CHAPTER 2

Literature reviews

2.1 Systematics and descriptive studies

The family Echinostomatidae constitutes a group of digeneans characterized by great confusion regarding their systematic classification, particularly within the genus *Echinostoma*. The trematode in the genus *Echinostoma* is a group of intestinal flukes. The higher taxa of this genus is based on Yamaguti (1958) and Kostadinova (2005).

Phylum Platyhelminthes
Class Trematoda
Subclass Digenea
Superorder Anepitheliocystidia
Order Echinostomata
Superfamily Echinostomatoidea
Family Echinostomatidae
Subfamily Echinostomatinae
Genus *Echinostoma*

In general, the Echinostomatidae has been viewed as a monophyletic taxon. However, the morphology and/or the diversity of the criteria adopted by different authors have led to its division into an impressive number of taxa. For taxonomic and systematic studies of echinostomes, the most important characters that traditionally used for identification and consideration at the generic or specific level are described as followings; the presence of the number, shape, arrangement and relative size of collar spines; the morphology of the male terminal genitalia (position of the cirrus, structure of the internal seminal vesicle, development of the pars prostatica, size of armament of the cirrus); the position of the ovary and testes; the location and structure of vitellaria, the

character of the tegument armament and the presence of the uroproct (Yamaguti, 1958; Kostadinova and Gibson, 2000). Considerable variation exists in size of echinostomes depending upon species, fixation procedures, definitive host, and crowding effect. Echinostomes are referred to as "small" if up to 5 mm in length, "medium" if 5-10 mm in length, and "large" if longer than 10 mm (Kostadinova, 2005; Esteban and Antoli, 2009)

Some additional features in terms of length/wide ratio, such as the size and shape of the body and the length of the fore body, uterine and post-testicular fields, have also been applied (Kostadinova, 2005). However, diagnoses only by microscopic examinations may result in improper identification due to similarities between many species of this group, which leads to great confusion regarding the echinostome taxonomy and systematics (Morgan and Blair, 1997a; Kostadinova and Gibson, 2000). Additionally, biological characteristics, including chaetotaxy (Toledo *et al*., 1998; Kostadinova, 1999; Toledo *et al*., 2000), cercarial morphology (Kanev *et al.,* 1995a), life cycle and life history (Lee *et al*., 1991; Esteban *et al.,* 1997; Sohn, 1998; Toledo *et al*., 2000), and molecular genetic characteristics (Fujino *et al*., 1997; Morgan and Blair, 1997b; Kostadinova *et al*., 2003; Saijuntha *et al*., 2010; Saijuntha *et al*., 2011a; 2011b), are also used for species identification and specific detection of echinostomes.

However, some difficulty was reported in identifying the species, because many different species with similar morphologies were reported among the 37-collar spined echinostome, the so-called "*revolutum*" group, and taxonomic problems exist among those species. Ten species of *Echinostoma* were listed as valid within the "*revolutum*" group by Fried and Graczyk (2004): *E. caproni* Richard, 1964, *E. trivolvis* (Cort, 1914), *E. paraensei* Lie & Basch, 1967, *E. revolutum* (Frölich, 1802), *E. friedi* Toledo *et al*., 2000, *E. miyagawai* Ishii, 1932, *E. echinatum* (Zeder, 1803), *E. parvocirrus* Nassy & Dupouy, 1988, *E. luisreyi* Maldonado *et al*., 2003 and *E. jurini* (Skvortzov, 1924).

2.2 Clinical features and pathogenesis

Human echinostomiasis infections may remain unapparent or become symptomatic depending on the worm burden. The symptoms, even when present, are often unspecific, making clinical diagnosis difficult, particularly in non-endemic areas. Local physicians with the knowledge of endemicity of these infections and greater familiarity with the

present signs and symptoms may, however, make clinical diagnosis (Haaseb and Eveland, 2000).

Major clinical symptoms due to echinostome infection may include abdominal pain, diarrhea, easy fatigue, and loss of body weight (Chai and Lee, 2002; Fried *et al*., 2004; Toledo *et al*., 2006). Clinical symptoms depend on the parasite load (Fried *et al*., 2004). Case reports on the clinical aspects of echinostomiasis have given insights into the manifestations of this disease. Chai *et al.* (1994) reported the case of *E. hortense* infection in human in Korea. The symptoms of echinostomiasis vary from none to abdominal pain, although a case in Korea showed duodenal ulceration and epigastric discomfort of several days duration (Chai *et al*., 1994; Cho *et al*., 2003). Several studies on *E. hortense* have shown that patients suffered epigastric and abdominal pain, acid belching, weight loss discomfort, anorexia, headache, nausea, and vomiting (Chai *et al*., 1994; Chang *et al*. 2005). Patients infected with *E. ilocanum* experienced intestinal colic and diarrhea (Chai, 2009).

Pathological changes were reported by Bindseil and Christensen (1984) in the small intestine of mice and congenitally athymic, nude mice infected with *E. caproni*. Pathological damage in human cases includes catharral inflammation, erosion, and even ulceration (Chai *et al*., 1994; Cho *et al*., 2003). Peripheral blood eosinophilia is commonly observed. In *E. hortense* infections, the level of peripheral blood eosinophilia was dependent on the worm burden (Chai, 2009). The clinical signs in echinostomiasis are poorly known.

2.3 Diagnosis and treatment

Laboratory diagnosis of echinostomiasis is based on demonstration of eggs in feces. The yellow to yellow-brown eggs are thin shelled with an operculum, which may be difficult to see, and a slight thickening of the shell at the abopercular end. They are unembryonated when passed in feces. The size of human-infecting echinostome eggs is in the range 0.066–0.145 mm in length 0.043–0.090 mm in wide (Chai, 2009). Upon careful microscopic observations with measurements of the eggs, specific diagnosis may be possible in known endemic areas with a single or mixed echinstome species infection (Chai, 2009). However, the clinical importance of each echinostomes, as well as specific

diagnosis is poorly understood. Diagnosis of echinostomiasis can be done by recovery eggs in the feces, but specific diagnosis is difficult due to the morphological similarity of the eggs. The determination of echinostome species, and even from other trematodes, on the basis of the egg morphology is difficult. Species diagnosis is based mainly on the morphological study of the adult worm which can be obtained after anthelmintic treatment was strongly recommended. Occasionally, human echinostomiasis has been revealed by gastroduodenal endoscopy performed in relation to severe epigastric symptoms and ulcerative lesions in the stomach and duodenum (Chai *et al*., 1994; Cho *et al*., 2003; Chang *et al*. 2005).

Praziquantel is the drug of choice for echinostomiasis. Echinstome infections can be treated successfully using 10-20 mg/kg praziquantel in a single oral dose. Albendazole may also be effective. Niclosamide 150 mg/kg for one or two days is less likely to be effective than praziquantel. Mebendazole and albendazole have also been shown to have an effect against echinostomiasis (Chai, 2009).

2.4 Life history studies

Echinostome adults are cosmopolitan hermaphroditic digeneans that live in the intestine and bile ducts of numerous vertebrate hosts, particularly aquatic or semi-aquatic birds and mammals, including humans. Echionostomes possess a life cycle following alternation of seven generations known as the adult, egg, miracidium, sporocyst, redia, cercaria and metacercaria, and including three hosts which have been recognized as the vertebrate definitive (final) host, and invertebrate first intermediate host (usually an aquatic gastropod mollusk), and a second intermediate host. Development and multiplication of the miracidium, sporocyst, redia and cercaria are limited in the first intermediate hosts. Second intermediate hosts are invertebrates and some amphibians which are available in the transmission localities of echinostomiasis (Esteban and Antoli, 2009; Toledo *et al*., 2009). Metacercariae are ingested by humans in raw or undercooked, fresh or brackish water, mollusks (pulmonate, opisthobranch snails or bivalves), freshwater fish (*Odontobutis obcura interrupta*, a second intermediate for *E. hortense*) (Chai, 2009), crustaceans, and amphibians (tadpoles or frogs) which constitute a substantial portion of the diet in endemic areas (Bandyopathy and Nandy, 1986).

However, it has been postulated that humans can also be infected through drinking untreated water containing echinostomes cercariae, which could become encysted when exposed to the human gastric juice (Xiao *et al*., 2005). The general life cycle of *Echinostoma* spp. are shown in Figure 2.1.

There are many reports on a first intermediate host (usually an aquatic gastropod mollusk) of *Echinostoma.* The second intermediate hosts are aquatic gastropod mollusks and some amphibians. The definitive hosts of echinostomes are birds and mammals including human. The list of intermediate hosts and definitive hosts of the 37-collar-spined *Echinostoma* in the *"revolutum"* group are shown in Table 2.1 and Table 2.2.

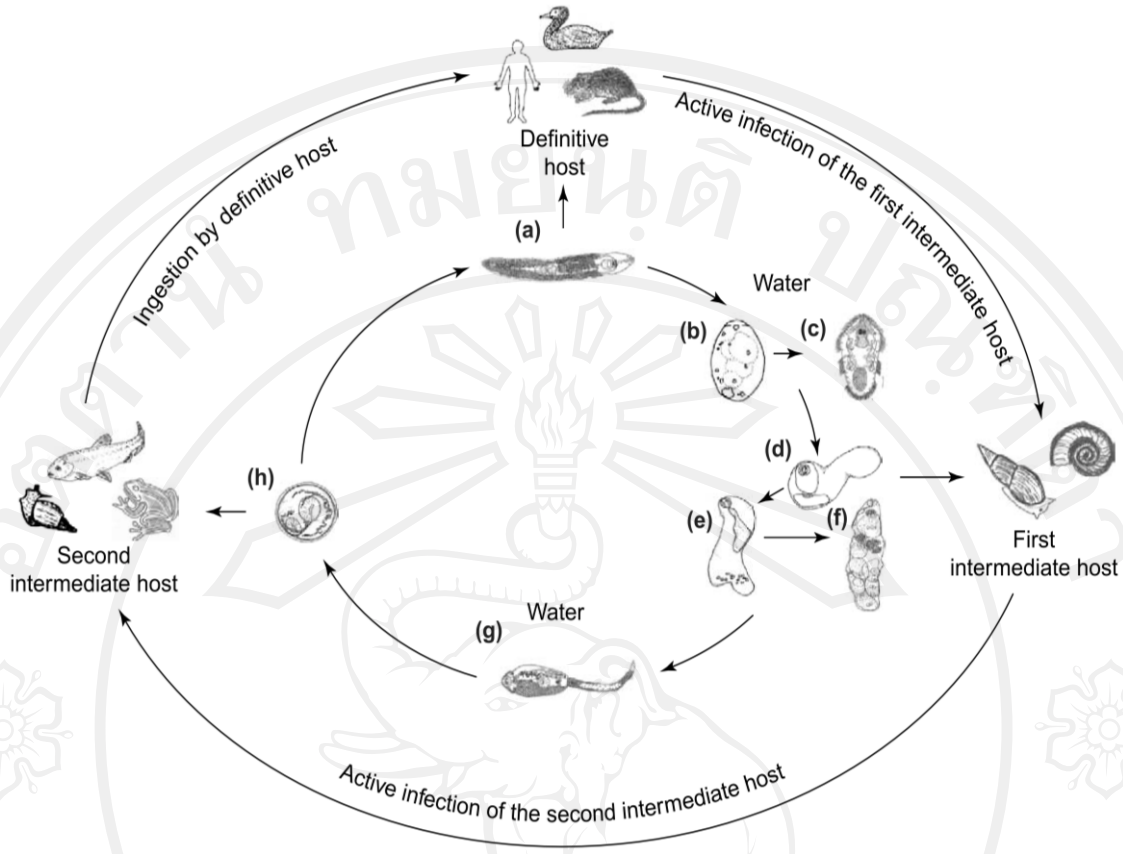

Figure 2.1 Generalized life cycle of *Echinostoma* spp. (a) Adult worms inhabit the small intestine of several vertebrate hosts, including humans. (b) Eggs are voided with the host faeces. (c) Miracidia hatch in fresh water and actively infect snails. (d) Sporocysts, (e) mother rediae and (f) daughter rediae are the intramolluscan stages. (g) Cercariae are released and swim to locate the second intermediate host (snails, amphibians, bivalves and fishes), in which they encyst to become metacercariae. (h) Metacercariae are ingested by the definitive host and excyst to become adults.

(Source: Toledo and Fried, 2005)

Table 2.1 The intermediate hosts of the 37-collar-spined *Echinostoma* in the *"revolutum"* group

Species	1st Intermediate hosts	2st Intermediate hosts	Reference
E. caproni	*Biomphalaria glabrata*	*B. pfeifferi*	Richard and Brygoo, 1978
		Bulinus spp., *Physa acuta, Planorbis corneus, Lymnaea stagnalis, Pisidium casertanum, Melania tuberculata, Rana temporaria* (frog), *Ptychadaena mascareniensis* (amphibian)	Huffman and Fried, 1990
		B. glabrata	Fried and Graczyk, 2004
E. trivolvis		Mussels (*Anodonta cygnea*); various freshwater snails, (*Viviparus* spp., *Lymnaea* spp., *Planorbis* spp., *Biomphalaria* spp., *Physa* spp., *Bithynia* spp.); frogs and tadpoles (*Rana* spp.); freshwater turtles (*Emys orbicularis*); planarians (*Planaria polychroa*)	Kanev *et al*., 1995b
	Helisoma trivolvis	various pulmonate and prosobranch snails, bivalves, planarias, fishes, frogs and tadpoles.	Fried and Graczyk, 2004
E. paraensei	*B. glabrata, Physa marmorata*	*B. glabrata*	Fried and Graczyk, 2004
	L. columella		Pinheiro *et al.*, 2004
E. revolutum	*Lymnaea* spp.	Freshwater snails (*Planorbarius corneus, Physa acuta, Lyrnnaea* spp.); frogs (*R. temporaria and R. ridibunda*); freshwater turtles (*Emys orbicularis*)	Kanev, 1994
	Lymnaea spp.		Fried and Graczyk, 2004
		Filopaludina sp.	Chai *et al.*, 2011
E. friedi	*L. corvusand, G. chinensis*	*L. peregra, L.corvus, G.chinensis, Physa acuta*	Toledo *et al.*, 2000
	Lymnaea peregra	*L. peregra, L. corvus, G. chinensis*	Fried and Graczyk, 2004
E. miyagawai	*P. planorbis*		Kostadinova, 1999
	P.planorbis	*L. stagnalis*	Kostadinova *et al*., 2000
	Planorbis planorbis, Anisus vortex		Fried and Graczyk, 2004
E. echinatum		*Corbicula lindoensis*	Huffman and Fried, 1990
	L. truncatula, Planorbis planorbis	*L. peregra, Physa acuta*	Kostadinova, 1995
	Lymnaea sp., *Planorbarius* sp., *Planorbis* sp., *Anisus* sp., *Gyraulus* sp., *Biomphalaria* sp., *Viviparus* sp.		Fried and Graczyk, 2004
E. parvocirrus	*Biomphalaria glabrata*	*Biomphalaria glabrata*	Fried and Graczyk, 2004
E. luisreyi	*Physa marmorata*	*Physa marmorata, Biomphalaria glabrata*	Maldonado *et al*., 20003
	Physa marmorata.	Physid snails, *Biomphalaria glabrata*	Fried and Graczyk, 2004
E. jurini	*Viviparus contectus, V.viviparus*	Snails: *Viviparus* spp., *Lymnaea* spp., *Planorbarius corneus, Planorbis planorbis, Biomphalaria* spp., *Physa* spp., *Bithynia* spp., *Unio crassus, Dreissena polymorpha;* frogs: *Rana* spp.; turtle: *Emys orbicularis*	Kanev *et al*., 1995

Table 2.2 The definitive hosts of the 37-collar-spined *Echinostoma* in the *"revolutum"* group

Species	Birds	Mammals	Reference
E. caproni	Domestic chick (*Gallus gallus*), Domestic duckling (*Anas bochas*), Pigeon (*Columba livia*), Finch (*Lonchura striata*), Falcon (*Falco newtoni*)	Rabbit (*Oryctolagus cuniculus*), Rat (*Rattus rattus*), Egyptian giant shrew (*Crocidura olivieri*), Golden hamster (*Mesocricetus auratus*), Mouse (*Mus musculus*)	Huffman and Fried, 1990
E. trivolvis	Duck (*Anus* spp., *Aythya* spp.), Goose (*Anser* spp., *Anseranas semipalmata*), Great-horned owl (*Bubo virginianus*), Rough-legged hawk (*Buteo lagopus*), Muscovy (*Cairina moschata*), Domestic pigeon (*Columba livia*), Domestic chick (*G. gallus*), Common scoter (*Oidemia nigra*), American flamingo (*Phoenicopterus*), Common grackle (*Quiscalus quiscula*), Mourning dove (*Zenaida macroura*)	Dog (*Canis familiaris*), Guinea-pig (*Cavia porcellus*), Cat, (*Felis catus*), Golden hamster (*Me. auratus*), House mouse (*M. musculus*), Muskrat (*Ondatra zibethica*), Rabbit (*O. cuniculus*), Norway rat (*R. norvegicus*), Pig (*Sus scrofa*), Red fox (*Vulpes vulpes*)	Huffman and Fried, 1990
E. paraensei		hamsters, albino rats, mice, rodent (*Nectomys squamipe*)	Huffman and Fried, 1990
E. revolutum	Sparrow, (*Passer domesticus*), Wild ducks (*Anas platyrhynchos*), Falcon (*Buteo buteo*), Thrush, (*Turdos philomelos*), white stork (*Ciconia ciconia*), Black-backed gull *(Larus dominicanus)*, Geese *(A. albitrons, A. tabalis, Branta canadensis*), Prey *(Accipiter gentilus, Aquila pomarina, Bueto buteo, B. lagopus,*	Golden hamsters (*Me. auratus*), Rats (*R. rattus*), Mice (*M. musculus*), Dog (*Canis* spp.), Cat (*Felis* spp.), Rabbit (*O. cuniculus*), Pig (*S. scrofa*), Muskrat (*Ondatra zibethica*), American Wigeon (*Mareca americana*)	Beaver, 1937 Huffman and Fried, 1990 Fried and Graczyk, 2004
E. revolutum	*Circaetus gallicus, Falco subbuteo, Acthene noctua, Strix alucoand, Tyto alba*) Pigeon (*Columba livia*), Domestic duck (*Anas borchas*), Domestic chick (*G. gallus*), Little cuckoo dove (*Macropygia rujkeps*), Blackheaded or Chestnut munia (*Lonchura ferruginosa*), Spotted munia (*L. punctulata*), Java sparrow (*Padda oryzivora*)		Beaver, 1937 Huffman and Fried, 1990 Fried and Graczyk, 2004
E. friedi	Chicks (*G. gullus*)	Rat (*R. norvegicus*), golden hamsters (*Me. auratus*)	Fried and Graczyk, 2004 Toledo *et al.*, 2000
E. miyagawai	pigeons		Kostadinova *et al*., 2000
E. echinatum	ducks, goose	Mice (*M. musculus*)	Huffman and Fried, 1990
E. parvocirrus			
E. luisreyi		Mice (*M. musculus*), Golden hamster (*Me. auratus*)	Maldonado *et al*., 20003
E. jurini		Golden hamsters (*Me. auratus*), Rats (*R. rattus*), Mice (*M. musculus*)	Kanev *et al*., 1995

2.5 Studies in first intermediate snail hosts

Echinostoma miracidia actively penetrate a specific snail host for the continuance of the life cycle. Miracidial development within the egg takes about 1-3 weeks in freshwater and the development is temperature dependent (Beaver, 1937). Miracidia of *Echinostoma* are typically digenean with uniform ciliation on the epidermal plates except in the suture areas; miracidia have an apical papilla at the anterior end followed by a primitive gut; the eyespots are located posterior to the gut; one pair of flame cells is present and germinal balls are located posterior to the eyespots (Beaver, 1937). Studies on site finding of *Echinostoma* miracidia are not available. The responses of *E. caproni* miracidia to gravity, light and chemicals were described by Behrens and Nollen (1992). *E. caproni* miracidia have a strong negative geotaxis and a strong positive phototaxis, with the light completely dominating the gravity response.

The Asiatic strain of *E. revolutum* miracidia infects both *Lymnaea ollula* and *L. swinhoei* (Lo and Cross, 1975). *L. ollulu* snails exposed to five miracidia were 95% infected, whereas *L. swinhoei* showed a 40% infection rate. Miracidial penetration of *L. ollula* took about 1 h and about 100-200 daughter rediae were present at 8 days post-infection. Cercariae emerged (about 350 cercariae per day per snail) from the mantle collar area and were negatively phototactic (Lo and Cross, 1975). In Korea, 3 freshwater snail species of the family Lymnaeidae have been reported, *Radix auricularia coreana*, *Austropeplea ollula* and *Fossaria truncatula*. In the experiments with the laboratory-bred snails, *F. truncatula* as well as *A. ollula* was susceptible to the *E. cinetorchis* miracidia with infection rates of 25% and 40%, respectively. It is evident that *A. ollulaacts* and *F. truncatula* as the first molluscan intermediate host of *E. cinetorchis* in Korea (Chung *et al*., 2001c).

The infectivity of *Echinostoma friedi* miracidia was studied experimentally in a range of laboratory-reared snails by Muňoz-Antoli *et al*. (2006). The snails in this study that coexist in the same natural locality and snails from different geographical origins acting naturally or experimentally as intermediate hosts of *Schistosoma* spp. Six species of snails were found to be susceptible, namely *R. peregra*, *L. fuscus*, *L. truncatula*,

Gyraulus chinensis, *Biomphalaria glabrata* and *Bulinus cernicus*, with the rate of infection ranging from 10.0 to 36.7%. The highest infection was detected in *R. peregra*.

The potential role of host cues in the timing of trematode egg development and hatching were described by Belden *et al.* (2009). The potential role of host chemical cues in mediating hatching of *E. trivolvis* miracidia were addressed by comparing hatching in response to cues from the first intermediate host (the snail *Planorbella trivolvis*), a non-host snail (the snail *Goniobasis proxima*), and a non-host invertebrate (earth-worm, *Lumbricus terrestris*). The hypothesized that in the presence of cues from their first intermediate host, *E. trivolvis* would hatch sooner and would be more synchronized than when host cues were absent. DeGaffĕ and Loker (1998) described the susceptibility of the snail *Biomphalaria glabrata* and digenetic trematode *E. paraensei*. The susceptibility of the snail *B. glabrata* to infection with this trematode was correlated with the ability of secretory-excretory products (SEP) derived from sporocysts of this parasite to interfere with the spreading behavior of host hemocytes in an in vitro assay. They are suggested that trematode larvae, and their SEP, can directly affect host hemocyte behavior (DeGaffĕ and Loker, 1998). The results suggest that parasite SEP-mediated effects on hemocyte spreading provide a reliable measure of the infectivity of parasites for their molluscan host and can account for patterns of age and strain related susceptibility to *E. paraensei* infection.

2.6 Studies in second intermediate hosts

Carcariae of *Echinostoma* emerges from the first intermediate host and encysts within the same or other snail hosts. Second intermediate hosts include snails, clams, tadpoles, fishes, other invertebrates, and natural products such as snail mucus (Beaver, 1937; Chai, 2009). Cercariae of *Echinostoma* are typically digenean, i.e. distomate, gymnocephalous with a prominent oral collar of spines and a simple tail. They are about 0.6 mm in length and are active swimmers showing typical wobble-like motion with constant flexion and extension of the tail. The tail of *Echinostoma* is often attenuated and bent at the distal tip and most *Echinostoma* have a pair of dorsal, ventral, and ventrolateral finfolds. Some finfolds are difficult to see by light microscopy, but can be seen by scanning electron microscopy (Huffman and Fried, 1990).

Although cercariae of *Echinostoma* encyst in a broad range of second intermediate hosts there are cercarial preferences for second intermediate hosts. For example, metacercariae of *E. revolutum* were encysted in *Filopaludina* spp., *Bithynia* spp., *Clea helena* and *Eyriesia eyriesi* (Chai *et al*., 2011, Chantima *et al*., 2013). Cercariae of *E. cinetorchis* encysted in second intermediate host snails, *Corbicula fluminea, Segmentina hemisphaerula* and *Pisidium coreanum* in Korea (Chung *et al*., 2001a; Chung *et al*., 2001b; Park *et al*., 2006a).

The cercariae of *Echinostoma* are short lived and rarely survive beyond 48 h at ambient temperatures. Temperature is a factor in survival and longevity and as expected survival is shorter at higher temperatures, e.g. 8 h at 40°C versus 75 h at 10°C (Evans, 1985). Schmidt and Fried (1996) examined the infection of *Helisoma trivolvis* (Colorado strain; CO) snails with cercariae of *E. trivolvis*. They determined that the CO strain of *H. trivolvis* can serve as an excellent experimental second intermediate host for *E. trivolvis*.

Although most cercariae of *Echinostoma* must enter a second intermediate host for the continuance of the life cycle, some ectopic encystment does occur, mainly on snail mucus or in vitro in a tissue culture medium (Huffman and Fried, 1990). The cercariae of *E. cinetorchis* were successfully cultured *in vitro* (Park *et al*., 2006b). The mixture of RPMI (Roswell Park Memorial Institute medium) 1640 plus 10% fetal bovine serum provided the best media for optimal cercariae encystment and metacercariae development.

Cercariae of *E. caproni* encyst in the heart, pericardium and kidney of their second intermediate host snails (Huffman and Fried, 1990). Five species of the genus *Bulinus*, and *Physa acuta* were very susceptible intermediate hosts, whereas three species of *Biomphalaria*, along with *Planorbarius corneus*, *L. natalensis* and *H. duryi* were less susceptible (Christensen *et al*., 1980)

Metacercarial cysts of *Echinostoma* are about 150 μm in diameter and contain a relatively wide and transparent outer cyst and a narrow but thicker inner cyst (Huffman and Fried, 1990). The larva within the cyst is transparent in viable organisms and excretory concretions and cephalic spines are visible; nonviable cysts are opaque and granular. Cysts may remain viable within second intermediate hosts for months.

Survival of *E. caproni* for 4 months and *E. trivolvis* for 6 months within planorbid intermediate host snails has been reported (Anderson and Fried, 1987). Cysts of some species are infective to definitive hosts within 4 h of encystment in the second intermediate host (Anderson and Fried, 1987).

2.7 Infectivity, growth and development in vertebrate hosts

2.7.1 Infectivity

The infectivity of *E. revolutum* in the domestic chick was reported by Fried, 1984. All chicks became infected with one to18 flukes/host and the recovery rate was 25%. Worm recovery in the domestic chick has been reported to be up to 44 days post-infection. Franco *et al*. (1986) reported that 100% of the hamsters became infected with *E. trivolvis* and worm recovery averaged 38%. Hamsters have remained infected for up to 123 days post-infection (Mabus *et al*., 1988). *E. liei* were infected 1-21 day post infection in domestic chicks (97%) with 28% of worm recovery rate (Fried *et al*., 1988b). Manger and Fried (1993) reported worm recovery rate of 100% of *E. caproni* in ICR mice. Worm recovery ranged from 18 to 95% per mouse with a mean infectivity of 45%. The ICR mice can be infected with metacercariae of *E. trivolvis*. The worm burden of ICR mice was 52% with a mean worm recovery of 11 to 41% (Gavet and Fried, 1994). Humphries *et al*. (1997) reported that 67% of domestic chicks became infected with *E. revolutum* and worm recovery averaged 32%. Srisawangwong *et al.* (2004) examined the infectivity of *E. malayanum* in mice. They found that the worm recovery rate was 70-84%.

2.7.2 Worm distribution

E. trivolvis occupies numerous sites in the intestine of the domestic chick including the ileum, rectum, cloaca, cecum and the bursa of Fabricius (Beaver, 1937). Fried (1984) examined the infectivity, growth and development of *E. revoltum* in the domestic chicks. All chicks were infected and flukes were recovered from the ilium caeca, rectum-cloaca and bursa of Fabricius. Huffman *et al.* (1986) reported *E. revolutum* heavy infections in golden hamster. The adult parasite could be found in the stomach, liver, gall-bladder and pancreas. Huffman *et al*. (1988) and Yao *et al*. (1991)

found that *E. caproni* were clustered mainly in the posterior third of the small intestine in golden hamsters, and mainly found in the posterior part of small intestine of ICR mice (Manger and Fried, 1993), whereas *E. liei* is mainly clustered in the lower ilium of domestic chick (Fried *et al*., 1988b). Gavet and Fried (1994) reported *E. trivolvis* distribution in the intestine of ICR mice from day 4 to 11 post-infection. The worms were located more anteriad in the gut early in the infections, and then moved more posteriad until day 11 post-infection.

2.7.3 Development in vertebrate hosts

In vivo development of *E. malayanum* in white rat was examined by Mohandas and Nadakal (1978). The percentage of development of flukes 18 h and 1-3 days post-exposure was 50-70 and in the remaining days 86-94. The developmental process was arbitrarily divided into four stages: organogeny, vitellogenesis, formation of Mehlis' gland complex and cirrus sac and oviposition. Moreover, the growth of *E. malayanum* in mice was described by Srisawangwong *et al*. (2004). The growth of the worms rapidly increased during the first three weeks after infection. The length increased from 1.43 mm at week 1 to 5.12 mm at week 3, and reached a peak at week 11. In contrast, the width of the worms increased very slowly and was nearly stable after 5 weeks. The body areas of the worms increased rapidly during the first seven weeks and fluctuated thereafter. The maximum was found at week 11. The worms became sexually mature and produced eggs, which were detected in feces as early as 2 weeks after infection.

The infectivity and growth of *E. revolutum* in the domestic chick were described by Humphrie *et al.* (1997). Worm length averaged 1.3 mm on day 6, 2.3 mm on day 8 and 3.6 mm on day 14. Mean body area averaged 0.29 mm^2 on day 6, 0.62 mm^2 on day 8 and 1.93 mm^2 on day 14. Worms first became ovigerous on day 12. Growth of *E. revolutum* in the chick was delayed compared to previous findings on *E. trivolvis*, a closely related species of 37-collar-spined echinostome in the *E. revolutum* complex.

The length of *E. revolutum* recovered from golden hamsters is similar to those recovered from domestic chicks. Fried (1984) reported that of the *E. revolutum* grew slowly to 1.0 mm by day 3 post-infection whereas in the golden hamster they averaged 0.77 mm. At 14 days, flukes from the domestic chick were 6 mm in length as compared

with 6.3 mm in the golden hamster. In the chick the mid acetabular width is equal to the greatest width, but in the hamster this is not the case. The ratio of length to midacetabular width of preovigerous worms was 6 in both the hamster and the chick. The ratio increased to 10 in ovigerous flukes from the chick, but remained at about 6 in the hamster. The overall result was a greater body area in flukes from the hamster versus the chick.

Fried *et al*. (1988b) reported the development of *E. liei* in the domestic chick. The fluke wet weights averaged 0.3 mg at 7 days, 2.5 mg at 14 days and 3.0 mg at 21 days; average dry weights for identical days were 0.1, 0.5 and 0.8 mg, respectively. Flukes were ovigerous by day 7. Many differences were noted in the infectivity, growth and development of *E. liei* in the domestic chick. Manger and Fried (1993) examined the growth of preovigerous adults of *E. caproni* in ICR mice. Mean body area of worms increased slowly until day 4 and then rapidly until day 8 post-infection. Distinction of the ovary and ootype was apparent by day 6 and uterine cooling was observed by day 8 post-infection. Only 2 of 50 worms showed eggs in the uterus on day 8 post-infection.

Gavet and Fried (1994) determined the effect of cyst dosage (100 metacercarial cysts) on *E. trivolvis* growth in ICR mice. Mean worm body area was about 0.3 mm^2 on day 4 post-infection and increased to about 2.0 mm^2 by day 9 post-infection. Beyond day 9, mean worm body area was variable with a low of about 0.6 mm^2 on day 10 post-infection and a high of 3.5 mm^2 on day 15. Overall body area increased about 6 or 7 times from day 4 today 17 post-infection.

2.7.4 Worm crowding

The ‘‘crowding effect’’ is a well-known phenomenon in parasitology (Bush *et al.*, 2001). The crowding effect has been studied most extensively in cestodes resulting in decreased size, weight, and number of eggs produced of individual tapeworms (Roberts, 2000; Stillson and Platt, 2007). Relatively few experimental studies have been done on the effects of intraspecific crowding of digeneans (Fried and Nelson, 1978; Mohandas and Nadakal, 1978).

Fried *et al.* (1988a) examined the effects of single and five worm infections of *E. revolutum* in the golden hamster. Their study confirmed the occurrence of self-

fertilization in single worms under conditions that precluded cross-fertilization. Worms in multiple infections were paired or clustered, but neither cirri protrusion nor cross-copulation was observed. There were no significant differences in body weight, body length and percentage of hatched eggs in worms from single versus multiple infections. Miracidia derived from single worms were as capable of infecting laboratory-reared *H. trivolvis* snails and producing patent rediae as were those from multiple infections. Single worms localized in the small intestine 15 to 22 cm anterior to the ileo-caecal valve, whereas multiple worms were more widely distributed (2-30.5 cm). Unlike a previous study on *E. revolutum* in the domestic chick, there were no reproductive advantages in multiple-versus single-worm infections of this parasite in the hamster.

These effects may be the products of a crowding effect facilitated by some form of intraspecific competition. Mohandas and Nadakal (1978) found that crowding reduced the length of *E. malayanum* in rats. Fried and Freeborne (1984) reported that under crowded conditions the number of eggs in the uterus of *E. rrivolvis* was about one-half that of worms in uncrowded sites.

Yao *et al*. (1991) reported the effects of crowding on adults of *E. caproni* in experimentally infected golden hamsters. They confirmed the crowding effects had occurred and indicated by the body area and ovarian and testicular areas at different intensities of infection are difference. The crowding effects were clearly observed from days 14 to 35 post-infection for body and ovarian areas. The tendency for these parasites to cluster may contribute to their lack of growth. Fried and Peoples (2007) determined the effects of a 300-metacercarial cyst inoculum on worm recovery and crowding of *E. caproni* in Balb/C mice. Their revealed the effects of crowding on the distribution of worms in the mouse gut. In crowded hosts, there was an anteriad shift of worms. In the presence of a heavy infection of *E. caproni*, competition for space and nutrient resources (niche competition) in the gut is probably a factor in causing this anteriad shift of worms.

2.8 Parasitic molecular study

Molecular genetic characterization is being used increasingly to distinguish among morphologically similar parasites. Many researchers have applied the polymerase chain reaction (PCR)–based technique for discriminating many parasites in taxonomically confusing status or specific diagnosis of some important parasites. PCR technology and DNA sequencing techniques permit the identification capability of species, strains and populations by using only a small quantity of tissues derived from such developmental stage in their life history (Morgan and Blair, 1997b). Therefore, DNA-based molecular biological tools for the differentiation between certain species of echinostomes have been developed. Several molecular approaches using PCR methods have been employed to support the newly developed approaches on taxonomy, systematics, diagnosis and accuracy detection. Random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis has also been used to differentiate species of *Echinostoma*. Fujino *et al.* (1995) applied RAPD-PCR techniques to demonstrate interspecific polymorphisms of genomic DNA of two closely related echinostomes, *E. trivolvis* and *E. caproni*. The PCR specific primers were used for the distinction between *E. caproni* and *E. trivolvis* (Fujino *et al*., 1997) and also for the differentiation of *E. caproni* and *E. paraensei* (Petrie *et al*., 1996). The PCR-restriction fragment length polymorphism (PCR-RFLP) is also available (Sorensen *et al*., 1998). Bowles and McManus (1993) recommended the PCR-RFLP as a simple and rapid method to distinguish parasites. However, these methods have been exclusively employed on laboratory samples. DNA sequencing has been mainly used over the last few years for taxonomic and phylogenetic studies which have allowed for the characterization of a number of echinostome species that were difficult to compare using traditional methods.

DNA sequencing has proved a useful tool for distinguishing among 37 collar-spined species of the genus *Echinostoma*. Morgan and Blair (1995) sequenced 1000 nucleotide bases of noncoding rDNA (ITS) from six echinostome species of this group and they differed from each other by 2.2% on average. The use of mitochondrial DNA genes showed a greater sequence divergence (Morgan and Blair, 1997a; 1997b). Sorensen *et al.,* (1998) detected high levels of intraspecific variation among ITS sequences of *E. revolutum* (0.9%) and *E. caproni* (0.6%). Kostadinova *et al*., (2003)

analyzed the phylogenetic relationships between several genera of Echinostomatidae. There are re-assessed the phylogenetic relationships of *Echinostoma* and related genera via morphological and mitochondrial DNA (mtDNA) analyses, including NADH dehydrogenase subunit 1 (ND1) and ITS sequences. By the way, the remaining sequences mainly represent molecules used in genetic variation, such as ribosomal (rDNA) or mitochondrial molecules (mtDNA) (Marcilla, 2009). The ITS sequences were used to study the genetic differentiation between two synonymous echinostomes species, *Artyfechinostomum malayanum* and *A. sufrartyfex* (Tantrawatpan *et al*., 2012). Their revealed that the two sibling species, *A. malayanum* and *A. sufrartyfex* are not synonymous. The variable nucleotide positions between *A. malayanum* and *A. sufrartyfex* in their ITS1 and ITS2 sequences could provide genetic markers for solving the taxonomy status of these closely related echinostomes. Moreover, the DNA sequencing of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene were used to genetically compare in four species of echinostomes of human health importance; *E. revolutum*, *E. malayanum*, *Echinoparyphium recurvatumand* and *Hypoderaeum conoideum* (Saijuntha *et al*., 2010). However, the conventional PCR approaches have several drawbacks, in that they require expensive equipments for amplification and detection of amplified products, such as the thermocycler and electrophoresis machine, and PCR requires time for the whole method at least 2-3 h.

Nowadays, a novel method termed loop-mediated isothermal amplification (LAMP) was developed by Notomi *et al*. (2000). This method can amplify DNA with high specificity, efficiency, rapidity and precision under isothermal conditions within 1 h, without the requirement of expensive equipments for amplification. LAMP method employs only a DNA polymerase and a set of six LAMP primers. The LAMP primers are designed to recognize a total of eight distinct regions on the sequence of the target DNA (Notomi *et al*., 2000; Nagamine *et al*., 2002). LAMP products can be easily observed under UV lamp by adding fluorescent dyes such as SYBR Green I (Goto *et al*., 2009; Izadi *et al*., 2012). The LAMP method is simple and easy to perform and has several advantages over the conventional PCR methods, including rapid performance, high sensitivity, cost and time effectiveness, and the products are visually detected with the naked eyes (Notomi *et al*., 2000).

The LAMP assay has been developed successfully for the diagnosis of pathogenic organisisms such as viruses (Parida *et al*., 2008; Kalvatchev *et al*., 2010), bacteria (Enosawa *et al*., 2003; Maruyama *et al*., 2003) and protozoa (Kuboki *et al*., 2003; Han *et al*., 2007; Liang *et al*., 2009). Moreover, the LAMP method has been applied for detection of trematodes including *Schistosoma* spp. (Abbasi *et al*., 2010; Xu *et al*., 2010), *Fasciola* spp. (Ai *et al*., 2010), *Clonorchis sinensis* (Cai *et al*., 2010), *Paragonimus westermani* (Chen *et al*., 2011) and *Opisthorchis viverrini* (Arimatsu *et al*., 2012; Le *et al*., 2012).

2.9 Epidemiology

Food-borne trematode infections represent important medical problems, which approximatly 40–50 million people are generally estimated to be infected worldwide (Abdussalam *et al*., 1995; Lima dos Santos, 1995; Fried *et al*., 2004). These infections occupy particularly Southeast Asia and Far East (Dixon and Flohr, 1997; Chai, 2009), and about 70 species of intestinal trematodes have been reported to infect humans (Yu and Mott, 1994). Almost one-half of these are belonging to the families of Heterophyidae and Echinostomatidae (echinostomes). Echinostomes are cosmopolitan intestinal parasitic trematodes, and some in habit occasionally in bile ducts or ureter, of reptiles, birds and mammals including humans (Yamaguti, 1958). The most frequently encountered species among the group of echinostomes are *E. trivolvis, E. caproni, E. echinatum, E. hortense, E. cinetorchis, E. malayanum, E. revolutum and Euparyphium ilocanum.* Anonymous (1995) described human echinostomiasis, attributed to at least 16 species and are endemic to Southeast Asia and the Far East. Haaseb and Eveland (2000) listed a total of 21 species infecting humans belonging to eight genera of Echinostomatidae (*Artyfechinostomum, Echinochasmus, Echinoparyphium, Echinostoma, Episthmium, Himasthla, Hypoderaeum* and *Paryphostomum*). Chai (2009) listed 20 species belonging to nine genera (*Acanthoparyphium, Artyfechinostomum, Echinochasmus, Echinoparyphium, Echinostoma, Episthmium, Himasthla, Hypoderaeum* and *Isthmiophora*) that have been found parasitizing humans. The species of Echinostomatidae infecting humans and their geographical distribution are summarized in Table 2.3 by Toledo and Fried (2013).

Although echinostomiasis occurs worldwide, most human infections are reported from foci in East and Southeast Asia. Echinostomiasis is relatively rare, yet the foci of transmission remain endemic owing to the local dietary preferences. Most of these endemic foci are localized in China, India, Indonesia, Korea, Malaysia, Philippines, Russia, Taiwan and Thailand (Haaseb and Eveland, 2000; Chai, 2009). Moreover, occasional cases have also been reported in other countries (Table 2.3).

The prevalence of echinostomiasis reported varied and ranged in different countries; 65% in Taiwan (Bundy *et al.*, 1991), 44% in the Philippines (Cross and Basaca, 1986), 5% in mainland China (Li, 1991), 22% in Korea (Ryang, 1990) and 50% in northern Thailand (Sanchaisuriya *et al.*, 1993). In Thailand, echinostomiasis is caused by *Echinostoma* spp. which commonly found in North and Northeastern parts of Thailand (Nithikathkul *et al*., 2008). Six species were reported to cause human infections; *E. malayanum*, *Hypoderaeum conoideum* (Bhaibulaya *et al*., 1964), *E. ilocanum*, *E. japonicus*, *E. revolutum* and *Episthmium caninum* (Radomyos *et al.*, 1982; Radomyos *et al.*, 1985; Radomyos *et al.*, 1994).

In endemic areas, the disease occurs focally and associates with common socio-cultural practices. Eating raw snails and tadpoles was identified as an important mode of transmission (Carney, 1991). Humans or animals are infected through ingestion of metacercariae encysted in the second intermediate host. Eating raw snails, clams, fishes, or over vegetation harboring metacercariae is the main practical mode of infection in humans (Chai, 2009) (Table 2.4).

Table 2.3 Geographical distribution of species of Echinostomatidae infecting humans clinical and pathological effects (Toledo and Fried, 2013)

Continent	Species	Country
Africa	*Echinoschasmus liliputanus*	Egypt
	Echinochasmus perfoliatus	Egypt
	Echinoparyphium recurvatum	Egypt
	Echinostoma revolutum	Egypt
America	*Himasthla muehlensi*	Colombia, USA
	Echinostoma echinatum	Brazil
	Isthmiophora melis	USA
Asia	*Acanthoparyphium tyonense*	Japan, Korea
	Artyfechinostomum malayanum	China, India, Indonesia, Malaysia, Philippines, Singapore, Thailand
	Artyfechinostomum mehrai	India
	Artyfechinostomum oraoni	India
	Echinoschasmus angustitestis	China
	Echinoschasmus fujianensis	China
	Echinoschasmus japonicus	China, Korea, Japan
	Echinoschasmus jiufoensis	China
	Echinoschasmus liliputanus	China, Palestina, Syria
	Echinochasmus perfoliatus	China, Japan, Taiwan
	Echinoparyphium recurvatum	Indonesia, Taiwan
	Echinostoma cinetorchis	China, Japan, Korea, Taiwan
	Echinostoma echinatum	Indonesia, Japan, Thailand
	Echinostoma hortense	China, Japan, Korea
	Echinostoma ilocanum	China, India, Indonesia, Malaysia, Philippines, Thailand
	Echinostoma japonicum	Japan, Korea
	Echinostoma macrorchis	Indonesia, Japan, Korea, Taiwan
	Echinostoma revolutum	Thailand
	Episthmium caninum	India, Thailand
	Hypoderaeum conoideum	Thailand
	Isthmiophora melis	China, Taiwan
	Paryphostomum sufrartyfex	Thailand
Europe	*Echinochasmus perfoliatus*	Denmark, Hungary, Italy, Romania, Russia
	Echinostoma echinatum	All Europe
	Echinostoma paraulum	Russia
	Echinostoma revolutum	All Europe
	Hypoderaeum conoideum	All Europe
	Isthmiophora melis	All Europe
Oceania	*Echinostoma revolutum*	Australia

Table 2.4 Possible source of human or animal infections with echinostomes (Chai, 2009)

Parasite species	Source of human or animal infections
Fish-borne	
Echinoschasmus fujianensis	Freshwater fish, *Pseudorasbora parva, Cyprinus carpio*
Echinoschasmus japonicus	Freshwater fish, *Pseudorasbora parva, Hypomesus olidus, Gnathopogon strinatus*
Echinoschasmus jiufoensis	Unknown
Echinoschasmus liliputanus	Freshwater fish, *Pseudorasbora parva,* goldfish
Echinochasmus perfoliatus	Freshwater fish, *Carassius* sp.
Echinoschasmus angustitestis	Freshwater fish
Echinostoma cinetorchis	Freshwater fish, *Misgurnus anguillicaudatus*
Echinostoma hortense	Freshwater fish, *Misgurnus anguillicaudatus, Misgurnus mizolepis, Odontobutis obscura interrupta, Moroco oxycephalus, Coreoperca kawamebari, Squalidus coreanus*
Episthmium caninum	Freshwater fish
Snail-borne	
Acanthoparyphium tyonense	Bivalve, *Mactra veneriformis, Solen grandis,* gastropod, *Neverita bicolor*
Artyfechinostomum malayanum	Snail, *Digoniostoma pulchella,* large snail, *Pila scutata, Lymnaea (Bullastra) cumingiana*
Echinoparyphium recurvatum	Freshwater snail, *Planorbis planorbis, Lymnaea* sp., *Lymnaea stagnalis*
Echinostoma cinetorchis	Freshwater snail, *Redix auricularia coreanus, Physa acuta, Cipangopaludina chinensis malleata*
Echinostoma echinatum	Mussel, *Corbicular lindoensis, Corbicular sucplanta, Idiopoma javanica,* freshwater snail, *Biomphalaria glabrata*
Echinostoma ilocanum	Large snail, *Pila conica, Viviparus javanicus*
Echinostoma macrorchis	Large snail, *Cipangopaludina malleata, Cipangopaludina japonica, Segmentina nitiella, Viviparus malleatus*
Echinostoma revolutum	Snail or clam, *Corbicular producta*
Himasthla muehlensi	Clams, *Venus mercenaria,* bivalve mollusk, *Mytilus, Mya* spp.
Hypoderaeum conoideum	Snail, *Lymnaea stagnalis, Lymnaea limosa, Lymnaea tumidae, Planorbis planorbis*
Amphibia-borne	
Echinoparyphium recurvatum	Tadpole and frog of *Rana temporaria*
Echinostoma macrorchis	Frog of *Rana* sp.
Echinostoma revolutum	Tadpole
Hypoderaeum conoideum	Tadpole
Isthmiophora melis	Tadpole

In summary, the epidemiology, life history and molecular biology of most digenetic trematodes have been documented, but the knowledge concerning such topics in echinostome flukes remains unclear. This study, therefore, aims to investigate the epidemiology of echinostome metacercariae, biological characteristics and molecular characters of *E. revolutum* which originated from the life history of this fluke in Chiang Mai Province. Data obtained from this study will provide the knowledge of the strategies for development of effective control measures for the disease, diagnosis and accurate detection. Information about the life history may also be important for future development in the study of taxonomy, systematics and host-parasite relationships.