nitrite compared to the uninfected cases (Srianujata et al., 1987). Praziquantel treatment and co-administration of ascorbic acid with proline showed enhanced immune responses to *O viverrini* and an increase of endogenous nitrosation (Oshima et al., 1994). Thus the aforementioned findings clearly demonstrate both exogenous and endogenous nitrosation may lead to DNA alkylation and deamination in predisposed and inflamed tissues (Miwa et al., 1987; Nguyen et al., 1992).

In Thailand, fish-borne trematode infections have been commonly found in the Northeastern and Northern regions of Thailand (Harinsuta & Vajrasthira, 1960; Wykoff et al., 1965; Vichasri et al., 1982). However, lack of data about the metacercariae; *O. viverrini* infection in Nakhon Ratchasima province, Thailand which is the largest province on the northeastern plateau and acts as a gateway to other provinces in the Northeastern region of Thailand. Therefore, we have constructed the cross-sectional research to survey and determined the metacercariae from the natural cyprenoid fish. This research data could be used to localize the risk areas to prevent the infection.

Materials and Methods

The study protocol was approved by Suranaree University Biotechnological Review Committee. Cyprinoid fish, the common intermediate host for *O. viveriini*, were collected from 32 districts of Nakhon Ratchasima province, Thailand. It is 259 kilometres from Bangkok and has an territory area of around 20,494 square kilometres, making it the biggest province in Thailand. Cyprinoid fish were collected at 32 water reservoirs; Mueang Nakhon Ratchasima, Khon Buri, Soeng Sang, Khong, Ban Lueam, Chakkarat, Chok Chai, Dan Khun Thot, Non Thai, Non Sung, Kham Sakaesaeng, Bua Yai, Prathai, Pak Thong Chai, Phimai, Huai Thalaeng, Chum Phuang, Sung Noen, Kham Thale So, Sikhio, Pak Chong, Nong Bun Mak, Kaeng Sanam Nang, Non Daeng, Wang Nam Khiao, Thepharak, Mueang Yang, Phra Thong Kham, Lam Thamenchai, Bua Lai, Sida, Chaloem Phra Kiat districts from February 2010 to February 2011 (Figure 1). These fish were collected by fisherman using nets (Figure 2), dead fish were kept in labeled plastic bags and transported in an ice box to the laboratory at the Parasitic Disease Research Unit, department of Pathology, Institute of Medicine, Suranaree University of Technology, Thailand, within a day after collection.

Figure 1. Collection of Cyprinoid Fish Collected from Natural Reservoirs of Various 32 Districts, Nakhon Ratchasima Province Thailand, by local Fishermans using nets.

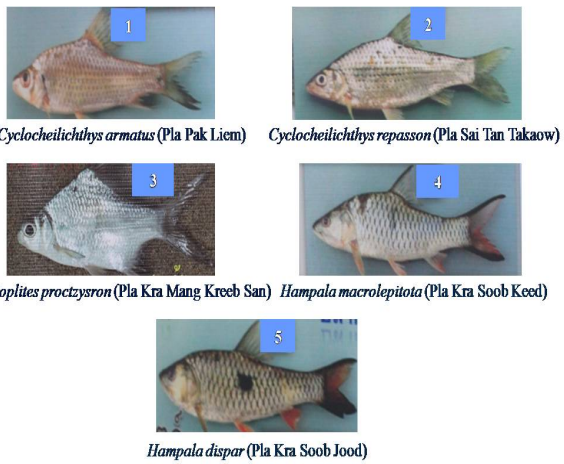

Figure 2. Cyprinoid Fishes were Collected from Various 32 Districts of Nakhon Ratchasima Province Thailand and Determined the *O. viverrini* Metaceria in this Study: 1) *Cyclocheilichthys Armatus*. 2) *C. Repasson*. 3) *Puntioplites Proctzysron*. 4) *Hampala Macrolepitota*. 5) *Hampala Dispar, Respectively*.

The fish were separated and categorized as species according to their morphological characteristics. For each species, fish samples were photographed and kept in a preserved state with labels, for further identification to species; the rest were used in the study. After thorough washing under tap water, and then inspected for metacercariae using the standard Pepsin-HCl digestion method (Sithithaworn et al., 1997; Srisawangwong et al., 1997). Fishes were digested using an acid pepsin solution (1 ml conc. hydrochloric acid, 1 g pepsin, 99 ml 0.85% sodium chloride solution for 1 1/2 hours at 37ºC. The digested material was then rinsed with 0.85% sodium chloride solution and examined for metacercariae. Encysted metacercariae were dissected from the cyprinoid fish under a stereomicroscope and placed in NSS. They were mounted individually on a glass slide, covered with a coverslip, and examined under amicroscope. Occasionally they were excysted by gentle pressing with the cover slip for more detailed examination in an extended condition. The metacercariae were identified with the aid of morphological criteria such as the size of cysts, folding body displaying vigorous movement within the cyst, prominent and clearly visible oral and ventral suckers (Vajrasthira et al., 1960; Scholz et al., 1991; Kaewkes, 2003). Finally, the numbers of cyprinoid fish were infected with *O. viverrini* metacercariae were recorded. The *O. viverrini* juveniles were removed, counted, fixed in 4% formalin, and stained with Borax's carmine or hematoxylin, dehydrated in an alcohol series, and mounted with Permount. The species were identified after Yamaguti (1958: 1963), Pearson (1964), Kliks & Tantachamrun (1974), Pearson & Ow-Yang (1982), Radomyos et al. (1990) and Kaewviyudth (1998).

The prevalence of *O. viverrini* metacercariae in fish was analyzed descriptively using Excel data sheet. The prevalence of infection was expressed as the percentage of the number of metacercariae-positive fish/total number of fish examined.

Results

A total of 640 cyprinoid fish classified into 5 species were collected during the study period. Among them, predominantly was *Cyclocheilichthys armatus* (140; 21.88%), and followed *Puntioplites proctzysron* (128; 20.00%), *C. repasson* (126; 19.68%), *Hampala macrolepitota* (124; 19.38%), and *Hampala dispar* (122; 19.06%) respectively (Table 1). The prevalence of infection of 5 cyprinoid fish species are summarized in Table 2.

The prevalence varied from 6.49% to 19.16% depending on fish species, with the mean prevalence of 12.34% in the total of 640 cyprinoid fishes. Among those, *Cyclocheilichthys armatus* and *C. repasson* showed relatively high (>15%) prevalence. The prevalence of other three cyprinoid species were <10%. Encysted metacercariae were oval in shape and covered with a thin single layer of cyst wall. The metacercariae had large oral and ventral suckers with a tubular excretory bladder at the posterior end, and were moving actively in the cyst (Figure 3).The excysted metacercariae; juvenile worms were lancet-shaped, about 0.24 mm long, and were morphologically identified as *O. viverini*. The morphological identification of metacercariae and the excysted worms was confirmed further by genetic analyses.

More than half (78.13%) of the natural reservoir sites were selected showed that cyprinoid fish were infected with *O. viverrini* metacercariae. The infection rates of *O. viverrini* in 32 districts for the year-round survey are

Table 1. Cyprinoid Fish Species Collected in and around Natural Reservoirs, 32 Districts of Nakhon Ratchasima Province Thailand

Scientific Name	Thai Name	No.
Cyclocheilichthys armatus	Pla Pak Liem	140(21.88%)
C. repasson	Pla Sai Tan Takaow	126(19.68%)
Puntioplites proctzysron	Pla Kra Mang Kreeb San	128(20.00%)
Hampala macrolepitota	Pla Kra Soob Keed	124(19.38%)
Hampala dispar	Pla Kra Soob Jood	122(19.06%)
Total		640(100%)

Table 2. The Prevalence of *O. viverrini* Metacercaria in 5 Cyprinoid Fish Species

Fish species	Number of fish infected/examined (%)
Cyclocheilichthys armatus	27/140 (19.16%)
C. repasson	23/126 (18.07%)
Puntioplites proctzysron	12/128 (9.64%)
Hampala macrolepitota	10/124 (8.13%)
Hampala dispar	7/122 (6.49%)

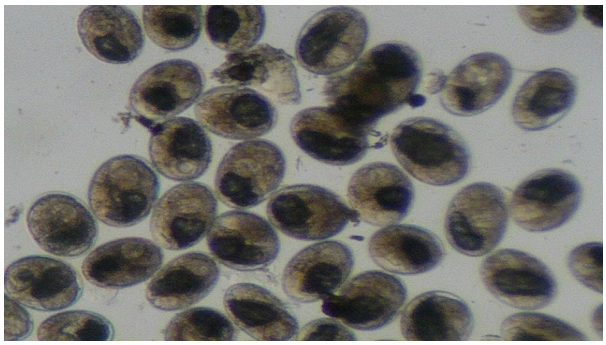

Figure 3. Encysted *O. viverrini* Metacercariae were Selected from the 5 Cyprinois Fish Species using Dissecting Microscope (Magnitude 400x)

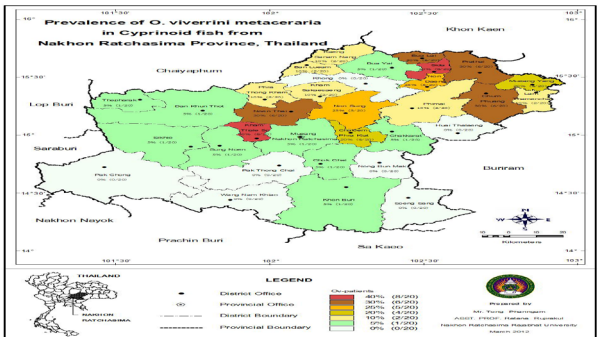

Figure 4. Prevalence of *O. viverrini* Metacercaria in Cyprinoid Fish from Various 32 Districts of Nakhon Ratchasima Province Thailand during February 2010-February

Table 3. The Prevalence of *O. viverrini* Metacercaria found in 5 Cyprinoid Fish Species Collected from Various 32 Districts, Nakhon Ratchasima Province Thailand

Reservoir sites	*O. viverrini* metacercarial infection
Mueang Nakhon Ratchasima	(1/20)
Khon Buri	(1/20)
Soeng Sang	(0/20)
Khong	(0/20)
Ban Lueam	(2/20)
Chakkarat	(1/20)
Chok Chai	(1/20)
Dan Khun Thot	(1/20)
Non Thai	(6/20)
Non Sung	(5/20)
Kham Sakaesaeng	(2/20)
Bua Yai	(1/20)
Prathai	(6/20)
Pak Thong Chai	(0/20)
Phimai	(2/20)
Huai Thalaeng	(0/20)
Chum Phuang	(6/20)
Sung Noen	(1/20)
Kham Thale So	(8/20)
Sikhio	(1/20)
Pak Chong	(0/20)
Nong Bun Mak	(0/20)
Kaeng Sanam Nang	(2/20)
Non Daeng	(5/20)
Wang Nam Khiao	(0/20)
Thepharak	(1/20)
Mueang Yang	(4/20)
Phra Thong Kham	(2/20)
Lam Thamenchai	(2/20)
Bua Lai	(6/20)
Sida	(8/20)
Chaloem Phra Kiat	(4/20)

shown in Table 3 and Figure 4. In Kham Thale So and Sida districts were found the highest infection rate, 8 of 20 fish in 5 species harbored metacercariae of *O. viverrini* while in 6 districts mainly Nong Bun Mak, Wang Nam Khiao, Huai Thalaeng, Pak Chong, Soeng Sang Khong, and Pak Thong Chai were not infected.

Discussion

Opisthorchis viverrini, fish-borne trematode infection causes a serious public-health problem in Southeast Asia mainly Thailand, Cambodia, and Laos PDR. In Thailand, opisthorchiasis is a major public health problem in the Northeastern and Northern region, and it is estimated that 5.5 million people are infected with the causative parasite (Sadun, 1955; Jongsuksuntigul & Imsomboon, 2003; Sripa et al., 2010). The infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis (Harinasuta et al., 1984). The experimental and epidemiological evidences strongly indicate the liver fluke infection in the etiology of cholangiocarcinoma (CCA); the bile duct cancer (Thamavit et al., 1978; IARC, 1994). Multi-factorial etiology of CCA, mechanical damage, parasite secretions, and immunopathology may enhance cholangiocarcinogenesis (Flavell, 1981; Sripa et a., 2007). Human have been infected *O. viverrini* by ingesting undercooked fish containing infective metacercariae, this is very common in the northeastern and northern regions particularly in rural areas. Three types of preparations contain uncooked, small and medium-sized, fish: *koi pla*, eaten soon after preparation; moderately fermented *pla som*; stored for few days to weeks; *pla ra* extensively fermented, highly salted fish, stored for at least 2-3 months (Sithithaworn et al., 1997). *O. viverrini* infection is commonly found in the northern and northeastern regions of Thailand. Several species of cyprinoid fish have been reported as the second intermediate hosts of *O. viviverrini* (Harinsuta & Vajrasthira, 1960; Wykoff et al., 1965; Vichasri et al., 1982). In our study, 5 species of cyprinoid fish were found infected with *O. viverrini* metacercariae. Among the metacercariae, *O viverrini* had the highest number found throughout the year-round survey. *O. viverrini* was predominantly found in several species of fish namely *Cyclocheilichthys armatus*, *C. repasson*, *Puntioplites proctzysron, Hampala macrolepitota,* and *Hampala dispar*, respectively. These figures showed the Similarity to the other research such as Khemphavanh et al (2009) concerning frequent infection in these cyprinoid fish species. Our study is the first report of *O viverrini* infected fish in Nakhon Ratchasima province Thailand that is different to other known reports. Nithiuthai et al. (2002) reported that no fish infected with metacercariae were found. However, they had surveyed in a small scale and specific sample with short period of time in the Lum Cha Muak stream, Nakhon Ratchasima province; the study was conducted during May 2000. Many factors can affect the prevalence of metacercariae infection in fish, including the setting, season, type, and number of parasites and fish species. Generally, the highest metacercarial infection rate in fish is found during late rainy season in October. The low prevalence in their study, in May, might therefore be lower than the actual peak prevalence in the late rainy season (Vichasri et al., 1982, Sithithaworn et al., 1997). Our study is set up to cover the areas of province and also select the samples throughout the year-round survey. Moreover, the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand have been reported that Nakhon Ratchasima is still the site to find the patients infected with *O. viverrini* (2010: 2011). Humans are not the only source of *O. viverrini*. Fish-eating animals can also be involved in their life cycle (Faust & Nishigori, 1926; Pearson 1964; Ditrich et al., 1990). Food-borne trematodes can be assessed by the prevalence of infections in several animal life cycles.

The distribution of food-borne trematodes depends on many factors, such as the relationship between host, parasite, environment, and traditional food habits (WHO, 1995). Kham Thale So and Sida district showed the highest prevalence of metacercarial infection in fish. It is possible that this area contained highest number of patients and it's also a high-risk area in Nakhon Ratchasima Province for *O. viverrini* infection. In addition, Thai traditional dishes, such as 'Pla-som' and 'Koy-Pla', are the most common sources of infection in people. These raw-fish

dishes cannot induce degeneration of the contaminated metacercariae a short period of time (Sukontason et al., 1998; Wiwanitkit et al., 2003), and there is a tendency that the prevalence of human infection will increase because of eating raw fish. We have no data concerning prevalence of *O. viverrini* infection in 32 districts, Nakhon Ratchasima province. Further study, to compare and determine the relationship, Should be done for screen the current status, their knowledge, attitude, and behavior of people to *O. viverrini*.

The result of this study was a practical understanding of fish-borne trematode infections in Nakhon Ratchasima Province, Thailand. Infection rates and distribution of *O. viverrini* metacercarial infection among certain species of cyprinoid fish were demonstrated in the Sikhio and Chaloem Phra Kiat reservoires. Further research studies in endemic areas of Nakhon Ratchasima Province, Thailand, should consider interventions using health-education and sanitation-improvement approaches in the liver-fluke control program, to change eating and defecation habits, and thereby reduce the occurrence of *O. viverrini* infection.

In conclusion, our study showed the update prevalence of *O. viverrini* metacercaria in cyprinoid fish in Nakhon Ratchasima Province. This may be caused by unsanitary rural latrines. Eggs and larvae of *O. viverrini* can contaminate reservoirs by running water and precipitation. *O. viverrini* can infect humans who eat undercooked fresh-water fish. The data from our study can help rural people who use this contaminated water to prevent parasitic infections in the future.

Acknowledgements

We would like to thank the Institute of Research and Development, Suranaree University of Technology for supported the grants Fiscal year 2010. We also thank the staff of the Tropical Disease Research Center, Department of Pathology, Faculty of Medicine, Khon Kaen University, in particular Mr.Suwit Balthaisong, Mr. Manop Sripa, for their assistance. We are grateful to Prof.Dr.Anuvat Roongpisuthipong for his consultations and editing the manuscript.

References

Bhamarapravati N, Thammavit W, Vajrasthira S (1978). Liver changes in hamsters infected with a liver fluke of man, *Opisthorchis viverrini*. *Am J Trop Med Hyg*, **27**, 787-94.

Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand (2010). Liver Fluke. Annual Report 2010. [Online]. [Accessed 10 February 2010]. Available at URL: http://www.boe.moph.go.th/

Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand (2011). Liver Fluke. Annual Report 2011 [Online]. [Accessed 10 February 2010]. Available at URL: http://www.boe.moph.go.th/

Flavell DJ (1981). Liver-fluke infection as an aetiological factor in bile-duct carcinoma of man. *Trans R Soc Trop Med Hyg*, **75**, 814-24.

Flavell DJ, Flavell SU (1986). *Opisthorchis viverrini*: pathogenesis of infection in immunodeprived hamsters. *Parasite Immunol*, **8**, 455-66.

Harinasuta T, Riganti M, Bunnag D (1984). *Opisthorchis viverrini infection*: pathogenesis and clinical features. *Arzneimittelforschung*, **34**, 1167-9.

Harinasuta C, Vajrasthira S (1960). Opisthorchiasis in Thailand. *Am J Trop Med Hyg*, **54**, 100-5.

IARC (1994). Infection with liver flukes (*Opisthorchis viverrini*, *Opisthorchis felineus* and *Clonrochis sinensis*). *IARC Monogr Eval Carcinog Risks of Hum*, **61**, 121-75.

Jinawath N, Chamgramol Y, Furukawa Y, et al (2006). Comparison of gene expression profiles between *Opisthorchis viverrini* and non-*Opisthorchis viverrini* associated human intrahepatic cholangiocarcinoma. *Hepatology*, **44**, 1025-38.

Jongsuksuntigul P, Imsomboon T (2003). Opisthorchiasis control in Thailand. *Acta Trop*, **88**, 229-32.

Kaewkes S (2003). Taxonomy and biology of liver flukes. *Acta Trop*, **88**, 177-86.

Kaewviyudth S (1998). Species and Distribution of Monogenean Parasite of Some Cyprinid Fish. Graduate School, Kasetsart University, 307 p. PhD Thesis.

Khemphavanh M, Chalit K, JitraW, Prayong R (2009). *Opisthorchis viverrini* Metacercariae in Cyprinoid Fish from Three Rivers in Khammouane Province, Lao PDR. *J Trop Med Parasitol*, **32**, 23-9.

Kim YI (1984). Liver carcinoma and liver fluke infection. *Arzneimittelforschung*, **34**, 1121-6.

Kliks M, Tantachamrun T (1974). Heterophyid (Trematoda). Parasites of cats in north Thailand, with notes on a human case found at necropsy. *Southeast Asian J Trop Med Public Health*, **5**, 547-55.

Migasena P, Reaunsuwan W, Changbumrung S (1980). Nitrates and nitrites in local Thai preserved protein foods. *J Med Assoc Thai*, **63**, 500-5.

Miwa M, Stuehr DJ, Marletta MA, Wishnok JS, Tannenbaum SR (1987). Nitrosation of amines by stimulated macrophages. *Carcinogenesis*, **8**, 955-8.

Nguyen T, Brunson D, Crespi CL, et al (1992). DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc Natl Acad Sci USA*, **89**, 3030-4.

Nithiuthai S, Suwansaksri J, Wiwanitkit V, Chaengphukeaw P (2002). A Survey of Metacercaria in Cyprenoid Fish in Nakhon Ratchasima, Northeast Thailand. *Southeast Asian J Trop Med Public Health*, **33**, 130-05.

Oshima Y, Kobayashi K, Hidaka C, Izu S, Imada N (1994). Differences in the drug-metabolizing enzyme activities among fish and bivalves living in waters near industrial and non-industrial areas. *Bull Environ Contam Toxicol*, **53**, 106-12.

Pairojkul C, Shirai T, Hirohashi S, et al (1991). Multistage carcinogenesis of liver-fluke-associated cholangiocarcinoma in Thailand. *Princess Takamatsu Symp*, **22**, 77-86.

Pearson JC (1964). A revision of the subfamily Haplorchinae looss, 1899 (Tremetoda : Heterophyidae). *Parasitology*, **54**, 601-76.

Pearson JC, Ow-Yang CK (1982). New species of Haplorchis from Southeast Asia, together with keys to the Haplorchis-Group of Heterophyid trematodes of the region. *Southeast Asian J Trop Med Public Health*, **13**, 35-60.

Radomyos B, Wongsaroj T, Wilairatana P (1998). Opisthorchiasis and intestinal fluke infections in northern Thailand. *Southeast Asian J Trop Med Public Health*, **29**, 123-7.

Sadun EH (1995). Studies on *Opisthorchis viverrini* in Thailand. *Am J Hyg*, **62**, 81-115

Satarug S, Haswell-Elkins MR, Sithithaworn P, et al (1998). Relationships between the synthesis of *N*-nitrosodimethylamine and immune responses to chronic

infection with the carcinogenic parasite, *Opisthorchis viverrini*, in men. *Carcinogenesis*, **19**, 485-91.

Sato M, Thaenkham U, Dekumyoy P, Waikagul J (2009). Discrimination of *O. viverrini*, *C. sinensis*, *H. pumilio* and *H. taichui* using nuclear DNA-based PCR targeting ribosomal DNA ITS regions. *Acta Trop*, **109**, 81-3.

Sithithaworn P, Pipitgool V, Srisawangwong T, Elkins DB, Haswell-Elkins MR (1997). Seasonal variation of *Opisthorchis viverrini* infection in cyprinoid fish in north-east Thailand: implications for parasite control and food safety. *Bull World Health Organ*, **75**, 125-31

Sriamporn S, Pisani P, Pipitgool V, et al (2004). Prevalence of *Opisthorchis viverrini* infection and incidence of cholangiocarcinoma in Khon Kaen, Northeast Thailand. *Trop Med Int Health*, **9**, 588-94.

Srianujata S, Tonbuth S, Bunyaratvej S, et al (1987). High urinary excretion of nitrate and *N*-nitrosoproline in opisthorchiasis subjects. *IARC Sci Publ*, **84**, 544-6.

Sripa B, Kaewkes S, Sithithaworn P, et al (2007). Liver fluke induces cholangiocarcinoma. *PLoS Med*, **4**, 201.

Sripa B, Kaewkes S, Intapan PM, Maleewong W, Brindley PJ (2010). Food-borne trematodiases in Southeast Asia: epidemiology, pathology, clinical manifestation and control. *Adv Parasitol*, **72**, 305-50.

Srisawangwong T, Sithithaworn P, Tesana S (1997). Metacercariae isolated from cyprinoid fishes in Khon Kaen District by digestion technique. *Southeast Asian J Trop Med Public Health*, **28**, 224-6.

Srisraluang T, Boriboon P (1998). Nitrosamines in meat products. *J Nutr Assoc Thai*, **21**, 163-85.

Srivatanakul P, Ohshima H, Khlat M, et al (1991). *Opisthorchis viverrini* infestation and endogenous nitrosamines as risk factors for cholangiocarcinoma in Thailand. *Int J Cancer*, **48**, 821-5.

Sukontason K, Methanitikorn R, Sukontason K, Piangjai S, Choochote W (1998). Viability of metacercariae in northern Thai traditional foods. *Southeast Asian J Trop Med Public Health*, **29**, 714-6.

Tansurat P (1971). Opisthorchiasis. In: Marcial-Rojas RA., editors. Pathology of Protozoal and Helminthic Diseases. Baltimore: Williams and Wilkins, 36-545.

Thamavit W, Bhamarapravati N, Sahaphong S, Vajrasthira S, Angsubhakorn S (1978). Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini*-infected Syrian golden hamsters. *Cancer Res*, **38**, 4634-9.

Thamavit W, Bramarapravati N, Sahaphong S (1978). Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini* infected Syrian golden hamsters. *Cancer Res*, **38**, 4634-9.

Vichasri S, Viyanant V, Upatham ES (1982). *Opisthorchis viverrini*: intensity and rates of infection in cyprinoid fish from an endemic focus in northeast Thailand. Southeast *Asian J Trop Med PublicHealth*, **3**, 138-41.

Viranuvatti V, Stitnimankarn T (1972). Liver fluke infection and infestation in Southeast Asia. In:Popper H, Schaffner F.,editors.Progress in Liver Disease. New York: Grune and Stration, 537–547.

WHO (1995). Control of foodborne trematode infections. WHO Tech Rep Ser, 157.

Wiwanitkit V, Nithiuthai S, Suwansaksri J (2003). Motility of minute intestinal fluke, *Haplorchinae* spp. Metacercariae in fish dishes prepared by different uncooked methods [Online]. [Accessed 29 September 2003]. Available at URL: http://www.medscape.com/viewarticle1429092.

Wykoff DE, Chittayasothorn K, Winn MM (1966). Clinical manifestation of *Opisthorchis viverrini* infection in Thailand. *Am J Trop Med Hyg*, **15**, 914-8.

Yamaguti S (1958). Systema helminthum. Vol I. The digenetic trematodes of vertebrates. Part I & II. New York: Interscience Pub.