CHAPTER I

INTRODUCTION

1. STATEMENT AND SIGNIGICANCE OF THE PROBLEM

Lymphatic filariasis still exists and affects more than 1.4 billion people in 73 countries worldwide. One of control strategies recommended by the World Health Organization (WHO) is based on the mass drug administration (MDA) with albendazole and either diethylcarbamazine citrate (DEC) or ivermectin to reduce circulating microfilariae below a threshold level and to break transmission by the disease vector. Mosquito species belonging to *Anopheles*, *Culex*, *Aedes*, *Mansonia*, *Coquillettidia* and *Ochlerotatus* genera are vectors of filarial parasites.

Mosquito-borne diseases continue to place tremendous health and economic burdens on a large percentage of the population of the world, thereby challenging the scientific community to develop new, more efficient means for control and treatment. Within the vector-biology community, one approach to this challenge has been to develop strategies to disrupt pathogen transmission by mosquito vectors. Conventional methodologies to control vector borne diseases with chemical pesticides are often associated with environmental toxicity, adverse effects on human health, and the emergence of mosquito resistance. Thus, development of novel approaches to fight mosquito vectors is urgently required.

In competent vectors, the parasites continue their life cycle when microfilariae are taken up by female mosquitoes during blood feeding on an infected host. For

normal development to proceed in such competent vectors, microfilariae must penetrate the mosquito midgut and traverse the hemocoel to invade the thoracic muscle cells, and then develop to the infective third larval stage. These migrate to the head and proboscis and enter a new host by penetrating the labellum of the proboscis when the mosquito takes another blood meal. However, there are various factors that may inhibit or prevent filarial infection. The geographical location, habitat type, compatibility of geographical strain variations in parasites and vectors, and inherent genetic selection and adaptation in both parasite strains and vector species influence to susceptibility or refractory.

Various previous studies examining development of *Brugia* spp. in susceptible (Black eye Liverpool) and refractory (Malayan, Bora-Bora, and Bankok) *Aedes aegypti* strains have been performed. However, very little is known about selective barriers for *Brugia* development at the midgut level in refractory *Ae. aegypti*. Therefore, the current study was undertaken in which a systematic investigation of the exsheathment and invasion of nocturnally subperiodic *B. malayi* microfilariae within the midgut of a refractory vector, *Ae. aegypti* (Thailand strain), was performed. In addition, the information might be useful for further studies on refractory mechanism (s) in other refractory vector (s) that aim to design of develop novel control program strategies for elimination of the lymphatic filariasis.

2. LITERATURE REVIEWS

2.1 Background and epidemiology of lymphatic filariasis

Lymphatic filariasis is a vector-borne disease caused by one of three parasitic nematodes, *Wuchereria bancrofti*, *Brugia malayi*, or *Brugia timori*. These are

transmitted by several mosquitoes within the genera *Mansonia, Anopheles, Culex* and *Aedes* (Schacher 1962; Guptavanij et al. 1978; Trpis 1981; Chang et al. 1991; Bangs et al. 1995; Kumar et al. 1998; Lek-Uthai and Tomoen 2005; Wada 2011). The disease is endemic in 73 countries worldwide with 1.4 billion people at risk and an estimated 120 million infected (http://www.who.int/mediacentre/factsheets/fs102/en/). WHO's African and South-East Asia regions harbour 95% of the population living in endemic areas, and 98% of the infected population. Of the total population requiring preventive chemotherapy for lymphatic filariasis, 866 million (62%) live in the South-East Asia region (9 endemic countries) and 464 million (33%) live in the African region (34 countries). The region of the Americas, Eastern Mediterranean region and Western Pacific region account for 5% of global distribution (http://www.who.int/gho/neglected_diseases/lymphatic_filariasis/en/) (Figure 1.1). Lymphatic filariasis, caused by *W. bancrofti*, is the most widespread human lymphatic filarial infection. The largest number of people both at risk, and infected, live in India, but the disease is a severe problem in many other Asian countries, notably Bangladesh, Burma, China, Indonesia, Malaysia, Papua New Guinea, the Philippines, Sri Lanka, Thailand and Viet Nam. *B. malayi* infections are found in Southern China, India Indonesia, Malaysia, the Philippines, the Republic of Korea, Thailand and Viet Nam while *B. timori* is localized in the Lesser Sunda Islands of eastern Indonesia (WHO 1987). In Thailand, lymphatic filariasis is endemic in the South and has been reported in Narathiwat province (http://www.thaivbd.org/n/contents/view/324451) (Figure 1.2).

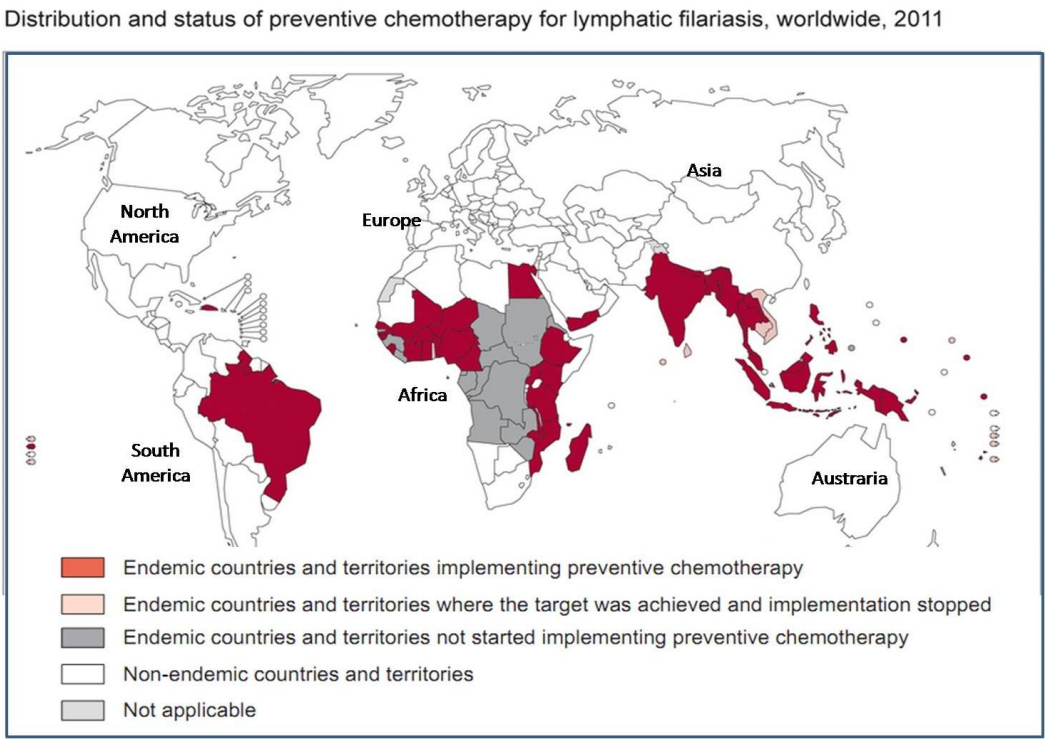

Figure 1.1 World map shows the global distribution and status of mass drug administration for lymphatic filariasis, 2011 (Modified from http://gamapserver.who.int/mapLibrary/Files/Maps/LF_2011.png).

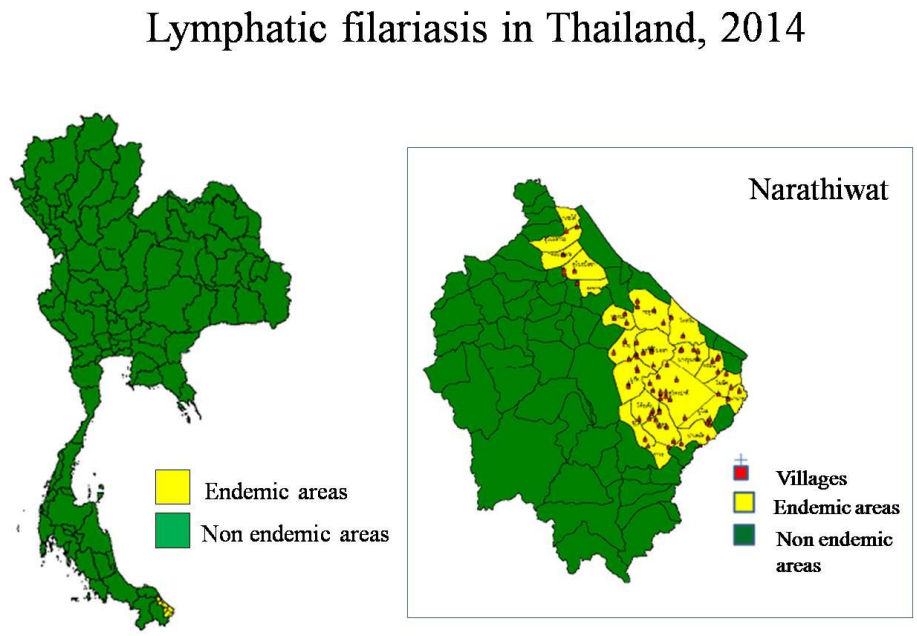

Figure 1.2 Province and villages affected with lymphatic filariasis in Thailand, 2014 (Modified from http://www.thaivbd.org/n/contents/view/324451).

2.2 Life cycle of parasite

Among the parasitic nematodes, filarial parasite has complicated life cycle in vertebrate host and vector. The parasite is transmitted by mosquitoes in which microfilariae develop to the infective stage. When the infective mosquito takes a blood meal, it introduces infective third-stage filarial larvae (L3) onto the skin (Figure 1.3a). These escape from the immune effectors at point of entry. A large number of L3 at the site of penetration are destroyed by the immune system and some of them reached the peripheral lymphatic vessels. The lymphatic system offers shelter to the larvae at periods when their developmental stages are the most vulnerable. Lymphatic vessels are able to contain large worms because of a capacity for dilatation. They allow the larvae to escape different molecules, leukocytes and provide easy routes of migration within the connective tissues, which rapidly react as assessed by leukocyte infiltrates (Bain and Babayan 2003). In the lymphatic vessels, L3 develops in the lymphatic system to the L4 stage, to the young adult stage (Figure 1.3b), and finally to the mature adult worm, male or female (Figure 1.4e). After fertilization, the female worms produce microfilariae (Figure 1.4a), which find their way from the lymphatic system to the bloodstream (Figure 1.4c). The pre-patent period (from the entrance of L3 to the appearance of microfilariae in the peripheral blood) is estimated at about 3 months for *Brugia* spp. and 9 months for *W. bancrofti*. The parasites continue their life cycle when microfilariae are taken up by female mosquitoes during blood feeding on an infected host. The mosquito acquires the infection by ingestion of the microfilariae in the blood meal (Figure 1.3d). Some of the microfilariae ingested by the mosquito shed their sheaths, penetrate midgut wall and traverse the hemocoel to invade the thoracic muscle cells and develop there without multiplication. The slender

active microfilaria transforms to the short-thick inactive sausage-stage or first-stage larva (L1). The L1 larva has a cuticle which forms a conspicuous slender tail. In the genus *Brugia* one or two nuclei are present inside the tail (Figure 1.4b). After the first molt, the larva grows rapidly in length and width and becomes potentially more active, although usually it does not move. It has a thin cuticle which can be seen at the caudal end forming a short tail. This second-stage larva (L2) or pre-infective larva is recognized by its short tail and the presence of one or two papillae at the caudal end (Figure 1.4c). After a second molt, the parasite no longer has a visible cuticle and is known as L3 (Figure 1.4d) grows further in length but not in width, moving actively in the hemocoel of the mosquito, first towards the abdomen and later to the head and proboscis. L3 enter a new host by penetrating the labellum of the proboscis when the mosquito takes another blood meal (Figure 1.3a) (WHO 1987).

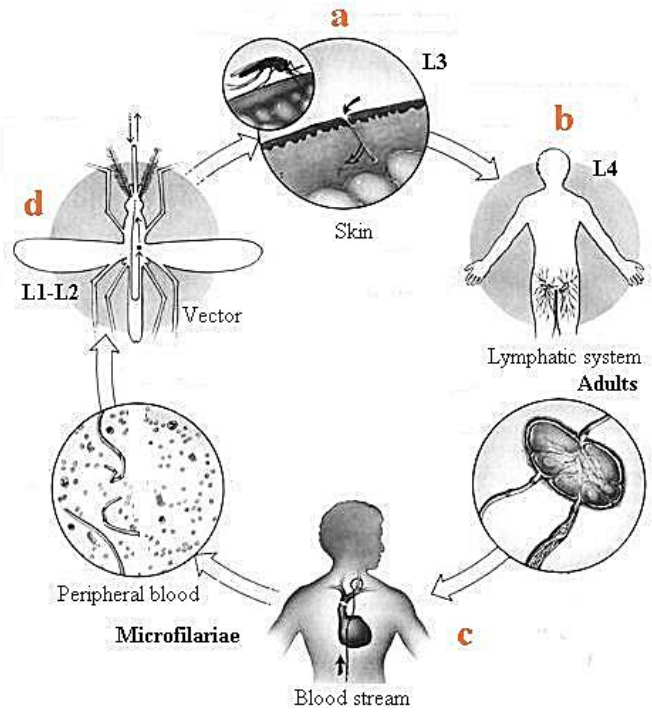

Figure 1.3 Life cycle of filarial parasite. (a) The infective third-stage filarial larva (L3) enters the host when a mosquito takes a blood meal. (b) The L3 migrates to lymphatic vessels and develops into the L4 stage, to the young adult stage and finally to the mature adult worm, male or female that commonly reside in the lymphatic system. (c) After mating, the gravid female produces sheathed microfilariae into lymph and enter the bloodstream reaching the peripheral blood. (d) After a mosquito acquires the infection by ingestion of an infected blood meal, microfilaria penetrates midgut wall and traverses the hemocoel to invade the thoracic muscle cells and then develops to the L3 which migrates to the head and proboscis and enters a new host by penetrating the labellum of the proboscis (Modified from http://intranet.tdmu.edu.ua/data/kafedra/internal/med_biologia/classes_stud/en/med/lik/ptn/medical%20biology/1%20course/Theme%2010.htm).

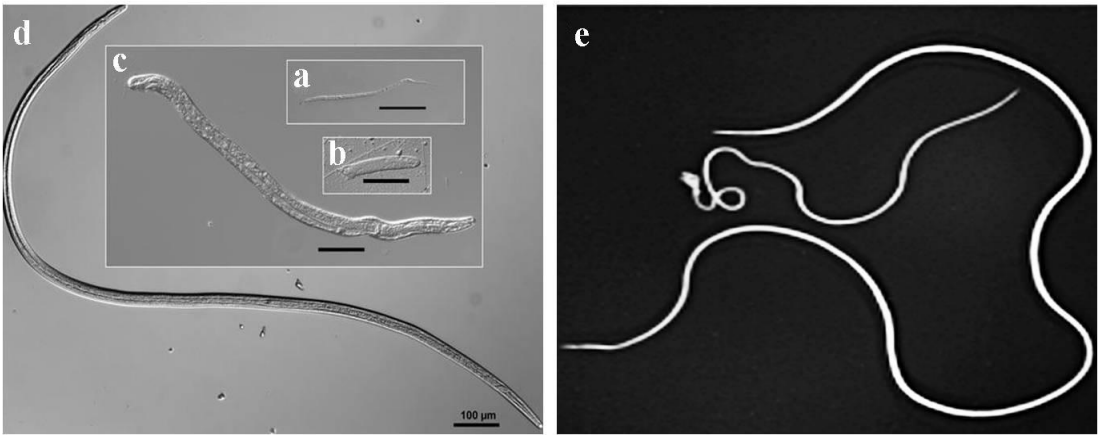

Figure 1.4 Morphology of different stages of *B. malayi*. (a) Microfilaria is ingested during blood feeding. (b) Parasite differentiates into non-feeding, L1 within mosquito indirect flight muscle cells. (c) After the first molt, L2 remains intracellular parasites which ingest cellular material into its newly developed digestive tract. (d) L3 leaves the muscle cells and migrates to the mosquito's head and proboscis where they will exit through the mosquito cuticle during blood feeding (Modified from Sara et al. 2009). (e) *B. malayi* adults. The adult male worm is considerably smaller than the female (Modified from http://www.metapathogen.com/lymphatic-filariasis/).

2.3 Pathogenesis and clinical manifestations of lymphatic filariasis

Lymphatic filariasis has a wide spectrum of clinical manifestations. The clinical presentations of filariasis can be divided into asymptomatic, acute, and chronic stages.

The majority of infections are asymptomatic. This is characterized by the presence of microfilariae in the circulating blood, although there are no clinical manifestations of filariasis (WHO 1987). Patients concerned have no inkling that their blood contains large numbers of microfilariae and this situation may persist for decades without any progression to overt clinical disease. Asymptomatic condition is not a benign phase, and that considerable occult lymphatic, tissue and organ damage may be occurring (Melrose 2002). However, they have some degree of subclinical disease that includes microscopic haematuria and/or proteinuria (Nutman and Kumaraswami 2001).

Acute filarial attacks can occur in both amicrofilaraemics and microfilaraemics, and are common in people with chronic filarial pathology. These clinical manifestations are characterized by recurrent attacks of fever associated with inflammation of lymph nodes (lymphadenitis) and lymph vessels (lymphangitis). In bancroftian filariasis, recurrent attacks of fever associated with lymphadenitis are less frequently seen than in brugian filariasis. The lymph nodes in the inguinal, axillary and epitrochlear regions, the lymphatic system of the male genitalia are frequently affected, leading to funiculitis, epididymitis or orchitis, or to a combination of these. In brugian filariasis, the affected lymph nodes are mostly situated in the inguinal and axillary regions, with inflammation along the course of the distal lymphatic vessels (WHO 1987). These conditions are inflammatory responses against incoming L3

larvae before the adult worm has become established (Melrose 2002). Acute infections are associated with establishment of parasite specific delayed-type hypersensitivity responses. With the onset of the development of adult worms and/or appearance of microfilariae in the blood, immune responses are profoundly altered. There is a diminution of parasite-specific lymphocyte proliferation, IL-2 and IFNg production, an increase in antifilarial IgG4 and the production of the regulatory cytokine IL-10 (Nutman and Kumaraswami 2001).There are at least two distinct mechanisms involved in the pathogenesis of acute attacks. The first is called acute dermatolymphangioadenitis, where there is development of a plaque-like lesion of cutaneous or sub-cutaneous inflammation, which may be accompanied by ascending lymphangitis and regional adenitis. Typical systemic manifestation of bacteraemics such as chills and fevers is usually present. There is often oedema of the affected limb. This may cause chronic lymphoedema. The other syndrome is called acute filarial lymphangitis result from an immunological reaction to dead or dying adult worms which have either been killed by the immune system or chemotherapy (Melrose 2002).

The chronic clinical manifestations are characterized by an absence of physical pain and suffering when there is no associated adenolymphangitis. During the chronic stage, microfilaria are usually absent in circulating blood. In bancroftian filariasis, the most common are hydrocele and swelling of the testis, followed by elephantiasis of the entire lower limb, the scrotum, the entire arm, the vulva, and the breast. In brugian filariasis, the leg below the knee is characteristically affected, and sometimes the arm below the elbow. Genital involvement has not been reported (WHO 1987). The pathological changes in chronic lymphatic filariasis result from

dysfunction of or inflammatory damage to the lymphatics. Adult worms live in the afferent lymphatics or sinuses of the lymph nodes and induce local changes that result in dilatation of the lymphatics and thickening of the vessel walls. Histologically, there is infiltration with plasma cells, eosinophils, and macrophages in and around the infected vessels. There is endothelial and connective tissue proliferation with tortuosity of the lymphatics and damaged or incompetent lymph valves. The overlying skin may show lymphoedema and chronic stasis changes with hard or brawny oedema. In the lymphatics, local immune responses directed toward the adult parasite cause the granulomatous and proliferative processes that precede total lymphatic obstruction. Death of the worm leads to local necrosis of a granulomatous reaction around the parasite. Fibrosis occurs and lymphatic obstruction develops (Nutman and Kumaraswami 2001).

2.4 Control of lymphatic filariasis

Lymphatic filariasis is one of five infectious diseases targeted for elimination by the WHO. The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was established in 1999 with the objective of interrupting transmission of the parasites in all endemic countries by 2020 (http://www.who.int/lymphatic_filariasis/disease/en/). The strategy proposed the WHO to achieve the goal of elimination comprises reduction of morbidity, reduction of transmission, and interruption of transmission (WHO 1987). Interruption of transmission recommended by the WHO is based on the mass drug administration (MDA) with albendazole and either diethylcarbamazine citrate (DEC) or ivermectin to reduce circulating microfilariae below a threshold level. The following recommended drug regimens are administered once a year for at least 5 years, with coverage of at least 65% of the total at-risk population

(http://www.who.int/lymphatic_filariasis/policy/en/). Within the vector-biology community, one approach to this challenge has been to develop strategies to disrupt pathogen transmission by mosquito vectors. However, conventional methodologies to control vectors with chemical pesticides are often associated with environmental toxicity, adverse effects on human health, and the emergence of mosquito resistance. Thus, development of novel approaches to fight mosquito vectors is urgently required.

2.5 Mosquito midgut

Various factors have been reported that may inhibit or prevent filarial infection and successful development in the vector, and singly or in combination these have been proposed to contribute to refractoriness. These factors include both physical obstacles (cibarial and pharyngeal armatures in the foregut) and physiological characteristics (peritrophic matrix formation and blood clotting in the midgut, direct toxicity and melanization/encapsulation responses in thoracic muscles) of the vector, and are manifested as compatibility/incompatibility of geographical strain variations in parasite and vector, and influenced by natural selection and adaptation in both parasite strain and vector species (Nelson 1964, Ewert 1965; Denham and McGreevy 1977; Owen 1979; Oda and Wada 1980; Townson and Chaithong 1991; Nayar and Knight 1995; Bangs et al. 1995; Christensen et al. 2005; Magalhaes et al. 2008).

The mosquito midgut is the primary site for digestion and absorption of food. This organ starts at the cardiac sphincter and ends at the pyloric sphincter. The midgut contains different cell type such as digestive cells, regenerative cells, endocrine cells and goblet cells. Blood feeding induces the release of proteolytic enzymes in the midgut, which leads to the degradation of blood meal proteins into peptides and

amino acids. These blood protein-derived peptides and amino acids are required for the synthesis of lipid and carbohydrate stores, and as a source of energy for egg production. The major classes of digestive enzymes in blood fed are trypsins, chymotrypsins, aminopeptidases and carboxypeptidases (Rascón et al. 2011).

In the midgut, the first vector microenvironment that filarial parasites encounter, early studies suggested that some microfilariae exsheathed either in the midgut itself (Esslinger 1962; Ewert 1965; Denham and McGreevy 1977) or during penetration of the midgut wall (Yamamoto et al. 1983; Christensen and Sutherland 1984; Agudelo Silva and Spielman 1985; Perrone and Spielman 1986). Exsheathment preceding migration from the midgut has been considered by some authors as a prerequisite for the complete development of certain microfilariae in their insect vectors (Esslinger 1962; Ewert 1965; Santos et al. 2006). Most previous studies of the development of *Brugia* parasites in competent vectors have only been performed using a susceptible strain of *Ae. aegypti* mosquitoes (Black eye Liverpool strain) (Erickson et al. 2009), and often these reached different conclusions. For example, Agudelo-Silva and Spielman (1985) studied the migration of *B. malayi* microfilariae in the Black eye Liverpool strain, and concluded that the microfilariae penetrated the midgut wall of the mosquito vector while still sheathed, and that the sheath remained protruding from the gut when entering the hemocoel, thus arguing against exsheathment as a prerequisite for migration. On the other hand, Chen and Shin (1988) have suggested that the exsheathment of *B. pahangi* microfilariae occurs both in the midgut and hemocoel of susceptible (Liverpool) and refractory (Bora-Bora) strains of *Ae. aegypti*.

Peritrophic matrix, a chitinous structure, is composed principally of chitin, a linear N-acetyl-D glucosamine polymer and glycoproteins (Tellam et al. 1999; Terra 2001). The roles of the PM are associated with the prevention of midgut microvilli from the midgut contents and against pathogens and abrasion by food particles. The PM of some insects has a function as a protection against the chemical attack of potentially toxic agents (Pascoa et al. 2002). The PM is secreted by the midgut epithelium upon blood feeding and encloses the blood meal (Figure 1.5), and this has to be transversed by microfilariae before they can access the midgut wall. It has been suggested that the PM is a barrier preventing microfilariae migrating towards the midgut and contributing to refractoriness (O'Connor and Beatty 1936; Iyengar 1936). In *Culex pipiens pipiens*, ingested *B. malayi* and *B. pahangi* microfilariae perish in the midgut soon after feeding, suggesting that the inability of *Cx. pipiens pipiens* to support the development and transmission of *Brugia* spp. occurs at the level of the midgut (Ewert 1965; Michalski et al. 2010). Christensen and Sutherland (1984) have reported that *B. pahangi* microfilariae freely traverse the PM of susceptible *Ae. aegypti* mosquitoes, and in addition approximately 75% retained their sheaths after midgut penetration and exsheathment of the microfilariae rarely occurred within the midgut. Further, Jariyapan et al (2013) have recently demonstrated that in a susceptible vector, *Ochlerotatus togoi* (formerly known as *Aedes togoi*) only sheathed microfilariae of noctunally subperiodic *B. malayi* invade across the PM through the midgut wall and these reach the hemocoel with their sheaths intact (Figure 1.6).

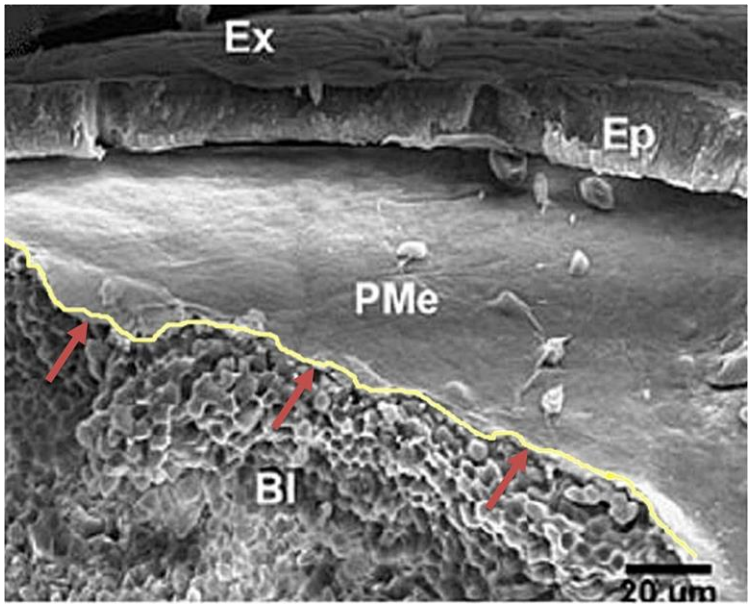

Figure 1.5 Fractured midguts and peritrophic matrix (PM) of *O. togoi* female after taking a blood meal. SEM micrograph shows an external face of the PM (*PMe*; arrows) that encloses the blood meal (*Bl*). The PM is completely formed and can be separated from the epithelium (*Ep*) (Modified from Jariyapan et al. 2013).

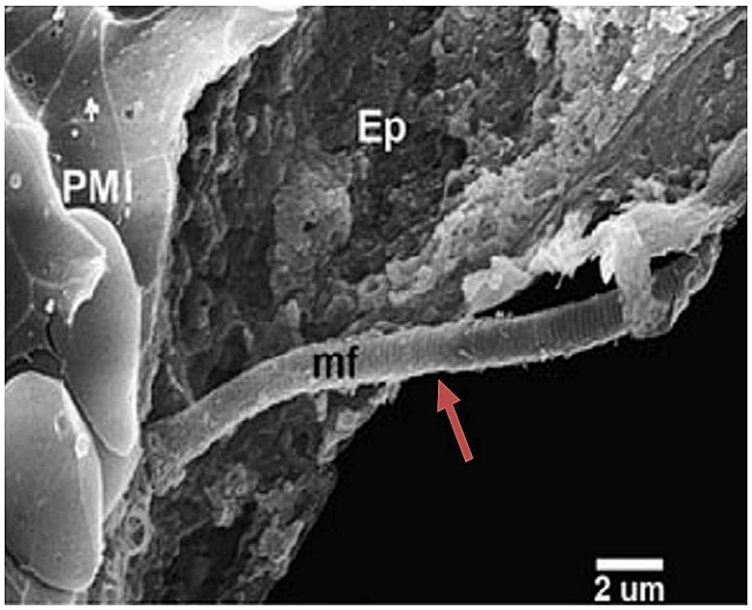

Figure 1.6 Fractured abdominal midguts of *O. togoi* female after taking an infected blood meal showing the invasion process of microfilariae (*mf*) from the midgut lumen into hemocoel. SEM showing a sheathed microfilaria (*mf*; arrow) penetrating across the internal face of the PM (*PM*) and epithelium (*Ep*) into hemocoel in the final stage of invasion (Modified from Jariyapan et al. 2013).

Various previous studies examining filarial parasites in refractory (Malayan, Bora-Bora, and Bangkok) *Ae. aegypti* strains have been performed (Kershaw et al. 1961; Chaithong 1976; Chen and Shin 1988; Pothikasikorn et al. 2008). However, very little is known about selective barriers for *Brugia* development at the midgut level in refractory *Ae. aegypti*. Therefore, the current study was undertaken in which a systematic investigation of the exsheathment and invasion of noctunally subperiodic *B. malayi* microfilariae within the midgut of a refractory vector, *Ae. aegypti* (Thailand strain), was performed.

3. OBJECTIVE

To investigate the exsheathment and midgut penetration of nocturnally subperiodic (NSP) *B. malayi* microfilariae in a refractory vector, *Ae. aegypti* (Thailand strain) using light microscope (LM) and scanning electron microscope (SEM).

4. USEFULNESS OF THE STUDY

Knowledge of the exsheathment and midgut penetration of *B. malayi* microfilariae in a refractory vector, *Ae. aegypti* (Thailand strain) would provide a better understanding of the invasion process of the parasite and possible factor(s) that affect the refractoriness. The information from this study might be useful for further studies on refractory mechanism(s) in other refractory vector(s) that aim to design or develop novel control program strategies for elimination of the lymphatic filariasis.