THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Veterinary Parasitology)

DEGREE

Veterinary Parasitology — **FIELD**

Parasitology — **DEPARTMENT**

TITLE: Fish-borne Trematodes in Cultured Nile Tilapia (*Oreochromis niloticus*) and Wild-Caught Fish from Thailand

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THESIS

FISH-BORNE TREMATODES IN CULTURED NILE TILAPIA (*OREOCHROMIS NILOTICUS*) AND WILD-CAUGHT FISH FROM THAILAND

BENJAMAPORN WIRIYA

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Veterinary Parasitology)
Graduate School, Kasetsart University
2013

Benjamaporn Wiriya 2013: Fish-borne Trematodes in Cultured Nile Tilapia (*Oreochromis niloticus*) and Wild-Caught Fish from Thailand. Master of Science (Veterinary Parasitology), Major Field: Veterinary Parasitology, Department of Parasitology. Thesis Advisor: Associate Professor Sathaporn Jittapalapong, Ph.D. 81 pages.

Fish-borne zoonotic trematode (FZT) infections affect the health of more than 18 million people around the world, particularly in Asian countries. Nile tilapia (Oreochromis niloticus) is a white meat fish for which there is an increasing national and international market. The objective of this study was to determining the prevalence of FZT metacercariae infections in Nile tilapia from cage and pond aquaculture systems and in wild-caught fish from Suphan Buri, Nakhon Pathom and Chachoengsao provinces, Thailand. Fish were collected from four cages in Suphan Buri and four ponds in Nakhon Pathom provinces between September-October 2011 and in April-July 2012 and wild-caught fish were collected in July 2012. All fish was examined for metacercariae by a pepsin digestion and the metacercariae were identified using morphological and molecular methods. During the first sampling of tilapia, the prevalence of metacercariae in fish which were 2.5% in cage culture and 10% from pond culture systems. During the second sampling, 2.0% of tilapia from cage culture whereas none of fish from ponds contained metacercariae. A total of 80 out of 150 wild-caught fish (53.3%) was found infected with metacercariae, mostly the zoonotic trematodes had species Stellantchasmus falcatus, Haplorchis pumilio and Procerovum varium. The results revealed a low risk for FZT in cultured Nile tilapia from cage and pond aquaculture systems. However, the high prevalence of FZT in wild-caught fish indicated a high potential for spillover from wild reservoir hosts and underscores the need for vigilance and good management practices by the aquaculture sector.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

For the successful completion of this work, I am deeply indebted to my committee members,

To Assoc. Prof. Dr. Sathaporn Jittapalapong, my advisor, for advice, encouragement, valuable suggestion for completely writing of thesis and his kindness.

To Dr. Kriengkrai Satapornvanit, my co-advisor, for every valuable suggestion, kindness and generous facilities. My research would not have been accomplished without the devoted and dedicated help from him.

To Dr. Jesper Hedegaard Clausen from SEAT project, Dr. Tawin Inpankaew from the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University and Dr. Urusa Thaenkham from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, who help for valuable guidance and comments, writing of thesis and our kindness.

I would like to thank the staff at Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University and laboratory colleagues whom gave me the generous help in everything and kind suggestion. I do thank my graduated friends from Veterinary Parasitology, Faculty of Veterinary Medicine, Kasetsart University, for their friendship, help, and cheerfulness.

This study was supported by the funding of the Graduate School and the EU funded FP7 project on Sustaining Ethical Aquaculture Trade (SEAT) especially the office in Thailand.

Finally, I sincerely thank my parents who always gave me the chance to do everything, wish well, support and encouragement to me all the time.

Benjamaporn Wiriya

June 2013

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LIST OF ABBREVIATIONS

et al	=	*et alli*
Lao PDR	=	The Lao People's Democratic Republic
N	=	Nitrogen
P	=	Phosphorus
ha	=	hectare
DM	=	dry matter
HP	=	Horsepower Aeration
m^2	=	meter square
m^3	=	cubic meter
kg	=	kilogram
etc.	=	et cetera
HCl	=	hydrochloric acid
g	=	gram
ml	=	milliliter
°C	=	degree Celsius
hrs	=	hours
mm	=	millimeter
min	=	minute
i.e.	=	id est
18S rDNA	=	small subunits of ribosomal DNA
28S rDNA	=	large subunits of ribosomal DNA
cm	=	centimeter
PCR	=	Polymerase chain reaction
µl	=	microliter
U	=	unit
mM	=	millimolar
$MgCl_2$	=	magnesium chloride

LIST OF ABBREVIATIONS (Continued)

dNTP	=	deoxynucleotide triphosphate
DNA	=	deoxyribonucleic acid
sec	=	second
P	=	Probability value
bp	=	base pairs
pH	=	negative logarithm of hydrogen ion activity
DW	=	distilled water

FISH-BORNE TREMATODES IN CULTURED NILE TILAPIA (*OREOCHROMIS NILOTICUS*) AND WILD-CAUGHT FISH FROM THAILAND

INTRODUCTION

Aquaculture is now accounting for half of all seafood products for human consumption, globally (FAO, 2012). Traditionally, fish has been an important and affordable source of animal protein in Southeast Asia including Thailand, and is now also becoming increasingly globally traded mostly as frozen fillets and frozen whole fish (Grundy-Warr *et al*., 2011). Nile tilapia (*Oreochromis niloticus*) is an important freshwater species for aquaculture in Thailand as a white meat fish which supplies high quality source of protein. Export of Nile tilapia from Thailand has increased from 7,622 tons in 2004 to 221,042 tons in 2009 out of a total of 521,880 tons produced in freshwater aquaculture (Hinsui *et al*., 2006; Suwanmanee *et al*., 2012). In Thailand, Nile tilapia is cultured in ponds, cages or pens where they are fed rice bran, plant material, and commercial pelleted feed.

Fish-borne zoonotic trematodes (FZT) can be transmitted to humans, to a wide range of mammals, and to fish-eating birds by eating of raw or improperly cooked fish containing the infective metacercarial stage of the parasite (Chai, 2007). Most FZT come from the families Heterophyidae (intestinal trematodes), Echinostomatidae (intestinal trematodes), and Opisthorchiidae (liver flukes) (Chai *et al*., 2005) which are highly prevalent in many countries, particularly in Thailand, Lao PDR, Cambodia and Vietnam where food traditions including eating raw or improperly cooked fish dishes (Touch *et al*., 2009; Thuy *et al*., 2010; Sripa *et al*., 2011). Food and human health hazards associated with FZT may have noticeable adverse economic and public health effects in these countries (Phan *et al*., 2010a).

Recent investigations have shown that the risk of FZT transmission in fish culture can be significantly reduced when implementing management practices that

reduce fecal pollution of ponds and control snail populations, in particular in nurseries where fingerlings often show high levels of infections (Clausen *et al*., 2012). Therefore, the production of FZT-free fish from aquaculture for human consumption should be a key objective for the aquaculture industry.

OBJECTIVE

To compare the prevalence and intensity of Fish-borne trematodes in Nile tilapia raised in cage and pond aquaculture systems and wild-caught fish from Suphan Buri, Nakhon Pathom and Chachoengsao provinces, Thailand.

LITERATURE REVIEW

1. Overview of Nile tilapia

1.1 History of Nile tilapia

The Nile tilapia (*Oreochromis niloticus*) was one of the first fish species cultured. Ancient picture from Egyptian tombs suggested that Nile tilapia were cultured more than 3,000 years ago. Tilapias have been called “Saint Peter’s fish” in reference to biblical passages about the fish fed to the multitudes. The Nile tilapia is still the most widely cultured species of tilapia in Africa (Popma and Masser, 1999).

1.2 Taxonomy

“Tilapia” is the generic name of a group of cichlids endemic to Africa. The group consists of three aquaculturally important genera *Oreochromis, Sarotherodon* and *Tilapia*. Several characteristics have been used to distinguish among three genera, but possibly the most critical relates to reproductive behavior. All Nile tilapia species are nest builders and their fertilized eggs are guarded in the nest by a brood parent. Species of both *Sarotherodon* and *Oreochromis* are mouth brooders; eggs are fertilized in the nest but parents immediately pick up the eggs in their mouths and hold them through incubation and for several days after hatching. In *Oreochromis* species, only females practice mouth brooding, while in *Sarotherodon* species, either male or both male and female are mouth brooders. During the last half century, fish farmers throughout the tropical and semi-tropical world have begun farming Nile tilapia. Today, all commercial Nile tilapia outside of Africa belong to the genus *Oreochromis,* and more than 90% of all commercial tilapia farms outside of Africa are Nile tilapia. Less common species are Blue tilapia (*O. aureus*), Mozambique tilapia (O. *Mossambicus*) and the Zanzibar tilapia (*O. urolepis hornorum*). The scientific names of Nile tilapia species have been revised for the last 30 years, creating some confusion.

The scientific name of the Nile tilapia has been named as *Tilapia nilotica*, *Sarotherodon niloticus*, and currently as *Oreochromis niloticu*s (Popma and Masser, 1999).

1.3 Physical characteristics

Nile tilapia (Figure 1) are shaped much like sunfish or crappie but can be easily identified by an interrupted lateral line characteristic of the Cichlid family of fishes. They are laterally compressed and deep-bodied with long dorsal fins. The forward portion of the dorsal fin is heavily spine. Spines are also found in the pelvis and anal fins. There are usually wide vertical bars down the sides of fry, fingerlings, and sometimes adults (Popma and Masser, 1999).

1.4 Feeding behavior

Nile tilapia ingests a wide variety of natural food organisms, including plankton, some aquatic macrophytes, planktonic and benthic aquatic invertebrates, larval fish, detritus, and decomposing organic matter. With heavy supplemental feeding, natural food organisms typically account for 30-50 % of tilapia growth. (In supplementally fed channel catfish only 5-10% of growth can be traced to ingestion of natural food organisms). Nile tilapias are often considered filter feeders because they can efficiently harvest plankton from the water. However, tilapias do not physically filter the water through gill rakers as efficiently as true filter feeders such as gizzard shad and silver carp. The gills of Nile tilapia secrete a mucous that traps plankton and then, the plankton-rich mucous, or bolus, is then swallowed. Digestion and assimilation of plant material occurs along the length of the intestine (usually at least six times the total length of the fish). The Mozambique tilapia is less efficient than the Nile tilapia at harvesting planktonic algae (Popma and Masser, 1999).

Figure 1 Nile tilapia

Source : Saowakontha (2005)

1.5 History of Nile tilapia in Thailand

On March 25, 1965, Emperor Akihito, as His Royal Highness Crown Prince of Japan at that time, sent, as the royal tribute to His Majesty the King of Thailand, 50 Nile-Tilapias (or called “Pla Nil” in Thai) (Pullin *et al*., 1987) with an average body length of 9 cm and average weight of 14 grams. At first, His Majesty the King allowed them to be fed in a pond with an area of approximately 10 square meters in Chitlada garden, Royal Palace. After more than 5 months, there seemed to be a lot of fry showing up. His Majesty then commanded royal garden staff to dig another 6 new ponds with an average area of approximately 70 square meters, which His Majesty consequently transferred the fish from the old ponds to these 6 new ponds by himself on September 1, 1965. Subsequently, His Majesty demanded the Department of Fisheries to send technical staff to monitor their growth on a monthly basis (Sukmanomon *et al*., 2012).

Figure 2 His Majesty King Bhumipol gives Nile tilapia fingerlings to the Director General, Department of Fisheries for breeding and distribution.
The fish, which were cultured in the Royal Palace for a year, were a gift from Prince Akihito of Japan.

Source : Na Mahasarakarm (2007)

1.6 Source of broodstock of Nile tilapia (DoF, 2013a)

The Department of Fisheries, Institute of Genetics and Development and the Nile tilapia fish has species called tilapia strain Chitralada which are the breeding of a new one of Nile tilapia strains as follows 3 strains.

1.6.1 Nile tilapia strain Chitralada 1

Nile tilapia strain Chitralada 1 is the breeding of Nile tilapia comes from Nile tilapia species selection within the family (within family selection) begin the process of breeding from 1985 to the current generation 7, the test species and found that the rate of growth was 22% higher than original Nile tilapia.

1.6.2 Tilapia strain Chitralada 2

Tilapia strain Chitralada 2 is the development of tilapia species from Nile tilapia species Chitralada 1 by modifying the genes in the chromosome as a breeder to have "YY" is called "YY - Male" or Super-male. Breed with the normal breeding male tilapia fish is called "Nile tilapia Chitralada 2 " which features a male who has sex chromosomes is "XY" headers smaller body width white thick and tight tasty aged 6 to 8 months to grow a size 2-3 per kilogram. The rate of growth was 45% higher than original Nile tilapia.

1.6.3 Tilapia strain Chitralada 3

Fish-breeding of tilapias were mixed in groups by adding the Nile tilapia hybrid groups resulting from mating between tilapia species Chitralada and tilapia species other seven species, including Egypt, Ghana, Kenya, Singapore Senegal, Israel and Taiwan, which is growing fast and have high survival rates. In different culture conditions were created a base population. By the way, the same as the current second generation, and called "Nile tilapia species Chitralada 3" which have features a head small body, large yellow, cream thick and tight tasty aged 6 to 8 months. Size can grow 3 to 4 per kg. The rate of growth was 40% higher than original Nile tilapia.

1.7 Ongrowing technique for production (FAO, 2013)

1.7.1 Ponds

Pond culture of tilapia is conducted with a variety of inputs such as agricultural by-products (brans, oil cakes, vegetation and manures), inorganic fertilizers and feed. Annual fish yields using tilapia in polyculture with other fish or shrimp, high levels of agricultural by-products and good stock management can reach or exceed 5 tons/ha. In monoculture tilapia systems, animal manures provide nutrients that stimulate the growth of protein-rich phytoplankton, which is consumed by filter feeding Nile tilapia. However, the nutrient content of manures varies. Buffalo manure

has much lower nutrient compared to duck or chicken manure. Obtaining sufficient nutrient from manures poses a danger of oxygen depletion from excessive loading of organic matter. Therefore, a combination of manures with inorganic fertilizers is used in low-input production systems. In Thailand, applying chicken manure weekly at 200-250 kg DM (dry matter)/ha and supplementing it with urea and triple super phosphate (TSP) at 28 kg N/ha/week and 7 kg P/ha/week produces a net harvest 3.4-4.5 tons/ha in 150 days at a stocking rate of 3 fish/m² or an extrapolated net annual yield of 8-11 tons/ha.

To reduce production costs for domestic markets in developing countries, two strategies are followed: delayed feeding and supplementary feeding. In Thailand, tilapia are stocked at 3 fish/m^2 and grown to 100-150 g in about 3 months with fertilizer alone, and then given supplemental feeding at 50% until the fish reach 500 g. Net harvest averages 14 tons/ha, which is equivalent to a net annual yield of 21 tons/ha. In Honduras, a yield of 4.3 tons/ha can be obtained with weekly application of 500 kg DM/ha of chicken litter and feed application of 1.5% of fish biomass for 6 days a week. However, this management regime is less profitable than the use of chicken litter and urea.

Many semi-intensive farms rely almost exclusively on high quality feeds to grow tilapia in ponds. Male tilapia are stocked at 1-3 fish/m^2 and grown to 400-500 g in 5-8 months, depending on water temperature. Normal yields range from 6-8 tons/ha/crop but yields as high as 10 tons/ha/crop are reported in northeast Brazil, where climate and water quality are ideal. Dissolved oxygen is maintained by exchanging 5-15% of the pond volume daily. Higher yields of large fish (600-900 g) are obtained in other regions by using high quality feed (up to 35% protein), multiple grow-out phases (restocking at lower densities up to three times), high water exchange rates (up to 150% of the pond volume daily) and continuous aeration (up to 20 HP/ha). Fish produced through these expensive methods are generally filleted and sold in export markets.

1.7.2 Floating cages

The culture of Nile tilapia at high densities in floating cages is practiced in large lakes and reservoirs of water. Mesh size has a significant impact on production and should be 1.9 cm or greater to maintain free circulation of water.

Cage culture offers several important advantages. The breeding cycle of Nile tilapia is disrupted in cages, and therefore mixed-sex populations can be reared in cages without the problems of recruitment and stunting. Eggs fall through the cage bottom or do not develop if they are fertilized.

Cages vary widely in size and construction materials. In Thailand, cage volumes and stocking densities range from 4 m^3 cages stocked at 200-300 fish/m^3 to cages 100 m^3 or larger stocked at 25-50 fish/m^3. Yields range from 50 kg/m^3 in 100 m^3 cages to 150 kg/m^3 in 4 m^3 cages. The fish are fed extruded feeds with 24-34% crude protein. Bacterial infections such as *Streptococcus* spp. are one of major problem that decrease the survival to 65%. Annual yields at final densities of 160-350 fish/m^3 are 76-116 kg/m^3.

1.8 Nutritional Profile of Nile tilapia

The nutritional profile of Nile tilapia contain a rich source of protein, phosphorus, potassium, selenium, niacin, vitamin B-12, saturated fat, omega-3 fatty acids, calories, carbohydrates, and sodium. Figure 3 provides a brief comparison of select nutrients between Nile tilapia, ground beef (85% lean meat/15% fat), and chicken breast (Mjoun *et al*., 2010).

1.9 Nile tilapia market in Thailand

Nile tilapia is a white meat fish that has the potential to export more. This fish is an important freshwater aquacultured fish in Thailand, exported to Japan, USA,

Italy etc. The export items are frozen whole fish, fresh and frozen fillet (Hinsui *et al*., 2006). Limitations of the operators to export are looking for nor off flavor, and the standard size and price. This can be taken as a guide for aquaculture industry development. Other than, requiring of fish culture is based on food safety, which means that farming must be followed a standard pattern of farm (DoF, 2013b).

The export items are frozen whole fish, fresh and frozen fillet. There is an increasing national and international market for Nile tilapia. In Thailand, the total volume of Nile tilapia export in 2003 and 2004 were 4,708 tons and 7,622 tons (Hinsui *et al*., 2006), whereas in 2009 the production of Nile tilapia was 221,042 tons with a total freshwater aquaculture production of 521,880 tons (Suwanmanee *et al*., 2012).

Nutrient, per 100 g	Tilapia	Beef	Chicken
Energy, kcal	96	215	114
Protein, g	20.1	18.6	21.2
Total fat, g	1.7	15	2.59
Minerals, mg			
Ca	10	15	5
P	170	171	210
Mg	27	18	26
K	302	295	370
Na	52	66	116
Se	41.8	15.8	32.0
Zn	0.33	4.48	0.58
Fe	0.56	2.09	0.37

Nutrient, per 100 g	Tilapia	Beef	Chicken
Vitamins			
Thiamin, mg	0.04	0.04	0.06
Niacin, mg	3.90	4.65	10.43
B-12, mcg	1.58	2.17	0.20
Fatty acids, g			
Saturated	0.77	5.87	0.57
Monounsaturated	0.65	6.55	0.76
Polyunsaturated	0.48	0.43	0.40
EPA	0.007	0	0.002
DPA	0.057	0	0.004
DHA	0.113	0	0.003
Cholesterol, mg	50	68	64

* Composition of raw tilapia, raw ground beef (85% lean meat/15% fat), and raw chicken breast; data adapted from ARS, 2009.

Figure 3 Nutrient composition of tilapia compared to other meats. * Cholesterol refers to total cholesterol

Source : Mjoun *et al*. (2010)

2. Fish-borne zoonotic trematodes (FZT)

The Fish-borne zoonotic trematodes (FZT) include many species, especially representatives of the families Heterophyidae, Echinostomatidae, and Opisthorchiidae. Although their metacercarial cysts are easily inactivated by heating at 60°C or freezing to –20°C, they are highly prevalent in many regions, especially in Asia where food traditions include eating raw or improperly cooked fish dishes. The fish-borne liver flukes *Clonorchis sinensis*, *Opisthorchis viverrini*, and *O. felineus* cause cholangitis, pancreatitis, and cholangiocarcinoma in humans. During the past 10-20 years, the second large group of FZT, the so-called minute intestinal flukes, has been increasingly recognized as widely distributed and a cause of illness. The exponential increase in aquaculture is the major cause of the emergence of FZT in east and southeast Asia. For example, in the People's Republic of China, the land devoted to aquaculture increased 75% (to 4.9 million hectares) since 1970, accompanied by a tripling of cases of infection with *C. sinensis*. The association of *O. viverrini* in Thailand and Lao People's Democratic Republic with fisheries has also been reported (Sripa *et al*., 2011).

The FZT live and develop to adult flukes in the liver or intestines of the final host and produce eggs that are excreted into the environment through faeces. Humans and animals acquire the FZT infection through consumption of raw, inadequately cooked, dried, salted or pickled fish that harbor infective metacercariae stages that shown in figure 4 and 5 (Sripa *et al*., 2011).

2.1 Biology of the fish-borne zoonotic trematodes

Generally, most of digenetic trematodes are hermaphroditic in which worms have both male and female reproductive organs. After fertilization, the zygote within the egg develops into a ciliated larva, the miracidium within the uterus. Embryonated eggs are released periodically and pass out with host excreta. In aquatic environment, where they hatch into a free swimming stage called a miracidum, which penetrates aquatic snails. Once in a snail, each miracidium produces numerous

embryos of the next larval stage, or redia. Each redia, in turn, contains numerous embryos of a third larval stage, which is either another redia or a swimming larval stage called a cercaria. This results in a kind of embryonic amplification in which hundreds, thousands, or even hundreds of thousands of cercariae are ultimately produced. Thus the snails serve as a kind of "incubator" for embryonic amplificiation. The cercariae exit the snail, in response to particular environmental stimuli (e.g. light and/or temperature), and they shed their propulsive tails swim vigorously around until they encounter a suitable second intermediate host, the freshwater fish. They are now called metacercariae, the infective stage. Within the cyst, the metacercariae have remained its potential to continue the development which will be completed when the cyst is consumed by their final hosts, fish-eating birds or mammals including humans that shown in figure 4 and 5 (Kaewkes, 2003; Sripa *et al.*, 2011).

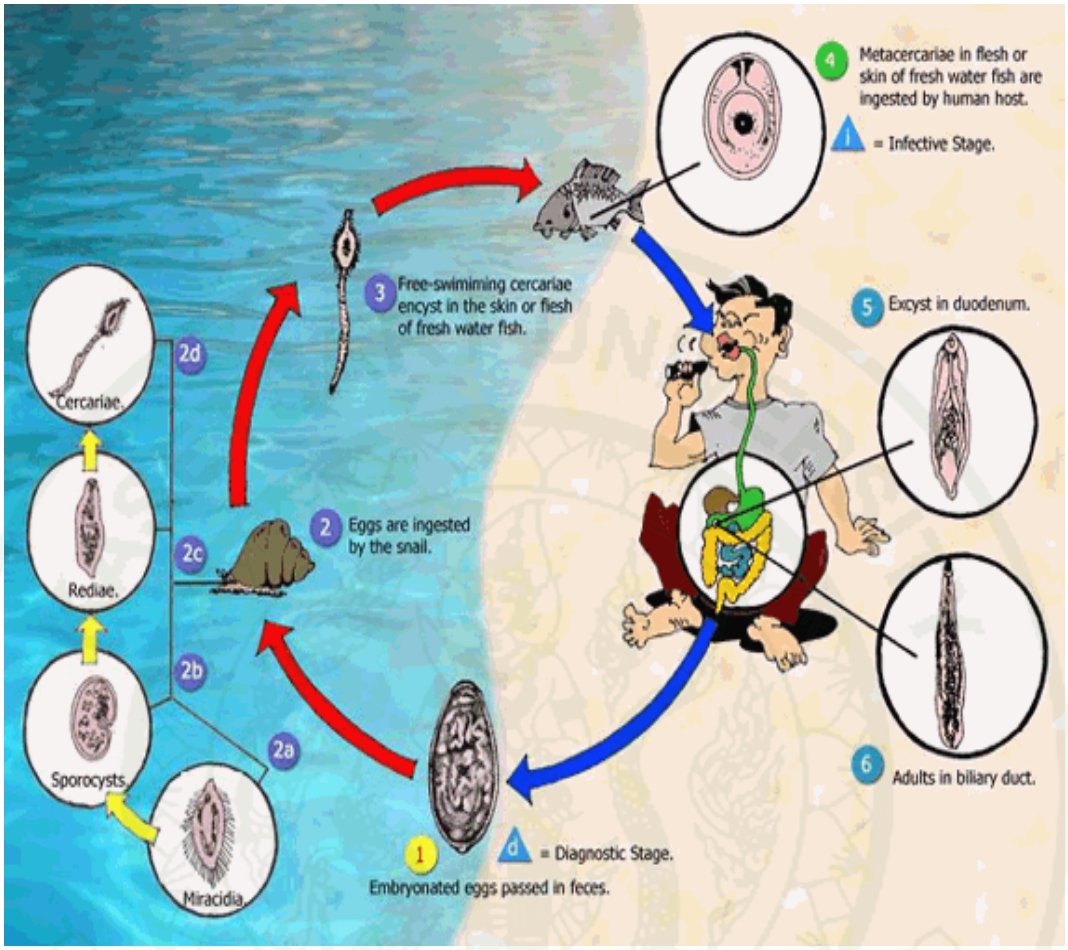

Figure 4 Life cycle of fish-borne zoonotic trematode (Liver fluke)

Source : Fibozopa (2005)

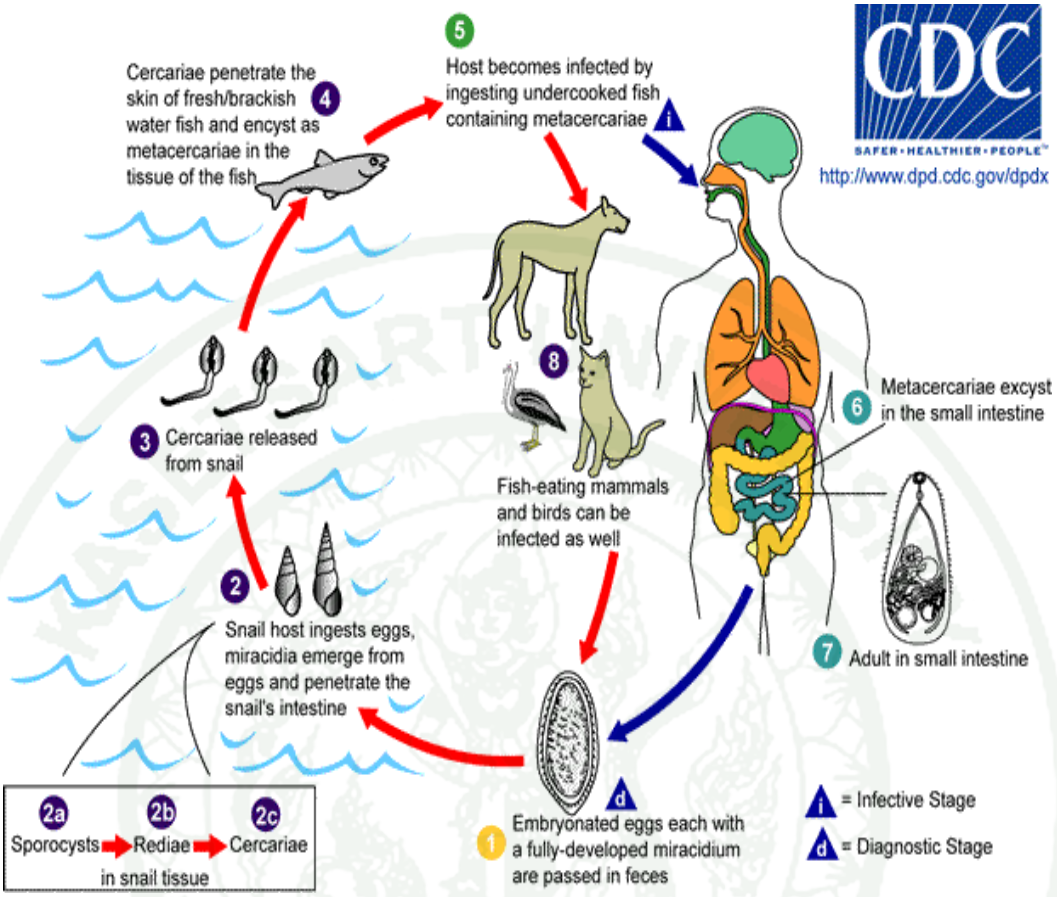

Figure 5 Life cycle of fish-borne zoonotic trematodes (Intestinal fluke)

Source : Laboratory Identification of Parasites of Public Health Concern (n.d.)

2.2 Epidemiology of fish-borne zoonotic trematodes

2.2.1 The liver flukes

The liver flukes are a closely related group of trematodes belonging to the family Opisthorchiidae (Table 1) and have similar life cycles and epidemiology. Liver flukes have long been known to cause serious disease in certain areas of the world. Cholangitis, choledocholithiasis, pancreatitis, and cholangiocarcinoma are the major clinical problems, associated with the long chronic pattern of these infections

(Chai *et al*., 2005). The causative agents of human infections include *Clonorchis sinensis* in 1907 (in East and Southeast Asia), *Opisthorchis viverrini* in 1896 (in Southeast Asia), *Opisthorchis felineus* in 1895 (in Russia and Eastern Europe), and *Metorchis conjunctus* in 1899 (in North America) (Chai *et al*., 2005), especially in Thailand, Lao PDR, Cambodia and Vietnam where food traditions include eating raw or improperly cooked fish dishes (Touch *et al*., 2009; Thuy *et al*., 2010; Sripa *et al*., 2011). A total of 17 million people around the world are estimated to be infected with these liver flukes (WHO, 1995).

2.2.2 The intestinal flukes

These minute intestinal flukes of the family Heterophyidae are parasites of birds and mammals (Table 2). These minute intestinal flukes of the family Heterophyidae are parasites of birds and mammals. A large number of species has been reported from humans, among which *Metagonimus yokogawai* and *Heterophyes heterophyes* are generally considered the most important species (Yu and Mott, 1994). However, because an extraordinary number of heterophyid species are zoonotic (about 35 species) and have very similar transmission patterns, this group is a very significant food safety and quality problem, but one has not attracted the interest of international agencies until recently. The importance of these flukes is being increasingly recognized through recent studies from the Philippines (Belizario *et al*., 2001) and from Korea on several species including *Heterophyes nocens* and *Metagonimus* spp. (Chai and Lee, 1991; 2002).

Table 1 Species of liver flukes reported from humans

Species	Molluscan and piscine hosts	Other definitive hosts	Geographic distribution
Clonorchis sinensis	Freshwater snails and fish	Dogs, cats, rats, pigs, badgers, weasels, camels, buffaloes	Korea, China, Taiwan, Vietnam, Russia
Opisthorchis viverrini	Freshwater snails and fish	Dogs, cats, rats, pigs	Thailand, Laos, Cambodia, Vietnam
Opisthorchis felineus	Freshwater snails and fish	Dogs, foxes, cats, rats, pigs, rabbits, seals, lions, wolverines, martens, polecats	Spain, Italy, Albania, Greece, France, Macedonia, Switzerland, Germany, Poland, Russia, Turkey, Caucasus
Metorchis conjunctus	Freshwater snails and fish	Dogs, cats, wolves, foxes, coyotes, raccoons, muskrats, minks, fishers	Canada, USA

Source : Chai *et al*. (2005)

Table 2 Species of intestinal flukes reported from humans

Species	Molluscan and piscine hosts	Other definitive hosts	Geographic distribution
Metagonimus yokogawai	Freshwater snails and fish	Dogs, cats, rats	Korea, China, Taiwan, Japan, Russia, Indonesia, Israel, Spain
Metagonimus takahashii	Freshwater snails and fish	(experimentally) mice, dogs	Korea, Japan
Metagonimus miyatai	Freshwater snails and fish	(experimentally) mice, dogs	Korea, Japan
Heterophyes heterophyes	Brackish water snails and fish	Cats, dogs, foxes, Wolves, pelicans	Egypt, Sudan, Palestine, Brazil, Spain, Turkey, Iran, India, Russia
Heterophyes nocens	Brackish water snails and fish	Cats	Korea, Japan, China
Haplorchis taichui	Freshwater snails and fish	Cats, dogs, foxes egret	Taiwan, Philippines, Bangladesh, India, Palestine, Egypt, Malaysia, Thailand, Laos, Vietnam, China
Haplorchis pumilio	Freshwater snails and fish	Cats, dogs, foxes, Wolves, pelicans	Thailand, Laos, China

Table 2 (Continued)

Species	Molluscan and piscine hosts	Other definitive hosts	Geographic distribution
Haplorchis yokogawai	Freshwater snails and fish	Cats, dogs, egret	Taiwan, Philippines, China, Malaysia, Indonesia, Thailand, Laos, India, Australia, Egypt
Pygidiopsis summa	Brackish water snails and fish	Cats	Korea, Japan

Source : Chai *et al*. (2005)

2.3 Epidemiology of fish-borne zoonotic trematodes in Thailand

The first intermediate hosts of intestinal flukes in Thailand contains three different hosts; the most important first intermediate host is *Melanoides tuberculata* (Pearson and Ow-Yang, 1982; Hernandez *et al*., 2003), the other species are *Thiara juncae* (Velasquez, 1973) and *Tarebia granifera* (Giboda *et al*., 1991a, 1991b; Kumchoo *et al*., 2005) and the first intermediate hosts of liver fluke in Thailand contains three different hosts; the most important first intermediate host is *Bithynia s. goniomphalos*, *Bithynia funiculate* and *Bithynia s. simensis* (Petney *et al*., 2012).

The second intermediate hosts are freshwater or brackishwater fish, which metacercariae are parasitized in different parts of fish depending on the species of parasites. The metacercariae of liver and intestinal flukes may also encyst in various fish belonging not only to different species but also to different families such as *Mugil* spp., *Hampala dispar*, *Puntius* spp., *Cyclocheilichthys armatus* and *Henicorhynchus*

lineatus (Srisawangwong *et al*., 1997; Kaewkes, 2003; Chai *et al*., 2005) that shown in table 3.

Sources of Thai human infected by fish-borne zoonotic trematode are caused by food traditions including eating raw or improperly cooked fish dishes that have three types of preparations contain uncooked, small and medium-sized, fish: (1) koi pla eaten soon after preparation (2) moderately fermented pla som stored for a few days to weeks; and, (3) pla ra extensively fermented, highly salted fish, stored for at least 2–3 months (Sadun, 1955). In the past, reports of consumption frequencies of koi pla were very high and up to 80% of the population in some communities were eaten every week (Grundy-Warr *et al*. 2011).

The frequencies of koi pla consumption have declined and are generally confined to special social occasions, while other under-cooked fish preparations like pla som and other moderately preserved fish are generally eaten several times a week (Elkins *et al*. 1991, Jongsuksuntigul, 2003). Fully preserved fish (e.g. pla ra and jaewbhong) is an important staple, consumed daily by 60–98% of northeasterners and lowland Laotians (Changbumrung *et al*., 1989). Several studies indicated that survival of infective stages depends on the concentration of salt and degree of fermentation (Tesana *et al*., 1986). Koi pla is probably the most infective, followed by fish preserved for <7 days, then pla ra and jaewbhong, in which viable metacercaria are rare (Sithithaworn and Haswell-Elkins, 2003).

Table 3 FZT reported from humans in Thailand

FZT-Species	The first intermediate host	The second intermediate host	Other definitive hosts	References
Opisthorchis viverrini	*Bithynia s. goniomphalos* *Bithynia funiculate* *Bithynia s. simensis*	*Cyclocheilichthys armatus* *Puntioplites proctzysron* *Hampala macrolepitota*	Dogs, cats, rats, pigs	Kaewkes, 2003 Chai *et al*., 2005 Petney *et al.* 2012 Kaewpitoon *et al*., 2012
Haplorchis pumilio	*Melanoides tuberculata*	*Hampala dispar* *Puntius gonionotus* *Pumtius leiacanthus*	Cats, dogs, foxes, wolves pelicans	Chai *et al*., 2005 Rattanathai, 2010 Srisawangwong *et al*. ,1997
Haplorchis taichui	*Melanoides tuberculata* *Tarebia granifera*	*Puntius gonionotus* *Pumtius leiacanthus* *Puntioplites proctozysron* *Hampala macrolepidota*	Cats, dogs, foxes, egret	Chai *et al*., 2005 Rattanathai, 2010 Srisawangwong *et al*. ,1997 Boonchot and Wongsawad, 2005
Haplorchis yokogawai	*Melanoides tuberculata*	*Mugil* spp. *Puntius* spp.,	Cats, dogs, egret	Chai *et al*., 2005 Rattanathai, 2010
Stellantchasmus falcatus	n.d.[1]	*Dermogenus pusillus*	Cats, dogs	Sripalwit *et al*., 2003 Saenphet *et al*., 2008

[1] n.d. = not data

2.3.1 The liver fluke

The liver flukes have long been known to cause serious diseases in Thailand. Cholangitis, cholelithiasis, pancreatitis, and cholangiocarcinoma are the major clinical problems, associated with the long chronic pattern of these infections. In Thailand, the first report of high prevalence of *O. viverrini* infection, reaching 100% in certain villages of the Northeast Thailand was by Sadun (1955). Almost 30 years later, a near 100% in prevalence and high intensity of *O. viverrini* was similarly reported in the Chonnabot district of Khon Kaen Province, confirming Khon Kaen to be one of the hot spots of the liver fluke infection in Northeast Thailand. The first nationwide survey of the four regions of Thailand during 1980–1981 revealed an overall prevalence of *O. viverrini* infection of 14%; the Northeast (34.6%), the Central (6.3%), the North (5.6%) and the South (0.01%) regions. As a result of intensive and continuous control programs and public health service activities, the average national prevalence of infection has declined to 9.4% in the year 2000 and went down further to 8.7% in the year 2009 (Sithithaworn *et al*., 2012). However, the high prevalence of the infection was still found in the Northeast (16.6%) followed by the North (10.0%), the Central (1.3%) and the South (0.01%) region of Thailand in 2009 as shown in Figure 6 (Sripa *et al*., 2011).

2.3.2 The intestinal flukes

The importance of these flukes is increasingly recognized through recent studies from Thailand on *Haplorchis taichui* (Waikagul, 1991; Sukontason *et al*., 2001). However, there are several heterophyid species, including *Stellantchasmus falcatus* which were not considered of significant clinical importance (Sripalwit *et al*., 2003). Therefore, these flukes infection are increasingly in human health problems (Sripalwit *et al*., 2003). Example, *S. falcatus* is one of the heterophyid flukes reported in humans in the North and Northeast of Thailand (Sripalwit *et al*., 2003), but its prevalence in fish hosts is rarely known.

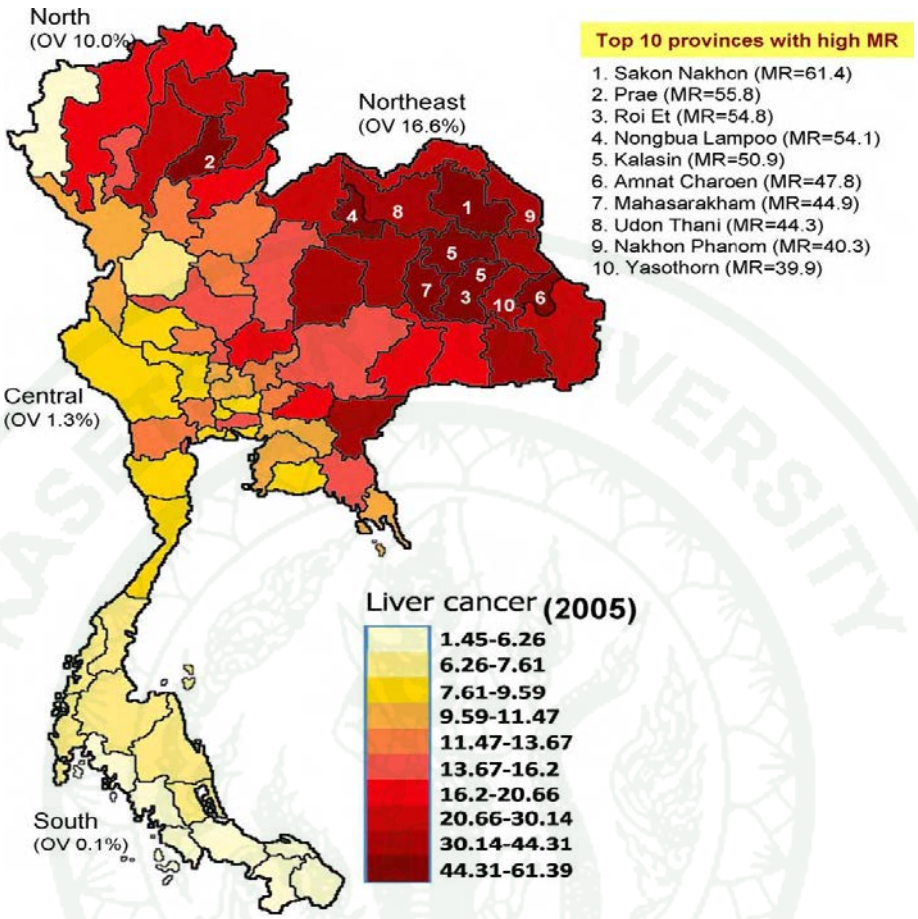

Figure 6 A distribution of *O. viverrini* infections of humans and the top 10 provinces with the highest mortality rate (per 100,000) of liver cancer deaths in Thailand in 2009

Source : Sripa *et al.* (2011)

3. Diagnosis tools

3.1 Liver and intestinal flukes in the second intermediated hosts

3.1.1 Digestion method

Several different diagnostic technique are available for fish, such as a pepsin digestion method that is reliable method for releasing metacercariae from infected fish (Thien *et al*., 2009; Sohn, 2009; Duenngai *et al*., 2012).

3.1.2 Molecular diagnosis

PCR allows the amplification of a specific DNA segment for which, at least, 18–30 bases of the extremity sequences are known. Following 30-40 PCR cycles, the DNA have been amplified approximately 1 billion times, and the resulting PCR products will be visualised on an agarose or polyacrilamide gel, after staining with ethidium bromide and exposing it under ultraviolet light. The specific size of the PCR product is evaluated by simultaneous migration of molecular size markers and a positive control. A negative control is run together to evidence any DNA contamination. In some instances, primers can amplify more than one tandem repeat which gives several specific bands. However, a sample is positive when, at least, the single product is observed. The actual sequence amplified can be analysed by sequencing, or checked by DNA probing, which may increase the sensitivity to detect as little as 1 μg of DNA. Generally, once the specificity of the primers has been established, the size of the PCR product is sufficiently characteristic for diagnostic purposes.

For DNA-based methods, a probe targeting repetitive DNA has been used for the detection of metacercaria from positive fish. DNA was separately extracted from metacercaria by using the Genomic DNA mini kit (Tissue) (Geneaid, Taiwan) according to the manufacturer's protocol. The PCR amplicons were amplified from different partitions, 18S rDNA, 28S rDNA, and ITS2 region in Table 4 that

unknown species of parasites, of the parasite genome by using the appropriate PCR conditions (Thaenkham *et al*., 2010).

3.2 Liver and intestinal flukes in other definitive hosts or humans

3.2.1 Parasitological (Faecal) diagnosis

Demonstration of eggs in feces, bile, or duodenal fluid or the recovery of fluke during transhepatic stent implantation or from the liver post mortem is considered the “gold standard” for diagnosis flukes (Sithithaworn *et al*., 1991). Fecal examination is the routine method for diagnosis of fluke infections due to ease, and low cost. This technique include the formalin-ether concentration (FEC), the modified Kato Katz thick smear, and Stoll’s dilution egg count (Viyanant *et al*., 1983; Elkins *et al*., 1990; Hong *et al*., 2003). Sensitivity and specificity varies by the method of examination and experience of the microscopist. The diagnostic value of these methods lies in the ability to detect light infections. Repeated examinations are needed to improve diagnostic sensitivity of faecal exams. However, even with repeated stool examination using a standardized method like FEC, there can remain discrepancy between egg count and worm detection, i.e. sufficient probability of a false negative diagnosis (Sripa *et al*., 2011).

Table 4 List of primers for detection of FZT

Primer	Primer sequences	PCR condition	PCR product size	References
18S rDNA	Uni 18S GCTTGTCTCAGAGATT AAGCC HET 18S ACGGAAACCTTGTTA CGA	94 °C 2 min 94 °C 30 sec; 56 °C 30 sec; 72 °C 2 min (35 cycle) 72 °C 5 min 4 °C α	≈1790 bp	Dzikowski *et al*., 2004
28S rDNA	LSU-5 TAGGTCGACCCGCTG AAYTTAAGCA 1500R GCTATCCTGAGGGAA ACTTCG	94 °C 2 min 94 °C 30 sec; 56 °C 30 sec; 72 °C 2 min (35 cycle) 72 °C 5 min 4 °C α	≈1280 bp	Thaenkham *et al* ., 2010
ITS2	3SF GGTACCGGTGGATCA CTCGGCTCGTG BD2R TATGCTTAAATTCA GCG GGT	94 °C 2 min 94 °C 30 sec; 48 °C 30 sec; 72 °C 45 sec (30 cycle) 72 °C 8 min 4 °C α	≈290 bp	Thaenkham *et al* ., 2010

3.2.2 Molecular diagnosis

For DNA-based methods, a probe targeting repetitive DNA has been used for the detection of eggs (Sirisinha *et al*., 1991). A PCR-based approach based on a pair of primers complementary to the same target DNA has shown utility for detection of fecal eggs (Wongratanacheewin *et al*., 2001, 2002).

Fecal samples were washed twice by DW to remove fixative, and frozen at -80 ◦C. The frozen feces were crushed three times with a glass bar to break any eggs. DNA was extracted from the broken eggs by Qiagen Stool kit (Qiagen, Taiwan) with half a tablet of inhibitEX. The DNA was resuspended with 50 µl DW and used as a template (Sato *et al*., 2009). For primers shown in Table 5 which known species of parasites from parasitological diagnosis.

Table 5 List of specific primers for detection of FZT

Specific primer	Primer sequences	PCR condition	PCR product size	Reference
O. viverrini	Trem25F ACAGGAAGTGGAA CCGTGTC OV25-4R AATGAACGGAAA TCGTGACC	95 °C 5 min 95 °C 1 min; 60 °C 1 min; 72 °C 1 min (35 cycle) 72 °C 5 min 4 °C α	229 bp	Parvathi *et al*., 2008
O. viverrini	OV-6F TGAATCTCTCGTT TGTTCA OV-6R GTTCCAGGTGAGT CTCTCTA	94 °C 5 min 94 °C 30 sec; 52 °C 30 sec; 72 °C 45 sec (30 cycle) 72 °C 10 min 4 °C α	330 bp	Wongratana-cheewin *et al*., 2001
H. taichui	ITS2-F CTTGAACGCACAT TGCGGCCATGGG ITS2-R GCGGGTAATCACG TCTGAGCCGAGG	94 °C 4 min 94 °C 1 min; 60 °C 30 sec; 72 °C 2 min (40 cycle) 72 °C 5 min 4 °C α	530 bp	Sato *et al*., 2009

Table 5 (Continued)

Specific primer	Primer sequences	PCR condition	PCR product size	Reference
H. pumilio	ITS2-F CTTGAACGCACAT TGCGGCCATGGG ITS2-R GCGGGTAATCACG TCTGAGCCGAGG	94 °C 4 min 94 °C 1 min; 60 °C 30 sec; 72 °C 2 min (40 cycle) 72 °C 5 min 4 °C α	380 bp	Sato *et al*., 2009

4. Strategies for prevention and control FZT infections

4.1 Prevention and control of FZT infection in the second intermediate hosts

In aquaculture systems, the main risk factors for FZT infection and transmission include contamination of pond environments with FZT eggs from infected hosts, i.e., humans, cats, dogs, pigs, and fish-eating birds (Figure 7). Factors that promote the diversity and population growth of snail intermediate hosts (families Thiaridae and Bithynidae) also increase a risk. In addition to local ecologic factors, this variation could also be influenced by flooding in snail distributions and migrations, leading to sudden rises in snail population (Clausen *et al*., 2012).

4.2 Prevention and control of FZT infections in human or other definitive hosts

Prevention and control approaches are similar for all liver flukes. The traditional habit of eating raw or improperly cooked freshwater fish is a major reason for sustaining the zoonosis in endemic areas and a seemingly intractable obstacle to control; health education efforts aimed at changing such habits have not been very successful (Guoqing *et al*., 2001). Currently, the major strategy for community prevention and control is by using the fecal examination with a treatment of

individual case by praziquantel (25 mg/kg, three times daily, for 2–3 days). Mass chemotherapy with praziquantel (40 and 50 mg/kg in a single dose) is highly efficient and generally feasible to distribute (Mairiang and Mairiang, 2003) and 40 mg/kg of praziquantel in naturally infected in reservoir hosts; dogs and cats (Anh *et al*., 2010; Clausen *et al*., 2012). Educated farmers to understand the risks associated with free-ranging dogs and cats and their indiscriminate defecation near the ponds was required (Clausen *et al*., 2012).

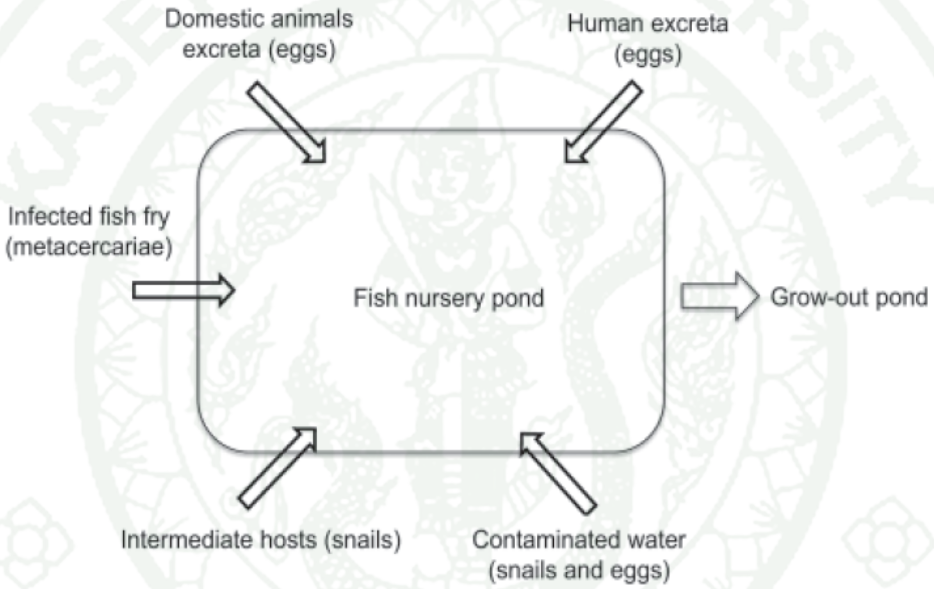

Figure 7 Main risk factors for transmission of fish-borne zoonotic trematodes in fish nurseries, Vietnam. Each risk factor (arrow pointing into pond) is also an intervention point.

Source : Clausen *et al*. (2013)

MATERIALS AND METHODS

Materials

1. cast net
2. hand net
3. weight
4. tape measure
5. beaker
6. concentrated HCl
7. pepsin
8. distilled water
9. mortar
10. pestle
11. electric grinder
12. incubator
13. 1 x 1 mm mesh sieve
14. 0.86% saline
15. scooping net
16. plastic containers
17. 5.5 cm diameter Petri dish
18. stereo microscope
19. 70% alcohol
20. Refrigerator
21. Genomic DNA mini kit (Tissue)
22. 18S rDNA gene
23. 28S rDNA gene
24. 10X PCR Buffer minus Mg
25. 5U of Taq DNA polymerase
26. 50 mM $MgCl_2$
27. dNTP

28. autoclaved distilled water
29. Thermal Cycler
30. 1% agarose gel
31. 0.5M TAE buffer
32. gel star®
33. horizontal plastic chamber
34. Gel Documentation
35. Ultra Clean™ GelSpin DNA purification Kit

Methods

1. Study areas

A cross-sectional survey of Nile tilapia was conducted in Suphan Buri, Nakhon Pathom and Chachoengsao provinces in Central Thailand. Market size fish were collected twice (September – October 2011 and April – May 2012) from same cages and ponds (15 Nile tilapia farms, representing ten pond and five cage culture systems). The two samplings represent two production cycles for market size fish. The cages and ponds were randomly selected among 200 tilapia farms.

2. Collection and analysis of fish

The fish was collected by cast net in ponds (Figure 8) and with hand net in cages (Figure 9). In each pond, the cast net was thrown once in each of the four corners and in the middle of the pond. If more than 10 fish were caught with the net, 10 fish were randomly selected for analyzes, and the rest released again. A total of 150 wild-caught fish was purchased from a nearby fresh market in the same area as the sampled farms of culture Nile tilpia. All fish collected were put on ice and transported within 12 hrs to the laboratory for analysis at Department of Parasitology, Kasetsart University, Bangkok Thailand. Identification of fish (Figure 10) was classified by morphological taxonomic criteria (FAO, 1999; Wongroj, 2004 ; Saowakontha, 2005).

Figure 8 The Nile tilapia farm (pond culture system) in Nakhon Pathom and Chachoengsao provinces

Figure 9 The Nile tilapia farm (cage culture system) in Suphan Buri province

Figure 10 Nile tilapia from study areas

3. Metacercarial analysis (Figure 11)

The fish were stored in a refrigerator at 4°C for no longer than five days before processing. The length and weight of each fish was recorded before the fish intestine was removed. To reduce the amount of fish flesh to be digested, each individual fish was divided into four different parts: (1) gills, (2) body muscle with dorsal fin, pelvic fin and pectoral fin, (3) a piece of body muscle with anal fin, and (4) caudal fin. The four different parts (subsamples) of each individual fish was ground all together in a mortar with pestle and then in an electric grinder (Kenwood Multi Pro FP730 series, China) and the total subsample weight was registered. All fins was included in the analysis as previous studies have shown that FZT has been found in fins as predilection sites (Sukontason *et al*., 2001; Krailas *et al*., 2004). The ground samples were transferred into a 500-ml beaker containing 200 ml of artificial gastric juice (8 ml of concentrated HCl + 6 g of pepsin (1:10,000) in 1,000 ml of distilled water) (Thien *et al*., 2009; Clausen *et al*., 2012). The sample solution was mixed by electric grinder and placed in a 37°C incubator for 2-3 hrs (or longer for hard parts like fin or scale), with occasional stirring. After incubation, 50 ml of water was added and the sample was poured through a 1 x 1 mm mesh sieve and washed with 0.86% saline then left to settle until sediment was easily observed at the bottom of the beaker. The

supernatant was carefully discarded and the sediment was kept. This supernatant was repeated at least five times until this was clear that shown in Figure 11.

Figure 11 The process of metacercarial analysis

4. Collection and analysis of snails

Snails were collected at the same time of the fish collection during the morning hours. The sampling was 15 minute picking snails by hand in ponds and scooping net in the cages (Figure 12 and 13). Snails were transferred to plastic containers and

transported alive to the laboratory where they were identified according to keys by Brandt (1974).

Figure 12 Snail were sampled by hand in Nile tilapia farm (pond culture system) in Nakhon Pathom and Chachoengsao provinces

Figure 13 Snail were sampled by scooping net in Nile tilapia farm (cage culture system) in Suphan Buri province

5. Cercarial shedding

Snail samples from each study area were sorted by species, cleaned and examined for trematode infection by observing their cercarial shedding. The cercarial shedding were done by placing individual snails in a small plastic container (3 cm×2.5 cm) half-filled with dechlorinated water (5 ml) and exposed to a strong illumination for about 2 hours at room temperature that were shown in figure 14. The emerged cercariae were examined and identified by morphological characters.

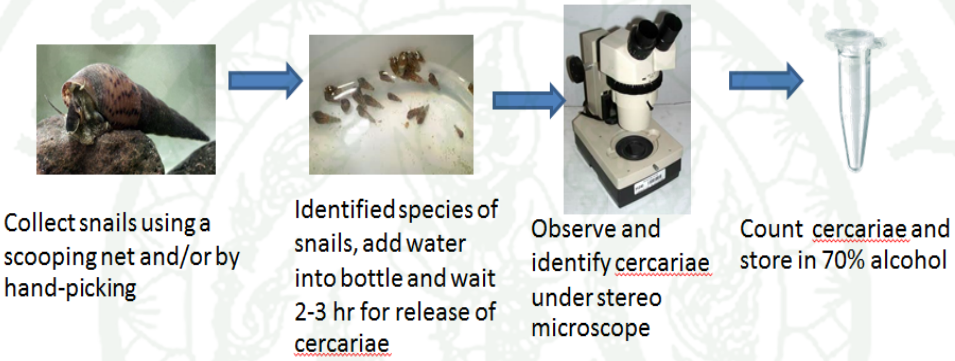

Figure 14 The process of cercarial shedding

6. Identification of metacercariae

Identification the type of metacercariae in fish was classified by morphological characteristics and confirmed by PCR using 18S and 28S rDNA as genetic markers.

6.1 For the morphological identification

The sediment from the digested fish samples were transferred into a 5.5 cm diameter Petri dish containing 6-7 ml 0.86% saline. Any metacercariae present was identified and counted under a stereo microscope (40x – 80x magnification) (Olympus SZ30, Japan) using morphological criteria as identification keys (Pande and Shukla, 1976; WHO, 1995; Chai *et al*., 2005; Sohn, 2009). Metacercariae were fixed and stored in 70% alcohol at 4°C for molecular analysis.

6.2 For the molecular identification

DNA was extracted for PCR from individual metacercaria using the Genomic DNA mini kit (Tissue) (Geneaid, Taiwan) (Thaenkham *et al*., 2010) according to the manufacturer's protocol. The PCR amplicons of partial targeted 18S rDNA (Dzikowski *et al*., 2004) and 28S rDNA genes (Thaenkham *et al*., 2010) were carried out. The 25 µl PCR mixture had a total volume (10X PCR Buffer minus Mg, 5U of Taq DNA polymerase (Invitrogen, Brazil), 50 mM $MgCl_2$, dNTP, primer mix, template DNA and autoclaved distilled water). The primers used for PCR and sequencing reactions are listed in Table 6.

The negative and positive controls were used in PCR analysis. The positive control, a known sample of metacercaria DNA (*Haplorchis taichui* and *Procerovum varium*) from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University and the negative control, a sample without DNA.

Table 6 PCR primers used for 18S and 28S rDNAs

DNA partition	Primer name	Primer sequence (5′-3′)	Amplification	References
18S rDNA	Uni 18S F	GCTTGTCTCAGAGATTAA GCC	PCR	Dzikowski *et al*., 2004
	HET 18S R	ACGGAAACCTTGTTACGA	PCR	Dzikowski *et al*., 2004
28S rDNA	LSU-5	TAGGTCGACCCGCTGAAY TTAAGCA	PCR and sequencing	Thaenkham *et al* ., 2010
	1500R	GCTATCCTGAGGGAAACT TCG	PCR and sequencing	Thaenkham *et al* ., 2010
	900F	CCGTCTTGAAACACGGAC CAAG	Sequencing	Thaenkham *et al* ., 2010

6.3 The amplification condition

The amplification condition for the 18S and 28S rDNA genes was consisted of an initial denaturation at 95 °C for 3 min, 40 cycles of 95°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 1 min, and a final extension at 72°C for 8 min in MyCycler™ Thermal Cycler (BioRad Laboratories, USA).

6.4 Agarose gel electrophoresis

The amplified PCR products were run on 1% agarose gel electrophoresis in 100 ml of 0.5M TAE buffer into the horizontal plastic chamber. After the gel become solid, 8 µl of amplified DNA from each sample was mixed with 2 µl of loading dye mixed with gel star® (Lab Focus INC, NY) and loaded into slots of gel. The electrophoresis was operated using 100 volt until loading dye nearly reach the end of the after that visualized on a Gel Documentation (SYNGENE BIO IMAGING, USA).

6.5 DNA purification and sequencing

PCR products were processed with Ultra Clean™ GelSpin DNA purification Kit (MO BIO LABORATORIES Inc, CA, USA) and submitted for sequencing (1st Base Laboratory, Malaysia).

6.6 DNA assembling and confirm species of the metacercariae

The DNA fragments were assembled into contigs with CAP3 (Huang and Madan, 1999). The obtained contigs were analyzed individually by The Basic Local Alignment Search Tool (BLAST) program to confirm the PCR target and aligned with the GenBank database (http://blast.ncbi.nlm.gov/Blast) to confirm the identification of the metacercariae.

7. Statistical analysis

Data was descriptively analyzed (means of number of metacercariae, infected fish) and the relationship between the cage and pond culture system and wild-caught

fish by Fisher's Exact Test using the Number Cruncher Statistical System (NCSS 2000) (Hintze, 1998).

RESULTS

Prevalence of metacercariae in cultured Nile tilapia and wild-caught fish

In the first sampling, 2.5% (1/40) of Nile tilapia from a cage and 10% (4/40) of Nile tilapia from two ponds were infected with metacercariae (Table 7). In the second sampling, only one infected fish, 1/50 (2.0%), was observed in one cage. The metacercariae from both cages and ponds were spherical with double walls and the larvae in the cyst were not visible (Figure 15) and could not be identified whereas PCR using 18S and 28S rDNA genes classified these metacercariae but did not yield any amplicons.

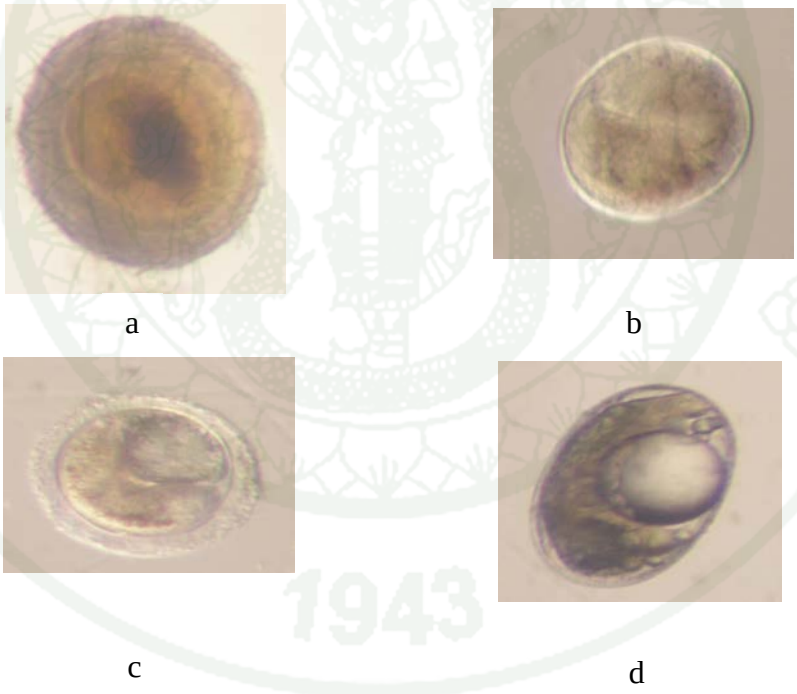

Figure 15 Metacercaria of Nile tilapia from cage culture system in Suphan Buri province (a), metacercaria from wild-caught fish in Suphan Buri province (b), metacercaria from wild-caught fish in Nakhon Pathom province (c) and metacercaria from wild-caught fish in Chachoengsao province (d) under a stereo microscope at a magnification of × 80.

The prevalence of metacercariae infection between fish ponds in the first and the second sampling and culture system and wild-caught fish in the second sampling were significantly different (P>0.06, P>0.001). The infection intensity (metacercariae/ g fish) in Nile tilapia from both samplings were low with a mean of 0.03 ± 0.02 metacercariae/g fish (The first sampling) and 0.04 ± 0.00 metacercariae/g fish (The second sampling).

The wild-caught fish sample was only done together with the second sampling of cultured Nile tilapia and consisted of 150 fish representing 11 different fish species infection and there was 1 species of wild-caught fish was Common snakehead (Channa striatus)) which wasn't infected. All species contained metacercariae (Figure 15) with a total prevalence of 80/150 (53.3%) fish infected. Fish species with the highest prevalence of metacercariae were *Henicorhynchus siamensis* (13/13), *Liza subviridis* (12/12), and *Osteochilus vittatus* (19/26). The intensity of infection in the wild-caught fish were ranged from 0.05 ± 0.01 to 0.62 ± 0.50 and the Greenback mullet *(Liza subviridis)* had the highest intensity (Table 8) and there was 1 species of wild-caught fish was Snakeheads (*Ophicephalus striatus*) which wasn't infected (0/30).

Three species of zoonotic metacercariae were identified by molecular identification in 11 species wild-caught fish including *Stellantchasmus falcatus*, *Haplorchis pumilio* and *Procerovum varium* and one non-zoonotic metacercariae *Prosorhynchoides ozakii* in Nile tilapia (*Oreochromis niloticus*). The metacercariae species were identified by morphological characteristics to family, Heterophydae. Sequence analysis of the 28S rDNA gene as obtained by PCR allowed identification of the metacercariae to species. The size of PCR amplicon of partial 18S and 28S rDNA genes from wild-caught fish were 1790 and 1280 bp (Figure 16 and 17). All fish analyzed had single species metacercarial infections with the four identified species of metacercariae.

Prevalence of snail infections in cultured Nile tilapia aquaculture systems

The total numbers of snails collected in the first and second sampling were 1,137 samples. The potential intermediate host snails recorded from the samples were from the Thiaridae group, especially *Melanoides tuberculata* and *Tarebia granifera*. A smaller number of *Bithynia* sp., was also found in a few pond systems (Table 9). High numbers of snails were found in pond systems. *Clea (Anentome) helena* snails were found only in cage systems. Both aquaculture systems had the high number of *Tarebia granifera.* Finally, no potential host snails were found positive with cercariae.

Table 7 Prevalence of metacercariae in cultured Nile tilapia[a] and wild-caught fish

Sampling period	Province (production system)	Total number of farms/ total number of fish	Prevalence of metacercariae[a]	Infection intensity (metacercariae per g fish)	Fisher's Exact Test
I.Sept-Oct, 2011	Suphan Buri (cage systems)	4/40[b]	1/40 (2.5%)	0.02±0.00	
	Nakhon Pathom (pond systems)	4/40[b]	4/40 (10.0%)	0.03±0.03	
II.Apr-July, 2012	Suphan Buri (cage systems)	5/50	1/50 (2.0%)	0.04±0.00	
	wild-caught fish	-	4/50 (8%)	0.07 ± 0.03	
	Nakhon Pathom (pond systems)	5/50	0/50 (0%)	-	$cage^{1st}$-$pond^{1st}$; P=0.36
	wild-caught fish	-	42/50 (84%)	0.33±0.11	$cage^{2nd}$-$pond^{2nd}$; P=0.33
	Chachoengsao (pond systems)	5/50	0/50 (0%)	-	$cage^{1st}$- $cage^{2nd}$; P=1.00
	wild-caught fish	-	34/50 (68%)	0.22±0.27	$pond^{1st}$-$pond^{2nd}$; P=0.006[c]

[a] It was not possible to determine the species of the metacercariae. [b] Due to flooding only 40 fish from four ponds and 40 fish from four cages could be sampled. [c] Probability value of Fisher's Exact Test between ponds in first and second sampling which was different with significant prevalence.

Table 8 Prevalence and intensity of trematode metacercariae in wild-caught fish

Fish species	Prevalence of infected fish (%)	Infection intensity[a] (metacercariae per g fish)
Long-fatty finned mystus (*Mystus singaringan*)	4/20 (20)	0.07 ± 0.03
Siamese mud carp (*Henicorhynchus siamensis*)	13/13 (100)	0.46 ± 0.18
Greenback mullet (*Liza subviridis*)	12/12 (100)	0.62 ± 0.50
Snakeskin gourami (*Trichogaster pectoralis*)	4/4 (100)	0.26 ± 0.13
Silver Barb (*Barbodes gonionotus*)	3/3 (100)	0.45 ± 0.63
Moonlight gourami (*Trichogaster microlepis*)	2/2 (100)	0.20 ± 0.11
Climbing perch (*Anabas testudineus*)	2/2 (100)	0.26 ± 0.04
Hard-lipped barb (*Osteochilus hasselti*)	19/26 (73.08)	0.33 ± 0.18
Catfish (*Clariidae* spp.)	11/18 (61.10)	0.13 ± 0.05
Nile tilapia (*Oreochromis niloticus*)	5/10 (50)	0.06 ± 0.07
Bagrid catfishes (*Bagaridae* spp.)	5/10 (50)	0.05 ± 0.01
Common snakehead (*Channa striatus*)	0/30 (0)	-

[a] Three species identified with zoonotic trematodes and one trematode include *Stellantchasmus falcatus, Haplorchis pumilio* and *Procerovum varium* and *Prosorhynchoides ozakii* was identified with non-zoonotic trematode.

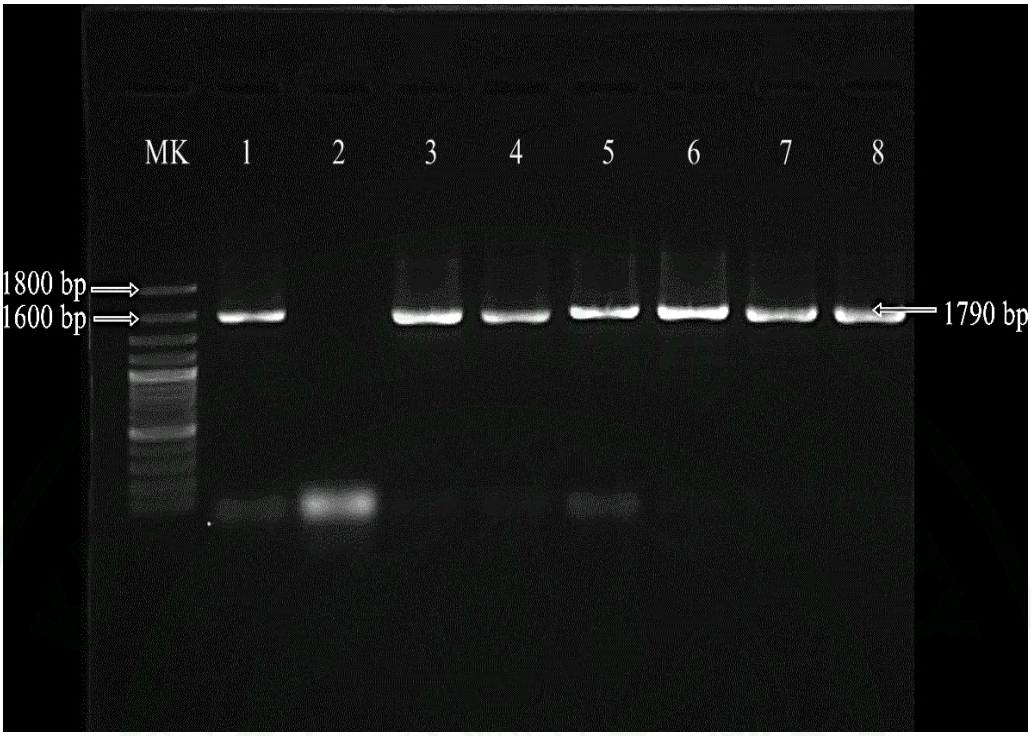

Figure 16 PCR assay of 1790 bp fragment of 18S rDNA in metacercariae. MK, the molecular size standard is a 100-bp ladder. Lane 1, positive control trematode (1790 bp); lane 2, negative control and lane 3-8 positive bands from wild-caught fish samples.

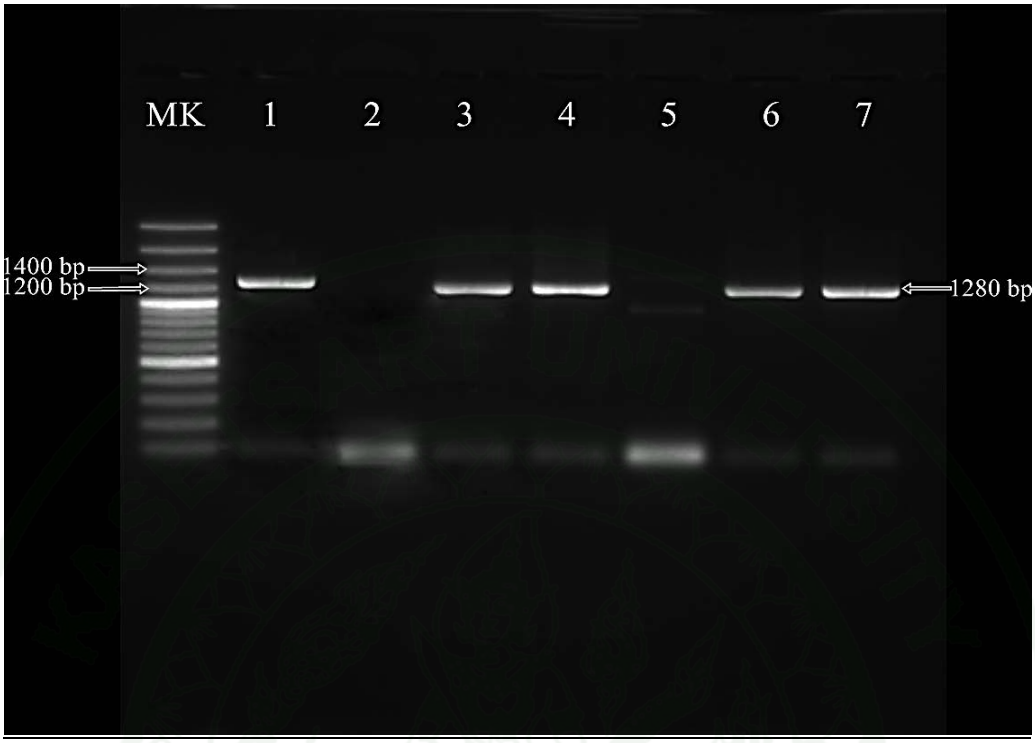

Figure 17 PCR assay of 1280 bp fragment of 28S rDNA in metacercariae. MK, the molecular size standard is a 100-bp ladder. Lane 1, positive control trematode (1280 bp); lane 2, negative control and lane 3-7 positive bands from wild-caught fish samples.

Table 9 The number of snail sampling in cultured Nile tilapia aquaculture systems

Snail species	pond systems	cage systems
Sept-Oct 2011		
Bithynia sp.	15	8
Melanoides tuberculata	15	12
Tarebia granifera	274	390
Lymnaea (Radix) auricularis rubiginosa	25	28
Clea (Anentome) helena	-	65
Apr-May 2012		
Pila polita	21	-
Tarebia granifera	-	284

DISCUSSION

The findings of this study demonstrated the low prevalence and intensity of metacercariae in cultured Nile tilapia from both cage and pond aquaculture systems compared to the higher prevalence and intensity of FZT in wild-caught fish from the same study area.

This is the first study looking at fish-borne trematode infections in Nile tilapia farms in Thailand, and the result indicated that there are none to low fish-borne trematode infection rates in Nile tilapia farms. This is somehow in contrast to Vietnam where there have been reports about higher infection rates of Nile tilapia in both from wild-caught and aquaculture sources may be due to the common practice of using night soil to fertilize ponds to increase growth of plankton, a major source of carp feed (Phan *et al*., 2010b). Nam Dinh Province, Vietnam also has a high human and domestic animal FZT prevalence making fecal waste from these hosts a highly risky fertilizer (Thien *et al*., 2009; Phan *et al*., 2010b). In Cambodia and Lao PDR where there have been reports about higher infection rates of wild-caught fish such as *Barbodes altus*, *Hampala dispar*, *Systomus orphoides*, *Cyclocheilichthys apagon*, *Dangila lineata*, *Cyclocheilichthys repasson*, *Cyclocheilichthys enoplos* and *Henicorhynchus lineatus* due to reservoir hosts including cats, dogs, and pigs, which may contaminate the environment with FZT eggs (Touch *et al*., 2009). In this area, cats, dogs, and pigs are not restrained, and can defecate anywhere in the village or floodplain area during the dry season (Touch *et al*., 2009) and another factor were low water levels in the rivers, slowly running water, the ease of catching fish, and the abundance of snail intermediate hosts in the rivers in the dry season (Manivong *et al*, 2009).

Due to heavy flooding in the central parts of Thailand in 2011 affected our study sites. Therefore, the sampling plan has been modified. It was only possible to collect samples from four ponds and four cages in Nakhon Pathom and Suphan Buri provinces in the first sampling period.

It was decided to compare wild-caught fish with the cultured fish in the second sampling to see if any difference between wild caught fish and cultured fish

Some farmed fish in this study were produced in environments which widely vary depending on the culture site, the culture system, and intensity. Also the level of management applied such as eliminating plants around pond culture system to reduce suitable habitat of snails that are recognized as the good practice in Nile tilapia farms. Moreover, improving growth has typically been done under favorable conditions where fish were fed high protein pelleted feed. Therefore, this might result in decreasing of contaminated parasites from animal manures.

Farm management practices to reduce water pollution and parasite levels. They can be a cost-effective way to recycle, clean, and store water supplies. They can even help rebuild wetlands and restock wild fisheries. Both cage and pond culture systems were a type of fish farm. Advantages of cage culture system contain many types of water resources including lakes, reservoirs, ponds, strip pits, streams, and rivers that could otherwise not be harvested. A relatively low initial investment is all that is required in an existing body of water. Harvesting is simplified and observation and sampling of fish is simplified but feed must be nutritionally complete and kept fresh and low dissolved oxygen (DO). This is an ever present problem and may require mechanical aeration (ISU fisheries extension, 2009). Advantages of pond culture system are consisted of water in pond that can provide some crops which were important nutrient in the family diet, as well as other aquatic organisms and easily catch fish when harvest period. However, this system has to manage water when dry season come and waste water is come from fish culture and ponds could also produce fish which prey on the snails that in some places serve as the parasite frequently associated with irrigation schemes (FAO, 2009). However, both culture systems have to have strategies for prevention and control FZT infections such as reduction in the number of snails (first intermediate host) by chemical or biological control and instructed to remove all vegetation from the pond to reduce snail habitat (Clausen *et al*., 2012).

The prevalence of metacercariae infection between fish ponds in the first and the second sampling and culture system and wild-caught fish in the second sampling were significantly different (P>0.006, P>0.001). This difference could be due to seasonal dynamics of snail populations and metacercariae infections in fish (Saenphet *et al*., 2008; Wiwanitkit, 2005).

Recently, the prevalence and species composition of FZT infections in dogs, cats, and pigs from a fish-farming community in Nghe An Province, Vietnam (Anh *et al*., 2009). Feces from 35 cats, 80 dogs, and 114 pigs contained small trematode eggs at 48.6%, 35.0%, and 14.4%, respectively; 7 species of adult FZT were recovered from these hosts. The study in Nghia Hung district, Nam Dinh province Vietnam, the prevalence of FZT varied significantly between cats (70.2%), dogs (56.9%) and pigs (7.7%) (Anh *et al*., 2009). Forty-nine of the egg-positive animals (25 dogs, 20 cats and 4 pigs) were necropsied to obtain adult trematodes for identification (Anh *et al*., 2009).

The first intermediate host of the intestinal flukes is *Melanoides tuberculata*, and other species are *Thiara* and *Terabia granifera* (Waikagul and Radomyos, 2004; Ukong *et al.*, 2007; Rattanathai, 2010). Freshwater snails in this study were found, but they were not infected with FZT. One of the reasons for this could be that most snails in this study were small in size. It has been suggested that most infected snails are found among developed and homologous adult stage of the snails (Ukong *et al*., 2007).

Wild-caught fish in Thailand are commonly known to be infected with FZT including *Haplorchis taichui*, *Centrocestus caninus*, and *Opisthorchis viverrini* (Nithikathkul *et al*., 2009; Boonchot and Wongsawad, 2005; Nithiuthai *et al*., 2002; Sithithaworn *et al*., 1997). The fish species including *Barbodes altus*, *Barbodes gonionotus*, *Henicorhynchus siamensis*, *Cirrhinus julleine*, *Cyclocheichthyes armatus*, *Cyclocheichthyes apogon*, *Hampala dispar*, *Trichogaster microlepis*, *Dermogenus pusillus* and *Mugil* sp. such as *Liza subviridis* and *Valamugil cunnesius* found to be infected with FZT and are all known to be second intermediate host for FZT

(Nithikathkul and Wongsawad, 2008; Saenphet *et al*., 2008; Pubua and Wongsawad, 2007; Waikagul and Radomyos, 2004; Krailas *et al*., 2004; Sripalwit *et al*., 2003). Wild-caught fish in the Northeastern and Northern regions may have high prevalence of metacercariae (Boonchot and Wongsawad 2005; Sripalwit *et al*., 2003 ; Nithiuthai *et al*., 2002; Sukontason *et al*., 2001). This corresponds with this research's results where more than 50% of the wild-caught fish analyzed contained FZT.

The highest prevalence of FZT in human are found in Central and Southern parts of Thailand, reaching levels of up to 60% (Sayasone *et al*., 2007; Sayasone *et al*., 2009; Touch *et al*., 2009; Thuy *et al*., 2010; Sripa *et al*., 2011). Conventionally, Laotian people have the unique food habit and some like to eat dishes containing raw freshwater fish, such as 'Koi pla', 'Pla som', and 'Som fak', and easily infect with FZT. Moreover, they have been engaged in aquaculture in the fish ponds with latrines which can play an important source of contamination (Rim *et al*., 2008). A total prevalence of *O. viverrini* in Khammuane, Saravane, and Savannakhet provinces infection was 32.2%, 21.5% and 25.9%, respectively with high prevalence. Additionally, there are several reports of *O. viverrini* infection in children and adults from1996 to present with high prevalence ranging from37 to 86% (Sithithaworn *et al*., 2012). A very recent survey in Champasack province, Southern Lao PDR has shown the prevalence of 64% for *O. viverrini* infection among 669 persons from 3 Districts. The prevalence of the infection was strikingly high in Khong (92%) and Mounlapamok Districts (90.9%) which are located in the lowlands, whereas the prevalence was low in the highland area such as Paksong (5.7%) (Sithithaworn *et al*., 2012). In Cambodia where has high infection in 2006, a stool survey was conducted by the National Center of Malaria, Parasitology and Entomology (CNM) of Cambodia in Ang Svay Chek Village in Takeo Province and Ampil Village in Kandal province, Southern Cambodia (Touch *et al*., 2009). The results showed 13.4% of the residents were positive for small trematode eggs correlated, with 12 ascites from the Director of Krang Yov Health Center, Kandal province (Touch *et al*., 2009). The high infection rate was 11% in school children in Kandal province, South of Phnom Penh. This correlates with the high prevalence of *O. viverrini* metacercariae found in 10 species of freshwater cyprinid fish with the infection rate ranging from 2.1-66.7% of the fish

captured from Kandal province (Sithithaworn *et al*., 2012). In Thailand, the first report of high prevalence of *O. viverrini* infection, reaching 100% in certain villages of the Northeast Thailand by report of Sadun in 1955 (Sadun, 1955). Almost 30 years later, a near 100% in prevalence and high intensity of *O. viverrini* was similarly reported in the Chonnabot district of Khon Kaen province, confirming Khon Kaen to be one of the hot spots of the liver fluke infection in Northeast Thailand. In 2009, the high prevalence of the infection was still found in the Northeast (16.6%) followed by the North (10%), the Central (1.3%) and the South (0.01%) region of Thailand was shown in Figure 6 (Sripa *et al*., 2011).

No *Opisthorchis viverrini*, metacercariae were found in this study, only the species from the Heterophyidae families. Whereas the public health and food safety issues with *Opisthorchis viverrini* are well known, there is less information available about the effects of Heterophyidae families on human health such as *Stellantchasmus falcatus, Procerovum varium, Haplorchis pumilio, Haplorchis taichui* and *Haplorchis yogokawai* (Waikagul and Radomyos, 2004; Sripalwit *et al*., 2003). There have been many reports of human infections with these parasites in the North, Northeast, and Central of Thailand which the clinical manifestations of intestinal flukes are less well described (Waikagul and Radomyos, 2004; Sripalwit *et al*., 2003). The mechanisms of pathogenesis are not clear but it has been reported as causing intestinal irritation accompanied by colicky pain and mucous diarrhea, with the production of excess mucus and superficial necrosis of the mucous coat. However, some of the heterophyid flukes perhaps wander in the deep layers of the intestinal wall, become imprisoned and dead (Sripalwit *et al*., 2003). Special report cases reported the eggs of the intestinal flukes including *Stellantchasmus falcatus* and *Haplorchis* spp. can cause significant pathology in humans (WHO, 1995).

Table 10 The reported of FZT (*Opisthorchis viverrini*) in other the definitive hosts of Thailand

Year of outbreak	Month/season	Other definitive hosts	Province	Diagnosis method	Prevalence	References
2007-2009	-	cats	Khon Kaen	formalin-ether sedimentation method	35.51%	Aunpromma *et al.*, 2012
2007- 2009	-	dogs	Khon Kaen	formalin-ether sedimentation method	0.37%	Aunpromma *et al.*, 2012
2010	-	cats	Khon Kaen	quantitative formalin ethyl acetate sedimentation	36.4%	Enes *et al.*, 2010
2010	-	dogs	Khon Kaen	quantitative formalin ethyl acetate sedimentation	3.8%	Enes *et al.*, 2010

Table 11 The reported of FZT (*Opisthorchis viverrini*) in the second intermediate host of Thailand

Year of outbreak	Month/season	Second intermediated host	Province	Diagnosis method	Prevalence	References
1991-1992	January-October /cool-rainy	*Puntius leiacanthus*	Khon Kaen	digestion technique	-	Sithithaworn *et al*., 1997
1991-1992	November-December /cool-cool	*Hampala dispar* *Puntius leiacanthus* *Cyclocheilichthys armatus*	Mahasarakham	digestion technique	-	Sithithaworn *et al*., 1997
1997	-	*Pumtius leiacanthus* *Cyclocheilichthyes armatus* *Mystacoleucus atridorsalis* *Cirrhinus julleine*	Khon Kaen	digestion technique	-	Srisawangwong *et al*., 1997
2003	-	*Cyclocheilichthys* sp. *Puntius* sp. *Hampala* sp.	-	-	-	Kaewkes, 2003

Table 11 (Continued)

Year of outbreak	Month/season	Second intermediated host	Province	Diagnosis method	Prevalence	References
2010-2011	February- February /cool-cool	*Cyclocheilichthys armatus* *Cyclocheilichthys repasson* *Puntioplites proctzysron* *Hampala macrolepitota* *Hampala dispar*	Nakhon Ratchasima	digestion technique	19.16% 18.07% 9.64% 8.13% 6.49%	Kaewpitoon *et al*., 2012

Table 12 The reported of FZT (The intestinal flukes) in the second intermediate host of Thailand

Year of outbreak	FZT species	Second intermediated host	Province	Diagnosis method	Prevalence	References
1997	*Haplorchis taichui*	*Hampala dispar* *Puntius gonionotus* *Pumtius leiacanthus* *Cyclocheilichthyes armatus* *Mystacoleucus atridorsalis* *Cirrhinus julleine*	Khon Kaen	digestion technique	-	Srisawangwong *et al*., 1997
1997	*Haplorchis pumilio*	*Hampala dispar* *Puntius gonionotus* *Pumtius leiacanthus* *Cyclocheilichthyes armatus* *Mystacoleucus atridorsalis* *Cirrhinus julleine*	Khon Kaen	digestion technique	-	Srisawangwong *et al*., 1997

Table 12 (Continued)

Year of outbreak	FZT species	Second intermediated host	Province	Diagnosis method	Prevalence	References
2001-2002	*Stellantchasmus falcatus*	*Dermogynus pusillus*	Chiang Mai	digestion technique	-	Saenphet *et al*., 2008
2001-2002	*Haplorchis taichui*	*Labiobarbus siamensis* *Systomus orphoides* *Barbonymus gonionotus* *Henicorhynchus siamensis* *Mystacoleucus marginatus* *Puntioplites proctozysron* *Amblyrhynchichthys trutcatus* *Ratamus guttatus* *Barbonymus schwanenfeldi* *Paralaubuca harmandi*	Chiang Mai	digestion technique	-	Saenphet *et al*., 2008

Table 12 (Continued)

Year of outbreak	FZT species	Intermediated host	Province	Diagnosis method	Prevalence	References
2001-2002	*Centrocestus caninus*	*Labiobarbus siamensis* *Systomus orphoides* *Tricopsis vittatus* *Barbonymus gonionotus* *Henicorhynchus siamensis* *Mystacoleucus marginatus* *Tricogaster microlepis* *Puntioplites proctozysron* *Rasbora parviei* *Rasbora tornieri*	Chiang Mai	digestion technique	-	Saenphet *et al*., 2008
2002	*Stellantchasmus falcatus*	*Dermogynus pusillus*	Chiang Mai	digestion technique	100%	Sripalwit *et al*., 2003

Table 12 (Continued)

Year of outbreak	FZT species	Intermediated host	Province	Diagnosis method	Prevalence	References
200-2003	*Haplorchis taichui*	*Puntioplites proctozysron* *Hampala macrolepidota* *Labiobarbus burmanicus* *Barbodes gonionotus* *Henicorhynchus siamensis*	Chiang Mai	digestion technique	-	Boonchot and Wongsawad, 2005
2008	*Haplorchis taichui*	*Barbodes gonionotus* *Hampala macrolepidota* *Mystacoleucus marginatus* *Puntioplites proctozysron* *Barbodes altus* *Osteochilus hasselti*	Chiang Mai	digestion technique	-	Nithikathkul and Wongsawad, 2008
2011	*Stellantchasmus falcatus*	*Puntioplites proctozysron* *Dermogenys pusillus*	Chiang Mai	HAT-RAPD PCR	-	Wongsawad, 2011

CONCLUSION

The present study showed the low prevalence and intensity of metacercariae in cultured Nile tilapia from both cage and pond culture systems compared to a higher prevalence and intensity of FZT in wild-caught fish from the same study area. This is the first study looking at FZT infections in farmed Nile tilapia in Thailand, and the result indicated that there are none to low FZT infection rates in farmed Nile tilapia. The result reveals the low risk for FZT in cultured Nile tilapia from cage and pond culture systems. However, the high prevalence of FZT in local wild-caught fish indicated a high potential for spillover from wild reservoir hosts and underscores the need for vigilance and good management practice to make aquaculture system bio-secure.

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APPENDICES

Appendix A

Reagents for laboratory experiment

Reagents for laboratory experiments

1. 0.86 % saline

NaCl	8.6 g
distilled water	1,000 ml

2. artificial gastric juice

concentrated HCl	8 ml
pepsin powder	6 g
distilled water	1,000 ml

3. 70 % ethanol

absolute ethanol	70 ml
add distilled water to a final volume of	100 ml

Appendix B

Reagents and buffers for agarose gel electrophoresis

Reagents and buffers for agarose gel electrophoresis

1. 10x TAE buffer, pH 8.0

10 mM Tris-HCl, pH 7.4-8.0	15.56 g
1 mM EDTA	3.72 g
add distilled water	500 ml
adjusted pH to 8.0	
add distilled water to a final volume of	1,000 ml
Autoclave before used	

2. 0.5M TAE buffer

10x TAE buffer	50 ml
distilled water	950 ml

3. 1% agarose gel

agarose powder	1 g
add TE buffer, pH 8.0 to a final volume of	100 ml

4. loading dye with mixed with gel star®

loading dye	270 μl
gel star®	30 μl

5. Marker

100 bp DNA ladder	90 μl
gel star®	10 μl

Appendix C

The sample pictures of fish

Apppendix Figure C1 During the heavy flooding in the central parts of Thailand in 2011

Apppendix Figure C2 Eliminating plants around pond culture system in Nakhon Pathom province

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