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THESIS

PREVALENCE OF *FASCIOLA* INFECTION IN CATTLE AND BUFFALOES AROUND SONGKHLA LAKE

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Boy Boonaue 2013: Prevalence of *Fasciola* Infection in Cattle and Buffaloes around Songkhla Lake. Master of Science (Veterinary Parasitology), Major Field: Veterinary Parasitology, Department of Parasitology. Thesis Advisor: Assistant Professor Burin Nimsuphan, Ph.D. 112 pages.

Fasciolosis is one of the most important parasitic diseases in ruminant and human health is caused by *Fasciola hepatica* and *F. gigantica*. The economic losses in limiting the development of beef and milk production and public health concern are caused by fasciolosis throught out the world. *F. gigantica* is identified in animals and humans in Thailand. The prevalence of bovine fasciolosis in Thailand was ranging from 0-85% depending on the areas. The referent method for diagnosis of fasciolosis is based on the fecal examination. Songkhla Lake is the largest lake in southern part of Thailand. There are cattle and buffaloes rearing nearby Songkhla Lake area. In the previous period, many cattle nearby the lake died and the post mortem found *Fasciola* in the liver. Heavy infection of *Fasciola* can be a cause of death in these cattle. There was the possibility of bovine fasciolosis distribution around the lake. The objective of this study is to determine the prevalence of *F. gigantica* infection in cattle and buffaloes nearby Songkhla Lake area by ES-Ag based on ELISA compared with the fecal examination. The results of ES-Ag-ELISA showed 29.3% (148/505) in cattle and 78.9% (75/95) in buffaloes were positive for *Fasciola* infection. Comparing with the fecal examination, 7.8% (39/500) in cattle and 30.1% (25/83) in buffaloes were found positive. The >7 years cattle had the higher infection rate than the <7 years cattle while the >7 years buffaloes had the lower positive rates than the <7 years buffaloes. Female cattle had the infection rates higher than male but the female buffaloes had the lower positive rates than male. Both of cattle and buffaloes were infected by *Fasciola* at the high level. Thus, Songkhla Lake is the natural reservoir for fasciolosis in cattle, buffaloes and humans.

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Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

°C	=	Degree of Celsius
µg	=	microgram
µl	=	microliter
µm	=	micrometer
ap.a.	=	apical papilla
c.	=	collar
c.f.r.	=	cilia free region
cm	=	centimeter
DW	=	Distilled Water
ES-Ag	=	Excretory-Secretory Antigen
ELISA	=	Enzyme-Linked Immnunosorbent Assay
e.s.	=	eye spot
et al.	=	et. alii (and others)
g	=	gram
L (l)	=	liter
l.c.	=	long cilia
LM	=	Light Micrograph
mg	=	milligram
mm	=	millimeter
No.	=	number
OD	=	Optical Density
ph.	=	pharynx
pH	=	negative logarithm of hydrogen ion activity
post.p.	=	posterior porcess
s.c.	=	short cilia
SEM	=	Scanning Electron Micrograph
SDS-PAGE	=	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

PREVALENCE OF *FASCIOLA* INFECTION IN CATTLE AND BUFFALOES AROUND SONGKHLA LAKE

INTRODUCTION

Fasciolosis is an important parasitic disease in animal and human health caused by *Fasciola hepatica* and *F. gigantica*. The distribution of *Fasciola* is different in each part of the world. *F. gigantica* causes outbreaks in tropical area of Africa, southern Asia and Southeast Asia, including Thailand. The total global economic loss attributed to fasciolosis has been estimated to be more than US$ billion per year (FAO, 1994) and its impact has been estimated in several studies. The case number of *Fasciola* infection diagnosed in the domestic animals and humans in Thailand and other countries has increased (Claxton *et at*., 1997; Kanoksil *et al*., 2006; Daryani *et al*., 2008).

Fasciola is a parasitic liver fluke categorized in family Fasciolidae, suborder Distomata, order Prosostomata, subclass Digenea, class Trematoda, phylum Platyhelminthes. Most important species are *F. gigantica* and *F. hepatica*. *Fasciola* can infect many mammals including humans but the main host is ruminant. *F. gigantica* is a mainly parasite in large ruminant such as cattle and buffaloes while *F. hepatica* is infected in small ruminant. The human can be infected with both of *F. gigantica* and *F. hepatica*. *Fasciola* infections occur by ingesting water plant or water contaminated with the metacercariae of *Fasciola*. The liver fluke infection rarely affects animals dramatically in the sense that they die or are obviously sick from the disease. Effects of fasciolosis are seen in reduced weight gain, low, slow reproduction, liver damage, lack of physical strength expressed as draught power and mortality. Fasciolosis leads to economic loss in decreasing production. The microscropic detection of egg in the feces is the referent method for diagnosis of *Fasciola* infection.

Songkhla Lake is the largest natural water reservoir in southern part of Thailand. The lake borders the provinces of Songkhla and Patthalung. The lake has stagnant water all year round. There are many domestic animals rearing nearby Songkhla Lake area such as goat, cattle and buffaloes. The traditional rearing system nearby the lake is freely grazing on the pastures. Moreover, in the previous period, many cattle nearby Songkhla Lake died and the post mortem of these cattle found numerous of *Fasciola* in the liver. Heavy infection of *Fasciola* may lead to the cause of death in these cattle. Then, there is a possibility of *Fasciola* infection in cattle and buffaloes throughout the area nearby Songkhla Lake.

OBJECTIVES

To detect *Fasciola* infections in cattle and buffaloes nearby Songkhla Lake using excretory-secretory antigen based on enzyme-linked immunosorbent assay and formalin-ethyl acetate sedimentation.

LITERATURE REVIEW

1. Morphology

Fasciola is one of the largest fluke which has the leaf shaped and grayish-brown in color (Figure 1) with a size of 3.5-7.5 cm in length and 0.65-1.2 cm in width. The oral sucker is small and located at the end of cone-shaped projection at the anterior end. The acetabulum is larger than the oral sucker and is anterior. The tegument is covered with large and scalelike spines. The intestinal caeca are highly dendritic and extend to near the posterior end of the body. The testes are large and greatly branched, arranged in tandem behind the ovary. The smaller, dendritic ovary lies on the right side, coiling between the ovary and the preacetabular is cirrus pouch. Vitelline follicles are extensive, filling most of the lateral body and becoming confluent behind the testes (Figure 2).

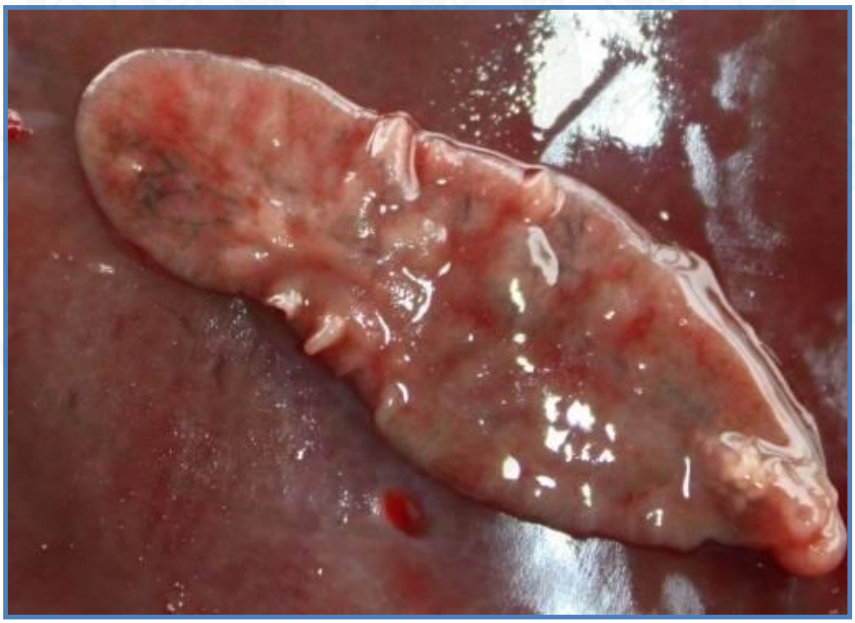

Figure 1 Adult *Fasciola* from slaughter house

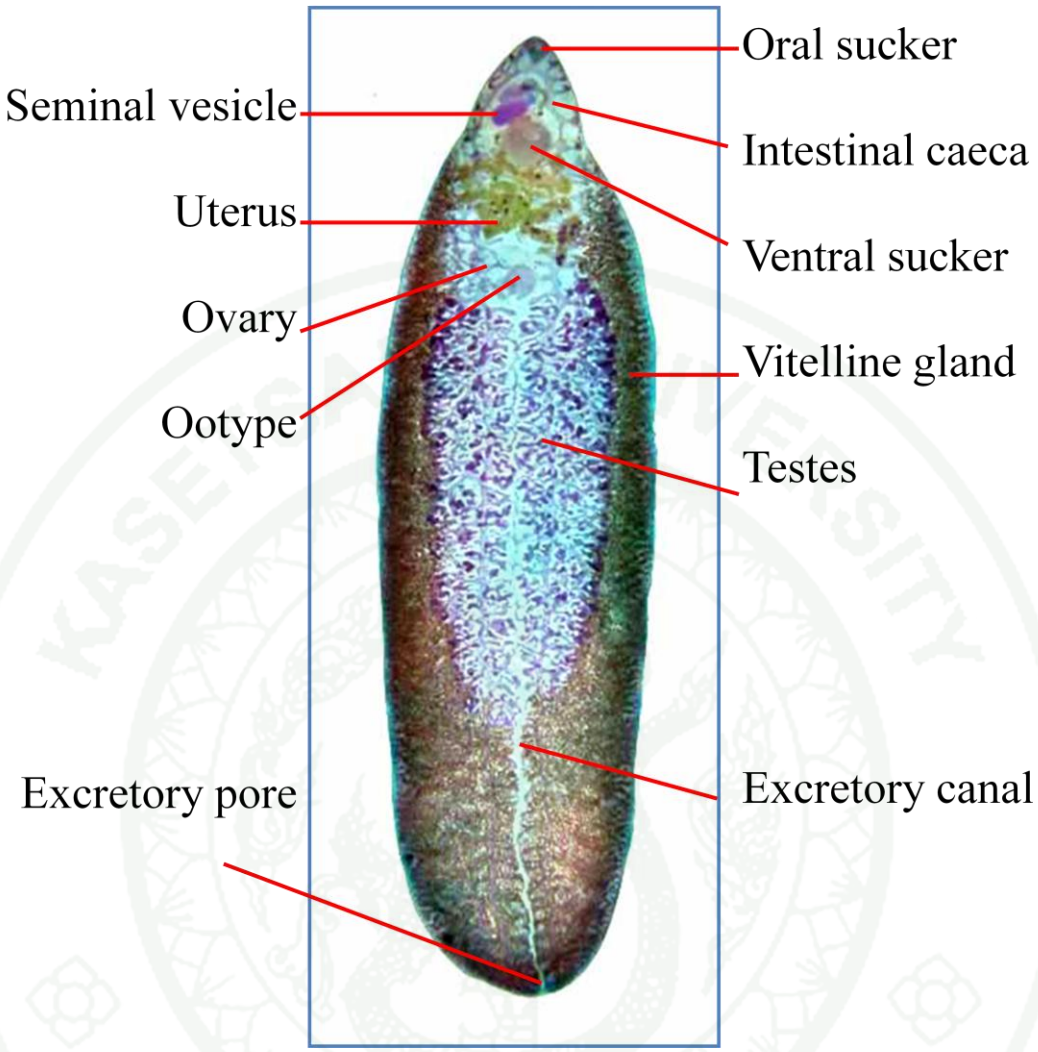

Figure 2 Morphology of *F. gigantica*.

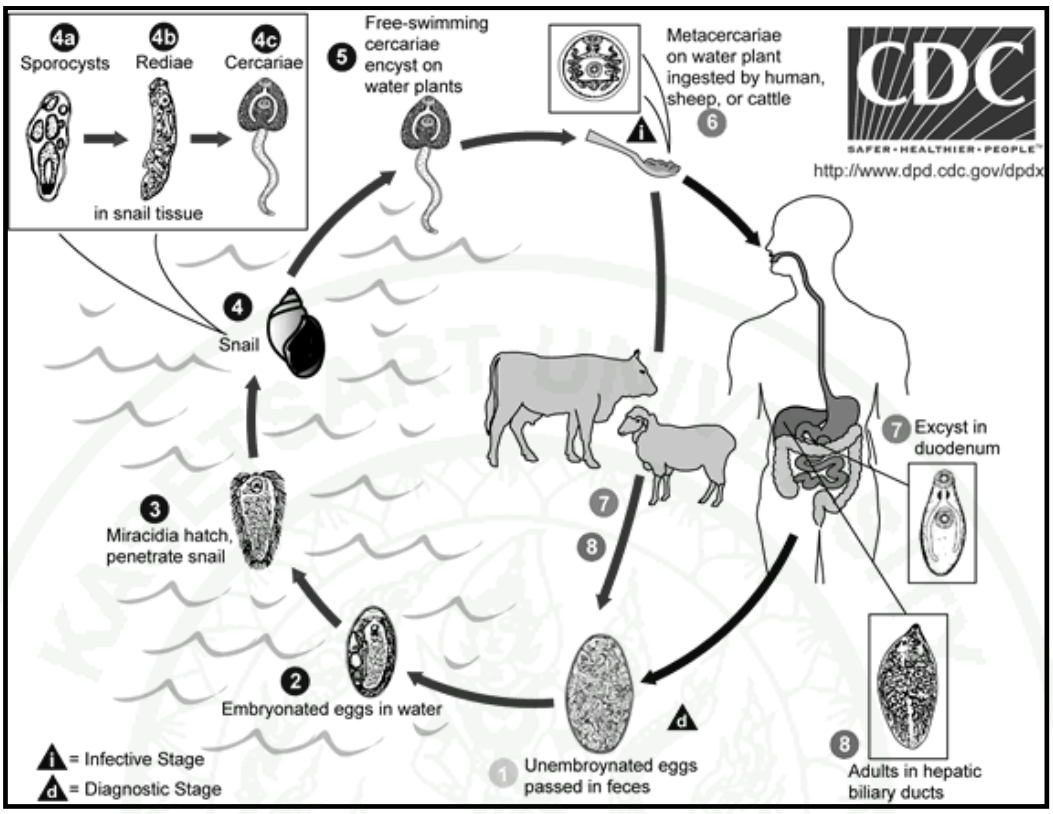

Figure 3 The life cycle of *Fasciola*

Source: CDC (2013)

2. Life cycle

Adult *Fasciola* lives in the bile ducts and gall bladder of the liver and young fluke lives in the liver parenchyma. The eggs of *Fasciola* enter the duodenum with the bile and leave the host in the feces. The rate of development and the hatching of liver fluke eggs depend on the surrounding environment's temperature, oxygen levels and humidity. At the temperature of 20-26°C eggs hatch in about 10-12 days producing the first larval stage, the miracidium. At over 40°C, eggs will die and in darkness, eggs develop well, though miracidia are not hatched out. Miracidium is about 0.15 mm in length, its head is covered with gland tissues for penetrating in to the intermediate host snail and with cilia surrounding it equipped for movement. In the outside environment, miracidia survive for 2-3 hours. In the event that miracidia do

not penetrate host snail *Lymnea*, they die. Following penetration, it casts off its ciliate covering and develops into the sporocyst, then rediae and cercariae. Development from miracidium to cercaria is 4-7 weeks. Cercariae leave the snail and within a few minutes to two hours the cercariae settle on blades of grass, water plants and rice stalks. Later, after casting off their tails, they secrete a covering from the cystogenous glands forming cysts at surface of the water which sink to the bottom. The encysted cercaria is called a metacercaria which is now infective. Cattle and buffaloes are infected by ingesting grass, water plant and rice stalk with metacercaria or swallow them in drinking water. In some cases, infection can occur from mother to the offspring via the placenta. Following ingestion of metacecariae into the intestine they become immature *Fasciola* which migrates to liver through two routes. The first route, the immature of *Fasciola* may migrate through the abdominal cavity and penetrate the liver capsule. They are later found moving through the liver parenchyma. Within three weeks, the immature flukes will reach the bile ducts. The second route, excystation of immature fluke occurs in the duodenum through the bloodstream across the intestinal vein. They then proceed to the bile ducts of the liver. The development of immature fluke to adult fluke will take for 2.5-4 months. *Fasciola* may live for 3-5 years in host.

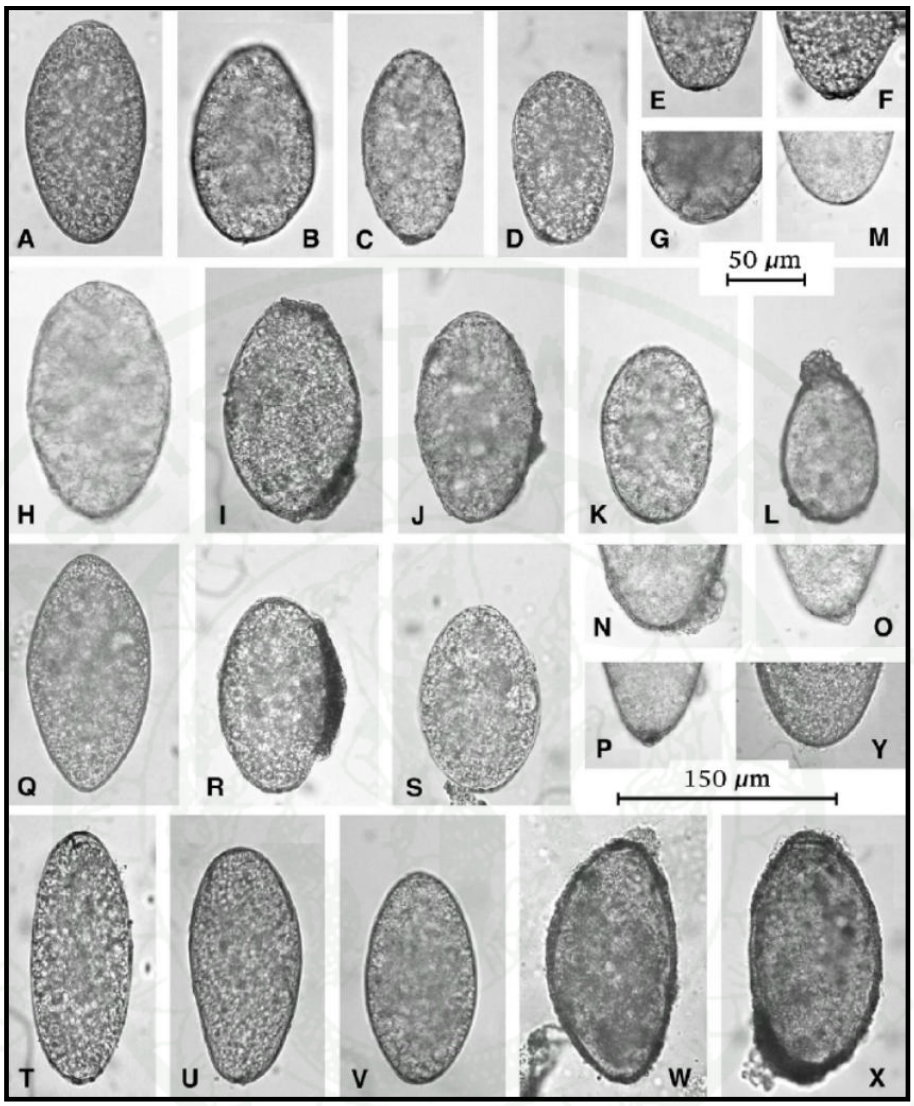

Figure 4 Shape and size variability of fasciolid eggs shed in human stools: (A-G) *F. hepatica* of Bolivia; (H-P) *F. hepatica* of Peru; (Q-S) *F. hepatica* of Georgia; (T-V) *F. hepatica* of Egypt; (W-Y) *F. gigantica* of Vietnam.

Source: Adela Valero *et al*. (2009)

2.1 *Fasciola* egg

The Liver fluke eggs are passed from the bile duct into duodenum and subsequently into feces. The eggs consist of a fertilized ovum surrounded by a large number of yolk granules. Eggs are large, yellowish brown in the color, oval in shape,

130-145 µm long by 70-90 µm wide and operculated with thin shell (Figure 4). It has a distinct, flat operculum and umbilicus-like invagination at the posterior end of the shell (Figure 5A). The eggs have shown the outer surface of the egg shell to be smooth and devoid of any microspines and highly conspicuous umbilicus-like invagination on the shell in the opposite side of the operculated end (Figure 5B and 5C). The eggs which are passed out in the feces on to pasture, are undevelop and undergo embryonation outside the host. Several physico-chemical factors, especially temperature, humidity and oxygen tension, are known to influence embryonation. The liver fluke eggs were found in the stools of infected animals between 9-13 weeks post infection. The highest egg counts (870-910 eggs per gram) were recorded at 24-25 weeks post infection (Martínez-Moreno *et al*., 1999).

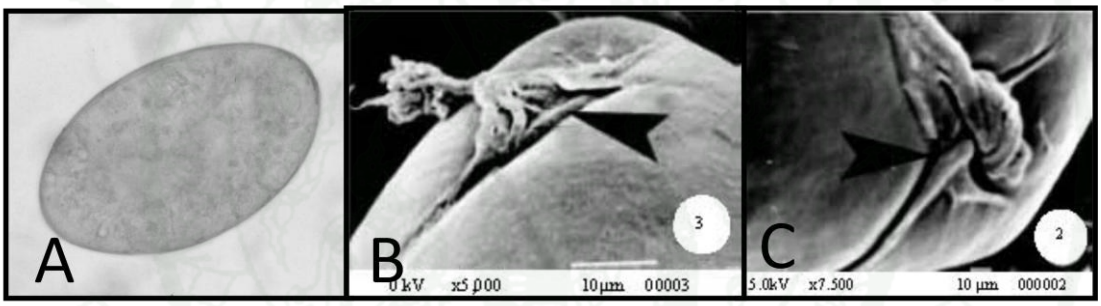

Figure 5 (A) Light micrograph (LM) of the egg.
(B) Scanning electron micrograph (SEM) of the egg showing an umbilicus-like invagination (arrowhead).
(C) SEM of the egg showing another shape of the umbilicus-like invagination (arrowhead).

Source: Hussein *et al*. (2010)

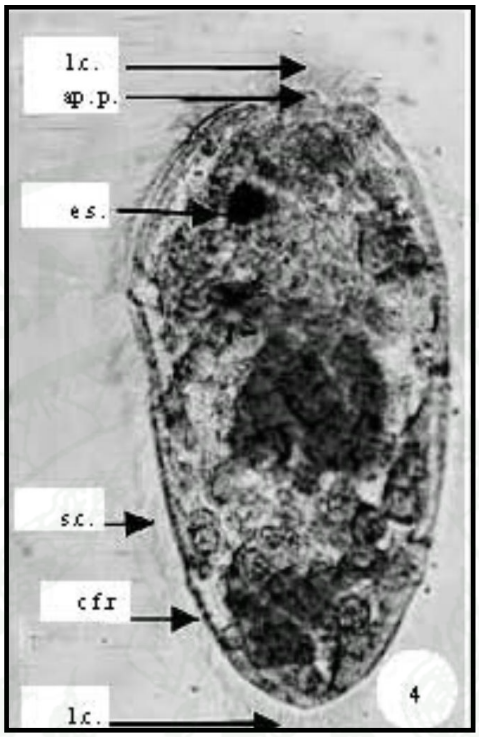

Figure 6 LM of miracidium showing apical papilla (ap. p.) and varied length of cilia, anterior and posterior long cilia (l. c.), eye spot (e. s.), short cilia (s. c.) and cilia free region (c. f. r.).

Source: Hussein *et al*. (2010)

2.2 Miracidium

The miracidium is about 130 µm in length. The emerged miracidium swims rapidly in aimless directions. It has an elongated conical body that has a broad anterior end and tapering posterior end. The surface is covered with numerous cilia, except in lateral connection regions of epidermal plates. These cilia are found to be characteristically longer on the apical part of anterior end and the posterior extremity than the cilia on the rest of the body. There is an apical or papilla, on which open one apical gland and two pair of penetration glands. There is one pair of eye spots at the

right side of midline of anterior part (Figure 6). The miracidium has one pair of flame cells situated at the end of second third of the body. Each flame cell leads to a tubule which goes down to the lateral side to end in an excretory pore. Germ cells are scattered in the posterior part. The miracidium with SEM has shown the typical pyriform shape of the body. The apical papilla is shown in the middle of the anterior broad part, while the whole surface of the body was illustrating dark with variable size (Figure 7). After hatched from the egg, the miracidium becomes active, starting to swim to find suitable hosts. They are short-lived. If they can't penetrate into the intermediate host within 24 hours, they will die.

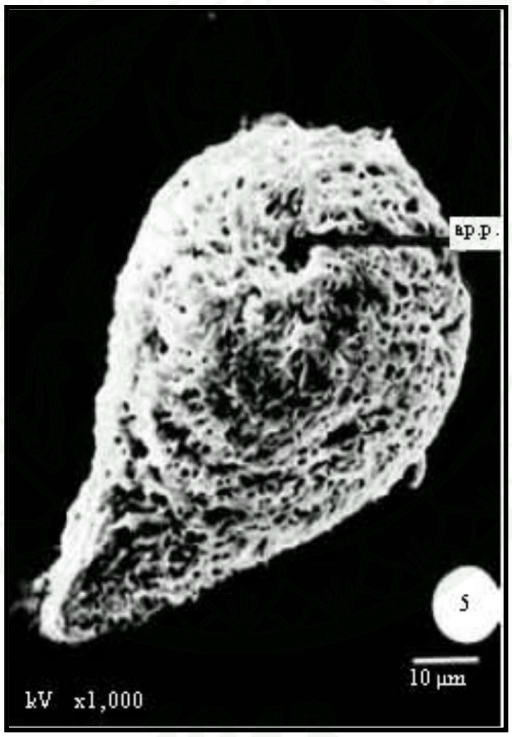

Figure 7 SEM of miracidium showing the conical body shaped, apical papilla (ap. p.) and many pits on the surface.

Source: Hussein *et al*. (2010)

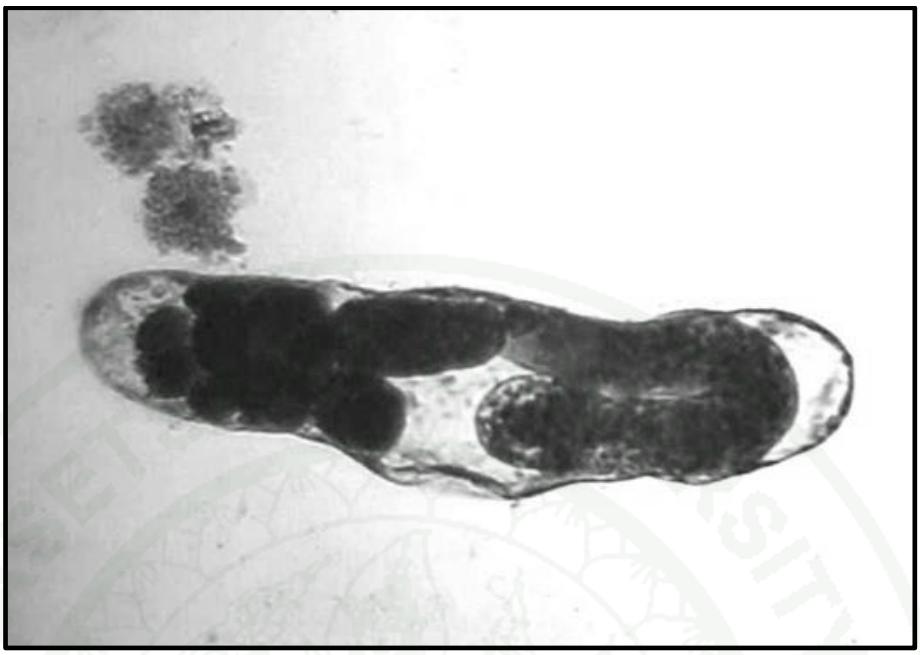

Figure 8 LM of sporocyst.

Source: Fox (2013)

2.3 Sporocyst

After penetrating the snail, miracidium loses its cilia and become the sporocyst (Figure 8). The sporocyst migrates via the blood vessels or lymph channels to the digestive gland, which is situated in the upper spirals of the shell. That place it begins to grow. Each germinal cell gives rise to a ball of new germinal cell from which the next stage of larvae, redia.

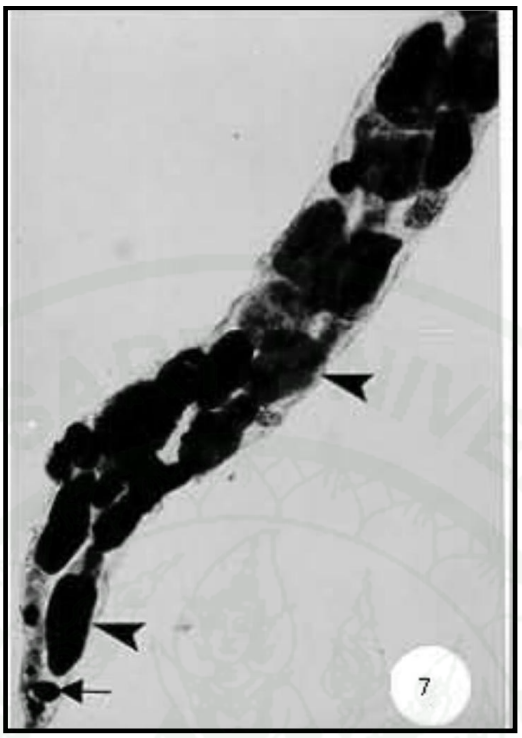

Figure 9 LM of daughter redia showing developing cercariae (arrowheads) and germ cells (short arrow).

Source: Hussein *et al*. (2010)

2.4 Redia

Mother redia has an elongated, flat body with an anterior projecting circular ridge or collar and ended with caudal papilliform process (Figure 9). It has a muscular pharynx followed by a simple sac-like intestine (gut). The mother redia contains undifferentiated structures and germ cells. Between 16 and 20 of these germinal balls are produced within each redia. Mother redia measures 1.47-1.86 mm in length, daughter redia has a long, cylindrical body but has not collar and the caudal papilliform process is inconspicuous. It has two posterior processes at the beginning of the posterior third of the body. It contains developing cercariae and germ cells. The alimentary canal begins with mouth, which leads to suctorial pharynx, followed by a short and simple gut. There is a birth pore at the anterior end through it the developed

cercariae emerge. Daughter redia measured 1.26-3.01 mm in length by 0.16-0.37 mm in width. It has a short gut that measures 0.42-1.02 mm in length. SEM study of daughter redia revealed the conspicuous suctorial pharynex with rounded muscular walls (Figure 10). The mature redia measure 1-3 mm in lengh and are capable of considerable movement. Their migrations can cause serious damage and in heavy infections, death of snail. When the cercariae are fully developed, they escape from the redia by way of the birth pore which is situated laterally behind the anterior collar.

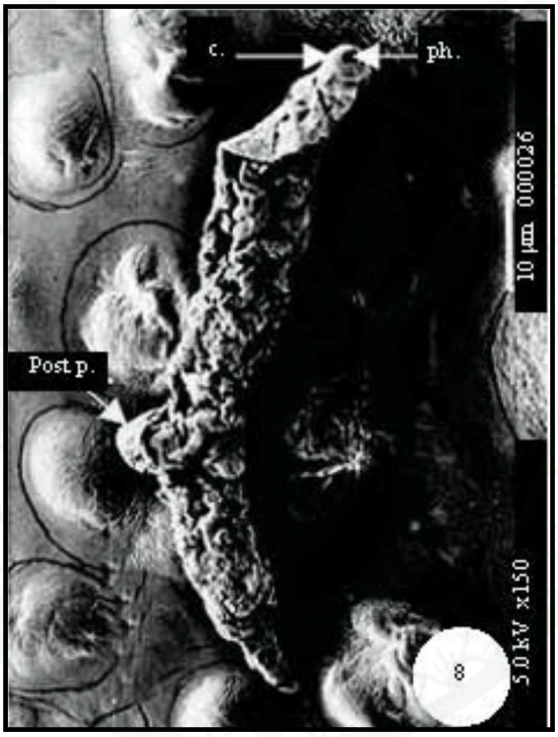

Figure 10 SEM of redia showing pharynx (ph.), collar (c.) and a posterior process (post. p.).

Source: Hussein *et al*. (2010)

Figure 11 LM of cercaria showing the general characters.

Source: Hussein *et al*. (2010)

2.5 Cercaria

The emerged cercaria in water swims actively in aimless direction. It is tadpole-like with a large heart shaped body and simple long tail. The body has a characteristically thick wall (Figure 11) and is surrounded by minute spines all over its surface. The body length ranges between 0.15-0.21 mm in length and 0.18-0.24 mm in width. The tail length is nearly three times as the body, as its length ranges between 0.56-0.7 mm, its width 0.35-0.56 mm. The ventral sucker is larger than the oral sucker. The former varies from 0.028-0.039 mm, while the latter 0.037-0.048 mm. The rudiment of the alimentary canal consists of a mouth followed by pharynx surrounding the oesophagus that leads to intestine. The latter bifurcates into two simple branches that extend around the ventral sucker to a level below the posterior border of the ventral sucker. The genital primordium is double-shaped with two unequal rounded or oval masses connected together with a longitudinal bar. It is located between the upper surface of the excretory vesicle and the upper border of the

ventral sucker. The body is full of numerous cystogenous glands. Due to the thick body wall and the densely deposited cytogenous glands, it is difficult to visualize the excretory system and flame cell formula. Study of the cercaria with two fin-like processes at its lateral sides (Figure 12). The cercariae generally leave the snail 4-7 weeks after infection by migrating through the tissues. Then, the cercariae are released from the snail. The cercariae swim freely in the water. They although tend to keep near the surface rather than going down into deeper water. They find the water plant or debris for attach and develop into the next stage, metacercariae.

After attachment of cercaria to a suitable object or even in the water, the tail is still lashing vigorously from side to side and a thin irregular wall starts to be secreted around the body from the cystogenous glands. Tail becomes separated from the body while the cystogenous glands secretions become more accumulated in several layers usually four around the more or less rounding-up body. At that stage, many of the internal structures of the cercarial body are easily seen through the cyst wall particularly the suckers and the genital primordium (Figure 13). The wall becomes more and more thick and the cercarial body becomes curled on itself in the cyst, which become slightly smaller in size but with a well developed cyst wall. At that stage it is difficult to differentiate any of the internal structures of the body.

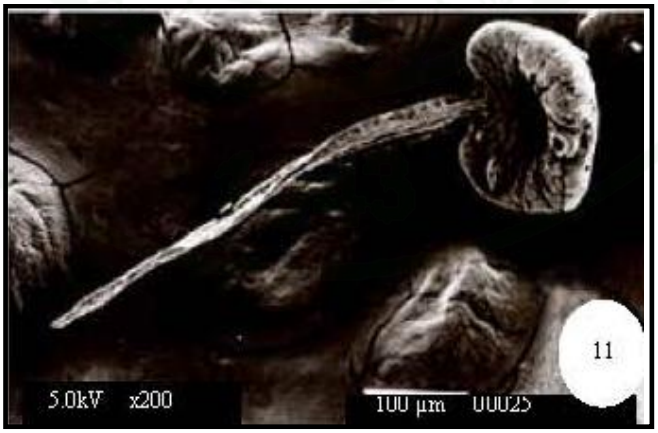

Figure 12 SEM of cercaria showing the general shape of the body.

Source: Hussein *et al*. (2010)

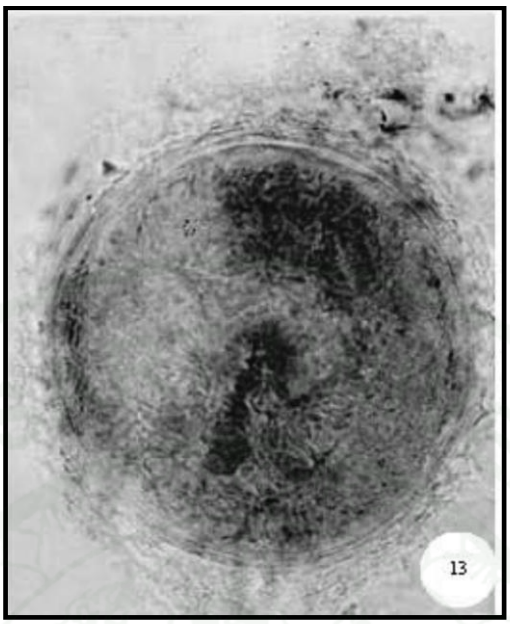

Figure 13 LM of metacercaria.

Source: Hussein *et al*. (2010)

2.6 Metacercaria

Metacercaria has a double thick cyst wall. The recently formed metacercaria has larger diameter than the mature one where the former diameter ranging between 0.224-0.272 mm and the latter's diameter ranging between 0.215-0.256 mm. Study of the metacercaria with SEM has shown that the outer layer consists of filament (Figure 14). The structure of the cyst wall is complex with consisting of an outer cyst and inner cyst. The outer cyst wall probably acts as a barrier against bacterial and fungal infections, and is also important for attachment to the substrate. Strong adhesion to blade of water plant for long times is important for the survival of metacercariae and the infection of the definitive hosts. As the cyst may survive for long period and remain infective if the outer wall is removed, the inner cyst wall must play a more important part in the survival of the metacercariae. The cyst is white when laid, after one or two day the cyst gradually becomes yellow in color due to the presence of quinine and darkens as it hardens. Definitive host infected

fasciolosis by ingestion of water plants or water carrying metacercariae. Metacercariae excyst in duodenum and the immature flukes penetrate through the intestine wall to the liver.

Figure 14 SEM of metacercaria.

Source: Hussein *et al*. 2010)

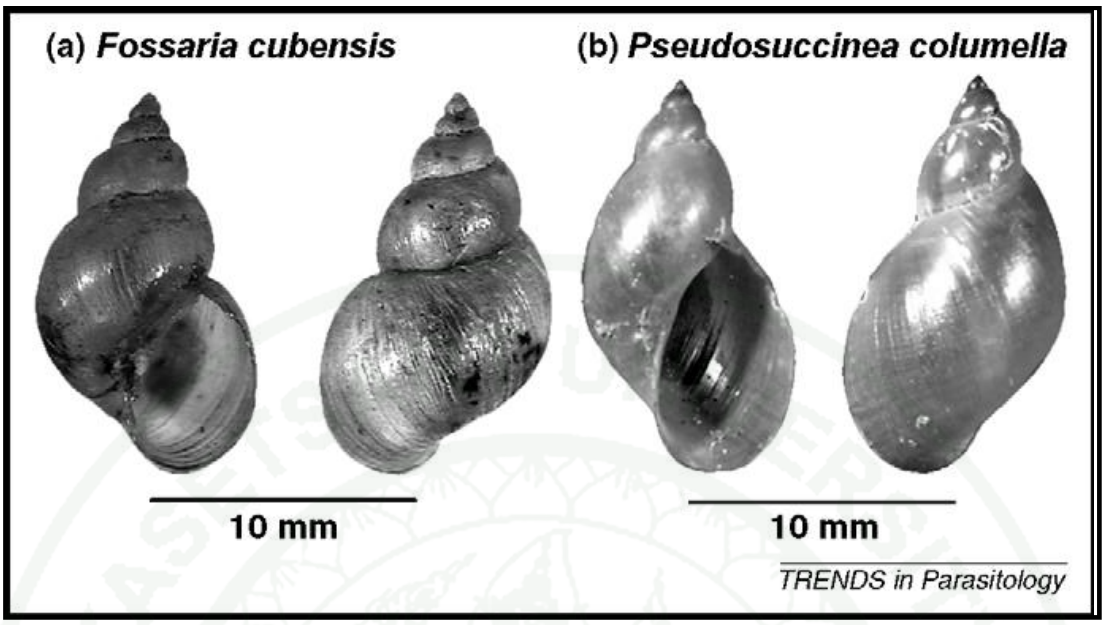

Figure 15 Lymnaeid snail

Source: Rojas *et al*. (2010)

2.7 Lymnaeid snail

Fasciola needs one intermediate host to complete the life cycle, a fresh water gastropod from the superfamily of pulmonate snail, family lymnaeidae. Lymnaeidae snail occurs worldwide. The water snails in the family lymnaeidae are major medical and veterinary importance problem. They act as the intermediate hosts of many trematodes including *Fasciola* spp. Several species of lymnaeidae including *Galba truncatula, Pseudosuccinea columella, Radix auricularia* have been described as potential vectors of fascioliasis. The ability of *Fasciola* spreading is related to capacity of liver fluke intermediate hosts to colonize and adapt to new environments. The larva development is occurred in the water snails. The *Fasciola* miracidium penetrate into the water snails and develop into sporocyst, rediae and cercariae, respectively. The *Fasciola* cercariae are discharged from the water snails. The prevalence of snail infections with liver flukes related to prevalence of liver fluke infection in domestic animals. *G. truncatula, P. columella* and *Austropeplea viridis*

snail was infected with *F. hepatica* (Mekroud *et al*., 2004; Molloy and Anderson, 2006). *R. rubiginosa*, *R. swinhoei* and *A. viridis* were reported in Thailand and infected with the *F. gigantica* larvae (Kaset *et al*., 2010). The areas occurring lymnaeid snails were the high prevalence of fasciolosis (Srihakim and Pholpark, 1991; Itagaki *et al*., 1988).

2.8 Water plant

After, cercariae are released from the water snails. They swim in water until encystment on the aquatic plants or debris. The *Fasciola* infection is occurred by ingesting the water plants or water contaminated with metacercariae of *Fasciola*. Many consumption water plants such as water caltrop (*Trapa natans*, *T. bispinosa* and *T. bicornis*) (figure 16), water chestnut *(Eleocharis tuberosa*) (figure 17), water hyacinth (*Eichhornia*) (figure 18), water bamboo (*Zizania*) (figure 19), water lotus (*Nymphaea lotus*), water lily (*Nymphaea*) (figure 20) and water morning glory (*Ipomoea aquatic*) (figure 21) can be attached with metacercariae.

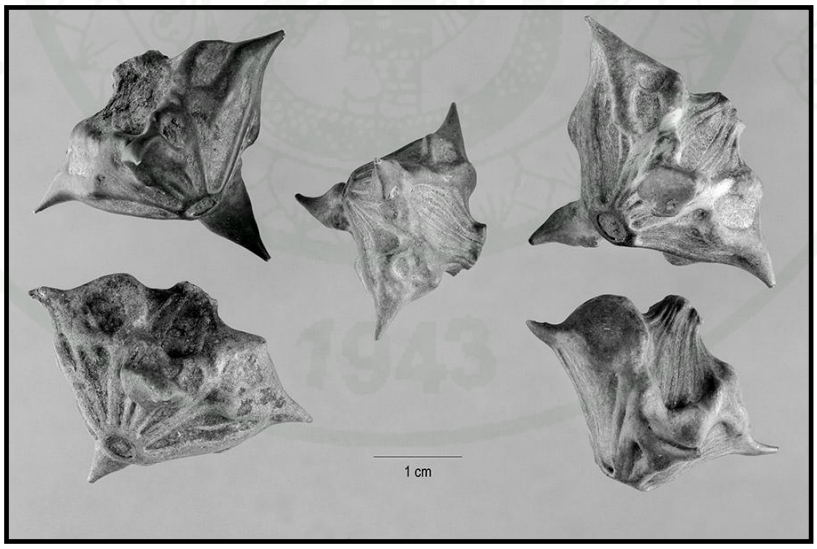

Figure 16 Water caltrop (*Trapa natans*, *T. bispinosa* and *T. bicornis*)

Source: USDA (2013)

Figure 17 Water chestnut *(Eleocharis tuberosa*)

Source: USDA (2013)

Figure 18 Water hyacinth (*Eichhornia*)

Source: USDA (2013)

Figure 19 Water bamboo (*Zizania*)

Source: USDA (2013)

Figure 20 Water lotus or water lily (*Nymphaea*)

Source: USDA (2013)

Figure 21 Water morning glory (*Ipomoea aquatic*)

Source: USDA (2013)

3. Epidemiology

The epidemiology of bovine fasciolosis had been reported in many countries around the world and the fasciolosis impact had been estimated in several studies. The epidemic areas of fasciolosis distributes in Andean countries (Bolivia, Peru, Chile and Ecuador), the Carribbean area (Cuba), Northern Africa (Egypt) and Western Europe (Portugal, France and Spain) (Claxton, 1997; Esteban, 1999; González-Lanza *et al*., 2011: Mas-Coma, 2005). In Asia (Turkey, Korea, Japan, Thailand, India, Yemen, Israel and Saudi Arabia), fasciolosis was sporadic. Some studies were reported the high prevalence in Iran (Kurdistan, Zandjan, Kerman shah, Mazandaran, Tehra, Azarbaijan and Gilan) (Moghaddam, 2004; Mas-Coma, 2005; Daryani, 2008).

There was the distribution of *Fasciola* infection in Southeast Asia and almost of fasciolosis was caused by *F. gigantica*. The liver fluke infection caused by *F. gigantica, F. hepatica* and *Gigantocotyle explanatum* were occurred in the liver of large ruminants and widespread on all the main islands in Indonesia (Suweta, 1991). The prevalences of bovine fasciolosis were found 6-90% in the both cattle and buffaloes in Indonesia and Malaysia (Partoutomo *et al*., 1985; Saleha, 1991). The survey of beef cattle at Nongdaung slaughterhouse, Vientiane capital in Lao PDR and Cambodia revealed between 5% and 94.7% infection rates with *F. gigantica* (Dorny *et al*., 2011; Phomhaksa *et al*., 2012). Fascioliasis was widespread and destructive parasitic disease of farm animals and still the leading cause of morbidity and mortality in ruminants in Vietnam. The survey on bovine fasciolosis was conducted in Vietnam and showed 28-72.2% positive rates in beef and dairy cattle (Geurden *et al*., 2008; Nguyen *et al*., 2011). The prevalence of *Fasciola* infection in cattle and buffaloes showed 0-85% and distributed in many parts of Thailand (Prasitirat *et al*., 1996-1997; Worasing, 2007; Gray *et al*., 2008; Neamjui *et al*., 2012). The percentage of infection in the northern part was the highest, while the lowest was found in the south (Tuntasuvan and kitikoon, 1996). Moreover, the human could be infected by *Fasciola* and there were the report of human fasciolosis in Thailand (Tesjaroen *et al*., 1988; Aroonroch *et al*., 2006; Kanoksil *et al*., 2006;). The infection rate of *Fasciola* was

found 0.36% in in-patient at Siriraj hospital from 1991-1995 (Tiewchaloren and Junnu, 1996).

Table 1 Prevalence of fasciolosis in cattle and buffaloes in the previous studies

Countries	Prevalence (%)	Authors and years
Indonesia	7-90	Partoutomo *et al*. (1985)
Colombia	18	Griffiths *et al*. (1986)
Spain	29.5	González-Lanza *et al*. (1989)
Malaysia	14.7-41.7	Saleha (1991)
Thailand	47.1	Sukhapesna *et al*. (1990)
Thailand	0-85	Srihakim and Pholpark (1991)
Thailand	0.36	Tiewcahloren and Junnu (1996)
Thailand	4-23.4	Tuntasuvan and Kitikoon (1996)
Thailand	8.9-13.9	Tomanakarn *et al*. (1998)
Mexico	60-80	Ibarra *et al*. (1998)
Peru	56.7-66.8	Neyra *et al*. (2002)
Spain	30.4-56	Paz-Silva *et al.* (2003)
Portugal	11-48	Conceição *et al*. (2004)
Algeria	6.3-27.3	Mekroud *et al*. (2004)
Iran	7.3-25.4	Moghaddam *et al*. (2004)
Australia	41.4	Molloy *et al*. (2005)
Britain	53.3	Pritchard *et al*. (2005)
Tanzania	28.4-63.8	Keyyu *et al*. (2006)
Australia	0-100	Molloy and Anderson (2006)
Switzerland	18	Rapsch *et al*. (2006)
Mexico	11.4-44.5	Munguía-Xóchihua *et al*. (2007)
Thailand	8.2	Worasing (2007)
Belgium	37.3	Bennema *et al*. (2009)
Ethiopia	24.3	Berhe *et al*. (2009)
Nigeria	29.4	Cadmus and Adesokan (2009)

Table 1 (Continued)

Countries	Prevalence (%)	Authors and years
China	24.9-44.7	Liu *et al*. (2009)
India	2.7-31.1	Garg *et al*. (2009)
Tanzania	16.9-52.6	Nonga *et al*. (2009)
Ethiopia	4.9-14	Abunna *et al*. (2010)
Pakistan	4.8-20	Athar *et al*. (2010)
Egypt	17.2-33.7	Hussein and Khalifa (2010)
Brazil	15.2-28.2	Bernado *et al*. (2011)
Nigeria	13.1	Alawa *et al*. (2011)
Combodia	5-20	Dorny *et al*. (2011)
France	17.8	Duscher *et al*. (2011)
Poland	21.2	Kozlowska-Lój (2011)
Tanzania	8.6	Mellau *et al*. (2011)
Netherland	0-41	Walker *et al*. (2011)
Thailand	3.7	Jittapalapong *et al*. (2011)
Thailand	3.3-13.9	Neamjui *et al* (2012)
Germany	45.1-57.1	Kuerpick *et al*. (2012)
Lao PDR	11.6	Phomhaksa *et al*. (2012)

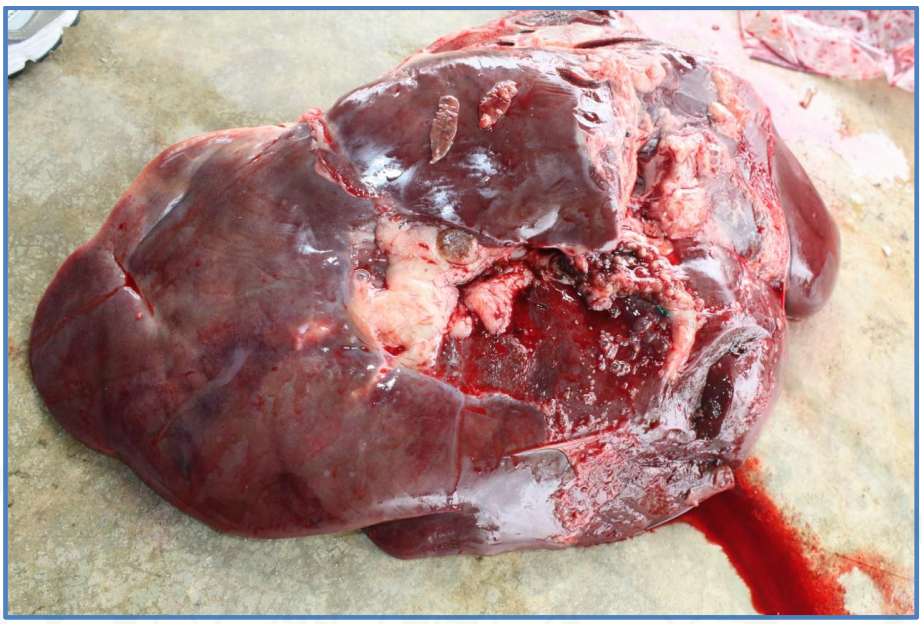

Figure 22 The infected liver with *F. gigantica*

4. Pathogenesis

Fasciola can naturally infect cattle, buffaloes, sheep, goats and human. It is a parasite of wildlife and zoo animals. The large ruminants are usually chronic *Fasciola* infection and the hosts express in poorly defined clinical signs. The farmers may have to be convinced that their domestic animals are affected by showing them specimens, slight shows or the improvements in body weight, draught performance or fertility that occur after treatment. The effect on animals is tissue damage, with resultant haematological and biochemical changes and immunological response. In general, the liver of host, the more relative damage caused by flukes and the less the functional reserve of the liver tissue. The disease occurs in three phases including pre-hepatic migration, the parenchyma during the migration of immature flukes through the liver parenchyma and biliary phase which coincides with their residence in the bile ducts. The infected livers are bigger and heavier than normal ones. Pale or haemorrhagic migratory tracts are present in the earlier stages. However, as the disease advances, the liver becomes generally paler and firmer and the bile ducts more prominent from dilated and thickened walls. There are fibrinous tags on the capsule of fibrous

adhesions between organs. Hepatic and mesenteric lymph nodes are reactive and enlarged.

Fasciolosis can be a spectacular disease. The acute terminal fasciolosis can be found causing the appearance of heavily infected animals with liver fluke. Pre-hepatic migration, newly excysted juveniles penetrate mucosa and through the abdominal cavity to the liver. After burrowing into the mucosa the juvenile fluke dissolves tissue in the submucosa and muscle layers of the small intestine and passes through the serosa into the abdominal cavity. Penetration is not associated with clinical disease perhaps because only relatively few cells are disrupted. The immature flukes may penetrate to other organs such as the diaphragm and lung. The pathology is more significant in heavy infections.

Maturation of flukes involves development and growth over 12-16 weeks during which time the immature fluke travels between and within organ. After penetration of the liver, immature flukes wander in the parenchyma for several weeks when their total effect is dose dependent. The individual fluke may pass through the same part of the liver twice or more during these peregrinations, fresh and resolving lesions caused by the sequential insults may be found in the same section of tissue. Histological changes occur in the following three phases which may coexist in fully developed cases. The first phase, necrotic and haemorrhagic tracts appear in sub-capsular and parenchyma areas. The second phase, there are cellular reactions with infiltration by eosinophil and later macrophages and lymphocytes. Vascular damage may lead to infarction. The developing flukes wander for several weeks in the liver parenchyma, tracts become larger. Finally, the immunological reaction of macrophage and lymphocyte infiltration merges with fibrotic healing of the necrotic areas. The portal areas also become fibrotic. The flukes enter the bile ducts from about eight weeks after infection and begin egg production about 12 weeks after infection. As the immature fluke grows, the size of its track through the liver increases as does the damage and the inflammatory response. The level of infection also affects the pathology. Smaller infection generally is a chronic fasciolosis. The heavy burden causes more severe pathology and earlier termination by death hepatic stages, flukes

are strong predilection for the liver tissue. The migration of the immature fluke through the liver tissue provides conditions conductive to multiplication of the bacterium and a necrotic hepatitis is usually found at post-mortem. Calves are susceptible to disease. The disease is characterized by weight loss, anaemia, hypoproteinaemia and death. Resistance develops with age so that adult cattle are quite resistant to infection although some benefits to weight gain have been reported following flukicide treatment. There is variation in the infection rates and the severity of disease with individual animals.

Surviving flukes confront hostile inflamatory reaction. The bile ducts thicken due to epithelial hypertrophy and subsequent fibrosis of the wall of the duct. Calcium deposits start to form in the duct wall after 8-12 weeks posted infection. As a result ducts enlarge up in diameter and become prominent on the surface of the liver. The lumina of the ducts are variously dilated and stenosed and the epithelium shows ulceration and haemorrhage. Few flukes reach the bile duct and few eggs are passed. Most flukes are lost by 12 weeks after infection.

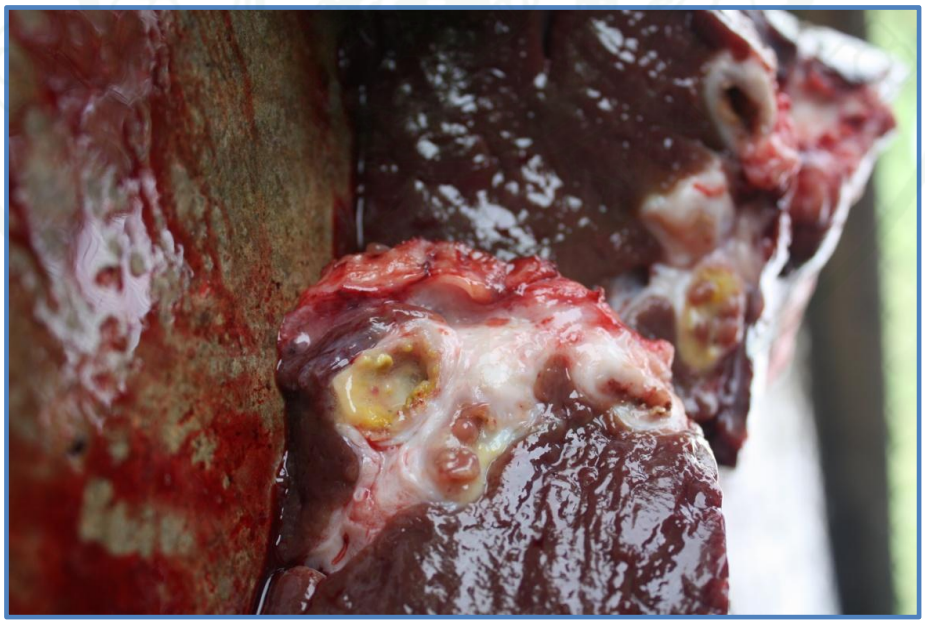

Figure 23 The dilatation of bile duct with stones and bile sladge.

5. Control measures and eradication

5.1 Treatment and control with drug

The drugs of benzimidazole group such as triclabendazole, albendazole and mebendazole are for the treatment and control of helminthic parasites including *Fasciola* in the domestic animals. These drugs are highly effective for the treatment and control of bovine fasciolosis. The timing of drug use is equally as important as their efficacy. The best time for treatment for control of *Fasciola* infection is when it is assessed that animals might be free from infection as the occurrence of fasciolosis is seasonal. The domestic animals were treated with triclabendazole (12 mg/kg for cattle and 24 mg/kg for buffaloes) at the beginning of the wet season.

5.2 Snail control

Molluscicides have mainly recommended for use in the pond to control the water snails which are the intermediate hosts. However, some drugs may effect on non-target animals and plants in the habitat. The ducks or geese, which eat the water snails, have also been proposed as a possible means for biological control of liver flukes. The effective control would need enough ducks to eat many water snails in habitat before they shed cercariae.

5.3 Feed management

The viability of metacercariae had up to 23 weeks when stored in water. The increasing temperature and humidity were effected the viability and the sunlight could kill metacercariae within 8 hours. Metacercariae immersed in water remained viable longer than those allowed to desiccate. The metacercariae on the desiccated stalks would survive less than 2 weeks (Suhardono *et al*., 2006). The fresh rice stalks were exposed by direct sunlight before storing or feeding them to the domestic animals.

6. Diagnosis

The diagnostic of *Fasciola* infection has many techniques including fecal examination, immunological method and molecular techniques. The conventional method is the fecal examination. This diagnostic test is finding the egg of parasites. The fecal techniques are achieved by both flotation and sedimentation of liver fluke eggs. The fecal methods base on formalin-ethyl acetate sedimentation, beads technique (Bonita and Taira, 1996) and direct smears (Srimuzipo *et al*., 2000). The flotation technique, which is generally used for concentrating parasite eggs, can also be used to float liver fluke eggs. Sedimentation techniques require little stools which can detect the infection of liver fluke and the number of parasitic egg. The formalin-ethyl acetate is the referent method for detection of *Fasciola* infection. However, the coproscopic examination was impractical because the small numbers of egg were passed in large amounts of feces, particularly in the early stage of fasciolosis (Intapan *et al*., 2003). The sensitivity and repeatability of the fecal technique are found to be low when used for detecting eggs of *F. gigantica* in bovine feces. The fecal examination could not detect the early fasciolosis and showed high false negative in the chronic infection. Moreover the morphology of egg was similar to egg of other flukes, it made to misidentification. However, they were different in color (Awad *et al*., 2009). Thus, the serological methods are developed to diagnose fasciolosis in the early infection.

The immunological method was used to diagnose subclinical infection, early, current and chronic infection. That assay was simple, sensitive and specific for *Fasciola* infection but it was less practical for field surveys (Intapan *et al*., 2003). The serological test was diagnosed by detecting circulating IgG against antigens. Moreover, ELISA could be used to diagnose the level of anti-*Fasciola* antibody in milk (Reichel *et al*., 2005; Salimi-Bejestani *et al*., 2007). The antigens were studied for immunological diagnosis which showed high sensitivity and specificity, such as crude somatic antigens, excretory-secretory antigen (ES-Ag), parasite eggs and larvae. The crude antigens from *F. gigantica* had 17 proteins such as 13.8, 15.7, 27.4, 30.1, 42.9, 53.3 and 178.4 kDa etc (Awad *et al*., 2009). The performance of crude somatic

antigen in ELISA had high sensitivity (93.3 – 100 %) and specificity (80 – 85.2 %). Some crude antigens had been studied for their characteristic. The 12 kDa was fatty acid binding protein (FABP) while the 45 kDa was glutathione S-transferases (Estuningsih *et al*., 1997).

There are many excretory-secretory antigens from *Fasciola* such as cathepsin cysteine proteases, glutathione S-transferase and fatty acid binding proteins. The molecular weights of ES-Ag were 28, 30, 40 and 60-62 kDa (Ridi *et al*., 2007). Other studies, ES-Ag showed protein bands with molecular weight of 15, 28, 31.6, 32.9, 39.4, 83.3 and 101.7 kDa. However, the 27 kDa antigen of ES-Ag was the specific antigen of *Fasciola* (Estuningsih *et al*., 1997).

The 27 kDa antigen was grouped of cathepsin cysteine proteases. This antigen was the main antigen in ES-Ag of *Fasciola* and detected in many organs of liver fluke including tegument, gut and reproductive organ. Cathepsin was produced from the midget epithelial cells of the parasite and the pH of cathepsin was 4.0-7.3 (Smith *et al*., 1993). Cathepsin proteases were found and differentiated function in each stage of the liver fluke. Cathepsin L was normally protease in each stages while, cathepsin B could find only in the juvenile stage (Robinson *et al*., 2009; Smooker *et al*., 2010). Cathepsin B and L helped the excystment in the metacercariae stage. Newly excysted juvenile produced cathepsin B and L to penetrating gastrointestinal tract, migration to liver, migration through liver parenchyma and feeding. Cathepsin L was the major proteases in the immature and adult fluke. The function of cathepsin L in that stage was the migration of immature fluke through liver parenchyma, migrating into bile duct and feeding. Moreover, cathepsin L could modulate the host immune response. Cathepsin protease was an important factor for damage of hosts causing *Fasciola* infection and modulation of host immune response (Oldham and William, 1985; Sajid and McKerrow, 2002).

Life stage	Major cathepsins secreted	Processes
 Metacercariae	Cathepsin B Cathepsin L	Excystment
 Newly excysted juvenile	Cathepsin B Cathepsin L	Penetration of gut Migration to liver Migration through liver parenchyma Immunomodulation Feeding (juvenile parasite)
 Adult	Cathepsin L	Migration through liver parenchyma Migration into bile ducts Feeding Immunomodulation

Figure 24 The life stage of liver fluke and the proposed contributions of protease to various essential functions

Source: Smooker *et al*. (2010)

The ES-Ag based on serological diagnosis had high sensitivity (93 – 100%) and specificity (96.3 – 100%) in experimentally infected sheep, cattle and buffaloes (Estuningsih *et al*., 1997; Cornelissen *et al*., 2001; Kumar *et al*., 2008), compared to coprological and bile examinations (Ridi *et al*., 2007). The high accuracy (96%) was obtained when used ES-Ag as antigen for ELISA (Awad *et al*., 2009). Cathepsin-ELISA (27 kDa) was high sensitivity (100%) and specificity (97.4). However, ELISA was no crossed reactivity with sera from animals infected with other worms such as *Paragonimus, Trichinella, Paramphistomum epiclitum, Schistosoma, Dicrocoelium*

and hydatid cyst (Cornelissen *et al*., 1999; Dixit *et al*., 2002; Intapan *et al*., 2005; Sriveny *et al*., 2006). The antibody against *Fasciola* in rabbit induced by 27 kDa ES-Ag could detect in 2 weeks post infection (Ghosh *et al*., 2005) and signals persisted for at least 20 weeks (Cornelissen *et al*., 2001). 27 kDa ES-Ag was evaluated to a commercially available ELISA for diagnose antibodies to *F. gigantica* and *F. hepatica* in cattle, sheep and buffaloes (Molly *et al*., 2005).

7. Information of Songkhla Lake

Songkhla Lake is the largest natural water reservoir in the southern part of Thailand. The lake covers the area of 1,040 km^2 and borders the provinces of Songkhla and Patthalung. This lake is divided into 3 parts of northern lake (Noi sea), southern lake (Luang sea) and southern end lake (Songkhla sea).The northern lake is the fresh water lake and covering the area of 27 km^2 in Patthalung province. The southern lake covers the area of 830 km^2. The upper southern lake is the fresh water lake but the lower southern lake is the brackish water. The southern end lake is the brackish water and covering the area of 183 km^2. It is said that Songkhla Lake is "Lake of the water" that is both fresh water, brackish water and salt water. The Lake of water is highly variable depending on the season. In the dry season, some of the fresh water flow in the lake or the salt water invasion, the water of lake will be explicitly that is the upper part is fresh water, center part s fresh-brackish water and lower part is brackish-salt water. In rainy season, some of fresh water almost completely drains salt water out of the lake; all of water in the lake is nearly fresh water except the mouth of the lake is brackish. Songkhla Lake has stagnant water all year round. Climate around Songkhla Lake is influenced from southeast and northeast monsoons. Climate is distinguished into 2 seasons including rain and summer. Summer begins February to mid of July while rain is between July and January.

There are several domestic animals of goat, cattle and buffalo rearing nearby Songkhla Lake area. The traditional rearing system of cattle and buffaloes nearby the lake is freely grazing on the pastures. Cattle graze on the pasture and the shallow water nearby the lake while buffaloes can graze both on the pasture, the shallow water

and deep water. Buffaloes are wider spread of grazing due to buffaloes can swim from the stay pasture to the other pastures and buffaloes have the long period of staying in Songkhla Lake. In the previous period, many cattle nearby Songkhla Lake died and the post mortem of these cattle found *Fasciola* in the liver. The dead cattle may be due to infections by *Fasciola*. There was the possibility of bovine fasciolosis distribution around the lake.

MATERIALS AND METHODS

1. Study areas

This study was conducted nearby Songkhla Lake at Sathing Phra (ST), Ranot (SR), Singha Nakhon (SS), Krasaesin (SA) and Khuan Niang (SK) districts in Songkhla province (Figure 25).

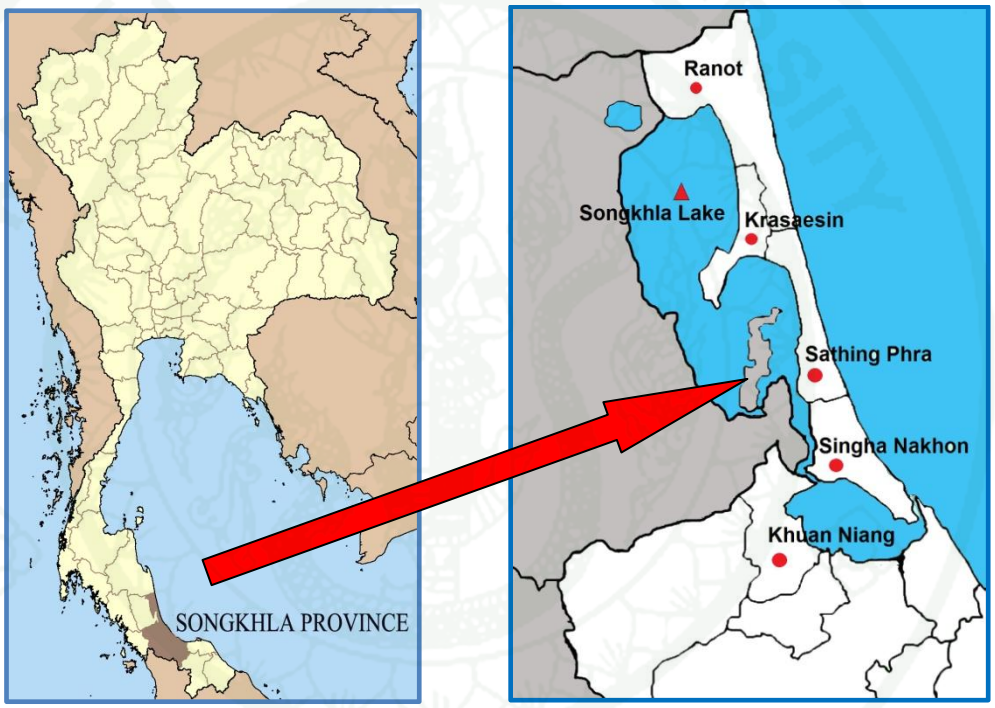

Figure 25 5 districts of study areas in Songkhla province nearby Songkhla Lake

2. Blood and fecal samples collection

505 and 95 blood samples of cattle and buffaloes, respectively were collected directly from jugular vein of animals. Blood samples were allowed to clot at room temperature for one hour, centrifuged at 1,448 G for 20 min, separated for sera, and stored at -20°C. 500 and 83 fecal samples of cattle and buffaloes, respectively, were collected directly from the rectum of the animals and stored at 4°C.

Figure 26 Collecting blood samples from jugular vein of buffalo

Figure 27 Collecting fecal samples from rectum of buffalo

3. Fecal examination by formalin-ethyl acetate sedimentation

Fecal samples were washed in normal saline, strained into centrifuge tube, and centrifuged at 1,448 G for 5 min. The supernatant was decanted, and 10%formalin was added to a volume of 10 ml into the tube and mixed. The suspension was added 2 ml with ethyl acetate, shaken and centrifuged at 1,448 G for 5 min. Finally, loosening the debris plugs at the top layer and 10%formalin were decanted. The remaining pellets were mixed with new 10%formalin, added a drop of suspension on a glass slide and examined under light microscope.

4. Collection of adult *F. gigantica* and isolation of ES-Ag

Adult worms of *F. gigantica* were collected from liver and bile ducts of cattle at the slaughter houses and transferred into phosphate buffer saline (pH 7.2). Alive flukes were washed with normal saline to remove bile contents. Thereafter, the flukes were washed five times in PBS (pH 7.2). Adult flukes were washed and cultured to obtaining ES antigen which followed the protocol by Sriveny *et al.* (2006). Briefly, the flukes were incubated in RPMI1640 (penicillin 100 unit/ml) at 37ºC overnight. One liver fluke was incubated in 1 ml of medium for each well. At the end of the incubation, the culture media containing ES-Ag product were harvested and centrifuged at 10,000 G for 30 min at 4ºC to remove the debris and parasite eggs. Then, the media supernatant was precipitated by two steps alcoholic precipitation. Chilled ethanol was added drop wise to the media supernatant to a final concentration of 60% (v/v) and protein precipitation was carried out overnight at -20ºC. The media supernatant at 60% (v/v) ethanol concentration was centrifuged at 10,000 G. The supernatant was taken to a final ethanol concentration of 75% (v/v) and protein precipitated overnight at-20ºC. The precipitate was retrieved by pelleting at 10,000 G for 30 min at 4 ºC. The pellet representing regurgitated protein was washed in absolute ethanol, air dried and resuspended in PBS, pH 7.2. ES-Ag was determined protein concentration by Bradford method, aliquotted and stored at -80ºC.

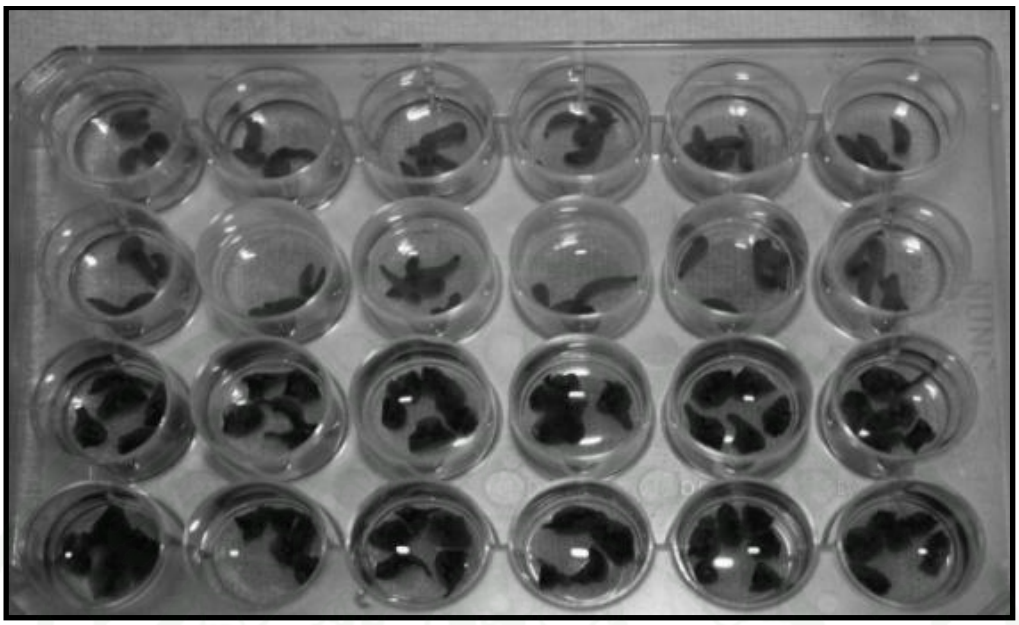

Figure 28 Incubated adult *F. gigantica* in RPMI1640

5. Enzyme-Linked Immunosorbent Assay (ELISA)

The sera from cattle and buffaloes were evaluated for anti-*F. gigantica* antibody by ELISA. The ELISA was performed following the method described by Raina *et al.* (2006) with some modifications. Briefly, ELISA plates were coated with 0.25 µg ES Ag per well in 100 µL of 0.1M carbonate buffer (pH 9.5), incubated at 4 ◦C overnight. The plates were washed five times with 200 µL/well washing buffer (0.05% Tween-20 in PBS) and blocked with 100 µL/well blocking buffer (3% skimmed milk powder in PBS) for 1 h at 37 ◦C. The plates were washed five times with washing buffer. One hundred µL of serum dilution (1:200 dilutions in blocking solution) was added into the wells and incubated at 37 ◦C for 1 h. The plates were washed five times. The anti-bovine IgG peroxidase conjugate was added into the wells and the plates were incubated at 37 ◦C. Finally for 1 h, after five washes, 100 µl of substrate (3,3'5,5'-tetramethyl benzidine, TMB) was added and plates were incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 100 µl of 1N HCl into each well. The absorbance was read at 450 nm by ELISA plate reader. The positive control was chosen from the positive sample tested

by fecal examination which the cattle showed single helminthic infection. The negative control was chosen from the sample which was not found any parasite in the stool. The cut-off point for the OD from the ELISA method was analyzed from the mean OD obtained from the sera of 32 negative samples diagnosed by formalin-ethyl acetate sedimentation. Three standard deviations of these measurements were added to the mean of negative samples (Hillyer *et al*., 1992).

Cut-off point = mean OD of negative samples + 3SD

6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE analysis of antigens of *Fasciola* was performed according to Laemmli (1970) and the obtained protein bands were stained with Coomassie blue R-250. The gels generally consist of acrylamide, bisacrylamide, SDS, and a Tris-Cl buffer with adjusted pH. Ammonium persulfate and TEMED were added when the gel was ready to be polymerized. Gels were polymerized in a gel caster. Firstly, the separating gel was poured and allowed to polymerize. Next, the loading gel was poured and a comb was placed to create the well. After the gel polymerization, the comb was removed and the gel was ready for electrophoresis. The electrophoresis apparatus was set up with cathode buffer covering the gel in the negative electrode chamber, and anode buffer in the lower positive electrode chamber. The denatured sample proteins were added into the wells with syringe or pipette. The apparatus was hooked up to a power source under appropriate running conditions to separate the protein bands. An electric current was applied across the gel, causing the negatively charged proteins to migrate across the gel towards the anode. Depending on their size, each protein would move differently through the gel matrix: short proteins would more easily fit through the pores in the gels while larger ones would have more difficulty (they encounter more resistance). After a set amount of time (usually a few hours- this depends on the voltage applied across the gel; higher voltages run faster but tend to produce somewhat poorer resolution), the proteins would have differentially migrated based on their size; smaller proteins would have traveled farther down the gel, while larger ones would have remained closer to the point of

origin. Therefore, proteins may be separated roughly according to their size (molecular weight), certain glycoprotein behave anomalously on SDS gels.

7. Statistical Analysis

The prevalence of fasciolosis was determined and analyzed by from the ratio of positive results and total number of animals, and data was analyzed using the chi-square test, according to age and sex by Number Cruncher Statistical System programs (NCSS) version 2000 (Kaysville, UT).

RESULTS AND DISCUSSION

Results

1. The serological examination using ES-Ag based on ELISA

The 32 negative samples were tested for ELISA and provided mean of 0.452 and SD of 0.089. The cut-off point = 0.454 + 3(0.089) = 0.719. A sample was positive when its OD was greater than 0.719. The seroprevalences of *Fasciola* infection were 29.2% (148/505) and 78.9% (75/95) in cattle and buffaloes, respectively (Table 2). The ELISA results in cattle were 49.4% (42/85), 34.2% (39/114), 15.3% (17/111), 22.8% (34/149) and 34.8% (16/46) in Singha Nakon, Sathing Phra, Krasaesin, Ranot and Khuan Niang districts, respectively (Table 2). The positive rates of *Fasciola* infection in buffaloes were 85.2% (23/27), 100% (29/29), 70% (14/20) and 52.6% (9/19) in Singha Nakon, Sathing Phra, Krasaesin and Ranot districts, respectively (Table 2). The end-point titer was varying between 1:100 and 1:6,400 in cattle while, the end-point titer in buffaloes varied between ranging from 1:200 to 1:12,800. The highest end-point titers of cattle and buffaloes were 1:400 and 1:6,400, respectively. The highest seroprevalence in cattle was Singha Nakon district (49.4%), while, the lowest was Krasaesin district (15.3%) (Table 2). Sathing Phra district (100%) was the highest positive rate of *Fasciola* infection diagnosed by ELISA and Ranot district (52.6%) was the lowest positive rate in buffaloes (Table 2).

Table 2 The prevalence of *Fasciola* infection in cattle and buffaloes checked by ELISA

Districts	No. of animals		Positive *Fasciola* (%)	
	Cattle	Buffaloes	Cattle	Buffaloes
Singha Nakon	85	27	42(49.4)	23(85.2)
Sathing Phra	114	29	39(34.2)	29(100)
Krasaesin	111	20	17(15.3)	14(70)
Ranot	149	19	34(22.8)	9(52.6)
Khuan Niang	46	-	16(34.8)	-
Total	505	95	148(29.2)	75(78.9)

2. The fecal examination using formalin-ethyl acetate sedimentation

The result of fecal examination showed the high prevalence of gastrointestinal parasite in both of cattle and buffaloes (92.8% in cattle and 85% in buffaloes) (Table 3). The present study found several parasites including *Capillaria*, *Eurytrema pancreaticum*, *Fasciola*, *Moniezia benedeni*, Rumen flukes, Strongyle worms and *Trichuris* (Table 4). These parasites distributed in each districts of Songkhla province nearby Songkhla Lake. The prevalences of *Fasciola* infection were 7.8% (39/500) and 30.1% (25/83) in cattle and buffaloes, respectively. The positive rates of *Fasciola* infection in cattle were 17.9% (15/84), 7.1% (8/113), 4.5% (5/112), 3.4% (5/145) and 13% (6/46) in Singha Nakon, Sathing Phra, Krasaesin, Ranot and Khuan Niang districts, respectively (Table 3). The prevalence of *Fasciola* infection in buffaloes were 18.5% (5/27), 65.4% (17/26) and 27.3% (3/11) in Singha Nakon, Sathing Phra and Ranot districts, respectively but Krasaesin did not find the infected buffaloes (Table 3).

Table 3 The prevalence of *Fasciola* infection in cattle buffaloes checked by fecal examination

Districts	No. of animals		Positive parasite (%)		*Positive Fasciola*(%)	
	Cattle	Buffaloes	Cattle	Buffaloes	Cattle	Buffaloes
Singha Nakon	84	27	76(90.5)	23(85.2)	15(17.9)	5(18.5)
Sathing Phra	113	26	108(95.6)	25(96.2)	8(7.1)	17(65.4)
Krasaesin	112	19	110(98.2)	17(89.5)	5(4.5)	-
Ranot	145	11	126(86.9)	6(54.5)	5(3.4)	3(27.3)
Khuan Niang	46	-	44(95.7)	-	6(13)	-
Total	500	83	464(92.8)	71(85)	39(7.8)	25(30.1)

3. The SDS-PAGE analysis of ES-Ag

Protein concentration of ES-Ag, measured using the Bradford assay, was estimated at 900 µg/ml. Banding patterns on SDS-PAGE for ES-Ag of *F. gigantica* was showed in figure 29. The one specific band was clustered at 27 kDa.

4. The statistic analysis of data

The data were analyzed using the chi-square test, according to age and sex. The >7 years cattle (42.7%) showed the highest positive percentage of *Fasciola* infection while the positive percentage in female cattle (33.5%) was higher than the male cattle (17.4%) (Table 5). Both age and sex group differences were significant ($p<0.001$) in cattle. The >7 years (75.9%) and female (78.6%) buffaloes showed the lowest positive rate of *Fasciola* infection. However, the age and sex group differences were not significant ($p=0.8$ in age group and $p=0.76$ in sex group) in buffaloes (Table 5).

Table 4 The prevalence of intestinal parasite infections in cattle and buffaloes in each districts checked by fecal examination

Districts	No. of animals	Positive parasite (%)	Parasite species														
			C	F	R	S	C+R	C+S	E+R	F+R	M+S	R+S	R+T	C+R+S	F+M+R	F+R+S	R+S+T
Singha Nakon																	
Cattle	84	76(90.48)	-	-	48	4	1	-	-	15	-	8	-	-	-	-	-
Buffaloes	27	23(85.18)	-	3	17	1	-	-	-	2	-	-	-	-	-	-	-
Sathing Phra																	
Cattle	113	108(95.58)	-	-	72	7	-	1	-	5	-	20	-	-	-	3	-
Buffaloes	26	25(96.15)	-	1	8	-	-	-	-	16	-	-	-	-	-	-	-
Krasaesin																	
Cattle	112	110(98.21)	-	-	76	4	-	-	1	4	-	23	1	-	-	1	-
Buffaloes	19	17(89.47)	1	-	16	-	-	-	-	-	-	-	-	-	-	-	-
Ranot																	
Cattle	145	126(86.90)	1	-	77	13	2	2	-	3	1	23	-	1	-	2	1
Buffaloes	11	6(54.54)	-	-	3	-	-	-	-	2	-	-	-	-	-	1	-
Khuan Niang																	
Cattle	46	44(95.65)	-	-	32	-	3	-	-	5	-	3	-	-	1	-	-
Total																	
Cattle	500	464(92.8)	1	-	30	28	6	3	1	32	1	77	1	1	1	6	1
Buffaloes	83	71(85)	1	4	44	1	-	-	-	20	-	-	-	-	-	1	-

Abbreviations: C = *Capillaria*, E = *E. pancreaticum*, F = *Fasciola*, M = *M. benedeni*, R = Rumen flukes, S = Strongyle worms, T = *Trichuris*

Table 5 The influential factors of age and sex on *Fasciola* infection in cattle and buffaloes

Parameters		No. of animals	Positive *Fasciola* (%)	Statistic
Cattle				
Age				
	<1	35	1(2.9)	X^2=21.0, df=2,
	1-6	367	103(28.1)	p<0.001
	>7	103	44(42.7)	
Sex				
	Male	132	23(17.4)	X^2=12.1, df=2,
	Female	373	125(33.5)	p<0.001
Buffaloes				
Age				
	<7	37	31(83.8)	X^2=0.06, df=1,
	>7	58	44(75.9)	p=0.8
Sex				
	Male	11	9(81.8)	X^2=0.09, df=1,
	Female	84	66(78.66)	p=0.76

*p≤0.05 is statistically significant

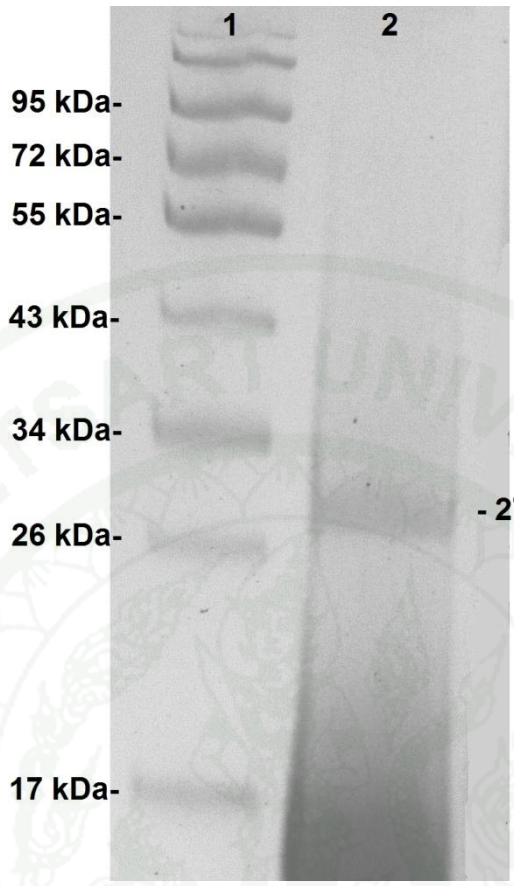

Figure 29 SDS-PAGE of ES-Ag stained with Coomassie blue: lane 1. Molecular weight marker (kDa) lane 2. ES-Ag

Discussion

The prevalence of bovine fasciolosis checked by fecal examination based on formalin-ethyl acetate sedimentation (7.8% (39/500) and 30.1% (25/83) in cattle and buffaloes, respectively) in the present study was similar to the previous studies in Nakhon Si Thammarat province. The fecal samples of beef and dairy cattle in Pakpanang river basin, Nakhon Si Thammarat province were diagnosed for gastrointestinal parasites by simple floatation technique and the result of this study showed 8.2% (237/2,898) in the positive rate of infection with *Fasciola*. However, there were several previous studies in many parts of Thailand and the results of these studies differentiated with the current study. Sukhapesna *et al*. (1990) studied the rate of liver fluke infection by fecal examination in buffaloes in Thailand. The infection rate in 240 buffaloes was 47.1% which was higher positive rate than the current study. The epidemiological investigation of liver fluke of cattle was carried out in Thailand from 1992-1995 by simple flotation method. The 14.4% of 7,209 cattle was found the *Fasciola* egg in the fecal samples (Prasitirat *et al*., 1996-1997). That study was higher positive rate of liver fluke infection than the current study. The prevalence of *Fasciola* infection was 3.7% (59/1,599) of dairy cows diagnosed by formalin-ethyl acetate sedimentation in Thailand (Jittapalapong *et al*., 2011). Compare to the current study showed the higher of infection rate than that study. Neamjui *et al*. (2012) investigated the prevalence and risk factor of liver fluke infection in cattle and buffaloes using the fecal sedimentation method in Buriram province and the results showed 3.3% (6/180) and 13.9% (25/180) of cattle and buffaloes, respectively. This study was lower prevalence of fasciolosis in both cattle and buffaloes than the current study.

The conventional method of diagnosis for fasciolosis is the fecal examination. These methods are simple, fast, low cost and practical for field survey. However, the coproscopic examination is impractical because the morphology of egg is similar to egg of other flukes such as *Fasciolopsis buski* and Rumen flukes, it makes to misidentification (Awad *et al*., 2009). The low sensitivity of the fecal examination may be due to low numbers of egg are passed in the large amounts of feces, possibly as a result of low parasite burden and the parasite eggs were not released in the early

stage of infection (Adedokun *et al*., 2008). Moreover, the coprological examination show high false negative in the chronic infection. ELISA was reliable tool for the early, current and chronic serodiagnosis of bovine and ovine fasciolosis (Awad *et al*., 2009; Cornelissen *et al*., 2001; Salam *et al*., 2009). The circulating antigens can be diagnosed as early as the first to third weeks after infection and the peak of antibody against *F. gigantica* at about 16 weeks after infection (Viyanant *et al*., 1997). ELISA was more sensitivity, specificity and accuracy than the fecal examination (Reichel *et al*., 2005). Moreover, 27 kDa ES-Ag based on ELISA could be used and showed as the sensitive and specific antigen for immunodiagnosis of human fascioliasis (Córdova *et al*., 1999; Tantrawatpan *et al*., 2003). 27 kDa antigen based on ELISA was no crossed reaction with Paramphistomes in bovine fasciolosis (Ghosh *et al*., 2005; Molloy, 2005; Yokananth *et al*., 2005) and *Schistosoma mansoni*, hydatidosis, cysticercosis and chagas disease in human patients (O'Neill *et al*., 1998). Then, ES-Ag based on ELISA is a recommendatory method for diagnosis bovine fasciolosis. However, there is the limitation of ES-Ag ELISA which has the false positive of *Fasciola* infection due to the cross-reactivity between *Fasciola* with other flukes.

Female dairy cattle was higher positive rate of fasciolosis than male which caused the practice of male dairy cattle under good management condition compared with the female for the breeding purpose (Bedarkar *et al*., 2000; Phiri *et al*., 2005; Salam *et al*., 2009). Some physiological peculiarities of the lactating mother cattle could reduce their immunity to infections and be weak or malnourished. Therefore, female cattle were more susceptible to the infection with parasites including *Fasciola* in the lactation period (Kuchai *et al*., 2011). However, in the study area, both male and female in cattle and buffaloes are the same practice which rears freely grazing on the pastures. Thus, sex is not influential factor of *Fasciola* infection in cattle and buffaloes nearby Songkhla Lake.

The prevalence of *F. gigantica* infection as observed through fecal egg counts was high in adult cattle compared to calves (Anderson *et al*., 1999). The older cattle were related to the longer exposure time and accumulation of flukes in the liver compared to young cattle (Vassilev, 1999). The 0-2 years of cattle grazed less

frequently than the older cattle, reducing possibility of infection with *Fasciola* metacercariae. Fasciolosis was mainly acquired when cattle were grazing pastures (Sánchez-Andrade *et al*., 2002). The high positive rate of liver fluke in adult cattle were attributed to their long exposure time leading to development of immunity against the pathogenic effects of the immature flukes but still having the mature flukes maintain their high capacity of egg production (Pfukenyi *et al*., 2005). Similarly, the highest prevalence was showed in the >7 years cattle while in the >7 years buffaloes showed lower positive rate than the <7 years buffaloes but it was not statistical significance (p=0.8). However, some studies reported the younger animals were more susceptible to infections than the older animals. The old animals may acquire immunity to the parasites through frequent challenge and expel the ingested parasite before they established infection (Dalton, 1999; Kuchai *et al*., 2011).

Buffaloes had lower of mature and immature flukes and fecal egg count than cattle. The flukes may be suppressed development, longer prepatent period or delayed migration in buffaloes. Buffaloes were likely to be more resistant to *F. gigantica* infection than cattle (Molina *et al*., 2005). The Indonesian swamp buffalo calves (*Bubalus bubalis*) were higher resistance and resilience to infection with *F. gigantica* than Ongole (*Bos indicus*) and Bali calves (*Bos sondaicus*) because the infected buffalo calves were the higher of immature and adult fluke counts, fecal *Fasciola* egg counts and weight gain than the infected cattle calves (Wiedosari *et al*., 2006). The naturally acquired fasciolosis in cattle and buffaloes was closely related to the husbandry system and the number of metacercariae occurred in the pastures (Sánchez-Andrade *et al*., 2002). Cattle were lower positive rates of *Fasciola* infection than buffaloes diagnosed by both fecal examination and ELISA in the current study Because of the wider grazing pastures in buffaloes. Cattle rear on the land or the shallow water but buffaloes can graze in all field in Songkhla Lake due to buffaloes can swim and dive to eat grass in the deep water. Therefore, buffaloes are more possibility of ingesting water plants contaminated with metacercariae than cattle.

Songkhla Lake has stagnant water all year round which influence for distributing of *Fasciola* due to the habitat nearby the lake appropriates the living and proliferation of freshwater snails and growth of water plants. Both freshwater snails and water plants are the important intermediate host for *Fasciola*. The flowing water into Songkhla Lake approves the migration of infected freshwater snails with *Fasciola* larva as same as the spreading of fasciolosis to other fields in the lake. The cycle of liver fluke is maintained in the areas because of the traditional rearing system around Songkhla Lake. The lake is contaminated with parasites from the feces of animal dropped on the ground or into the water and spreading around the lake from water current. Moreover, the animal migration around the lake is also a factor of parasite spreading. Although, the data of human fascioliasis in Songkhla province has never reported in the area but the data in the present study shows high prevalence of bovine fasciolosis. There is a possibility of *Fasciola* infection in human because human and domestic animals ingest water plants growing in the lake and they share the environment or habitat with each other. From the current study, Songkhla Lake is the natural reservoir for parasitic disease of cattle, buffaloes and human. Therefore, the people nearby the lake should avoid ingesting raw or uncooked water plants which grow in this area. And cattle and buffaloes should be treated by triclabendazole administered in twice times of 10 mg/kg per treatment (Mas-Coma *et al.*, 2007).

CONCLUSION

Both of cattle and buffaloes were infected with *F. gigantica* in every district in this study area. Particularly, buffaloes showed the high level of *Fasciola* infection (78.9%). The <7 years cattle was the higher positive rate of infection with *Fasciola* than the >7 years and the male cattle showed lower infection rate than the female. Both of age and sex group differences were significant ($p<0.001$) in cattle while there were not found statistic significance in buffalo. Thus, Songkhla Lake is the natural reservoir for fasciolosis in cattle, buffaloes and possibly humans. Humans should avoid eating raw and uncooked water plants growing in Songkhla Lake.

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APPENDICES

Appendix A

The figure of Songkhla Lake area and helminthic eggs were found in current study

Appendix Figure A1 The habitat nearby Songkhla Lake

Appendix Figure A2 The habitat nearby Songkhla Lake

Appendix Figure A3 The habitat nearby Songkhla Lake

Appendix Figure A4 The habitat nearby Songkhla Lake

Appendix Figure A5 The habitat nearby Songkhla Lake

Appendix Figure A6 The pen of buffaloes nearby Songkhla Lake

Appendix Figure A7 The habitat nearby Songkhla Lake

Appendix Figure A8 The habitat nearby Songkhla Lake

Appendix Figure A9 The habitat nearby Songkhla Lake

Appendix Figure A10 The habitat nearby Songkhla Lake

Appendix Figure A11 The habitat nearby Songkhla Lake

Appendix Figure A12 The rearing cattle in the pen nearby Songkhla Lake

Appendix Figure A13 The rearing cattle in the pen nearby Songkhla Lake

Appendix Figure A14 The buffaloes stayed at the pen in the morning nearby Songkhla Lake

Appendix Figure A15 The buffaloes reared nearby Songkhla Lake

Appendix Figure A16 The buffaloes reared nearby Songkhla Lake

Appendix Figure A17 The traditional rearing system of cattle nearby Songkhla Lake

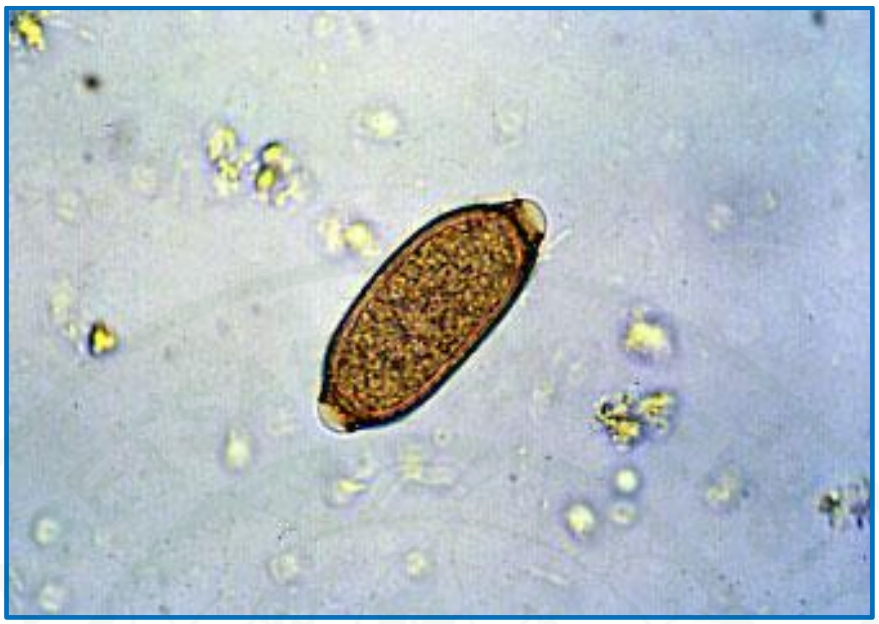
Appendix Figure A18 The traditional rearing system of buffaloes nearby Songkhla Lake

Appendix Figure A19 The traditional rearing system of buffaloes nearby Songkhla Lake

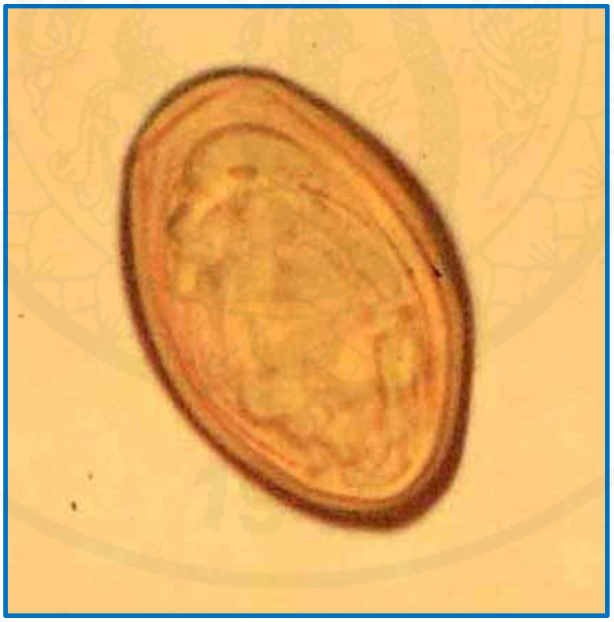
Appendix Figure A20 The freshwater snail nearby Songkhla Lake

Appendix Figure A21 The water plant nearby Songkhla Lake

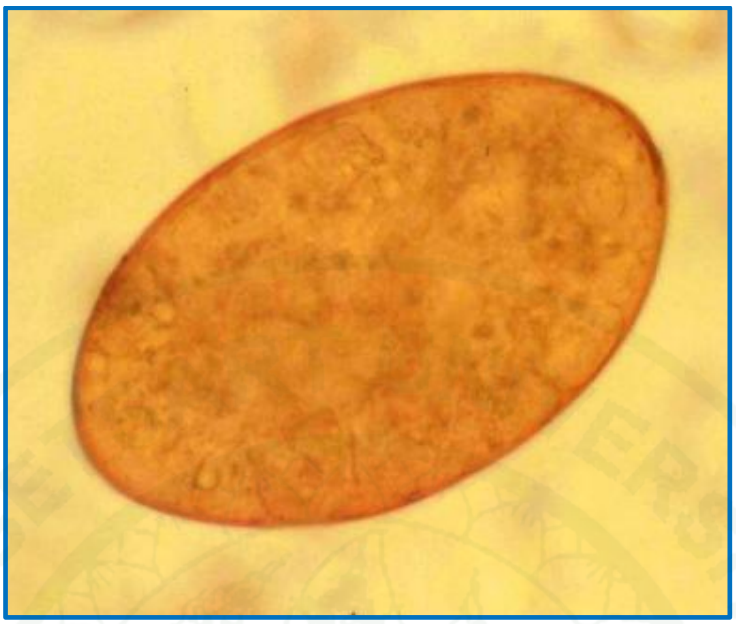
Appendix Figure A22 The carcass of cattle was discarded into the pond nearby Songkhla Lake



Appendix Figure A23 *Capillaria* egg

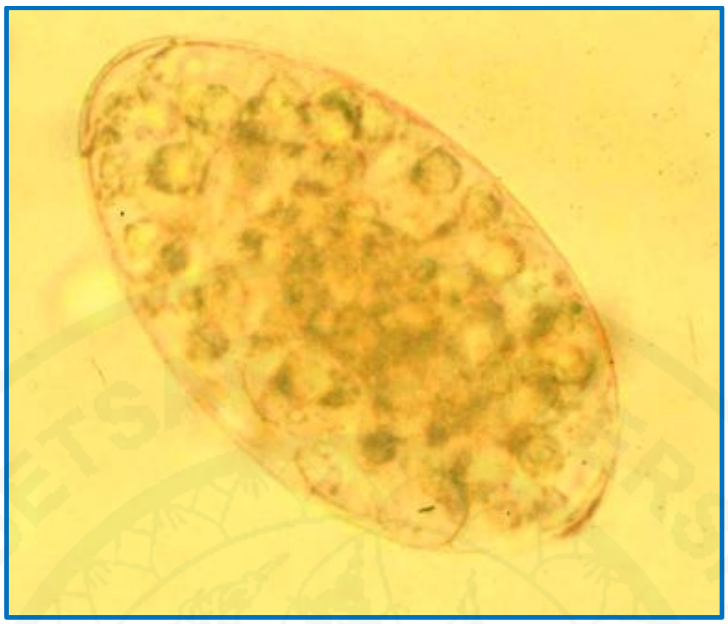


Appendix Figure A24 *Eurytrema pancreaticum* egg

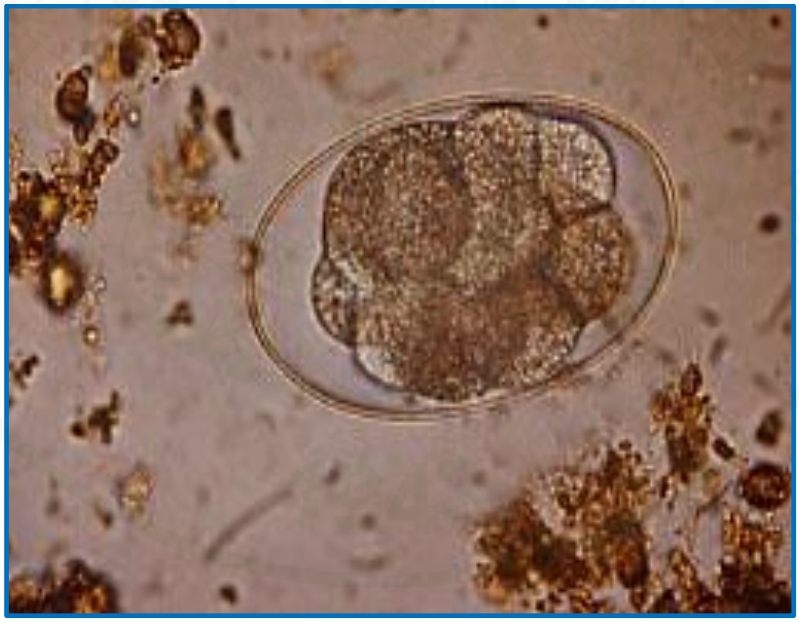


Appendix Figure A25 *Fasciola* egg

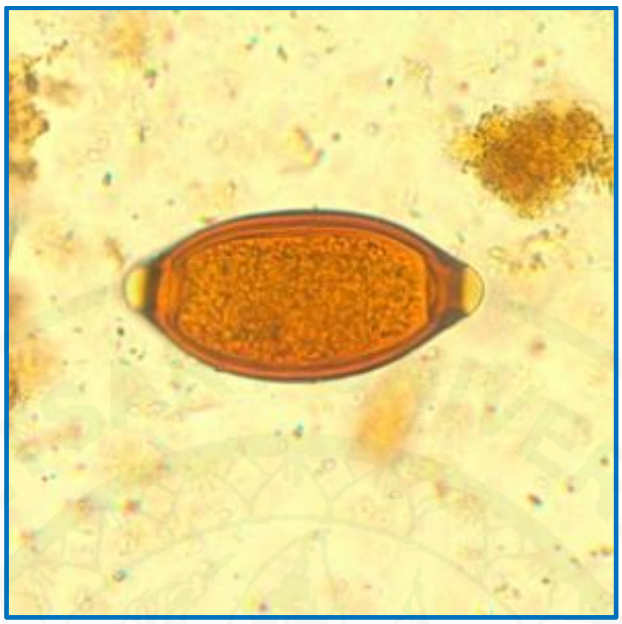
Appendix Figure A26 *Moniezia benedeni* egg



Appendix Figure A27 Rumen fluke egg



Appendix Figure A28 Strongyle worm egg



Appendix Figure A29 *Trichuris* egg

Appendix B

The raw data

Appendix Table B1 The end point titer of infected cattle with *F. gigantica*

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
SRC1	0.86	0.746	0.546	0.335	0.169	0.126	0.1	0.078
SRC17	1.527	1.058	0.731	0.464	0.325	0.207	0.131	0.099
SRC22	1.357	1.169	1.088	0.797	0.51	0.345	0.205	0.132
SRC23	1.191	0.907	0.666	0.477	0.322	0.214	0.145	0.093
SRC25	1.337	0.908	0.712	0.515	0.297	0.198	0.124	0.081
SRC27	1.408	0.975	0.773	0.532	0.434	0.293	0.168	0.113
SRC29	1.286	0.881	0.592	0.486	0.411	0.305	0.178	0.1
SRC30	1.106	0.735	0.72	0.562	0.408	0.218	0.139	0.104
SRC31	1.161	0.931	0.846	0.728	0.592	0.387	0.219	0.117
SRC48	0.875	0.717	0.552	0.435	0.185	0.113	0.085	0.07
SRC79	0.899	0.769	0.551	0.461	0.213	0.175	0.123	0.08
SRC80	1.421	1.14	0.848	0.453	0.289	0.217	0.13	0.119
SRC81	1.136	0.825	0.567	0.338	0.214	0.147	0.119	0.09
SRC85	2.221	1.973	1.645	1.249	0.926	0.706	0.43	0.264
SR1/1	2.01	1.642	1.222	0.793	0.531	0.336	0.223	0.137
°SR2/1	2.582	2.168	1.731	1.385	1.121	0.806	0.534	0.342
°SR2/2	1.757	1.315	0.858	0.586	0.379	0.228	0.15	0.117
SR2-27	1.386	1.211	0.798	0.53	0.344	0.226	0.147	0.11
SR2-28	1.424	1.148	0.873	0.578	0.392	0.25	0.157	0.112
SR2-29	1.406	1.219	0.896	0.58	0.386	0.257	0.164	0.118
SR2-32	1.438	1.168	0.857	0.542	0.345	0.231	0.159	0.108
SR2-33	1.382	1.208	0.781	0.543	0.343	0.231	0.15	0.118
SR2-37	2.238	1.542	1.068	0.683	0.789	0.319	0.175	0.14
SR2-38	2.244	1.559	1.175	0.777	0.502	0.332	0.222	0.14
SR2-39	2.281	1.566	1.125	0.75	0.474	0.315	0.226	0.135
SR2-40	1.691	1.051	0.752	0.489	0.303	0.223	0.166	0.122
SR2-41	2.746	2.135	1.74	1.209	0.924	0.635	0.446	0.262
SR2-42	2.572	2	1.45	1.011	0.68	0.398	0.264	0.188

Appendix Table B1 (Continued)

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
SR2-43	2.528	2.036	1.438	1.009	0.699	0.468	0.307	0.183
SR2-44	2.493	1.873	1.39	0.93	0.728	0.406	0.257	0.153
SR2-45	2.94	2.369	2.051	1.602	1.176	0.695	0.488	0.294
SR2-46	2.642	2.019	1.609	1.198	0.814	0.516	0.347	0.21
SR2-48	2.265	1.539	1.144	0.803	0.516	0.309	0.198	0.13
SR2-49	2.004	1.661	1.406	1.097	0.686	0.48	0.332	0.204
SAC7	1.115	0.751	0.555	0.338	0.223	0.165	0.12	0.094
SAC9	1.131	0.768	0.644	0.431	0.279	0.187	0.129	0.099
SAC12	1.263	1.05	0.764	0.473	0.303	0.236	0.148	0.107
SAC31	1.231	0.941	0.684	0.428	0.29	0.189	0.133	0.099
SAC32	1.393	0.98	0.768	0.465	0.299	0.201	0.134	0.106
SA1/1	1.046	1.018	0.859	0.575	0.413	0.285	0.193	0.126
SA1/2	1.677	1.545	1.343	1.071	0.767	0.538	0.358	0.226
SA1/4	1.578	1.391	1.277	0.955	0.589	0.369	0.257	0.153
SA1/5	1.673	1.37	1.246	0.874	0.619	0.395	0.25	0.164
SA1/7	1.816	1.659	1.413	1.02	0.667	0.46	0.298	0.18
SA2/3	1.895	1.549	1.25	0.82	0.613	0.391	0.268	0.183
SA2/4	2.284	2.177	1.994	1.52	1.219	0.768	0.534	0.343
SA2/5	1.783	1.442	1.168	0.707	0.476	0.298	0.193	0.116
SA3/1	1.895	1.664	1.281	0.773	0.57	0.328	0.196	0.119
SA4/1	2.064	1.588	1.425	1.019	0.792	0.483	0.276	0.146
SA6/4	1.528	1.209	0.98	0.657	0.464	0.274	0.151	0.097
SA2-20	1.736	1.537	1.256	0.768	0.562	0.299	0.182	0.102
2.1	1.955	1.562	1.205	0.829	0.566	0.302	0.175	0.103
2.2	2.199	1.687	1.477	1.087	0.803	0.464	0.295	0.152
3.2	1.242	0.817	0.629	0.396	0.267	0.153	0.11	0.074
5.1	2.681	2.413	2.337	2.101	1.653	1.189	0.84	0.441
6.1	1.27	1.001	0.754	0.513	0.289	0.21	0.158	0.161

Appendix Table B1 (Continued)

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
6.2	1.095	0.843	0.63	0.49	0.267	0.162	0.164	0.047
7.1	1.143	0.773	0.698	0.432	0.295	0.182	0.079	0.053
7.2	1.002	0.847	0.557	0.493	0.102	0.103	0.076	0.055
7.3	1.266	1.007	0.78	0.491	0.371	0.168	0.071	0.055
8.4	1.048	0.954	0.697	0.309	0.212	0.112	0.067	0.063
8.5	0.963	0.767	0.619	0.319	0.139	0.103	0.07	0.051
SS1/3	1.168	1.044	0.819	0.482	0.463	0.241	0.139	0.073
SS1/4	0.948	0.751	0.62	0.389	0.171	0.167	0.053	0.047
SS1/5	0.909	0.763	0.624	0.394	0.172	0.165	0.058	0.045
SS2/1	0.971	0.772	0.685	0.424	0.193	0.182	0.056	0.047
SS2/2	1.052	0.919	0.759	0.525	0.408	0.242	0.17	0.104
SS2/3	0.975	0.876	0.62	0.534	0.259	0.183	0.11	0.059
SS2/4	1.137	0.942	0.736	0.529	0.388	0.254	0.18	0.102
SS3/1	1.132	0.907	0.578	0.431	0.25	0.193	0.097	0.07
SS3/2	0.85	0.718	0.536	0.458	0.212	0.082	0.066	0.055
SS3/3	0.869	0.712	0.565	0.47	0.225	0.183	0.064	0.06
SS4/1	0.868	0.707	0.523	0.437	0.213	0.176	0.169	0.052
SS4/2	1.214	0.928	0.744	0.561	0.437	0.288	0.193	0.11
SS4/3	1.032	0.819	0.67	0.56	0.284	0.122	0.075	0.06
SS5/1	1.481	1.259	1.056	0.827	0.602	0.374	0.248	0.143
SS5/2	1.312	1.028	0.782	0.613	0.429	0.295	0.177	0.113
°SS1/1	1.293	1.049	0.796	0.518	0.399	0.269	0.158	0.108
°SS3/2	0.956	0.699	0.598	0.302	0.222	0.16	0.117	0.082
SS2-2	0.955	0.732	0.581	0.487	0.311	0.108	0.181	0.068
SS2-3	0.958	0.837	0.61	0.462	0.377	0.326	0.294	0.167
SS2-4	1.243	0.977	0.762	0.502	0.342	0.241	0.133	0.102
SS2-5	1.084	0.905	0.691	0.586	0.498	0.437	0.386	0.18
SS2-6	1.198	1.035	0.822	0.662	0.485	0.402	0.326	0.285

Appendix Table B1 (Continued)

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
SS2-7	1.062	0.864	0.62	0.444	0.354	0.315	0.175	0.162
SS2-13	1.517	1.278	0.797	0.535	0.309	0.197	0.114	0.083
SS2-14	0.903	0.747	0.656	0.568	0.515	0.301	0.075	0.062
SS2-18	0.918	0.777	0.662	0.565	0.309	0.191	0.075	0.06
SS2-19	0.883	0.753	0.561	0.387	0.172	0.16	0.053	0.045
SS2-20	1.441	1.036	0.989	0.842	0.653	0.396	0.172	0.151
SS2-25	1.046	0.828	0.758	0.611	0.582	0.374	0.155	0.144
SS2-26	1.142	0.978	0.49	0.322	0.181	0.163	0.093	0.061
SS2-32	0.937	0.694	0.447	0.242	0.121	0.101	0.066	0.045
SK5/2	0.815	0.79	0.556	0.427	0.251	0.176	0.099	0.066
SK6/1	0.851	0.7	0.417	0.368	0.203	0.135	0.075	0.047
SK6/2	1.043	0.911	0.815	0.738	0.593	0.27	0.35	0.143
SK7/3	0.965	0.879	0.77	0.718	0.691	0.472	0.354	0.145
SKC1	1.233	0.987	0.801	0.767	0.688	0.47	0.257	0.45
SKC5	0.864	0.785	0.714	0.69	0.568	0.267	0.148	0.047
SK2-2	2.389	1.3	0.816	0.418	0.188	0.185	0.111	0.074
SK2-3	1.471	1.051	0.628	0.22	0.177	0.141	0.094	0.089
SK2-6	1.281	0.961	0.567	0.281	0.202	0.131	0.108	0.073
SK2-8	0.977	0.602	0.37	0.258	0.154	0.147	0.086	0.071
SK2-9	1.29	0.943	0.545	0.334	0.175	0.123	0.098	0.061
SK2-13	1.194	1.021	0.514	0.379	0.244	0.142	0.101	0.079
SK2-17	1.041	0.735	0.359	0.259	0.161	0.124	0.084	0.06
SK2-18	1.76	1.292	0.717	0.405	0.251	0.157	0.099	0.076
SK2-20	1.774	1.141	0.562	0.312	0.274	0.187	0.104	0.077
SK2-30	1.933	1.281	0.453	0.37	0.215	0.104	0.096	0.067
STCA4	2.128	1.649	0.527	0.287	0.18	0.123	0.073	0.062
STCA5	2.988	2.721	2.224	1.67	1.059	0.614	0.33	0.235
STCA7	1.684	1.259	0.89	0.536	0.34	0.235	0.179	0.124

Appendix Table B1 (Continued)

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
STCA8	2.183	1.566	1.021	0.688	0.417	0.322	0.21	0.15
STCA9	1.846	1.418	0.907	0.537	0.383	0.271	0.198	0.132
STCA10	1.447	0.949	0.603	0.447	0.321	0.222	0.142	0.11
STCA11	1.203	0.865	0.61	0.381	0.271	0.22	0.146	0.127
STCA12	1.89	1.526	0.994	0.659	0.441	0.247	0.185	0.136
STCA13	1.974	1.403	0.991	0.539	0.386	0.247	0.176	0.135
STCA15	1.545	1.103	0.688	0.408	0.274	0.176	0.13	0.111
STCB1	1.693	1.203	0.825	0.49	0.304	0.203	0.153	0.117
STCB2	2.434	1.882	1.279	0.684	0.487	0.294	0.201	0.154
STCB3	1.013	0.813	0.688	0.384	0.225	0.193	0.173	0.055
STCB4	1.159	0.956	0.753	0.431	0.257	0.112	0.081	0.063
STCB5	1.301	1.069	0.896	0.556	0.371	0.119	0.085	0.062
STCB6	1.063	0.825	0.608	0.359	0.101	0.109	0.063	0.055
STCB7	0.982	0.743	0.538	0.379	0.119	0.087	0.07	0.055
STCB8	1.055	0.81	0.657	0.389	0.233	0.184	0.074	0.242
STCB9	1.014	0.871	0.625	0.493	0.353	0.207	0.153	0.091
STCB10	0.92	0.769	0.552	0.364	0.26	0.157	0.11	0.066
STCD2	1.294	1.055	0.824	0.519	0.251	0.105	0.074	0.06
STCD3	0.971	0.765	0.514	0.359	0.102	0.08	0.056	0.048
STCD4	0.94	0.734	0.588	0.236	0.092	0.078	0.058	0.048
STCD6	0.927	0.746	0.628	0.511	0.443	0.226	0.08	0.071
STCD7	1.293	1.085	0.823	0.601	0.442	0.361	0.215	0.078
STCD8	1.239	1.044	0.924	0.666	0.5	0.395	0.332	0.195
STCD9	0.804	0.753	0.686	0.634	0.507	0.388	0.18	0.063
STCD11	0.979	0.744	0.607	0.583	0.474	0.264	0.158	0.057
ST2-29	0.816	0.711	0.656	0.608	0.488	0.279	0.165	0.163
ST2-35	0.972	0.885	0.737	0.523	0.242	0.209	0.182	0.069
ST2-37	0.816	0.726	0.663	0.416	0.286	0.176	0.161	0.055

Appendix Table B1 (Continued)

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
ST2-41	1.216	1.018	0.857	0.677	0.522	0.216	0.137	0.09
ST2-42	0.941	0.751	0.596	0.458	0.359	0.32	0.19	0.069
ST2-46	1.261	1.082	0.781	0.584	0.44	0.348	0.2	0.093
ST2-47	0.955	0.871	0.627	0.452	0.207	0.204	0.169	0.07
ST2-48	0.995	0.812	0.636	0.424	0.256	0.115	0.089	0.062
ST2-49	0.941	0.739	0.573	0.266	0.211	0.094	0.069	0.058
ST2-50	1.171	0.925	0.585	0.327	0.211	0.149	0.099	0.072
ST2-54	0.948	0.872	0.742	0.658	0.625	0.394	0.171	0.159

Note: The underline number presents the end point titer.

Abbreviations: SRC, SR, °SR = Cattle in Ranot district
SAC, SA = Cattle in Krasaesin district
SS, °SS = Cattle in Signha Nakhon district
SK, SKC = Cattle in Khuan Niang district
STC, ST = Cattle in Sathing Phra district

Appendix Table B2 The end point titer of infected buffaloes with *F. gigantica*

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
SSB1	1.322	1.086	0.845	0.637	0.425	0.302	0.18	0.137
SSB2	1.285	0.942	0.713	0.478	0.313	0.226	0.156	0.112
SSB3	1.159	0.831	0.549	0.356	0.234	0.167	0.118	0.093
SSB4	2.125	1.953	1.553	1.206	0.88	0.584	0.388	0.254
SSB5	2.169	1.996	1.727	1.322	0.994	0.696	0.395	0.256
SSB6	1.598	1.251	0.814	0.611	0.39	0.259	0.168	0.122
SSB7	1.545	1.32	1.017	0.764	0.55	0.362	0.24	0.16
SSB8	2.579	2.427	2.243	2.118	1.679	1.214	0.85	0.577
SSB9	1.879	1.927	1.668	1.312	1.073	0.631	0.492	0.31
SSB10	1.728	1.646	1.421	1.077	0.716	0.492	0.277	0.169
SSB11	1.733	1.506	1.291	1.014	0.686	0.448	0.263	0.14
SSB13	2.002	1.905	1.713	1.382	1.032	0.741	0.486	0.305
SSB14	1.172	0.907	0.708	0.521	0.423	0.361	0.121	0.093
SSB15	1.189	1.002	0.749	0.575	0.439	0.281	0.113	0.09
SSB16	1.079	0.87	0.651	0.506	0.417	0.262	0.122	0.09
SSB17	1.315	1.183	0.94	0.728	0.604	0.47	0.17	0.12
SSB18	1.134	0.944	0.758	0.614	0.505	0.313	0.148	0.118
SSB19	1.324	1.108	0.843	0.677	0.519	0.402	0.138	0.098
SSB20	1.218	0.99	0.536	0.437	0.271	0.133	0.102	0.087
SSB21	1.178	0.944	0.622	0.427	0.28	0.134	0.1	0.085
SSB22	1.383	0.978	0.54	0.375	0.256	0.175	0.128	0.091
SSB26	1.502	1.71	0.623	0.31	0.229	0.164	0.121	0.096
SSB27	1.227	0.967	0.782	0.546	0.427	0.243	0.097	0.076
SRB1	1.42	1.145	0.878	0.618	0.476	0.371	0.112	0.083
SRB2	1.557	1.279	1.059	0.724	0.533	0.411	0.128	0.109
SRB3	2.402	2.405	2.269	2.028	1.567	1.264	0.889	0.576
SRB5	1.902	1.71	1.506	1.145	0.791	0.501	0.27	0.167
SRB9	1.951	1.846	1.655	1.389	1.053	0.69	0.428	0.254

Appendix Table B2 (Continued)

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
SRB10	1.733	1.56	1.33	1.003	0.755	0.508	0.3	0.19
SRB13	1.23	0.973	0.705	0.424	0.295	0.175	0.122	0.092
SRB15	1.167	0.889	0.702	0.534	0.442	0.259	0.111	0.097
SRB16	2.044	1.801	1.704	1.449	1.129	0.756	0.485	0.287
SAB1	1.869	1.742	1.546	1.143	0.874	0.59	0.337	0.203
SAB2	2.155	2.008	1.716	1.686	1.389	0.987	0.758	0.51
SAB3	1.839	1.603	1.266	0.947	0.649	0.394	0.253	0.161
SAB4	1.001	0.785	0.579	0.476	0.287	0.138	0.108	0.092
SAB5	1.4	1.286	1.056	0.964	0.652	0.411	0.26	0.195
SAB6	1.292	1.109	0.834	0.612	0.378	0.223	0.161	0.14
SAB7	0.988	0.785	0.599	0.408	0.161	0.116	0.093	0.093
SAB8	1.559	1.309	1.108	0.969	0.731	0.482	0.336	0.246
SAB9	1.375	1.251	1.061	1.017	0.819	0.596	0.417	0.311
SAB15	1.179	0.83	0.625	0.489	0.201	0.144	0.104	0.126
SAB16	1.571	1.386	1.004	0.791	0.48	0.304	0.197	0.17
SAB17	1.068	0.882	0.657	0.541	0.359	0.128	0.096	0.111
SAB19	0.902	0.84	0.657	0.472	0.357	0.211	0.135	0.167
SAB20	1.067	0.854	0.566	0.408	0.276	0.112	0.147	0.313
STB1	1.527	1.248	0.946	0.671	0.548	0.523	0.245	0.175
STB2	1.837	1.593	1.249	0.906	0.687	0.52	0.438	0.351
STB3	1.649	1.385	1.019	0.793	0.626	0.457	0.279	0.178
STB4	1.524	1.307	1.066	0.891	0.73	0.488	0.397	0.31
STB5	1.69	1.444	1.002	0.852	0.624	0.363	0.23	0.143
STB6	1.501	1.319	1.051	0.799	0.617	0.368	0.233	0.142
STB7	1.898	1.626	1.331	1.241	1.136	0.945	0.691	0.47
STB8	1.845	1.665	1.333	1.179	0.886	0.648	0.386	0.248
STB9	1.672	1.494	1.212	1.027	0.438	0.295	0.262	0.196
STB10	1.725	1.563	1.311	1.174	0.906	0.629	0.456	0.304

Appendix Table B2 (Continued)

Code	End point titer							
	100	200	400	800	1,600	3,200	6,400	12,800
STB11	1.415	1.269	1.033	0.774	0.52	0.388	0.252	0.179
STB12	1.512	1.336	1.085	0.805	0.61	0.408	0.265	0.175
STB13	1.642	1.43	1.193	0.972	0.668	0.535	0.318	0.212
STB14	1.461	1.212	0.987	0.704	0.463	0.312	0.205	0.134
STB15	1.163	0.899	0.643	0.451	0.294	0.194	0.124	0.105
STB16	1.565	1.195	0.884	0.723	0.451	0.323	0.206	0.135
STB17	1.199	1.038	0.835	0.709	0.668	0.491	0.331	0.238
STB18	1.28	1.073	0.869	0.723	0.561	0.393	0.215	0.155
STB19	1.781	1.693	1.43	1.329	1.252	1.1	0.919	0.878
STB20	1.541	1.452	1.187	1.02	0.899	0.722	0.487	0.368
STB21	1.258	0.874	0.547	0.465	0.206	0.2	0.149	0.104
STB22	1.505	1.135	1.04	0.865	0.658	0.451	0.301	0.181
STB23	0.994	0.835	0.558	0.657	0.461	0.315	0.163	0.119
STB24	1.892	1.53	1.351	1.222	1.016	0.741	0.383	0.295
STB25	1.49	1.308	1.384	0.815	0.548	0.334	0.251	0.227
STB26	1.451	1.202	0.837	0.751	0.28	0.248	0.158	0.243
STB27	1.135	0.78	0.62	0.525	0.427	0.272	0.322	0.175
STB28	1.443	1.211	0.986	0.721	0.59	0.422	0.255	0.139
STB29	1.571	1.392	1.228	1.042	0.758	0.576	0.345	0.16

Note: The underline number presents the end point titer.

Abbreviations: SRB = Buffaloes in Ranot district

SAB = Buffaloes in Krasaesin district

SSB = Buffaloes in Signha Nakhon district

STB = Buffaloes in Sathing Phra district

Appendix C

Chemical reagents and substrates

Reagents and buffers for ELISA

1. Coating buffer

Na_2CO_3	1.59 g
NaHCO3	2.95 g

2. PBS (Phosphate buffer solution), pH 7.2

NaCl	8.00 g
KCl	0.20 g
$NaH_2PO_4 . 12H_2O$	2.90 g
KH2PO4	0.20 g
Distilled water	1000 ml

3. Washing buffer

PBS,pH 7.2	1000 ml
Tween-20	500 µl

4. Blocking solution

Skimmed milk powder	3.00 g
PBS,pH 7.2	100 ml

5. Substrate (Peroxidase substrate)

3,3’5,5’-tetramethyl benzidine, TMB (K Blue® TMB substrate, Neogen Europe ltd, Scotland, UK)

Reagents and buffers for polyacrylamide gel electrophoresis (PAGE)

1. 10% Polyacrylamide gel

Distilled water	4.2	ml
30% Acrylamide/Bis	3.3	ml
1.5 M Tris-HCl	2.5	ml
10% SDS	0.1	ml
10% APS	100	µl
TEMED	10	µl

2. Sample buffer (SDS reducing buffer)

0.5 M Tris-HCl, pH 6.8	1.25	ml
Glycerol	2.50	ml
10% SDS	2.00	ml
0.5% Bromophenol blue	0.20	ml
Distilled water	3.55	ml

3. 10X PAGE buffer, pH 8.3

Tris base	30.3	g
Glycine	144	g
SDS	10	g

4. 1X PAGE buffer

10X PAGE buffer	100	ml
Distilled water	900	ml

Appendix D

The proceeding paper in the 51th Kasetsart University Annual Conference

KASETSART UNIVERSITY

This is to certify that
the research report for

Prevalence of a Liver Fluke Infection *(Fasciola)* in Cows and Buffaloes nearby Songkhla Lake

By

Boy Boonaue Wissanuwat Chimnoi
Verachai Virochasaengaroon Tassanee Mungmuang
Sathaporn Jittapalapong and Burin Nimsuphan

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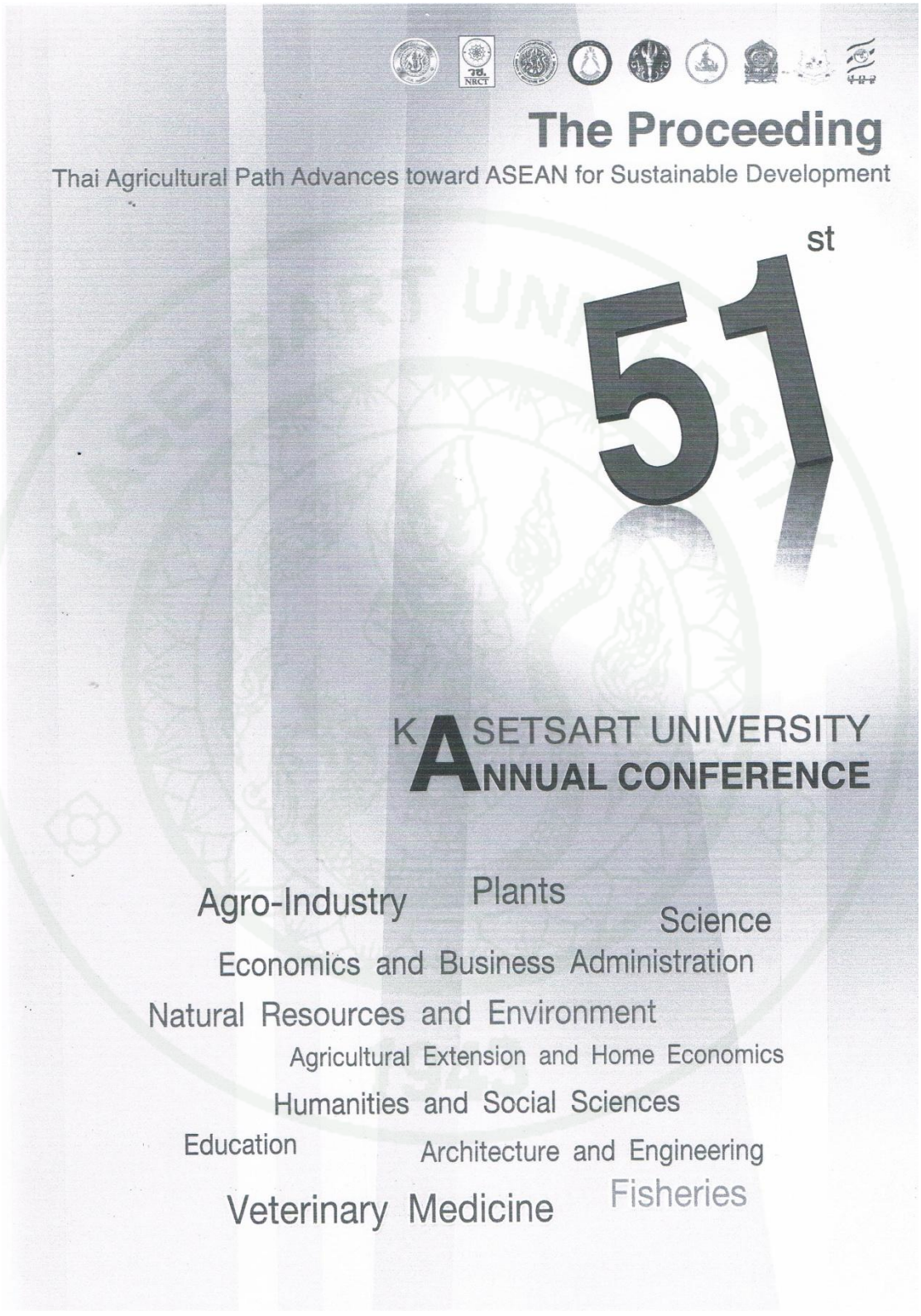
(Associate Professor Dr. Siree Chaiseri)
Vice President for Academic Affairs
51st Kasetsart University Annual Conference Committee Chairman



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Prevalence of a liver fluke infection (*Fasciola)* in Cows and Buffaloes nearby Songkhla Lake

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บทคัดย่อ

โรคพยาธิใบไม้ตับชนิด fasciolosis เป็นโรคที่มีความสำคัญโรคหนึ่งในปศุสัตว์และมีผลต่อสุขภาพในคนทำให้เกิดการสูญเสียทางด้านเศรษฐกิจและสาธารณสุข การศึกษาครั้งนี้เพื่อหาความชุกของการติดพยาธิใบไม้ตับชนิด *Fasciola* ในโคและกระบือรอบทะเลสาบสงขลา โดยทำการเก็บตัวอย่างเลือดและอุจจาระจากโคและกระบือจำนวน 277 และ 95 ตัว ตามลำดับในพื้นที่รอบทะเลสาบสงขลา ผลการตรวจอุจจาระด้วยวิธี Formalin-ethyl acetate sedimentation พบโคและกระบือมีการติดพยาธิใบไม้ตับคิดเป็นร้อยละ10.3 (28/272) และ 30.1 (25/83) ส่วนการตรวจซีรัมด้วยวิธี ELISA พบการติดพยาธิในโคและกระบือร้อยละ 34.3 (95/277) และ 78.9 (75/95) โคที่มีอายุมากมากกว่า 7 ปี พบการติดพยาธิมากกว่าโคอายุน้อยกว่า 7 ปี แต่ในกระบือพบว่ากระบืออายุมากกว่า 7 ปีมีการติดพยาธิน้อยกว่ากระบืออายุน้อยกว่า 7 ปี ทั้งในโคและกระบือให้ผลที่สอดคล้องกันในเรื่องเพศคือตัวเมียตรวจพบการติดพยาธิใบไม้ตับสูงกว่าในตัวผู้

ABSTRACT

Bovine fasciolosis is one of the most important parasitic diseases in domestic animals and human health. The great economic losses and public health concern are caused by fasciolosis. The objective of this study is to determine the prevalence of *Fasciola* infection in cows and buffaloes nearby Songkhla Lake areas. Fecal samples and blood of 277 cows and 95 buffaloes were collected. The results of fecal examination by formalin-ethyl acetate sedimentation method shown 10.3% (28/272) of cows and 30.1% (25/83) in buffaloes were positives. However, the serological test by ELISA showed 34.3% (95/277) in cows and 78.9% (75/95) in buffaloes were positive for *Fasciola* infection. The older cows had the higher infection rate than young cows. However, the older buffaloes had the lower positive rate than young buffaloes. Both female cows and buffaloes had the highest *Fasciola* infection.

Keyword: *Fasciola*, cow, buffalo, Songkhla Lake
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INTRODUCTION

A liver fluke infection is an important health problem for animals and humans. *Fasciola* is one of the liver flukes causing fasciolosis in ruminant and many mammals including humans. The *Fasciola* infections are transmitted by ingesting water plants or water contaminated with metacercaria. In Thailand, there were reports of *F. gigantica* infection in cattle and humans (Intapan *et al.*, 2005; Kanoksil *et al.*, 2006). The prevalence of fasciolosis in Thailand was 12% (Gray *et al*, 2008) and varied between provinces ranging from 0–85%. The northern part had the highest infection (23.4%), while the south had the lowest (4%) (Tuntasuvan and Kitikoon, 1996). In Pakpanang river basin, Nakhon Si Thammarat province had 8.2% of *Fasciola gigantica* infection (Worasing, 2007). Twenty-five cases of human fasciolosis were occurred in Thailand from 1967-1990. Since then, at least 10-20 new human fasciolosis cases have been confirmed in Khon Kaen University Hospital each year (Gray *et al*, 2008). The infection rate of *Fasciola* was found 0.36% in in-patient at Siriraj hospital from 1991-1995 (Tiewchaloren and Junnu, 1996).

There were many techniques for diagnose *Fasciola* infection including fecal, immunological and molecular techniques. The faecal examination is the referent method for detection of *Fasciola* infection. This method based on formalin-ethyl acetate sedimentation (Bonita and Taira, 1996). The test was diagnosed by finding the egg of parasites. The serological methods such as ELISA were developed to diagnose fasciolosis in early infection. The assay was more sensitive for *Fasciola* infection than fecal examination but it was less practical for the field survey (Intapan *et al*., 2003). Several antigens were used including crude antigen, tegument, egg and excretory-secretory antigen (ES Ag). There were many ES Ag from *Fasciola spp.* The molecular weight of ES protein was 15-101 kDa (Awad *et al*., 2009). However, the

twenty-seven kDa proteins of ES protein was the specific Ag of *Fasciola spp*. The 27 kDa ELISA had high sensitivity (93-94.9%), high accuracy (96%) and no cross reaction between *Fasciola gigantica* and *Paramphistomum epiclitum*, but that assay was not practical in the field diagnosis (Estuningsih *et al.*, 1997; Cornelissen *et al.*, 1999; Dixit *et al.*, 2002).

Songkhla Lake is the largest lake in Thailand and has the environment facilitating *Fasciola* life cycle. The lake promoted increasing population and freely spreading in the pasture of water snails, and vegetating of water plants. Many cows and buffaloes were reared nearby the lake. There were cattle in nearby Songkhla Lake areas, which died from *Fasciola* infection. In the present study, we aim to detect *Fasciola spp*. infection in cows and buffaloes by using fecal technique and serological method (ELISA) and determine the risk factor for the transmission of *Fasciola* infection in nearby Songkhla Lake areas.

MATERIALS AND METHODS

Blood Samples and Study areas

Five districts including Sathing Phra (ST), Ranot (SR), Singha Nakhon (SS), Krasaesin (SA) and Khuan Niang (SK) districts of Songkhla province (Figure 1) were assigned and 277 blood samples of cows (ST=54, SR=99, SS=49, SA=59 and SK=16) and 95 of buffaloes (ST=29, SR=19, SS=27 and SA=20) were collected. Blood samples were allowed to clot at room temperature for one hour, centrifuged at 1,448 G for 20 min, separated for sera, and stored at -20°C until tested.

Coprological examination

Two hundred and seventy-two fecal samples of cows and 83 fecal samples of buffaloes were collected directly from rectum of the animals in the study areas and examined for the presence of helminthic eggs by formalin-ethyl acetate sedimentation method. Fecal samples were washed in normal saline, strained into 15 mL centrifuge tubes, and centrifuged at 1,448 G for 5 min. The supernatant was decanted, and 10%formalin was added to a volume of 10 mL into the tube and mixed. The suspension was added 2 mL with ethyl acetate, shaken and centrifuged at 1,448 G for 5 min. Finally, loosening the debris plugs at the top layer and 10%formalin were

decanted. The remaining pellets were mixed with 10%formalin, added a drop of suspension on a glass slide and examined under light microscope.

Collection of adult worms

Adult worms of *Fasciola* were collected from liver and bile ducts of cattle at the slaughter houses and transferred into phosphate buffer saline (PBS, pH 7.2). Alive flukes were washed with normal saline to remove host contents. Thereafter, the flukes were washed five times in PBS (pH 7.2) and processed for obtaining adult worm regurgitant for isolation and purification of *F. gigantica* ES proteins

Isolation of *Fasciola* excretory/secretory antigen

Adults of *Fasciola* were washed cultured to obtaining ES antigen which followed the protocol by Raina *et al.*, 2006. Briefly, the flukes were incubated in RPMI1640 (100 unit/mL penicillin) at 37 º C overnight. The media containing ES Ag product was collected and centrifuged at 10,000 G for 30 min at 4 º C. Then, the supernatant was precipitated by two steps alcoholic precipitation. Finally, this product was resuspensed with PBS. The suspension was aliquotted and stored at -80 º C until test. Protein concentration of each prepared antigens was determined by Bradford method.

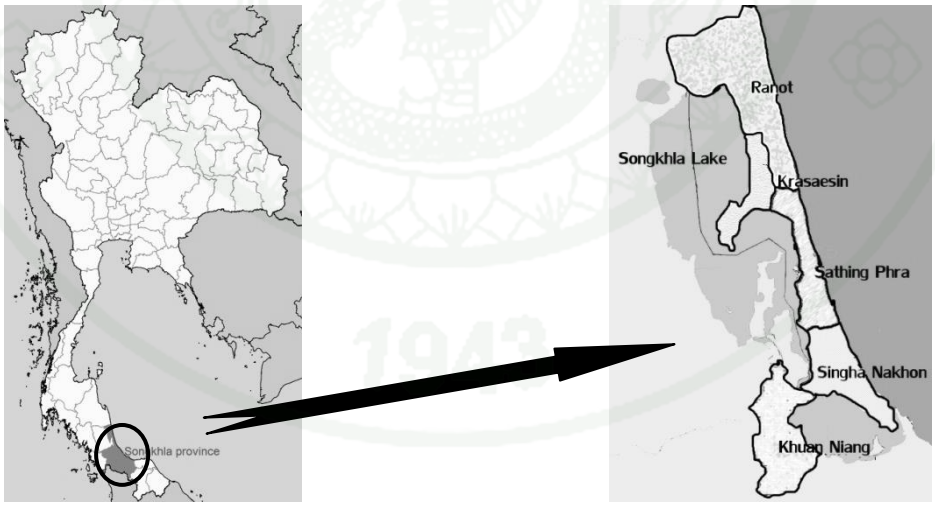

Figure 1 Map of Thailand and Songkhla Lake in Songkhla province

Enzyme linked immunosorbent assay (ELISA)

The sera from cows and buffaloes were evaluated by ELISA. The ELISA was performed as per the method described by Raina *et al.* (2006) with some

modifications. Briefly, ELISA plates were coated with 100 µL of 0.25 µg ES Ag in 0.1M carbonate buffer (pH 9.5) per well, incubated at 4 ◦C overnight. The plates were washed five times with 200 µL/well washing buffer (0.05% Tween-20 in PBS) and blocked with 100 µL/well blocking buffer (3% skimmed milk powder in PBS) for 1 h at 37 ◦C. The plates were washed five times with washing buffer. One hundred µL of serum dilution (1:200 dilutions in blocking solution) was added into the wells and incubated at 37 ◦C for 1 h. The plates were washed five times. The anti-bovine lgG peroxidase conjugate was added into the wells and the plates were incubated at 37 ◦C. Finally for 1 h, after five washes, 100 µl of substrate (3,3'5,5'-tetramethyl benzidine, TMB) and plates were incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 100 µl of 1N HCl into each well. The absorbance was read at 450 nm by ELISA plate reader. The positive control was chosen from the positive sample tested by fecal examination which the cattle showed single helminthic infection. The negative control was chosen from the sample which was not found any parasite in the stool.

Statistical Analysis

The prevalence of fasciolosis was determined and analyzed by from the ratio of positive results and total number of animals, and data were analyzed using the chi-square test, according to age and sex by Number Cruncher Statistical System programs (NCSS) version 2000 (Kaysville, UT).

RESULTS

The prevalence of *Fasciola* infection in cows and buffaloes was 10.3% (28/272) and 30.1% (25/83), respectively by the fecal exam (Table 1). Compared to the fecal examination, the seroprevalence was increasing to 34.3% (95/277) and 78.9% (75/95) in cows and buffaloes by ELISA (Table 1). The *Fasciola* infection occurred in all districts. The percentage of infected cows were 17.5% (11/63) in male cows and 39.25% (84/214) in female cows (Table 1). The male cows were lower positive numbers than the female cows but in buffaloes, the male (81.8%) was higher than the female (78.6%). The old cows (46.9%) showed more infected numbers than the young cows (31.4%). In buffaloes, the young buffaloes showed the highest positive percentage of *Fasciola* infection. The ages and sex group differences were

significant (p=0.0013 and p=0.0032, respectively) in cows. However, in buffaloes, the ages and sex group differences were not significant (p=0.8 and p=0.76, respectively).

Table 1 Seroprevalence of *Fasciola* infection using fecal examination and ELISA in cows and buffaloes

Parameter				**Statistic**	
Fecal examination					
cows			272	28(10.29)	
buffaloes			83	25(30.12)	
ELISA					
cows	ages	<1	9	1(11.1)	X^2=10.26, df=1, p=0.0013
		1-7	204	64(31.4)	
		>7	64	30(46.9)	
	sex	Male	63	11(17.5)	X^2=6.89, df=2, p=0.0032
		Female	214	84(39.3)	
	total		277	95(34.3)	
buffaloes	ages	<7	37	31(83.8)	X^2=0.06, df=1, p=0.8
		>7	58	44(75.9)	
	sex	Male	11	9(81.8)	X^2=0.09, df=1, p=0.76
		Female	84	66(78.6)	
	total		95	75(78.9)	

DISCUSSION

Songkhla Lake, which is the largest lake in Thailand, covered area of 1,040 km^2 and bordered Songkhla and Pathalung provinces. There are many wildlifes living in this area. Several domestic animals were reared nearby and in Songkhla Lake. In the morning most domestic animals including cattle, buffaloes and goats were grazed in the field neaby the lake (fig 2), and at night, cows and buffaloes came back into the pens by themself. The animals lived, fed, defecated and slept nearby Songkhla Lake.

The feces contaminated with parasitic eggs dropped on the ground or into the water in the lake. The parasites were spread around the lake by the flowing of water (Patz *et al*., 2000). Then, the lake was the reservoir of parasite. Humans lived nearby Songkhla Lake which ate vegetable from the lake and the domestic animals could graze water plants nearby the lake, which were a potential risk to the infection of *helminthic parasites including Fasciola gigantica*. Cattle and humans nearby Songkhla Lake areas had many risk factors for *Fasciola* infection such as fresh water snails and water plants (Claxton *et al*., 1997). The environment of Songkhla Lake, where was large lake and had water all the year round, promoted proliferation and spreading of fresh snails and water plants. The fresh water snails and water plants had importance for the transmission of liver flukes.

Figure 2 The environment nearby Songkhla Lake.

The previous study nearby Pakpanang river, Nakhon si thammarat province in the south of Thailand had low prevalence (8.2%) of *Fasciola* infection (Worasing, 2007). The prevalence of bovine fasciolosis (34.3% and 78.9% in cows and buffaloes, respectively) in the present study was greater than the previous study in Nakhon si thammarat. The water in Pakpanang river flowed all times. The flow of running water in river might have carried some the infected snails (Rondelaud *et al*, 2005). That habitat was not appropriate for distributing of fasciolosis. However, in Songkhla Lake, the water was flowed in the lake and had water all year round. The migration of the infected snails was in the same area. The geography of Songkhla Lake supported the fasciolosis distribution but no data on human fasciolosis in Songkhla province was reported. However, the data in the present study showed high prevalence of bovine

fasciolosis. The people nearby Songkhla Lake had risked to the liver flukes infection. Therefore, they should avoid ingesting raw water plants which grew nearby the lake.

The positive result of immunological assay in our study was higher than fecal examination. The fecal examination was simple method for detecting the intestinal parasites and the formalin-ethyl acetate sedimentation was practical for field survey. The disadvantage of fecal examination was not detecting in the early stage of fasciolosis and showed high false negative in chronic infection (Awad *et al*., 2009). ELISA could diagnose fasciolosis in the early stage, current and chronic infection (Arias *et al.*, 2007). The infected buffaloes with *Fasciola gigantica* were detected in three week post infection by using 27 kDa Ag iELISA (Kumar *et al.*, 2008). The ES-ELISA had high sensitivity (100%) and specificity (near 100%), compared to coprological and bile examinations (Ridi *et al*., 2007). ELISA was high sensitivity, specificity and no cross reaction with others flukes (Intapan *et al*., 2003). Then, ELISA is a recommendatory method for diagnosis bovine fasciolosis than fecal examination.

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