รายงานวิจัยฉบับสมบูรณ์

โครงการการจำแนกทางอณูวิทยาและสายวิวัฒนาการของพยาธิปอดหนูในประเทศไทยด้วยการใช้ลำดับเบสของยีน cytochrome c oxidase subunit I

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กรกฎาคม 2558

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยนเรศวร

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) กองทุนสนับสนุนการวิจัย (สกว.) และมหาวิทยาลัยนเรศวร (มน.) ที่ให้การสนับสนุนทุนในการทำวิจัยในครั้งนี้จนเสร็จสิ้น และขอขอบคุณ รศ.ดร.พารณ ดีคำย้อย ภาควิชาปรสิตหนอนพยาธิ คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล ที่คอยแนะนำให้คำปรึกษาในการทำวิจัย ขอขอบคุณนางสาววิไลวรรณ ผลสุทธิ์ นายอนุชา วงศ์มา นายณรงค์ฤทธิ์ ศรีสงคราม นางสาวจิตรานุช ทิพย์โรจน์ และนางสาวทัชชา ยิ้มถิน และนางสาวชไมพร ฟักรักษา ที่ช่วยเหลือในการเก็บตัวอย่าง สุดท้ายนี้ผู้วิจัยขอขอบคุณภาควิชาจุลชีววิทยาและปรสิตวิทยา คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร ที่เอื้อเฟื้อสถานที่และอุปกรณ์ในงานวิจัย

อภิชาติ วิทย์ตะ

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Abstract

Project Code : MRG5580044

Project Title : Molecular identification and phylogeny of *Angiostrongylus* from Thailand based on cytochrom c oxidase subunit I (COI) sequence

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Project Period : July 2012 –July 2015

Since the basic problem of identification of *Angiostrongylus* into species level is the ambiguity of morphological characters, therefore alternative methods are needed. Molecular techniques provide a useful alternative approach for identifying nematodes that can be applied to both adult and juvenile worms. The objective of this research was to identify and study phylogeny of *Angiostrongylus* collected from natural hosts in Thailand. A total of 14,032 mollusks, namely, *Filopaludina* sp., *Pomacea canaliculata*, *Achatina fulica*, *Cryptozona siamensis*, *Cyclophorus* sp. and *Megaustenia siamensis*, were collected from 19 provinces of Thailand. The larvae of *Angiostrongylus* were isolated by artificial digestion methods following Bearmann's techniques. The genomic DNA of larvae was extracted and partial sequence of cytochrome c oxidase subunit I (COI) was amplified by polymerase chain reaction. It was found that 11 isolates of *Angiostrongylus* from 5 provinces were identical to *Angiostrongylus cantonensis* ranging from 92-99% of identity. Phylogenetic analysis separated 11 isolates of *Angiostrongylus* into 3 groups. Group I contained 1 isolate of *Angiostrongylus* isolated from *A. fulica* of Tak province and included sequence belonging to *A. cantonensis* from Bangkok, Thailand. Group II contained 5 isolates of *Angiostrongylus* from Phetchabun and Kamphaeng Phet Provinces and a reference sequence of *A. cantonensis* from Zhejiang Wenzhou, China. Group III contained 5 isolates of *Angiostrongylus* from Kalasin, Tak, and Phitsanulok Provinces and no reference sequence. This is the first record of *C. siamensis* as natural intermediate hosts for *A. cantonensis* in Thailand. The prevalence of *Angiostrongylus* in intermediate hosts will be useful for surveillance of angiostrongyliasis in Thailand.

Keywords : *Angiostrongylus*, Phylogenetic tree, Cytochrome c oxidase subunit I

บทคัดย่อ

รหัสโครงการ : MRG5580044

ชื่อโครงการ : การจำแนกทางอณูวิทยาและสายวิวัฒนาการของพยาธิ *Angiostrongylus* ในประเทศไทยด้วยการใช้ลำดับเบสของยีน cytochrome c oxidase subunit I

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ปัญหาพื้นฐานของการจำแนกชนิดของพยาธิ *Angiostrongylus* คือ การมีรูปร่างลักษณะที่เหมือนกัน จึงจำเป็นต้องหาวิธีการอื่นเป็นทางเลือก ซึ่งเทคนิคทางด้านอณูวิทยามีประโยชน์มากในการจำแนกชนิดของหนอนตัวกลมโดยสามารถจำแนกได้ทั้งในระยะตัวเต็มวัยและระยะตัวอ่อน วัตถุประสงค์ของงานวิจัยครั้งนี้คือ จำแนกชนิดและศึกษาสายสัมพันธ์ทางวิวัฒนาการของพยาธิ *Angiostrongylus* ที่เก็บจากโฮสต์ในธรรมชาติในประเทศไทย จากการเก็บตัวอย่างหอยทั้งหมด 14,032 ตัว จาก 19 จังหวัดของประเทศไทย ได้แก่ *Filopaludina* sp., *Pomacea canaliculata*, *Achatina fulica*, *Cryptozona siamensis*, *Cyclophorus* sp. และ *Megaustenia siamensis* ระยะตัวอ่อนของพยาธิ *Angiostrongylus* ถูกแยกออกด้วยวิธีย่อยด้วยน้ำย่อยเทียมและวิธี Bearmann แล้วแยกสกัดดีเอ็นเอจากตัวอ่อน เพื่อเพิ่มปริมาณและวิเคราะห์หาลำดับเบสบางส่วนของยีน cytochrome c oxidase subunit I (COI) ด้วยวิธี polymerase chain reaction พบว่าพยาธิ *Angiostrongylus* จำนวน 11 ไอโซเลตที่แยกได้จาก 5 จังหวัด มีความเหมือนกับพยาธิปอดหนู *Angiostrongylus cantonensis* มากถึง 92-99% การวิเคราะห์ทางสายสัมพันธ์ทางวิวัฒนาการแยกพยาธิ *Angiostrongylus* จาก 11 ไอโซเลตเป็น 3 กลุ่ม คือ กลุ่มที่ 1 ประกอบด้วย *Angiostrongylus* 1 ไอโซเลต ที่แยกได้จากหอย *A. fulica* จากจังหวัดตาก มีความใกล้ชิดกับพยาธิ *A. cantonensis* จากกรุงเทพของประเทศไทย ส่วนกลุ่มที่ 2 ประกอบด้วย *Angiostrongylus* 5 ไอโซเลต ที่แยกได้จากจังหวัดเพชรบูรณ์และกำแพงเพชร มีความใกล้ชิดกับพยาธิ *A. cantonensis* จากเมือง Zhejiang Wenzhou ในประเทศจีน สำหรับกลุ่มที่ 3 ประกอบด้วย *Angiostrongylus* 5 ไอโซเลต จากจังหวัดกาฬสินธุ์ ตาก และพิษณุโลก ไม่มีความใกล้ชิดกับ *A. cantonensis* ที่เป็นอ้างอิง งานวิจัยนี้เป็นรายงานครั้งแรกที่พบ *C. siamensis* เป็นโฮสต์ตัวกลางในธรรมชาติของ *A. cantonensis* ในประเทศไทย ความชุกของพยาธิปอดหนูในโฮสต์ตัวกลางมีประโยชน์สำหรับการวางแผนควบคุมโรคพยาธิปอดหนูในประเทศไทย

Keywords : พยาธิปอดหนู, สายสัมพันธ์ทางวิวัฒนาการ, Cytochrome c oxidase subunit I

สารบัญ

CHAPTER I

INTRODUCTION

Rationale and background

Human angiostrongyliasis is a food-borne parasitic zoonosis caused by larval stage of *Angiostrongylus cantonensis* or the rat lungworm. It has been reported from several parts of the world, particularly from Southeast Asia to the Pacific Islands (Alicata and Jindrak, 1970). To date, at least 2,872 cases of the disease have been documented worldwide and over half of these cases (1,337) were reported from Thailand (Wang et al., 2008). Although four species of Metastrongyloidea are found in Thailand, namely, *A. cantonensis* (Chen, 1935), *A. siamensis* (Ohbayashi et al., 1979), *A. malaysiensis* (Bhaibulaya and Cross, 1971) and *Thaistrongylus harinasuti* (Ohbayashi et al., 1979), *A. cantonensis* is the only known cause of the disease in the country. It is considered to be the primary causative agent of eosinophilic meningitis or meningoencephalitis in humans infected with helminth parasites. Moreover, this parasite can occasionally be the cause of ocular angiostrongyliasis (Sinawat et al., 2008).

Humans are an accidental host who become infected by ingesting third-stage larvae (L3) in infected snails or slugs or contaminated uncooked vegetables. Upon ingestion, L3 larvae migrate to the brain, spinal cord and nerve roots, causing eosinophilia in both the spinal fluid and peripheral blood. Because humans are not the normal definitive host, larvae are unable to complete development to the adult stage, thus no progeny larval worms are seen microscopically during examination of human fecal samples. However, adult worms are occasionally reported in young children (Yii et al., 1976; Sonakul, 1978). The most common clinical manifestations of angiostrongyliasis are severe headache, vomiting, paresthesia, weakness and occasionally visual disturbances. Most patients recover fully, although severe infections can lead to coma and even death (Chotmongkol and Sawanyawisuth, 2002). For example, In March 2011, one severe case of angiostrongyliasis with eosinophilic meningoencephalitis was reported on news from a woman in Khon Kaen province (http://www.youtube.com/watch?v=nAr2YtUBr2Q).

Accurately identifying medically important nematodes such as *A. cantonensis* is crucial in diagnosing and controlling the diseases that they cause (Casser and Newton, 2000). It is, however, difficult to identify worms to the species level due to a lack of suitable morphological characters in both the adult (Newton et al., 1998: Roberts and Janovy, 2005) and juvenile

stages (Newton et al., 1998). For example, species identification of order Strongylida such as *Angiostrongylus* relies heavily on the morphological analysis of the copulatory bursa which is found only in adult males (Newton et al., 1998: Roberts and Janovy, 2005). Complicating identification is the difficulty involved in setting apart juvenile worms because of the ambiguity of their morphological characters (Newton et al., 1998). For example, the 3rd stage larva of *A. cantonensis* and *A. vasorum* are very similar in size and body shape (Ubelaker, 1986) and can only be differentiated from one another based on subtle differences in the way their tails terminate; *A. cantonensis* has a fine point termination whereas *A. vasorum* has a digitiform termination (Ash, 1970). Whether juveniles of other *Angiostrongylus* species can also be distinguished is not known. With such difficulties involved in identifying *A. cantonensis*, a viable alternative is clearly needed. Molecular methods provide a useful alternative approach for identifying nematodes that can be applied to both adult and juvenile worms (Newton et al., 1998; Bhadury et al., 2006). Caldeira et al. (2003) developed a PCR-RFLP approach using the ITS2 and CO1 to distinguish *A. cantonensis*, *A. costaricensis* and *A. vasorum*. However, this technique can not detect nucleotide substitutions outside the restriction site.

Cytochrome c oxidase is a 13-subunit protein complex located on the inner mitochondrial membrane that catalyzes electron transfer, proton translocation processes, production of up to 95% of the energy of eukaryotic organism (Saraste, 1999; Johnston 2006), thus directly influence metabolic performance. Cytochrome c oxidase subunit I is the most highly conserved among 3 genes coding for cytochrome oxidase therefore has been employed in several phylogenetic studies (Traversa et al., 2007).

Objectives of the research

The main objective is to identify *Angiostrongylus* in Thailand by using molecular technique

Specific objectives are as the followings:

1. To survey of *Angiostrongylus* in both land and fresh water snails collected from Thailand
2. To sequence and analyze cytochrome C oxidase subunit I of *Angiostrongylus*
3. To study evolution relationship among *Angiostrongylus* from different regions of Thailand by phylogenetic tree

Scope and limitation of the research

This research is the experimental study that begin with the collection of intermediate hosts for *Angiostrongylus* spp. in Thailand. The experiment were perfomed in Department of Microbiology & Parasitology, Faculty of Medical Science, Naresuan University. Genomic DNA from worms was extracted and amplified by polymerase chain reaction. The gene COI from *Angiostrongylus* was sequenced and analyzed by bioinformatic software. Number of intermediate host collection depended on the season

Anticipated outcomes

It is expected that the result from this research will be useful in development molecular technique for identification of *Angiostrongylus*. The sequence of CO I of *Angiostrongylus* will be served as the database on NCBI server. The results in this project will be useful for identifying *Angiostrongylus*.

CHAPTER II

LITERATURE REVIEW

Genus *Angiostrongylus*

The genus *Angiostrongylus* is placed in phylum Aschelminthes, class Nematoda, subclass Secernentae, superfamily Metastrongyloidea, and family Strongylidae. Until now, 20 species of this genus have been described in various animals from several localities (Table 1).

Table 1 Various species of the genus *Angiostrongylus* found in several animals.

Species	Animals	Location in animals	Locality	Reporter/year
1. *A. vasorum*	Domestic dog	Pulmonary arteries, right side of the heart	Europe, South America, Australia	Baillet/1866
2. *A. raillieti*	Crab-eating dog	Pulmonary arteries, right ventricle	Brazil	Travassos/1927
3. *A. tateronae*	Kemp jerboa	Stomach	Ibadan, West Africa	Baylis/1928
4. *A. ondatrae*	Muskrat	Lung	USSR	Schulz et al./1928
5. *A. cantonensis*	Rodent	Pulmonary arteries, right side of the heart	Indo-Asian Pacific regions	Chen/1935
6. *A. ten*	Black footed marten	Heart	Japan	Yamaguti/1941
7.*A. gusbernaculatus*	Badger, Skunk	Heart	California, USA	Dougherty/1946
8. *A. blarini*	Short-tailed shrew	Lung	Illinois, USA	Soltys/1953
9. *A. soricis*	Shrew (*Sorex minutus*)	Lung	Poland	Ogren/1954
10. *A. chabaudi*	Wild cat (*Felis silvestris*)	Pulmonary arteries,	Central Italy	Biocca/1957
11. *A. sciuri*	Squirrel (*Sciurus vulgalis*)	Pulmonary arteries	Turkey	Merdivenci/1964
12. *A. michiganensis*	Shrew	Bronchioles	Michigan, USA	Ash/1967
13. *A. sandarasae*	Rodent	Pulmonary arteries	Mozambique,	Alicata/1968
14. *A. mackerrasae*	Rat (*Rattus fuscipes*)	Pulmonary arteries, right side of the heart	Queensland, Australia	Bhaibulaya/1968
15. *A. dujardini*	Wild rodent	Lung	South France	Drozdz and Doby/1970
16. *A. schmidti*	Rice rat	Pulmonary arteries	Florida, USA	Kinsella /1971
17. *A. malaysiensis*	*Rattus jalorensis*	Pulmonary arteries	Malaysia	Bhaibulaya and Cross/1971
18. *A. costaricensis*	*Sigmodon hispidus*	Mesenteric arteries	Costa Rica, Central American	Morera and Cespedes/1971
19. *A. minutus*	Japanese shrew mole	Lung	Japan	Obayashi et al./1973
20. *A. siamensis*	Rodent	Pulmonary arteries	Thailand	Obayashi, Kamiya and Bhaibulaya/1979

The most important species of metastrongylidae is *Angiostrongylus cantonensis*. Therefore, this species is the most review.

Historical discovery of *Angiostrongylus cantonensis*

In 1935*, Angiostrongylus cantonensis* was first described under the name of *Pulmonema cantonensis* by Chen who discovered it in the lungs of rats in Canton, China. In 1937, Yokogawa described this parasite under the name of *Haemostrongylus ratti*. In 1946, Dougherty demonstrated that the genus *Pulmonema* was synonymous with *Angiostrongylus* (Kamenskii, 1905) and changed the genus name to *A. cantonensis*. Soon after *H. ratti* also was found to the same as *A. cantonensis*, and the name was synonymized with *A. cantonensis*. In 1945, the first case of human angiostrongyliasis was reported by Nomura and Lin who found the larvae of *A. cantonensis* in the spinal fluid of a 12-year old boy with meningitis from central Taiwan (Beaver and Rosen, 1964). In 1955, the life cycle of *A. cantonensis* and its migration through the brain of vertebrate hosts were described by Mackerras and Sandars. In 1962, Rosen and colleagues discovered *A. cantonensis* in the brains of a fatal case of eosinophilic meningoencephalitis. As a result of this finding, *A. cantonensis* is now considered one of the causes of eosinophilic meningoencephalitis. Up to the present, this disease has been reported from several areas of the world. However, the most cases continue to be reported from Southeast Asia to the Pacific islands, especially from Taiwan and Thailand.

Morphology of *Angiostrongylus cantonensis*

The body of adult *A. cantonensis* is a delicate filiform worm that tapers slightly at both ends. It has a length of 17 to 25 mm and a maximum diameter of 0.26 to 0.36 mm. It has a pallid hue in the living state. The cuticle is smooth and slightly thickened at the both ends. The cephalic end is simple with three lips; one dorsal lip, with two submedian papillae and two subventral lips, each with one submedian papilla. In addition, there are four pairs of papillae displayed symmetrically at quadrant distances from one another on the external border of the head. A buccal capsule is absent and the mouth opens directly into a muscular esophagus (Eamsobhana, 2006).

Male worm (Figure 1A): The length of male worm is about 18 to 25 mm, and the width is 0.2 to 0.4 mm. The length of esophagus is 0.31 to 0.32 mm. The intestinal tract is straight and runs parallel to the genital tube. It has two spicules, which are slightly sub-equal in size

and measure from 1.02 to 1.25 mm in length. The right spicule is longer than the left. Caudal bursa (Figure 1B) at the end of the body is well-developed and kidney-shaped. The arrangement of the bursal rays (Figure 1B) is divided into 4 groups. First, the two ventral rays originate from a common trunk; the ventro-ventral is slightly shorter and thinner than the latero-ventral rays. Second, the three lateral rays also originate from a common trunk; the antero-lateral ray is shaped like a thumb, the medio-lateral ray is longer than the postero-lateral ray. Third, a single externo-dorsal ray is located between the lateral and dorsal rays. The latest bursal ray is dorsal ray, which is short and stout and usually ends in three small digitiform structures (Eamsobhana, 2006). A gubernaculum is located at the posterior end and measures approximately 95 μm in length (Alicata, 1968).

Female worm (Figure 1A): Generally, the body size of the adult female worm is larger than that of the male. The length is from 21 to 34 mm, and the width, 0.34 to 0.56 mm. The esophagus is 0.35 to 0.46 mm long. The posterior tip ends bluntly with the sub-terminal anus on the ventral surface. In living worms, the blood-filled intestine and milky-white uterine tubes are spirally wound in the characteristic “barber’s pole” pattern. The vulva opens posteriorly near the anus (Eamsobhana, 2006). Egg: The eggs in uterus are unembryonated, thin-shelled, transparent, elliptical shape and 46-48 × 68-74 μm in size. A single female may lay up to 15,000 eggs daily (Beaver et al., 1984). The eggs of *A. cantonensis* develop into five larval stages, which are found in several hosts. The first-stage larvae are found in the lung and feces of the definitive host, whereas the second-stage and the beginning of the third-stage larvae are found in the snail intermediate or paratenic hosts. The remainder of the third-stage, the fourth-stage and fifth-stage (young adult) larvae are found in mammalian definitive hosts. The morphology of each stage is as the following:

First-stage (L1) larva (Figure 1C): The body size is 0.014-0.017 × 0.26-0.31 μm with slender and cylindrical shape. The rhabditoid esophagus is slender with a broader posterior portion and occupies about the anterior half of the body. The small genital primordium is located anterior to the middle part of the intestine. The tail is sharply pointed and has a prominent notch on the dorsal surface (Eamsobhana, 2006).

Second-stage (L2) larva: This stage closely resembles the first-stage larva, but the size (approximately 0.42-0.48 × 0.03 mm) is larger. The esophagus is approximately one-third of the body length. The genital primordium is located at the middle of the body (Eamsobhana, 2006).

Third-stage (L3) larva (Figure 1D): The body the L3 larva is long, thin and delicate (0.46-0.52 mm long and 0.029-0.038 mm wide). It has two chitinous rods at the anterior end and the cuticle possesses delicate transverse striation. The rhabditiod esophagus is 0.17-0.19 mm long. The genital primordium is located at the middle of the body. The tail is cone-shaped, slightly curved and pointed (Eamsobhana, 2006).

Fourth-stage (L4) larva: The body length and width of this stage is 0.54-0.56 mm and 0.03 mm, respectively. The chitinous rods are absent, and the larva possesses a small buccal capsule. The cuticle is smooth and the esophagus is elongated. After ingested by host, the larva is distinguishable as to sex. In male worm, the cuticle at the tip of tail becomes expanded while the female worms still retains their conical shape (Eamsobhana, 2006).

Fifth-stage (L5) larva or young adult (Figure 1E): About 10 days after ingestion by the definitive host (rodent), larvae have completed their fourth and final molt to become young adults. The body size of male worm is 1.7-1.8 × 0.05 mm and it possesses a well formed bursa (Figure 1F), whereas the female worm is 2.5-2.7 × 0.06 mm. Young adults live in the subarachnoid space of rat brain for 3 weeks and then they migrate to the lungs where they develop to mature adult stages (Eamsobhana, 2006).

Life cycle and biology of *Angiostrongylus cantonensis*

The life history of *A. cantonensis* was first demonstrated by Mackerras and Sandars (1955). Up to the year 1975, Bhaibulaya showed that the species used in his experiment was most likely to be *A. mackerrasae*. The differences of two species are as follows: (1) the molting times of *A. cantonensis* in the definitive host occurred a few days earlier than those of *A. mackerrasae* and (2) the growth rate of *A. cantonensis* was more rapid than that of *A. mackerrasae*. The life cycle of *A. cantonensis* is shown in Figure 2. To complete the life cycle, *A. cantonensis* requires the definitive and intermediate hosts. Several species of rodents (Table 2) from different parts of the world have been reported as the natural definitive hosts (reviewed by Alicata and Jindrak, 1970). Some of them that served as definitive hosts in Thailand are also found in Chiang Mai, in the north (Namue and Wongsawad, 1997) and northeastern (Pipitgool et al., 1997) regions.

Adult male and female worms live in the pulmonary arteries and the right side of the heart of rodents. After copulation, female worms lay unembryonated eggs, 42-45 days after infection, which lodge in the small capillaries of the lungs. Embryonation of eggs occurs within 6

days (Weinstein et al., 1963). The first-stage larvae hatch from the eggs and migrate up the bronchial tree. Then their migrations reach to the esophagus and then are swallowed to the alimentary tract and pass out with the feces. Outside the host, they can survive only a few hours in dry feces and one or two weeks in stagnant water with fecal pollution either in freshwater or in seawater (Richards and Merritt, 1967). The first-stage larvae can infect the molluscan intermediate host either by ingestion (Cheng and Alicata, 1964) or by penetration (Harris and Cheng, 1975). Several species of mollusks, which are documented by Alicata and Jindrak (1970), are recorded as the natural intermediate hosts (Table 3) or experimental intermediate hosts (Table 4) of *A. cantonensis*. In Thailand, Pipitgool and co-workers (1997) reported that *Pila polita* and *Achatina fulica* were the main intermediate hosts of *A. cantonensis* in the northeast area. Upon entry of first-stage larvae into the intermediate host, the larvae migrate to the muscular tissue of the body. Within a few days, the intestine of the worm becomes completely dark due to the occurrence of food granules in the intestinal cells and then they molt between 7 to 9 days after infection, giving rise to second-stage larvae but without casting off their sheaths. The second-stage larvae do not grow much in size. After 12 to 16 days of infection, they molt to third-stage larvae which remain enclosed in the sheaths of the first two stages. The third-stage larvae are usually spirally coiled and partially encapsulated in the muscular portion of the foot of the snail host (Bhaibulaya, 1975). At this point in development they are infective to the definitive host. The time required for the first-stage larvae to develop to the third-stage depends on the species of mollusk infected (Richards and Merritt, 1976). Rodents become infected by ingestion of raw mollusks containing infective third-stage larvae. After the rodents ingest the infective larvae, the third-stage larvae are released from host tissue by digestion and exsheath in the intestine. The exsheathed larvae penetrate the intestinal mucosa and enter the blood circulation via mesenteric veins and lymph glands (Alicata and Jindrak, 1970). The larvae reach the lungs via the pulmonary artery as early as one hour after infection. From the lungs, they are carried to various parts of the body by way of the arterial circulation before reaching the brain. They are found in the cerebral hemisphere between one and two days of infection. After 4-6 days of infection, the third-stage larvae molt (third molt) to the fourth-stage larvae (Bhaibulaya, 1975) and then migrate to the subarachnoid space of the brain where they molt to become young adults (L5) 7-9 days after infection. Ten days after the fourth molt, the young adults leave the sheath in the subarachnoid space and migrate to the pulmonary arteries where they attain sexual maturity. Recovery of the first-stage

larvae in feces of rodents can be accomplished 42 to 45 days post infection (Eamsobhana, 2006).

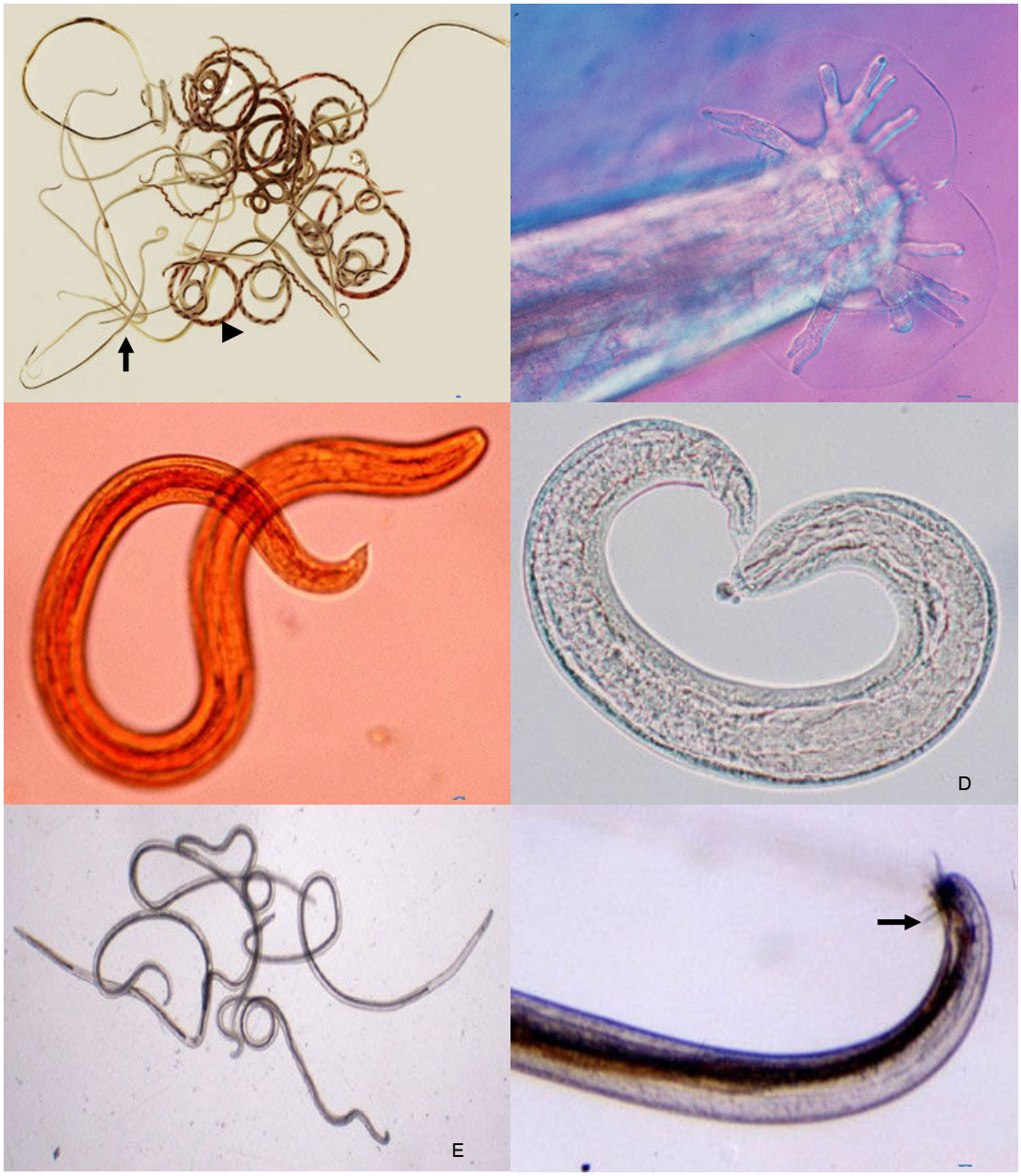

Figure 1 Photomicrographs of *Angiostrongylus cantonensis* showing prominent morphological features. A: adult male (arrow) and female (arrow head), B: the posterior end of adult male showing caudal bursa and bursal rays, C: the first-stage larva, D: the third-stage larva, E: the fifth-stage larva or young adult and F: the posterior end of young adult male showing caudal bursa (arrow). (from: http://research.md.kku.ac.th/comm/comrru/meningitis/main02.html)

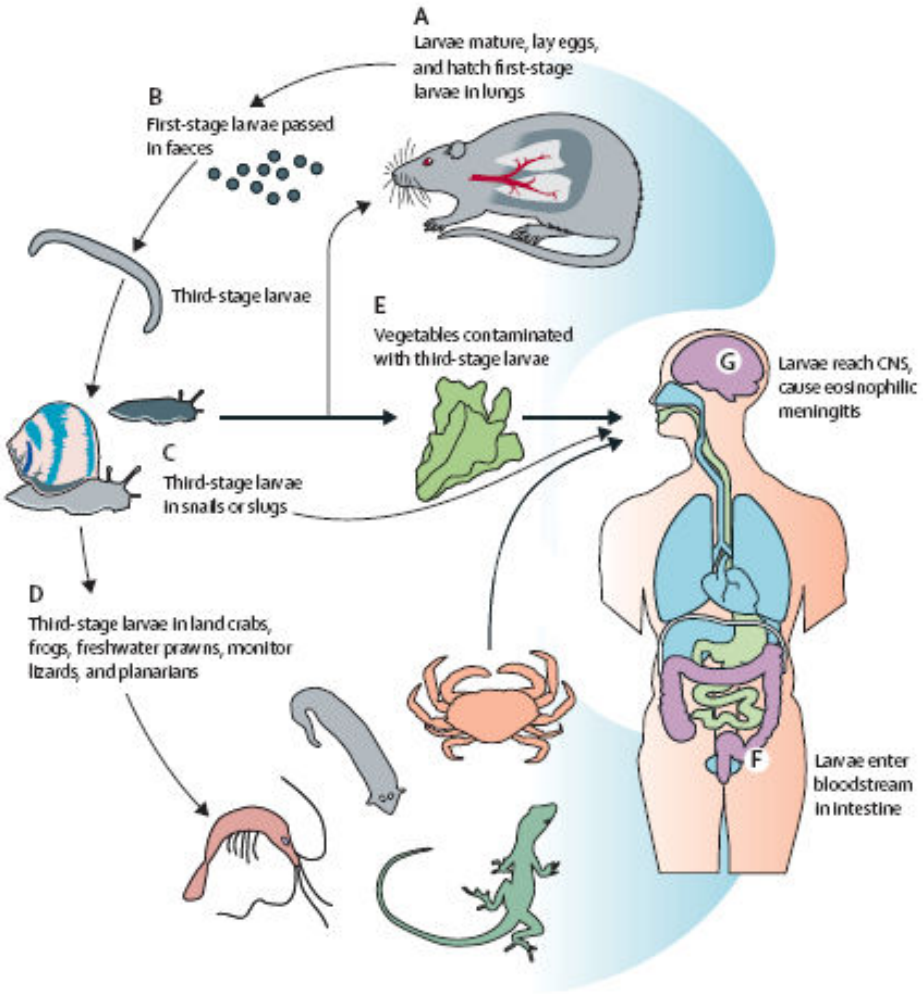

Figure 2 The life cycle of *Angiostrongylus cantonensis* (Wang et al., 2008)

Table 2 Rodent species that served as natural definitive hosts of *Angiostrongylus cantonensis*.

Rodents	Locality reported	Reporter/year
Bandicota indica	India, Taiwan, Thailand	Kuntz and Myers/1964, Parmeter and Chowdhury/1966, Pipitgool et al.,/1997
B. malabarica	Ceylon	Alicata/1996
Melomys littoralis	Australia	Mackerras/1985
Rattus annandalei	Malaya	Lim et al.,/1965
R. argentiventer	Malaya	Lim et al.,/1965
R. assimilis	Australia	Mackerras/1985
R. bowersi	Malaya	Lim et al.,/1965
R. conatus	Australia	Mackerras/1985
R. coxinga	Taiwan	Kuntz and Myers/1964
R. exulans	Malaya, Melanesia, Micronesia, Polynesia	Alicata/1963, Jackson/1962, Lim et al.,/1965, Wallace and Rosen/1965
R. jalorensis	Malaya, Sumatra	Kwo and Kwo/1968, Lim et al.,/1965
R. losea	Taiwan	Kuntz and Myers/1964
R. muelleri	Malaya	Lim et al.,/1965
R. norvegicus	Australia, Ceylon, China, Japan, Malaya, Mauritius, Melanesia, Micronesia, the Philippines, Polynesia, Ryukyu	Alicata/1965, Alicata/1966, Chen/1933, Lim et al.,/1965, Mackerras and Sandars/1955, Nishimura/1966, Nishimura and Yogore/1965, Wallace and Rosen/1965
R. rattus	Australia, China, Madagascar, Malaya, Mauritius, Melanesia, Micronesia, Polynesia,	Alicata/1963, Alicata/1966, Chen/1933, Harinasuta/1965, Jackson/1962, Kuntz and Myers/1964, Mackerras/1985,
R. rattus diardi	Sarawak, Taiwan, Thailand Malaya, Sumatra	Mackerras and Sandars/1955, Wallace and Rosen/1965 Kwo and Kwo/1968, Lim et al.,/1965

Table 3 Natural intermediate hosts of *Angiostrongylus cantonensis*.

Mollusks	**Locality**	**Reporter/Year**
Terrestrial snails		
Achatina fulica	Pacific islands, Malaya, Thailand	Alicata/1962, Chang et al.,/1968, Chiu et al.,/1968, Harinasuta et al.,/1965, Lim et al.,/1965
Bradybaena similaris	Pacific islands	Alicata/1962
Macrochlamys resplendens	Malaya	Lim et al.,/1965
Opeas javanicum	Pacific islands	Alicata/1965
Pupina complanata	Pacific islands	Alicata/1965
Subulina octona	Pacific islands	Alicata/1962
Aquatic snails		
Bellamya ingallsiana	Malaya	Lim et al.,/1965
Cipangopaludina chinensis	Taiwan	Chang et al.,/1968
Indoplanorbis exustus	Malaya	Lim et al.,/1965
Pila ampullacea	Thailand	Punyagupa/1965
P. gracilis	Thailand	Harinasuta et al.,/1965
P. polita	Thailand	Harinasuta et al.,/1965
P. scutata	Malaya	Lim et al.,/1965
P. turbinis	Thailand	Harinasuta et al.,/1965
Sinotiana martensiana	Thailand	Harinasuta et al.,/1965
Terestrial slugs		
Deroceras laeve	Pacific islands	Alicata and Brown/1962, Alicata and McCarthy/1964
Girasia peguensis	Malaya	Lim et al.,/1965
Microparmarion malayanus	Malaya	Lim et al.,/1965
Deroceras laeve	Pacific islands	Alicata and Brown/1962, Alicata and McCarthy/1964
Veronicella alte	Pacific islands, Malaya	Alicata/1965, Lim et al.,/1965

Table 4 Experimental intermediate hosts of *Angiostrongylus cantonensis.*

Mollusks	Reporter/Year
Terrestrial snails	
Allopeas kyotoensis	Yanagisawa/1967
Euglandina rosea	Ash/1962
Euhadra peliomphala	Yanagisawa/1967
E. quaesita	Yanagisawa/1967
Fruticicola despecta sieboldiana	Yanagisawa/1967
Helicina orbiculata	Ash/1962
Mesodon thyroidus	Ash/1962
Zonitoides arboreus	Yanagisawa/1967
Aquatic snails	
Biomphalaria glabata	Richards and Merritt/1967
B. heliophila	Richards and Merritt/1967
B. obstructa	Richards and Merritt/1967
B. pallida	Richards and Merritt/1967
B. pfeiferi	Richards and Merritt/1967
B. straminea	Richards and Merritt/1967
B. temagophila	Richards and Merritt/1967
Bulinus forskalii	Richards and Merritt/1967
B. globosus	Richards and Merritt/1967
B. senegalensis	Richards and Merritt/1967
B. tropicus	Richards and Merritt/1967
Drepanotrema simmonsi	Richards and Merritt/1967
Ferrissia tenuis	Richards and Merritt/1967
Galba viridis	Weinstein et al.,/1963
Helisoma sp.	Richards and Merritt/1967
Lymnea swinhoe	Chang et al.,/1968
L. volutata	Richards and Merritt/1967
Marisa cornuarietis	Richards and Merritt/1967
Physa sp.	Richards and Merritt/1967
Physa acuta	Yanagisawa/1962
Pila angelica	Harinasuta et al.,/1965
Plesiophysa lubendicki	Richards and Merritt/1967
Segmentina hemisphaerula	Chang et al.,/1968
Sinotaia quadrata	Chang et al.,/1968
Terrestrial slugs	
Deroceras reticulatum	Weinstein et al.,/1963
Limax flavus	Ash/1962
L. maximus	Weinstein et al.,/1963

Prevalence of *Angiostrongylus cantonensis* in host animals

A. cantonensis has been found in both intermediate and definitive hosts in various areas of the world. In Asian countries, the environment is suitable for development of its hosts including rats, snails and slugs. The high susceptibility of its hosts is considered to be an important factor in the widespread distribution *A. cantonensis* throughout these areas.

In Thailand, Harinasuta et al. (1965) reported the infection rate of *A. cantonensis* in edible snails from Bangkok and several provinces in northeast Thailand to be 90.5% of *Achatina fulica*, 0.5% of *Pila polita*, 20% of *P. turbinnis*, 21.7% of *P. gracilis* and 3.4% of *Sinotiana martensiana*. Crook et al. (1967) reported that the prevalence of *A. cantonensis* in rats collected from seven areas of the country was 2.9%. They also suggested that *Pila ampullaceal* could be the intermediate host of this parasite. In 1968, a survey of natural infections of *A. cantonensis* in rodents and molluscan intermediate hosts from various parts of Thailand, indicated the incidence of infection among *Bandicota indica*, *B. bengalensis*, *Rattus rattus*, *R. norvegicus*, and *R. berdmorei* were 8.58%, 3.87%, 1.43%, 2.03% and 2.32%, respectively, and for the intermediate hosts *A. fulica*, *Pila turbinnis*, *P. gracilis*, *P. angelica*, *P. polita*, *P. scutata* and *Sinotaia martensiana* infection rates were 94.4%, 5.71%, 4.13%, 1.1%, 2.29%, 1.99% and 1.51%, respectively (Setasubun et al., 1968). The prevalence of angiostrongyliasis in rats (*Rattus* spp.) from northern Chiang Mai was 42.1% (Namue and Wongsawad, 1997), while infections of this parasite in rats, *Rattus norvegicus* and *Bandicota indica,* were 3.8% and 1.4% , respectively, from another five provinces (Ubon Ratchathani, Udon Thani, Kalasin, Chaiyaphum and Khon Kaen) in northeastern Thailand (Pipitgool et al., 1997). Pipitgool et al. (1997) also recorded that the prevalence of this parasite in *Pila polita* and *Achatina fulica* to be 0.9 and 36.4%, respectively, whereas the *P. ampullacea* was negative for larval infection. The yellow tree monitors (95.5%) collected from five provinces of the country, namely Lumpang, Phitsanulok, Kamphaeng Phet, Tak and Prachin Buri, also were found infected with *A. cantonensis* (Radomyos et al., 1994). Recently, 12.38% of naturally infected *A. fulica* collected from Phitsanulok were demonstrated (Vitta et al., 2011).

In Indonesia, Kwo and Kwo (1968) demonstrated that five out of 13 *Ruttus tiomanicus jalorensis* were naturally infected with adult *A. cantonensis* in northern Sumatra. Ten of 18 *Rattus rattus diardi* examined also carried this parasite. In molluscan hosts, *Achatina fulica* and *Pila scutata* are also recorded to harbor this parasite (Margono, 1970). In a survey of rats and molluscs in Jakarta and suburbs, *A. cantonensis* is found in 2.4% of *R. rattus diardi*, 21.4% of

R. argentiventer and 18.1% of *R. norgegicus*, while infection rates in *A. fulica*, *Laevicaulis alte* and *Pila scutatu* were 35.1%, 35.5% and 5.9%, respectively (Margono and Ilahude, 1974). *Rattus rattus diardi* is also found to be the host of this parasite in Bogor, western Java, (Wiroreno, 1975, 1978). In 1976, Stafford et al. reported that *Bandicota indica setifera* was considered to be a natural definitive host in Ancol, Jakarta. In their study of the geographic and host occurrence in Indonesia, Carney et al. (1978) reported that western and southern Sumatra, Lampung, western and central Java, northern Sulawesi and eastern Nusa Tenggara were the endemic areas of *A. cantonensis*. In Japan, from a study in Ryukyu Islands, namely Okinawa, Miyako-jima, Ishigaki-jima and Iriomte-jima, Nishimura (1966a) reported that 15.8% of rats on average from all 4 islands were infected with *A. cantonensis*, with *Laevicaulis alte* and *Achatina fulica* serving as natural intermediate hosts of the parasite. In 1978, Noda et al. also reported infection rates for *A. cantonensis* in *Rattus norvegicus* of 19.2% and 23.1% in Kogoshima New Port and Port for Lumber, Kagoshima prefecture, respectively. *Limax marginatus* serves as the intermediate host in these areas (Noda et al., 1978). In 2004, a survey of *A. cantonensis* infections of intermediate hosts in Okinawa, it was found that *Achatina fulica*, *Parmarion martensi*, *Veronicella alte*, *Limax ralentians*, *Platyademus manokwari* and *Bufo asiaticus* had prevalences of 10.1%, 20.3%, 13.8%, 3.8%, 14.1% and 5.6%, respectively (Asato et al., 2004). Toads and frogs in Okinawa also were found to be the paratenic hosts of *A. cantonensis* (Asato et al., 1978). Many species of snails, *Zonitoides arboreus*, *Allopeas kyotoensis*, *Frutcicola despecta sieboldiana*, *Euhadra peliomphala*, *Euhadra quaesita* and *Physa acuta*, were found to be experimentally infected with this parasite (Yanagisawa, 1967).

In Kuala Lumpur, Malaysia, the natural hosts of *A. cantonensis* include 7 species of rats (*R. jalorensis*, *R. argentiventer*, *R. exulans*, *R. norvegicus*, *R. rattus diardi*, *R. muelleri* and *R. bowersi*), 3 species of land slugs (*Microparmarion malayanus*, *Girasia peguensis* and *Laevicaulis alte*), 2 species of land snails (*Macrochlamys resplendens* and *Achatina fulica*) and 3 species of freshwater snails (*Pila scutata*, *Bellamya ingallsiana* and *Indoplanorbis exustus*) (Liat et al., 1965). In Sarawak, *R. rattus diardi*, *R. jalorensis* and *R. exulans* were also found infected with this parasite (Lim, 1967). Lim (1970a) reported the infection of *A. cantonensis* in various rodent species: 5 of 109 *R. rattus diardii*, 11 of 76 *R. tiomanicus jalorensis* and 3 of 8 *R. argentiventer*. In this survey several natural intermediate hosts, e.g., land mollusks (*Achatina fulica*, *Macrochlamys resplendens*, *Microparmarion malayanus* and *Laevicaulis alte*) and aquatic snails (*Pila scutata*) were identified. The infection rates of *A. cantonensis* in *Achatina fulica* collected around Kuala Lumpur and the rural villages of Bukit, Tinggi, Pahang state were 26.5%

and 40%, respectively (Bisseru and Verghese, 1970). *Rattus tiomanicus* is one of the definitive hosts of *A. cantonensis* in the East Coast of Peninsular Malasia (Liat, 1975).

In Vietnam, three of 183 *R. norvegicus* and 5 of 93 *R. exulans* collected from the greater Saigon area were found naturally infected with *A. cantonensis* (Nhuan and Hendricks, 1974), while in Manila, the Philippines, and two out of 51 *R. norvegicus* harbored infections (Nishimura and Yogore, 1965).

In Taiwan, snails, *Cipangopaludina chinensis* were found to be a natural intermediate host for *A. cantonensis* and were thought to be the source of infection in a case of eosinophilic meningitis in a Taiwanese child (Chang et al., 1968). In rodent hosts, 99 of 1,650 rats collected from Taiwan and offshore islands were infected with *A. cantonensis*. Of these, *Bandicota indica nemorivaga*, *R. coxinga coxinga*, *R. losea*, *R. norvegicus*, *R. rattus mindanensis* and *R. rattus* subsp. served as final hosts (Kuntz and Myers, 1964).

In India, the highest intensity of *A. cantonensis* in the land mollusk, *Laevicaulis alte*, was observed in the rainy season (June-November) (Mahajan et al., 1992).

In Haiti, although angiostrongyliasis in human has not been reported, *R. norvegicus* and *R. rattus* are naturally infected with this parasite. This indicated that *A. cantonensis* should be considered to be a potentially new public health problem in the country (Raccurt et al., 2003).

In the Pacific islands, twenty-three of 43 frogs (*Hyla aurea*) in Noumea, New Caledonia were found to be naturally infected with infective-stage larvae of *A. cantonensis*. Sea snakes (*Laticauda colubrina*) can be experimentally infected with this parasite in New Caledonia (Ash, 1968). These findings showed that frogs and sea snakes could be considered as paratenic hosts of *A. cantonensis* in this location. In Papua New Guinea, the infection rate of the parasite in rats ranges from 12.7% at Port Moresby to 32.4% at Kimbe in West New Britain. One-hundred of 847 snails (*A. fulica*) collected from several areas of the country also were found to be infected with *A. cantonensis* (Scrimgeour, 1984).

The natural infection also have been reported from Louisiana wildlife, namely lemur (*Varencia variegate rubra*), wood rat (*Neotoma floridanus*) and opossums (*Didelphis virginiana*) (Kim et al., 2002), although to date no human infections have been recorded.

In experimental studies, *A. cantonensis* has been shown to infect monkeys such as *Maccacus rhesus* (Alicata, 1963; Weinstein et al., 1963) and *Macaca fascialaris* (Lim, 1970b), the mongoose such as *Herpestes urva* (Wood, 1965) and Malaysian mongoose (Liat, 1970), the tree-shrew *Tupaia glis* (Lim, 1970b) and slow loris *Nycticebus coucang*.

Routes of infection by *Angiostrongylus cantonensis*

It is known that the main causative agent of angiostrongyliasis or helminthic eosinophilic meningitis or meningoencephalitis in man is *A. cantonensis*. Man is an accidental host and can be infected with this parasite in the following ways:

(1) Ingestion of raw or improperly cooked snails or slugs, which served as the intermediate hosts containing the infective third-stage larvae.

(2) Ingestion of raw or improperly cooked crustaceans or yellow tree monitors, which served as the paratenic hosts harboring the infective third-stage larvae.

(3) Ingestion of raw vegetable contaminated with the infective third-stage larvae.

However, the routes of infection in man vary by the eating habits characterizing different regions of the world. In Asian countries, the most common route of the infection is by eating raw snails (Hongladarom and Indarakoses, 1966; Punyagupta, 1965; Punyagupta et al., 1975; Bhaibulaya, 1979; Tsai et al., 2001), while in Tahiti and other Pacific islands; infections are mainly acquired by eating raw freshwater prawns and terrestrial crabs (Yii et al., 1975). In some cases the sources of the infection are difficult to determine. These might be infection through the accidental eating of intermediate hosts or paratenic hosts or eating of infective third-stage larvae in their mucus contaminating salad vegetables (Slom et al., 2002). Recently, eating of raw frogs (*Rana plancyi*) has been reported as the infectious source of human angiostrongyliasis in Taiwan (Lai et al., 2007).

Epidemiology of Angiostrongyliasis

Epidemiology of the disease depends on the relationship between the parasites, hosts and environments. The majority of human infections have been reported for the most part in the tropical region, which lies between latitude 23º N and latitude 23º S (Figure 3). This area is characterized by mild climate and moderate to heavy rainfall. These factors favor the propagation and maintenance of rodents, mollusks and certain paratenic hosts, thus providing suitable conditions for the transmission or spread to the human population. Angiostrongyliasis and eosinophilic meningitis or meningoencephalitis are reported from all over the world. The most common endemic areas are Asia and Pacific Islands. Table 5 shows the cases of human angiostrongyliasis reported by countries and regions.

In the early 2000s, three outbreaks of eosinophilic meningitis due to *A. cantonensis* were reported from 3 countries. The first outbreak occurred in Kaosiung, Taiwan involving 17

Thai laborers who eat raw golden apple snails (Tsai et al., 2001). In the second outbreak, 12 American travelers returning from the Caribbean were found to be infected by eating Caesar salad contaminated with the infective stages of *A. cantonensis* (Slom et al., 2002). In addition, a case of this disease in a Swiss traveler returning from Cuba also has been recorded (Bartschi et al., 2004). This is a reminder that travelers heading to the endemic areas, such as the Caribbean, Southeast Asia and the Pacific islands, should be warned to avoid eating unwashed products and undercooked mollusks and paratenic hosts. The latest outbreak occurred in China involving 55 cases from Zhejiang and Fujian (Chen et al., 2005).

In Thailand, from 1992 to 2001, more than 5,000 cases of helminthic eosinophilic meningitis or meningoencephalitis were reported by Division of Epidemiology, Ministry of Public Health, Thailand, 2001. Most of them were from the northeastern part of the country. Recently, 231 cases of this disease were also reported. Of these, 121, 27, 18, 15 and 14 cases were reported from Loei, Chaiyaphum, Udontanee, Kalasin and Burirum, respectively. Most cases occurred in adults and were more prevalent among male (Division of Epidemiology, Ministry of Public Health, Thailand, 2004).

Clinical manifestation of Angiostrongyliasis

The severity of the disease depends on the number of worms present, their location in the body and the tolerance of the patient. Clinical manifestations of eosinophilic meningitis or meningoencephalitis, which have been reported by several authors, are shown in Table 6. The most common symptoms of the infection are severe headache, nausea, vomiting, neck stiffness, seizures, and neurological abnormalities. Clinical manifestations of ocular angiostrongyliasis include redness, pain, diminished vision (Malhotra et al., 2005), loss of vision accompanied by feeling of irritation (Ketsuwan and Pradatsundarasar, 1966) and watering of the eye (Prommindaroj et al., 1962).

Table 5 Number and prevalence of cases of human angiostrongyliasis reported in countries or regions (Wang et al., 2008).

	Cases (%)
Thailand	1337 (47·33)
China (including Taiwan and Hong Kong)	769 (27·22)
Tahiti, French Polynesia	256 (9·06)
USA	116 (4·11)
Cuba	114 (4·04)
New Caledonia	72 (2·55)
Japan	63 (2·23)
Australia	24 (0·85)
Vanuatu	19 (0·67)
India	10 (0·35)
Vietnam	8 (0·28)
Malaysia	6 (0·21)
Mayotte	6 (0·21)
Réunion island, France	4 (0·14)
Egypt	3 (0·11)
Sri Lanka	3 (0·11)
Cambodia	2 (0·07)
Samoa	2 (0·07)
Fiji	2 (0·07)
Belgium	1 (0·04)
Costa Rica	1 (0·04)
Germany	1 (0·04)
Indonesia	1 (0·04)
Jamaica	1 (0·04)
Italy	1 (0·04)
Côte d'Ivoire	1 (0·04)
New Zealand	1 (0·04)
Papua New Guinea	1 (0·04)
Switzerland	1 (0·04)
UK	1 (0·04)
Total	2827

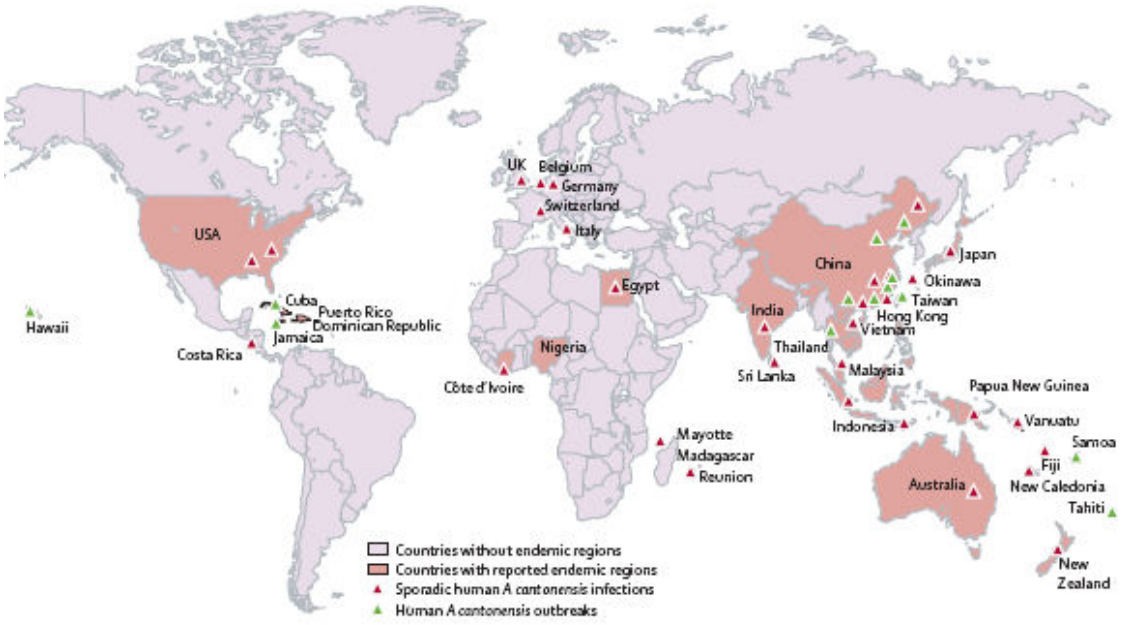

Figure 3 Geographic distributions of human angiostrongyliasis in tropical and subtropical regions (Wang et al., 2008).

Table 6 Symptoms of patients with eosinophilic meningitis or meningoencephalitis.

Symptoms	Percent of patients				
	Yii, 1976 (125 cases)	Tsai et al., 2001 (17 cases)	Slom et al., 2002 (12 cases)	Chen et al., 2005	
				Outbreak 1 (47 cases)	Outbreak 2 (8 cases)
Headache	86	100	100	93.6	100
Nausea or Vomiting	83	24 or 24	or 67	or 19.1	or 100
Sominolence or Lethargy	82	-	-	8.5	87.5
Blurred vision or Diplopia	10	12	92	-	-
Fever	80	65	42	-	-
Constipation	76	-	-	-	-
Malaise	71	-	-	-	-
Anorexia	64	-	-	-	-
Cough	54	-	-	-	-
Subjective neck stiffness	40	47	83	-	75
Abdominal pain	34	18	-	-	-
Paresthesia	28	12	50	63.8	37.5
Weakness of extremity	17	-	-	-	-
Muscle twitching	13	-	-	-	-
Strabismus	10	-	-	-	-
Sneezing	10	-	-	-	-
Coma	10	-	-	-	-
Irritation	8	-	-	-	-
Paralysis of extremity	6	-	-	-	-
Urinary incontinence or intention	6	-	-	-	-
Rhinorrhea	5	-	-	-	-
Diarrhea	-	-	17	-	-
Profuse salivation	4	-	-	-	-
Convulsion	3	-	-	-	-
Muscle weakness		47	37	-	-
Orbital/retro-orbital pain	-	41	-	-	-
Ataxia	-	6	-	-	-
Skin rash	-	24	-	-	-
Hyperesthesia	-	-	75	-	-
Fatigue	-	-	83	14.9	87.5
Muscle pain	-	-	50	91.5	100
Skin eruption	-	-	-	21.3	-
Skin itch	-	-	-	27.7	-

Pathogenesis and pathology of Angiostrongyliasis

The pathogenesis of human angiostrongyliasis is associated with the migration of the infective-stage larvae and the resulting host inflammatory response; however, most of the evidence comes from studies conducted in experimental animals (Hung and Chen, 1988; Cross, 1994). The larvae invade the gastric mucosa, pass to the blood vessels, and are carried to the liver. This may cause gastroenteritis and hepatomegaly. The larvae leave the liver and are carried to the right heart, then to the lung, from which they reenter the blood through the walls of the pulmonary veins. In rats, the larvae are carried directly to the central nervous system (CNS) or to skeletal muscles where they destroy muscle fibers, find a small nerve and then travel to the CNS. These observations in animals explain myalgia, pain and paresthesia in human. In the CNS, the larvae invade neural tissue; some die, elicit inflammatory responses and become surrounded by granulomatous reactions. Others survive, emerge onto the surface of the brain, and become preadults (Cross, 1994).

In human, the presence of the worm on the brain surface produces an inflammatory reaction. Pathological changes can be seen both in the brain and the spinal cord. There are infiltrations of the meninges and around intracerebral vessels by varying proportions of lymphocytes, plasma cells and eosinophlis. Numerous tracks and microcavities can be observed both in the brain and the spinal cord. Fourth or fifth stage larvae of *Angiostrongylus*, alive or dead, are often found in the meninges and brain tissue (Sonakul, 1978). Dead parasites are often found within granulomas (Tangchai et al., 1967). Worms may migrate into the eye chamber from the brain through spaces between the optic nerve and its sheath. Larvae in the spinal cord are rarely seen (Kliks et al., 1982).

At autopsy in humans, the brain is congested and there is inflammation of the leptomeninges. The inflammation reaction consists of leukocytes, including eosinophils, and plasma cells. Isolated giant cells may be present throughout the subarachnoid spaces. Dead worms and fragments of worms may be found in the brain and spinal cord surface. Tracts may contain debris, glitter cells and Charcot-Leyden crystals (Tangchai et al., 1967).

Diagnosis of Angiostrongyliasis

Specific diagnosis can be made by findings the worm at the site of infection, but this only occurs in some patients. A past history of eating raw or improperly cooked snails, slugs, prawns, crabs, frogs and yellow tree monitors or eating raw vegetables contaminated with

infective third-stage larvae may aid in diagnosing this disease. So far, many methods have been reported for diagnosis, recovery of the worm from infected organs (Punyagupta, 1979; Jaroonvesama et al., 1985 Mehta et al., 2006), laboratory findings (Punyagupta, 1979; Bhaibulaya, 1991; Sagaya et al., 1997), Computerized tomography scans (Weller and Liu, 1993), Magnetic resonance imaging scans (Tsai et al., 2001; Podwall et al. 2004; Jin et al., 2005), Complement fixation test (Anderson et al. 1962), Indirect hemagglutination test (Tungkanak et al., 1972; Kamiya et al. 1973; Sato and Otsuru, 1983; Chen 1975), Immunoelectrophoresis test (Bouthemy et al., 1972; Kamiya, 1975; Tharavanij, 1979 Sato and Otsuru 1983), Enzyme-linked fluorescent assay (Shih and Chen, 1991), Enzyme-linked immunosorbent assay (Cross 1978; Cross and Chi 1982; Jaroonvesama et al. 1985; Chen 1986; Nuamtanong, 1996; Dekumyoy et al., 2000; Intapan et al. 2002; Yen and Chen 1991; Chye et al. 2000; Eamsobhana et al., 1995; Eamsobhana et al., 1997; Chye et al., 1997; Eamsobhana et al., 2006), and immunoblot (Akao et al., 1992; Nuamtanong 1996; Maleewong et al. 2001; Intapan et al. 2003; Eamsobhana et al., 2004; Vitta et al., 2010)

Treatment of Angiostrongyliasis

To date, there are no specific treatment for angiostrongylasis and its accompanying eosinophlic meningitis or meningoencephalitis (Sawanyawisuth and Sawanyawisuth, 2008), although there are several reports of trial treatments for this disease. Several *in vitro* and *in vivo* animal studies have been conducted to determine the effects of various drugs on both larval and adult stages of this parasite. For example reduction of larval and adult stages of infected rats treated with albendazole was observed by Lakwo et al. (1998), while good candidates for treatments of human angiostrongyliasis have been suggested including PF1022A, pyrantel and flubendazole (Akyol et al., 1993; Mentz and Graeff-Teixeira, 2003).

In clinical angiostrongyliasis, Punyagupta et al. (1975) found no difference in the duration or severity of illness in patients treated with analgesics alone, analgesics and glucocorticosteroids, or analgesics and antibiotics. Treatment with prednisolone at 60 mg/day for 14 consecutive days demonstrated good efficacy in the treatment of this disease (Chotmongkol et al., 2000). In ocular angiostrongyliasis, effective treatment can be attained by elimination of the worm from eyeballs (Mehta et al., 2006). In addition, repeated lumbar punctures can also used to decrease intracranial pressure of patients (Sawanyawisuth et al., 2006).

Prevention and Control of Angiostrongylus cantonensis

Angiostrongylasis, a food-born parasitic zoonosis, is difficult to control in nature. Control of the natural definitive hosts such as rats and natural intermediate hosts such as snails, may be possible in some areas. However, because changing of human eating habits is often difficult, preventing the consumption of paratenic hosts such as prawns, shrimps and crabs is not an easy task in control programs. Prevention should include elimination of the mucus containing active third-stage larvae on vegetables through thorough washing, and of course, the best prevention of the infection is proper cooking of foods before eating.

Molecular aspects of *Angiostrongylus cantonensis*

Molecular genetic research on parasite has had a major impact on many fundamental and applied areas of medical and veterinary parasitology. Gene technology has been led to some progresses for this group of parasites, particularly in studying parasite systematic, drug resistance and population genetics, and in the development of diagnostic assays and the characterization of potential vaccine and drug targets (Mulhardt, 2007).

Only very few studies on the molecular aspects of *A. cantonensis* have been conducted; therefore little information on the molecular biology of the parasite is available. Joshua and Hsieh (1995) studied stage-specifically expressed genes of *A. cantonensis* by using differential display. Five stages, including first-stage larvae, third-stage larvae, fifth-stage larvae, immature adult worms and adult worms were used in this study. Total RNA of these specimens was extracted followed by reverse transcrition and subsequent amplification of cDNAs by polymerase chain reaction (PCR) using pairwise combinations of primers (3′ primer-T_{12} MG and 5′ primer-GCAAGGAGTC and 3′ primer-T_{12} MC and 5′ primer-CGTGGCAATA), and then electrophoresis in a 6% acrylamide urea sequencing gel. Twenty-nine cDNA bands were cut from gel, purified and re-amplified by using the same primer sets and confirmed by dot blot and Northern blot analyses. The results showed 14 cDNA bands were over expressed or specifically expressed in RNA from specific parasitic stages. DNA were cloned, sequenced, and blasted against the GenBank and EMBL databases of cDNA sequences. They found that the sequences from clones of immature-stage and third stage larvae showed 65% similarity with the *Caenorhabditis elegans* leucine aminopeptidase (LAP) gene and 42% and 85% identity, respectively. The homology to *C. elegans* LAP is interesting because LAP is thought to be involved in degrading the cuticle after molting, and might be a target for therapeutic or

prophylactic intervention. Furthermore, a sequence from a fifth stage larval clone showed 52% nucleotide and 60% amino-acid similarity to a *C. elegans* serine-threonine kinase gene, whereas the other cDNAs of developmentally expressed genes showed no clear similarity to previously identified sequences. Bessarab and Joshua (1997) also reported the characterization of a gene designated Ac-fmp-1 expressed only in adult worms. Full length cDNA of 1.5 kb terminated at the 5′ end with the conserved nematode spliced leader (SL) sequence and contains one open reading frame coding for a putative protein of 417 amino-acids. The recombinant protein expressed from this open reading frame was shown to be antigenic in the infected host and polyclonal antibodies raised against the recombinant protein recognized a 66 kDa protein present only in adult female worms. This protein localized to the muscle cell membranes adjacent to the pseudocoelom.

The PCR-restriction fragment length polymorphism (PCR-RFLP) method was used for differentiation of DNA profiles in the rDNA second internal transcribed spacer (ITS2) and mtDNA cytochrome oxidase I (COI) regions of *A. cantonensis*, *A. costaricensis* and *A. vasorum* (Caldeira et al., 2003). The PCR systems used the primer LCO (forward; 5′-GGCTAACAAATCATAAAGATATTGG-3′) and primer HCO (reverse; 5′-TAAACTTCAGGGTCACCAAAAATCA-3′) for amplification of COI region of mtDNA and primer NC1 (forward; 5′-ACGTCTGGTTCAGGGTTGTT-3′) and primer NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCTCCGCT-3′) for amplification of ITS2 of rDNA of the samples. The PCR products were digested with restriction enzymes, *Rsa*I and *Cla*I, whose fragments demonstrated the most discriminating profiles for differentiation of the COI regions of mtDNA and ITS2 region of rDNA, respectively.

CHAPTER III

MATERIALS AND METHODS

Study design and study sites

A cross sectional study was conducted in several regions of Thailand between 2012 and 2014.

Collection of intermediate hosts

Land snails (Figure 4) and fresh water snails were randomly collected from several regions of Thailand. The snails were transported and maintained in plastic cage in Department of Microbiology & Parasitology, Faulty of Medical Science, Naresuan University, Phitsanulok. They were feed with lettuce leaf.

Figure 4 *Cryptozona siamensis* collected from Kamphaeng Phet province

Artificial digestion and Bearmann'techniques

Figure 5 show the artificial digestion methods and Bearmann'techniques. Intermediate host snails were artificially digested using 1% pepsis solution. The snails were sacrificed by chopping them up into small pieces and mixing the tissue with 1% acid pepsin solution, then incubating in a 37ºC water bath for 1 hour. The Bearmann's apparatus were made up from a grass funnel connected to a short piece of rubber tubing at the outlet. Several layers of gauze were laid over a wire screen positioned at the bottom surface of the funnel. With the outlet closed, tap water was poured into the funnel until the fluid level touched the wire screen. The digested juice was placed on the gauze surface and the apparatus was left standing for 30 minutes to allow larvae migrate into the surrounding fluid, and then into the rubber tube. At the end of this period, the fluid containing mainly enriched L3 larvae were released into a Petri dish. More tap water was added to the funnel and the same procedure was repeated if more larvae were needed from the same batch of digested juice. Following collection the L3 larvae were ready to use for further experiments. They were keep at -20 $^{\circ}$C.

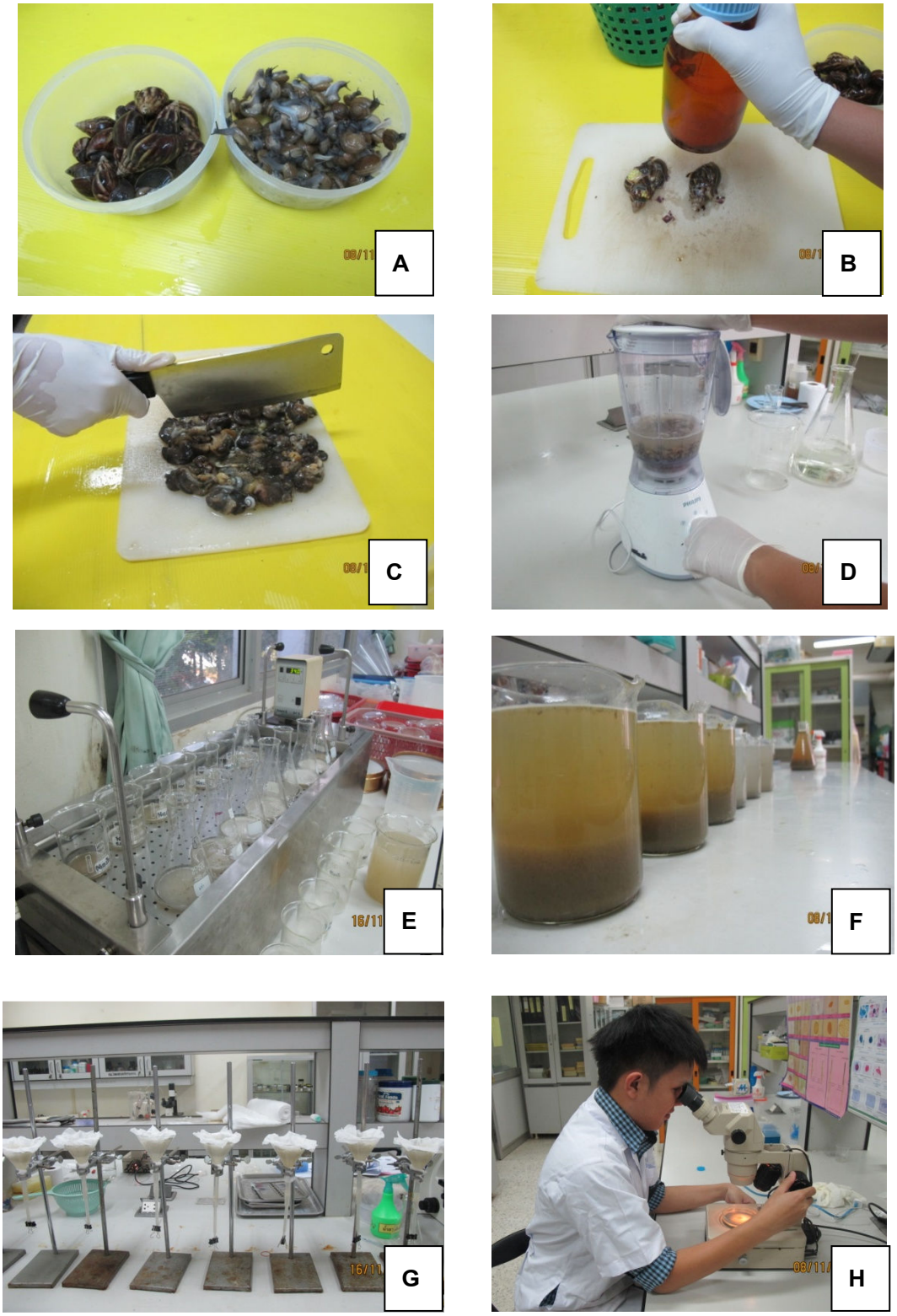

Figure 5 Artificial digestion methods and Bearmann'techniques: Clean, Identification and separation of snail species (A), Shell of snails were removed (B), Snails were chopped into small pieces (C), Snails were blended and mixed with 1% pepsin solution (D), The mixtures were incubated in water bath at 37ºC (E), The solution was sedimented with 0.85% NaCl (F), Bearmann'technique was used for isolating larvae (G), Collection of larvae under stereomicroscopic (H).

DNA Extraction from 3rd stage larvae of *Angiostrongylus*

Genomic DNA of *Angiostrongylus* was extracted from 3rd stage larvae. Genomic DNA from worms were extracted by a modified method from Homonick, et al., (1997). Each specimen were put in a 1.5 ml microcentrifuge tube with 200 µl of ATL tissue lysis buffer. The worms were ground with a 200 µl sterile tip. Twenty microliters of proteinase K (10 mg/ml) were added to the tube. The lysate was frozen at -80°C for 10 min to lyse the cells completely. After freezing, the tube was incubated at 65°C for 1 h and 95°C for 10 min to digest the proteins, and inactivate proteinase K, respectively. Subsequently, the tube was placed on ice and the lysate was centrifuged at 12,000 rpm for 2 min. The supernatant containing DNA was collected. Five microliters of purified DNA was analyzed by 0.8% agarose gel electrophoresis in 0.5X TBE buffer at 80V. After completion, the gel was stained with ethidium bromide (10 µg/ml) for 1 min and destained with the distilled water for 30 min. The DNA band was visualized and photographed under UV light and products were compared to 100 bp molecular size marker. The concentration of purified DNA was measured by spectrophotometer. The DNA solution was kept at -20°C for further experiment.

PCR amplification

Polymerase Chain Reaction (PCR) was performed following the previous describe; primers; CO1_F 5' TAAAGAAAGAACATAATGAAAATG 3' and CO1_R 5' TTTTTTGGGCATCCTGAGGTTTAT 3' for amplifying a partial regions of COI gene (Bowles et al., 1993; Hu et al., 2002; Jefferies et al., 2009). The reaction was made up in 10 µl volumes which was consisted of 1 µl of 10X buffer, 1.4 µl of 25 mM $MgCl_2$, 0.2 µl of 200 mM dNTPs, 0.4 µl of 5 µM of each Primer, 0.2 µl of 1 unit Taq DNA Polymerase, 2.5 µl of DNA template and 3.9 µl of distilled water. PCR was performed using a Thermal Cycler with an initial denature step of 94°C for 5 min., followed by 30 cycles of denaturation of 94°C for 1 min., annealing temperature of 55°C for 30 sec., and extension of 72°C for 1 min. and a final extension of 72°C for 7 min. One microliter of PCR products was analyzed by 1.2% agarose gel electrophoresis in 0.5X TBE buffer at 100V. After completion, the gel was stained with ethidium bromide (10 µg/ml) for 1 min and destained with distilled water 30 min. The DNA band was visualized and photographed under UV light. The products size will be compared to a 100 bp molecular size marker.

DNA sequencing

For sequencing, total volume of 30 µl of PCR reaction was performed. PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan). Briefly, PCR product (30 µl) was transferred into a 1.5 ml microcentrifuge tube. Five volumes of Gel/PCR buffer was added to the tube and vortex. The mixture was then transferred into a DFH column in a 2 ml collection tube and centrifuged at 14,000 g for 30 sec. followed by discarding the flow-through. The tube containing DFH column was centrifuged at 14,000 g for 3 min. to dry the column matrix. The DFH column was transferred to a new 1.5 microcentrifuge tube. Twenty µl of elution buffer was added into a center of the column matrix. The tube was allowed to stand for 2 min in order to completely absorb. To elute purified DNA, the tube was then centrifuged at 14,000 g for 2 min. Purified PCR product was checked by running 1.2% agarose gel electrophoresis in 0.5X TBE buffer at 100V and was compared to a 100 bp standard ladder. The gel were stained with ethidium bromide for 1 min, destained with distilled water for 30 min and visualized and photographed under UV light. Purified PCR product was sent to Korea for sequencing by Macrogen Inc. (Korea). Sequencing was performed by both direction.

Analysis of CO I sequences

Contiq assembly, chromatograms, sequence ambiguity resolution was visually checked using Lasergene software, Madison, WI, USA. Multiple sequences were aligned using Clustal W (Higgins et al., 1994). Phylogenetic distance trees were calculated using the Kimura two-parameter model (1980) and the Neighbor-Joining module (Saitou and Ney, 1987) of MEGA version 4.0 (Tamura et al., 2007).

CHAPTER IV

RESULTS

Survey of *Angiostrongylus* in intermediate hosts

A total of 14,032 fresh water and land snails were collected from 19 provinces including Chiang Mai, Phare, Lampang, Lamphun, Phitsanulok, Tak, Phichit, Sukhothai, Kamphaeng Phet, Uthai Thani, Bueng Kan, Chaiyaphum, Maha Sarakham, Nakhon Ratchasima, Phetchabun, Kalasin, Khon Kaen, Mukdahan and Nonthaburi (Table 6). Snails from 5 provinces including Phitsanulok, Kamphaeng Phet, Kalasin, Tak and Phetchabun were positive for *Angiostrongylus*. Six species of snails were collected e.g., *Filopaludina* sp., *Pomacea* sp., *Achatina fulica*, *Cryptozona siamensis*, *Megausatenia siamensis* and *Cyclophorus* sp (Figure 6).

Prevalence and intensity of *Angiostrongylus cantonensis* in intermediate hosts

Pooled snails collected from Phitsanulok, Kalasin, Tak and Phetchabun were articially digested with 0.3% pepsin solution. Low intensity of *A. cantonensis* in snails was demonstrated (Table 7)

Individual snail collected from Kamphaeng Phet was articially digested with 0.3% pepsin solution. A total of 2,228 fresh water and land snails were collected. It consisted of 1,119 *Filopaludina* spp., 409 *Pomacea caniculata*, 275 *Achatina fulica* and 425 *Cryptozona siamensis*. Table 8 and 9 show the prevalence and intensity of *Angiostrongylus cantonensis* 3rd stage larvae (Figure 7) in natural hosts. Low prevalence and intensity was observed in *Achatina fulica*. High prevalence and intensity was found in *Cryptozona siamensis* which was the first record of intermediate host for *A. cantonensis* in Thailand.

Table 6 No. of snail collected form 19 provinces of Thailand

Provinces	***Achatina fulica***	***Cryptozona siamensis***	***Pomacea* sp.**	***Filopaludina* sp.**	***Megausatenia siamensis***	***Cyclophorus* sp.**	**Total**
Chiang Mai	12	46	0	0	0	0	**58**
Phare	0	35	0	0	0	0	**35**
Lampang	10	89	0	0	0	0	**99**
Lamphun	8	42	0	0	0	0	**50**
Phitsanulok	138	745*	0	0	0	0	**883**
Phichit	0	343	0	0	0	0	**343**
Sukhothai	0	109	0	0	0	0	**109**
Kamphaeng Phet	275*	425*	409	1119	0	0	**2228**
Uthai Thani	0	0	0	128	0	0	**128**
Tak	425*	1411	136	0	936*	44	**2952**
Phetchabun	2087*	1673*	0	27	0	0	**3787**
Bueng Kan	2	0	9	17	0	0	**28**
Chaiyaphum	90	280	0	0	0	0	**370**
Maha Sarakham	0	0	137	270	0	0	**407**

Nakhon Ratchasima	8	0	0	0	0	0	**8**
Kalasin	555*	703*	84	22	0	67	**1431**
Mukdahan	101	750	0	0	0	0	**851**
Khon Kaen	11	32	42	35	0	0	**120**
Nonthaburi	0	0	3	142	0	0	**145**
Total	**3722**	**6683**	**820**	**1760**	**936**	**111**	**14032**

*Positive for *Angiostronylus* larvae

Figure 6 Mollusks collected from Thailand for searching of *Angiostrongylus* larvae; *Achatina fulica* (A), *Cyclophorus* sp. (B), *Megaustenia siamensis* (C), *Cryptozona siamensis* (D), *Filopaludina* sp., (E) and *Pomacea* sp. (F)

Table 7 Intensity of *A. cantonensis* in snails collected from Phitsanulok, Kalasin, Tak and Phetchabun Provinces.

Province	Snails	No. of snails collected	No. of larvae	Intensity
Phetchabun	*A. fulica*	2087	2263	1.08
	C. siamensis	1673	123	0.07
	Filopaludina sp.	27	0	0
Kalasin	*A. fulica*	555	920	1.65
	C. siamensis	703	54	0.07
	Cyclophorus sp.	67	0	0
	Filopaludina sp.	22	0	0
	Pomacea sp.	84	0	0
Tak	*A. fulica*	425	1841	4.33
	C. siamensis	1411	0	0
	Cyclophorus sp.	44	0	0
	M. siamensis	936	13	0.01
	Pomacea sp.	136	0	0
Phitsanulok	*A. fulica*	138	10	0.07
	C. siamensis	745	4	0.005
Total		9004	5228	0.58

Table 8 Prevalence and intensity of *Angiostrongylus cantonensis* in natural hosts in Kamphaeng Phet Province

Natural hosts	**No. of Positive**	**No. of Examined**	**Prevalence (%)**	**No. of worm in each infected snail**	**Average intensity**
Filopaludina spp.	0	1119	0	-	-
Pomacea canliculta	0	409	0	-	-
Achatina fulica	3	275	1.1	5, 8, 15	0.1
Cryptozona siamensis	45	425	10.6	1, 1, 1, 1, 1, 2, 3, 3, 4, 4, 5, 6, 6, 9, 10, 17, 18, 18, 21, 22, 25, 26, 31, 31, 34, 43, 53, 56, 56, 61, 72, 85, 86, 107, 112, 145, 195, 202, 305, 316, 330, 479, 766, 1147, 4858	23
Total	48	2228	2.1	9774	

Table 9 Intensity of *Angiostrongylus cantonensis* 3rd larvae in *Cryptozona siamensis* and *Achatina fulica* collected from Kamphaeng Phet Province

No. of larvae/snail	***Cryptozona siamensis***		***Achatina fulica***	
	No. of snail (%)	**No. of larvae (%)**	**No. of snail (%)**	**No. of larvae (%)**
0	380(89.4)	0	272(98.9)	0
1-10	15(3.5)	57(0.6)	2(0.7)	13(46.4)
11-100	18(4.2)	755(7.7)	1(0.4)	15(53.6)
101-1000	10(2.4)	2957(30.3)	0	0
1001-10000	2(0.5)	6005(61.4)	0	0
Total	**425(100)**	**9774(100)**	**275(100)**	**28(100)**

Identification of *Angiostrongylus cantonensis*

Preliminary identification of *A. cantonensis* was made by morphological study of 3rd stage larvae. It was characterized by thin and delicate body (0.46-0.52 mm long and 0.029-0.038 mm wide). It has two chitinous rods at the anterior end. The tail is cone-shaped, slightly curved and pointed (Figure 7).

Polymerase chain reaction was performed to amplify a partial gene cytochrom c oxidase subunit I (COI) using a set of primer CO1_F 5' TAAAGAAAGAACATAATGAAAATG 3' and CO1_R 5' TTTTTTGGGCATCCTGAGGTTTAT 3'. Ten isolates of *Angiostrongylus* L3 can be amplified a partial COI gene. Genomic DNA from adult worms collected from natural rats in Kamphaeng Phet Province was used as a positive control. After electrophoresis of PCR products, approximately 450 bp. was demonstrated in all isolates (Figure 8).

BLASTN analysis of 261-264 bp. from COI gene revealed that 11 isolates of *Angiostrongylus* were identical to *Angiostrongylus cantonensis* with identity ranged 92-99% (Table 10).

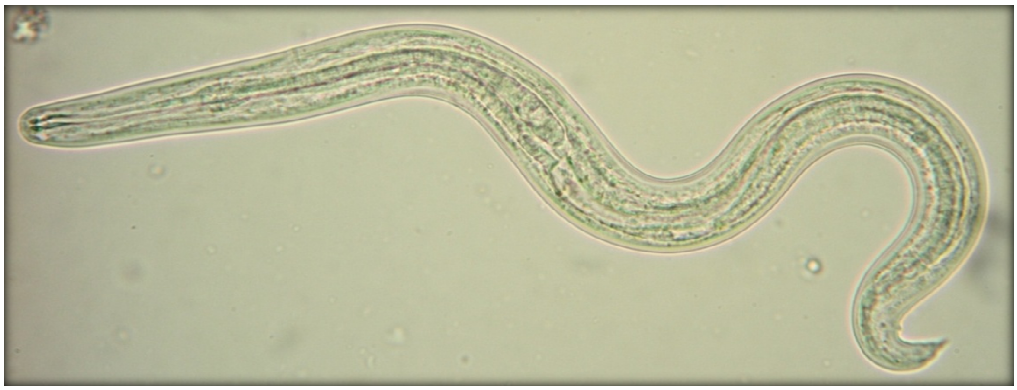

Figure 7 Third stage larva of *Angiostrongylus cantonensis* isolating from *Cryptozona siamensis* collected from Kamphaeng Phet Province (40X magnification under light microscope).

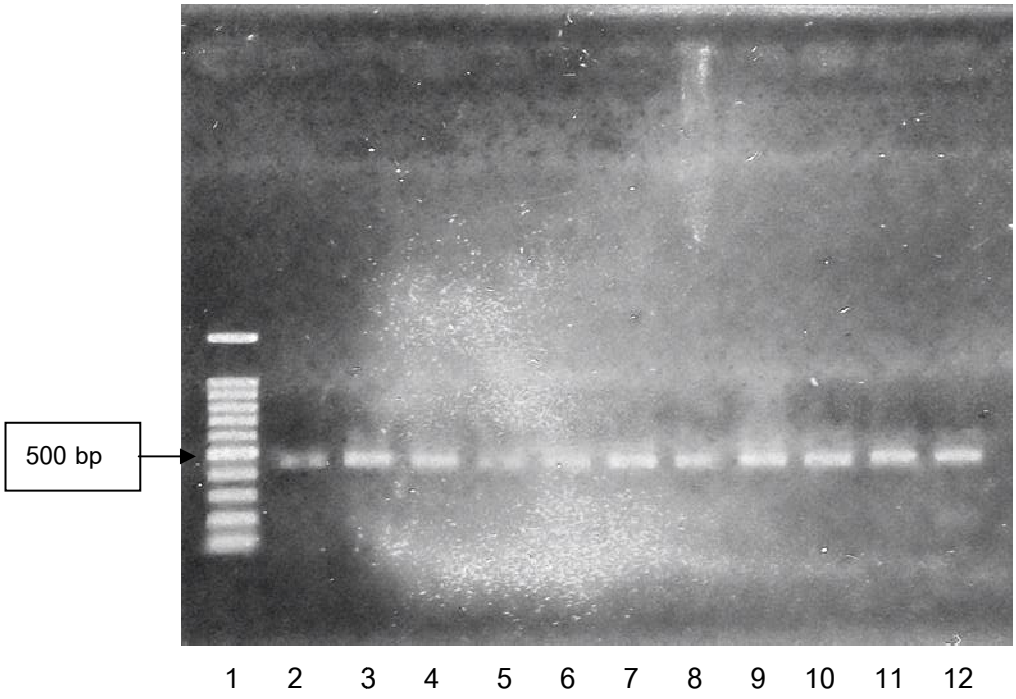

Figure 8 PCR products of a partial COI gene of ten isolates of *Angiostrongylus* were separated on a 1.2% agarose gel.

Lane 1 100 bp. ladder

Lane 2 Third stage larvae of *Angiostrongylus* isolated from *C. siamensis* of Kamphaeng Phet Province

Lane 3 Third stage larvae of *Angiostrongylus* isolated from *A. fulica* of Kamphaeng Phet Province

Lane 4 Third stage larvae of *Angiostrongylus* isolated from *C. siamensis* of Tak Province

Lane 5 Third stage larvae of *Angiostrongylus* isolated from *M. siamensis* of Tak Province

Lane 6 Third stage larvae of *Angiostrongylus* isolated from *C. siamensis* of Phitsanulok Province

Lane 7 Third stage larvae of *Angiostrongylus* isolated from *C. siamensis* of Kalasin Province

Lane 8 Third stage larvae of *Angiostrongylus* isolated *A. fulica* of Kalasin Province

Lane 9 Third stage larvae of *Angiostrongylus* isolated *A. fulica* of Phetchabun Province

Lane 10 Third stage larvae of *Angiostrongylus* isolated *A. fulica* of Phetchabun Province

Lane 11 Third stage larvae of *Angiostrongylus* isolated *C. siamensis* of Phetchabun Province

Lane 12 Third stage larvae of *Angiostrongylus* isolated *C. siamensis* of Phetchabun Province

Table 10 Alignment score of *Angiostrongylus* isolates as calculated by NCBI-BLAST program

Isolate description	Maximum identity to	Accession No.	E-value	Query coverage	Identity
A. cantonensis TH Kamphaeng Phet_Adult	*Angiostrongylus cantonensis* Thailand: Bangkok, haplotype: ac4	AB684368.1	3e-134	99	99%
*A. cantonensis*_TH Kalasin isolate 1_AF	*Angiostrongylus cantonensis* Japan: Nagoya, haplotype: ac7	AB684376.1	5e-107	100	94%
*A. cantonensis*_TH Kalasin isolate 2_AF	*Angiostrongylus cantonensis* Japan: Aichi, Nagoya, haplotype: ac7	AB684376.1	4e-104	100	92%
*A. cantonensis*_TH Phetchabun isolate 1_AF	*Angiostrongylus cantonensis* Thailand: Bangkok, haplotype: ac4	AB684368.1	4e-137	100	99%
*A. cantonensis*_TH Phetchabun isolate 1_CS	*Angiostrongylus cantonensis* Thailand: Bangkok, haplotype: ac4	AB684368.1	3e-116	100	97%
*A. cantonensis*_TH Phetchabun isolate 2_AF	*Angiostrongylus cantonensis* Thailand: Bangkok, haplotype: ac4	AB684368.1	1e-133	100	99%
*A. cantonensis*_TH Phetchabun isolate 2_CS	*Angiostrongylus cantonensis* Thailand: Bangkok, haplotype: ac4	AB684368.1	1e-134	100	99%
*A. cantonensis*_TH Phitsanulok_AF	*Angiostrongylus cantonensis* Japan: Aichi, Nagoya, haplotype: ac7	AB684376.1	1e-100	100	92%
*A. cantonensis*_TH Tak isolate 1_AF	*Angiostrongylus cantonensis* Japan: Aichi, Nagoya, haplotype: ac7	AB684376.1	4e-104	100	92%
*A. cantonensis*_TH Tak isolate 2_AF	*Angiostrongylus cantonensis* Thailand: Bangkok, haplotype: ac4	AB684368.1	9e-135	100	99%
*A. cantonensis*_TH Tak isolate 3_AF	Angiostrongylus cantonensis Japan: Aichi, Nagoya, haplotype: ac7	AB684376.1	2e-102	100	92%

Phylogenetic tree analysis

A maximum likelihood tree reconstructed using the 11 *Angiostrongylus* sequences together with sequences downloaded from GenBank are shown in Figure 11. The sequences from 11 isolates of *A. cantonensis* in the present study were distributed as groups on 3 branches of the tree. Group I contained 1 isolate of *A. cantonensis* isolated from *A. fulica* of Tak province and included sequence belonging to *A. cantonensis* from Bangkok, Thailand. Group II contained 5 isolates of *A. cantonensis* from Phetchabun and Kamphaeng Provinces and a reference sequence of *A. cantonensis* from Zhejiang Wenzhou, China. Group III contained 5 isolates of *A. cantonensis* from Kalasin, Tak, and Phitsanulok Provinces and no reference sequence.

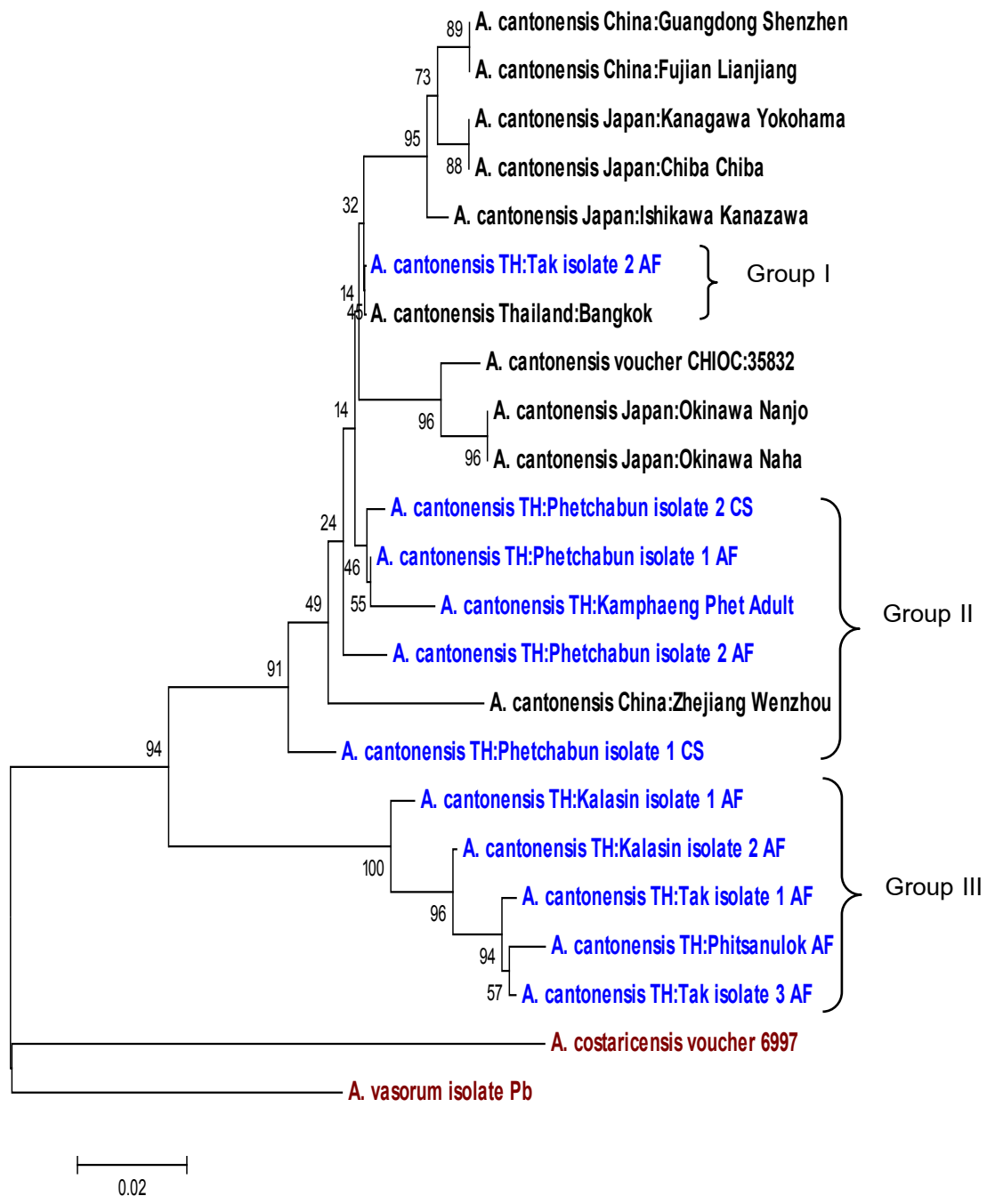

Figure 11 Maximum likelihood tree based on a 261-264 bp. from COI gene for 11 isolates of *Angiostrongylus* from Thailand together with *Angiostrongylus cantonensis* sequences downloaded from the GenBank database (shown in black). Bootstrap values are based on 1,000 replicates.

CHAPTER V
DISCUSSION AND CONCLUSION

Discussion

The present study demonstrated that low prevalence and intensity of *Angiostrongylus cantonensis* in *Cryptozona siamensis*, *Achatina fulica*, and *Megaustenia siamensis* collected from Phitsanulok, Kalasin, Tak, Phetchabun and Kamphaeng Phet Provinces. *A. fulica* is a common natural intermediate host of *A. cantonensis* with variable prevalence from 7.55-94.4% (Harinasuta et al., 1965; Setasubun et al., 1968; Pipitgool et al. 1997; Tesana et al., 2009; Vitta et al., 2011). In the other hand, fresh water snails, namely, *Pila polita*, *Filopaludina martensi martensi*, *Filopaludina sumatrensis polygramma*, *Pila pesmei*, *Clea helena*, *Bithynia siamensis goniomphalos* have been reported as low prevalence as less than 5% (Setasubun et al., 1968; Pipitgool et al. 1997; Tesana et al., 2009) while *P. ampullacea* was negative for larval infection (Pipitgool et al. 1997). Our result agreed with those mentioned above as none of *Pomacea canliculta* and *Filopaludina* spp. was infected with *A. cantonensis*. This may be due to low susceptible host of these two snails.

In Kamphaeng Phet Province, prevalence of *A. cantonensis* in *C. siamensis* was 10.6% and average intensity of larvae in this snail was 23. *C. siamensis*, a land snail was considered as novel intermediate host for *A. cantonensis* in Thailand. Two species of this genus; *Cryptozana imperator* and *Cryptozana bistrialis* was reported as the host of *A. cantonensis* (Ko 1991; Cross and Chen, 2007). Although this snail was not cooked as favorite dish for Thai people, it can transmit to the natural hosts. This snail closely resides to the agricultural area of human. This may be the source of infection to man.

In Tak Province, *Angiostrongylus* larvae were isolated from *Megaustenia siamensis*. The worms from this host were not identified into species level. Interesting, *M. siamensis* might be the first record of natural intermediate host for *A. cantonensis*. However, more study on identification of *A. cantonensis* in *M. siamensis* was needed.

In this study, we have identified *Angiostrongylus* larvae by morphology and molecular technique. By morphological identification, *Angiostrongylus* larvae were characterized by (1) thin and delicate body, (2) two chitinous rods at the anterior end and (3) The tail is cone-shaped, slightly curved and pointed (Eamsobhana, 2006). In this study, eleven isolates of

Angiostrongylus larvae isolated from different hosts from 5 provinces of Thailand were morphologically identified. A partial sequence of COI gene was selected as a tool for molecular identification and study on phylogenetic relationships. In the present study, 5 isolates of *Angiostrongylus* showed low identity (92-94%) with *A. cantonensis* after BLASTN search. This might be due low variation of this gene. However, this gene has proved to be a powerful marker in study phylogenetic tree for closely related *Angiostrongylus* species (Eamsobhana et al., 2010; Tokiwa et al., 2012). A nuclear small subunit (SSU) rRNA sequences show little variation within nematode species, but substantial divergence among species, allowing for species identification (Fontanilla and Wade, 2008).

Phylogenetic tree analysis based on a partial sequence of COI gene revealed that 3 groups of *Angiostrongylus* were closely related to *A. cantonensis* from Bangkok, Thailand and Zhejiang Wenzhou, China. This indicated that *Angiostrongylus* Thai isolates showed distinct lineages. Similar result was observed among *A. cantonensis* collected from Thailand, Hawaii and Mainland China (Eamsobhana et al., 2010). Also, *A. cantonensis* from Brazil and Mainland China were distinct lineages (Simoes et al., 2011). This indicates that difference geographical isolates showed distinguishing lineages and *A. cantonensis* has been spreading across the Pacific. However, it has been difficult to infer the phylogeographical patterns due to the paucity of information.

Conclusion

A total of 14,032 fresh water and land snails were collected from 19 provinces including Chiang Mai, Phare, Lampang, Lamphun, Phitsanulok, Tak, Phichit, Sukhothai, Kamphaeng Phet, Uthai Thani, Bueng Kan, Chaiyaphum, Maha Sarakham, Nakhon Ratchasima, Phetchabun, Kalasin, Khon Kaen, Mukdahan and Nonthaburi. Snails from 5 provinces including Phitsanulok, Kamphaeng Phet, Kalasin, Tak and Phetchabun were positive for *Angiostrongylus*. Six species of snails were collected e.g., *Filopaludina* sp., *Pomacea* sp., *Achatina fulica*, *Cryptozona siamensis*, *Megausatenia siamensis* and *Cyclophorus* sp. *Angiostrongylus cantonensis* larvae were isolated from three species of land snails, e.g., *Achatina fulica*, *Cryptozona siamensis*, and *Megausatenia siamensis*. *C. siamensis* is the first record for natural intermediate host of *A. cantonensis* in Thailand. Prevalence of *Angiostrongylus* was 10% in *C. siamensis*.

Based on a partial sequence of COI gene from 3rd stage larvae of *Angiostrongylus* isolated from natural intermediate hosts, all of 10 isolates were closely related to *Angiostrongylus cantonensis*. Phylogenetic tree analysis demonstrated that 3 groups of *Angiostrongylus* Thai isolates were closely related to *A. cantonensis* from Thailand and China.

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Output

1. นำเสนอ Poster เรื่อง “ความชุกและความหนาแน่นของพยาธิปอดหนู (*Angiostrongylus cantonensis*) จากโฮสต์ตัวกลางในธรรมชาติในจังหวัดตาก” งานประชุม the 4th MSAAM 2014 เมื่อวันที่ 26-27 ธันวาคม 2556 คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร
2. นำเสนอ Poster เรื่อง “ความชุกและความหนาแน่นของพยาธิปอดหนู (*Angiostrongylus cantonensis*) จากโฮสต์ตัวกลางในธรรมชาติในจังหวัดกาฬสินธุ์ และมุกดาหาร” งานประชุม the 4th MSAAM 2014 คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร
3. นำเสนอ Poster เรื่อง “Molecular identification and phylogeny of Angiostrongylus from Thailand based on cytochrome c oxidase subunit I (COI) sequence” งานประชุม “นักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส” ครั้งที่ 14 ณ โรงแรมแอมบาสซาเดอร์ ซิตี้ จอมเทียน จังหวัดชลบุรี
4. Manuscript in preparation เรื่อง “First report of *Cryptozona siamensis* as natural intermediate host of *Angiostrongylus cantonensis* in Thailand “ สำหรับลงตีพิมพ์ในวารสาร Southeast Asian Journal of Tropical Medicine and Public Health
5. Manuscript in preparation เรื่อง “Phylogeny of *Angiostrongylus cantonensis* based on cytochrome c oxidase subunit I sequence in Thailand” สำหรับลงตีพิมพ์ในวารสาร Tropical Biomedicine