CHAPTER I

INTRODUCTION

GENERAL CONSIDERATION

Canine dirofilariosis (canine heart worm disease) is caused by *Dirofilaria immitis*. It is a worldwide distribution and endemic in America, Africa, Asia, Australia and southern Europe (Schrey and Trautvetter, 1998). Adult worms were found in the right ventricle and pulmonary artery. Occasionally, heartworms were found in the epidural space (Blass et al., 1989), in the brain (Hamir, 1987), anterior chamber of the eye (Brightman et al., 1977) and systemic arterial system (Frank et al., 1997). It has been reported to infect the various species of animal such as dog, dingo (*Canis dingo*), wolf (*Canis spp.*), timber wolf (*C. floridanus*), fox (*Vulpes vulpes*), domestic cat (*Felis catus domestica*), tiger (*F. tigris*), jaguar (*F. onca*), seal (*Phoca vitulina*), ferret and horse (Faust et al., 1941; Sasai et al., 2000). Heartworm is distributed in tropical and subtropical areas and the southern zones of temperate regions. The disease has been distributed by the mosquito vector. At first time, *Dirofilaria immitis* was described in 1850 in "*Filaria canis cordis*" and changing to *D. immitis* (Leidy, 1856). In vertebrates the genus *Dirofilaria* includes at least 7 species, which are dwelling in general in the subcutaneous tissue, only *D. immitis* in the heart (Anderson, 1992)

MORPHOLOGY OF DIROFILARIA

The morphological feature of the *Dirofilaria immitis* is fairly uniform. Generally, adult worm are long, slender, thread-like and creamy white in color. Female worms are bigger than males. In both sexes, the anterior end is plain and devoid of any cuticular ornamentation. In contrast, the posterior end of the male is spirally coiled, and the tail is short. The tail has caudal alae supported by large, pendunculated papillae that are most frequency arranged in pairs in preanal, adanal, and postanal position. The number and arrangement of papillae are useful for taxonomic characteristics. In the male, paired, unequal, and dissimilar spicules have structural features that are also used in taxonomy. The structure of the cuticle and its surface, the hypodermis, and the musculature (essentially the body wall) are among the features most useful for identification (Georgi and Georgi, 1992). Female worms are ovoviviparous shed unsheathed motile embryonic stage called microfilariae in the blood stream (Levine, 1968). Chaowanaprecha (1976) studies the gross morphology of *D. immitis* and can be summarized as follow:

THE MALE

The male generally has 10 pairs of caudal papillae and one pair of plasmids. It has usually five pairs of large papillae, oval shaped, unequal sized, symmetrically or sub symmetrically arranged with 4-5, frequently 4, of precloacal and one of post cloacal papillae on each side of the caudal part, two adanal pairs of finger-shaped, smaller than above on lateral and posterior to the cloacal opening, and three of postcloacal pairs of very small conical papillae on line with precloacal papillae situated near the tip of the tail. Plasmids situated on lateral position next to the tip of

the tail. Spicules are unequal and dissimilar in both size and length. Tail spirally coiled in three to four turns, usually three, broad caudal alae. Adult male are measure12 to20 cm long and 0.7 to 0.9 mm wide (Suttajit, 1993; Schrey and Trautvetter, 1998).

THE FEMALE

Adult females measure 25 to 31 cm in length and 1.0 to 1.3 mm in width (Suttajit, 1993; Schrey and Trautvetter, 1998). Vagina vera is modified into a highly muscularized long slender ovijector with a bulbous, vagina uttering directed posterior, occasionally looped or coiled. Uterine branch paired, straight and turn twist together at irregularly intervals, along the most length of body and nearly filling the body cavity. The distal ends of both uterine tubes are modified to large and prominent tube of seminal receptacle, which are connected to the small tubes, the oviduct. Ovaries are long, coiled filling and extending almost to posterior part of rectum.

MICROFILARIA

Microfilariae of *D. immitis* measure on the average 308-micrometer in length (Suttijat, 1993) and 6 to 7 micron in diameter (Georgi and Georgi, 1992). The feature exhibit a relatively straight, fully extended body with a conical anterior end and straight posterior end by modified Knott's preparation.

LIFE CYCLE

Heartworm disease is distributed by several species of mosquitoes. Grassi and Noe (1900) found that the mosquitoes serve as the intermediate host. In Thailand, Suttajit (1993) reported, that the natural vectors of *D. immitis* in Amphoe Muang,

Chiang Mai are *Aedes aegypti*, *Culex quinquefasciatus*, *Mansonia unifomis* and *Ae. Albopictus*. The specific mosquito species are the primary vector, with the season and feeding habit of the species affecting the timing of infection in susceptible dogs (Rawlings and Calvert, 1995). The female mosquitoes are the intermediate hosts and taken the microfilariae (L1) during the blood meal from the infected dogs, which have more circulating microfilariae in the warm ambient temperature (Dillon). The larvae undergo three developmental stages in the mosquito by 2 molts, that happening at the end of first and second larval stage. Suddenly after the blood meal, the undigested blood with undeveloped microfilariae (L1) passes through the midgut and still in the midgut for the first 24 hours. The number of microfilariae taken in by the mosquito was directly proportional to the number of microfilariae in the blood and the volumes of blood that taken by the mosquito (Hendrix et al., 1985). During the next 24 hours, the microfilariae migrate to the mulpighian tubules, and dwelling in the tubular cells for 6 to 7 days. For this time, the undeveloped microfilariae (L1) show an increase in diameter and reduction in length became the " sausage stage larvae" (220 to 240 micrometer in length and 20 to 25 micrometer in diameter). By approximately 10 days after the first molt, the sausage stage larvae have developed into the second stage larvae by increase in length and diameter. The tapering tail of the first stage larvae is lost and the posterior end of the second stage larvae becomes broadly rounded. The second stage larvae migrate from the cell of malpighian tubules into the lumen of the tube. The third stage follows the second molt. The third stage larva is 700 to 1100 micrometer in length and has a tapered anterior end and a blunt posterior end. They are found either within the malpighian tubules or migrating within the hemocoel of the abdomen, thorax, head and proboscis. When the third stage larva is found within the

head or proboscis, it is classified as and infective stage larvae (Hendrix et al., 1985). Altogether, the microfilaria takes 2 to 2.5 weeks in the mosquito for develop to infective stage larvae (L3). The rate of development can be as short as 8 days at 30 °C or as long as 28 days at 18 °C (Dillon).

The third stage larvae are deposited by the infected mosquito into the haemolymph produced by the puncture wound in the dog's skin and approximately 20 to 30% of the infective larvae left the mosquito during the blood meal. A single mosquito can transmitted 10 to 12 infective larvae at least and only a few of them were capable of penetrating the skin and even fewer eventually reached the right ventricle and pulmonary arteries (Hendrix et al., 1985). The larvae enter through the cutaneous wound and migrate to either subcutaneous or subserosal tissue, such as, muscle and fat. Within 1 week of entering the dog, the L3 molt to L4 stage and L4 later develop to L5 or young adult stage in approximately 85 to 120 days. During the time, young adult worms enter the vascular system by penetration of peripheral veins resting a few days before arriving the right ventricle and pulmonary arteries. Young adults initially go to the caudal lung lobe, especially the right caudal lung lobe (Rawlings, 1986).

Heartworms grow to mature size during the next 3 to 4 months of their residence within pulmonary arteries. Female heartworms are gravid in 5 to 6 months, whereas circulating microfilariae being detected 6 to 7 months after infection (Rawlings, 1986). The number of microfilariae in the blood circulation usually increases markedly over the next 6 months; thereafter the microfilarial frequency declines (Rawlings and Calvert, 1995). The microfilariae survive for up to 2 to 2.5 years in the blood circulation (Schrey and Trautvetter, 1998) and adult heartworm can live 3 to 5 years.
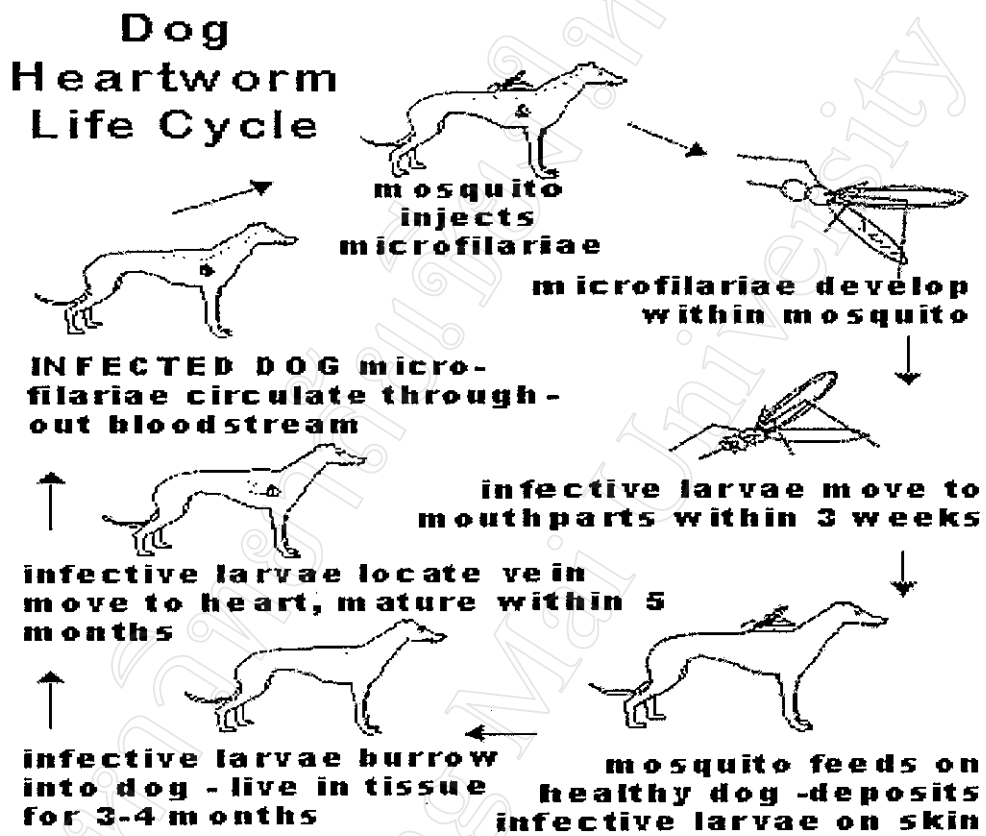
**Dog
Heartworm
Life Cycle**

mosquito
injects
microfilariae

microfilariae develop
within mosquito

INFECTED DOG micro-
filariae circulate through-
out bloodstream

infective larvae move to
mouthparts within 3 weeks

infective larvae locate vein
move to heart, mature within 5
months

infective larvae burrow
into dog - live in tissue
for 3-4 months

mosquito feeds on
healthy dog -deposits
infective larvae on skin
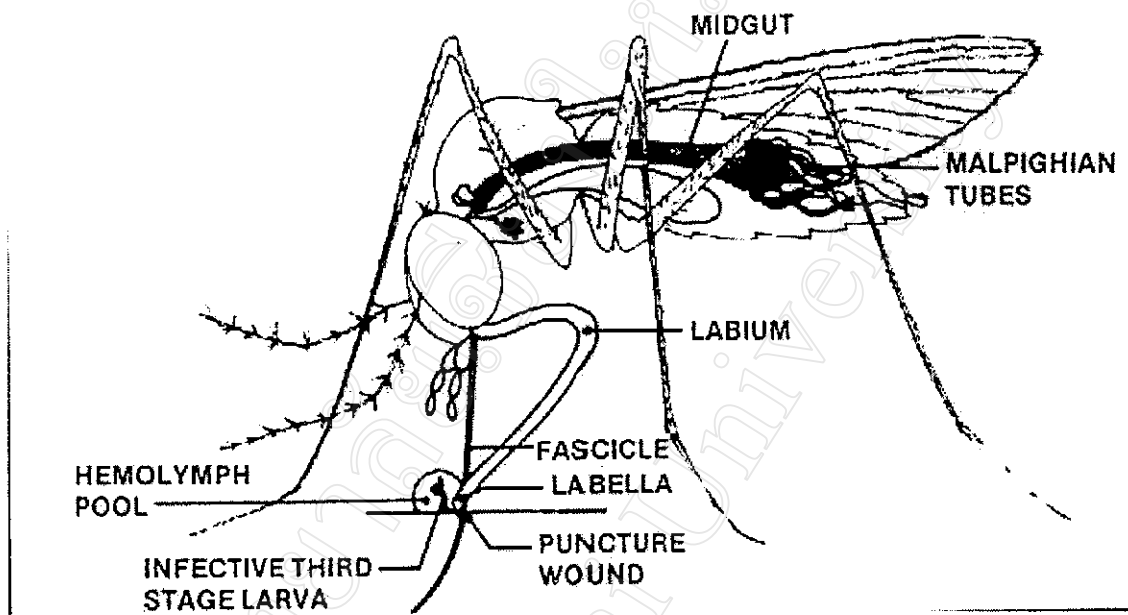
Figure1: Life cycle of heartworm disease.

Figure2: Structure of mosquito intermediate host. (Hendrix et al., 1985)

EPIDEMIOLOGY

There are many studies about the prevalence of canine heartworm in the world. Ahid et al. (1999) reported the prevalence of 12,8% of canine heartworm disease in Sao Lui Island, Northeastern, Brazil between 1991-1994. During February to June 1995, Souza et al. studied the prevalence of dirofilariosis in Belem city, Brazil that representing a prevalence of 10.74% (89.66% in males and 10.34% in females). The prevalence of canine heartworm infection in the Rio de Janeiro region was 29.7% (Labarthe et al., 1998). In the Pernambucco-area/Brazil only 2.3%(14/611) of dogs could be identified microfilariae positive but at necropsy 57.1% of heartworm infected dogs were found, a high rate of occult infections (Alves et al., 1999).

In northwest Spain, the prevalence of heartworm infections in dogs was 12.3% with a higher prevalence in the irrigated areas (Perez-Sanchez et al., 1989). The infection rate in dogs in Canary Islands between 1994-1996 raised up to 43,81% (Montoya et al., 1998).

In north Taiwan, 60.6% of dogs were infected with filarial worms, which 55% of these were *D. immitis (Wang, 1997)*.

In South Korea, the infection rate among German Shepherds dog was 28.3% (Lee et al., 1996).

Rossi et al. (1996) found that the prevalence of *D. immitis* in Northwest Italy had increased 4-fold between 1971 and 1996 and the prevalence in the north of Italy in 1996 was 44-45 %.

In the southeastern United States, data suggests that all dogs over one year of age, with access to outdoor and not incorporated in a heartworm-prevention program are infected. The estimated incidence of heartworm infection in North Georgia is

assumed with 5%. In Northern California 8.3-27.3% of coyotes were infected with heartworms found in post mortems (Acevedo and Theis, 1982).

In Thailand, a prevalence of 39.47% in domestic dog was reported by animal hospital, Chulalongkorn University, whereas the infection rate in stray dogs of the same area was 24.58 % (Sangkavaranont 1981). A similar was found in stray dogs (24.71%) in Amphur Muang, Chiang Mai (Choochote et al., 1992).

SEASONALITY

Heartworm transmission requires populations of infected and susceptible hosts, abundant vector-competent mosquitoes and sufficient environmental temperature to allow the development to the infective larval stage in the vectors. Maturation of microfilariae to the infective stage larvae following ingestion by a mosquito can only occur when the ambient temperature exceeded 18 °C. Development requires the accumulation of warmth applied over an extended period of time. The incubation period shortens from 29 days at an average daily temperature of 20 ° C to about 8 days at 30 ° C. Higher temperatures may be lethal for the larvae. In regions of temperate climate where the average daily temperature remains at or below 17 ° C over a longer period (autumn, winter, spring) no maturation to the infective third stage larvae occur (Knight and Lock, 1998).

The vector density and behavior also affected the transmission potential. In winter period, the humidity is the critical factor that affected emergence of the mosquitoes.

ZOONOSIS

Dirofilariosis is not only found in canines but also in humans (Orihel and Eberhard, 1998). Human pulmonary dirofilariosis caused by *Dirofilaria immitis* is an important zoonosis in several parts of the world. The infective stage of the filarial worm inoculated by an infected vector invades human tissue. Larvae develop first in subcutaneous tissues; later on they migrate to the right side of heart. There, they either dies or become moribund and are swept into the pulmonary artery, blocking a small-caliber vessels and producing infarcts and ultimately, a granulomatous coin lesion (Makiya et al., 1988). Chest radiographs of patients infected with *D. immitis* revealed solitary, small, peripheral, non-calcified nodular lesion (Levinson et al., 1979; Jagusch et al., 1984; Watson et al., 1991) and the incidence of pulmonary coin lesions as a result of *D. immitis* seems to be increasing (Fleisher et al., 1988). Most infected individuals (more than 60%) are asymptomatic, while the remainder complain of cough, chest pain, or hemoptysis (Orihel and Ash, 1995). In high endemic areas human dirofilariosis should be considered as a differential diagnosis for solitary pulmonary coin lesions (Makiya et al., 1987; Knauer, 1998).

In the literature, Echeverri et al. (1999) reported approximately 150 human cases worldwide and Shah (1999) reviewed 37 of human pulmonary dirofilariosis, found that the youngest patient was a 33-year-old woman, and the oldest was a 79-year-old man (mean age, 57.3 years), 43.2% were symptomatic, 51.4% were asymptomatic, and 5.4% were not recorded. Solitary lesions were found in 89.7%, with a mean diameter of 1.9 cm (Shah, 1999). In humans the disease causes solitary pulmonary nodules usually identified incidentally by chest radiography in asymptomatic patients (Prioleau et al., 1976; Merrill et al., 1980; Diaz et al., 1991;

Shah, 1999). The lesions are often mistaken for a primary or metastatic lung tumor, and the diagnosis is not often established until thoracotomy with excisional lung biopsy (Asimacopoulos et al., 1992). In the Peoria, Illinois area, the dog heartworm, *D. immitis*, has been diagnosed by thoracotomy as the etiology of neoplastic-appearing nodules in two patients (Bailey et al., 1990).

In general a single nodule is present, but two or more nodules have occasionally been reported in the some patient. In humans, the parasites do not usually reach the adult stage and microfilaremia is absent (Muro et al., 1999). The prevalence of pulmonary dirofilariosis in humans appears to be increasing (Asimacopoulos et al., 1992; Fleisher et al., 1998). The first report of dirofilariosis in human bladder was found in 1985 by Nelson and Thomas (1985). In New Zealand the first case of human dirofilariosis was reported in 1984 (Jagusch et al.), in India 1989 (Bahde and Sahne) and in Australia 1999 (Narin et al.)

In Thailand, a case of pulmonary dirofilariosis was reported at the Maharaj Nakorn Chiang Mai Hospital (Choochote et al., 1992) and in the Siriraj Hospital 1998 (Sukpanichnant). Human dirofilariosis is not seen only in the lung but also in subcutaneous tissue, the eye, abdominal cavity, and bladder.

LABORATORY DIAGNOSIS OF HEARTWORM INFECTION

The diagnosis of heartworm infection is traditionally based upon the presence circulating *Dirofilaria immitis* microfilariae. Since many infected dogs do not have circulating microfilariae, an immunodiagnosic test is proposed to diagnose adult *D. immitis* infection. Technique for laboratory diagnosis can be summarized as follow:

☐ **Microfilarial detection**

- Direct smear technique

- Thin blood smear

- Thick blood smear

- Haematocrit capillary tube (Buffy coat method)

- Modified Knott's technique

- Filter technique

☐ **Immunologic heartworm tests (Serological test)** (Goodwin, J-K, 1998)

- Indirect fluorescens antibody test (IFA)

- Haemagglutination test (HA)

- Enzyme linked immunosorbent assay (ELISA)

- Immunochromatographic test

Direct smear technique

This method is simple, quick and least expensive (Georgi and Georgi, 1992). A drop of heparinized fresh venous blood is placed on the microscopic slide and covered with a coverslip and then examined under the low magnification. If many microfilariae are present in the blood, the wet smear technique will reveal their presence and provide information that is useful to differentiate *Dirofilaria immitis* from *Dipetalonema reconditum* microfilaria (Rawlings, 1986). Microfilaria makes their presence obvious by vigorously agitating the erythrocytes in their immediate neighborhoods. *D. immitis* microfilariae tend to remain more or less on place and gradually beat the erythrocytes away, so that if slides are left standing any length of

time, the microfilariae will be found in clear pools of plasma undulating in place without forward motion (Georgi and Georgi, 1992). In contrast, *Dipetalonema reconditum* microfilariae tend to move quite and rapidly and progress across the field. This wet preparation thus supplies an accurate differential diagnosis without recourse to measurements or morphologic criteria (Georgi and Georgi, 1992). The absence of microfilariae is meaningless as it may be due to a low microfilarial concentration. It seems that microfilarial counts of greater than 1000/mL frequently provide a positive diagnosis by wet blood smear technique (Rawlings, 1986). Recently, Courtney and Zeng (2001) reported that the sensitivity of direct smears is very low, when 10 or less than 10 microfilariae/mL are present. Sensitivity generally increased with increasing microfilarial count, and no microfilariae were missed by directed smear when more than 50 microfilariae/mL was present.

Thin and thick blood smear (Hendrix et al., 1985)

Thin blood smear technique used small amount of blood and prepared in the same procedure as a blood smear prepared for a white blood cell differential count. A thin blood smear cannot be used for accurate differentiation of the microfilariae of *D. immitis* from *D. reconditum*. Using small amount of blood is the major disadvantage of this technique, unless the parasites are present in very large numbers, they may be easily overlooked. A thick blood smear allows examination of larger amount of blood than a thin blood smear.

Haematocrit capillary tube (Buffy coat method)

The buffy coat method is a concentration technique for detection of microfilariae in blood samples. The buffy coat is the layer of white blood cell located between the red blood cell and the clear plasma formed by the centrifugation of whole blood. The specific gravity of microfilariae causes them to gravitate to the upper surface of the buffy coat layer. The haematocrit tube was examined under the microscope using 40 – 80 fold magnification between the buffy coat layer and the plasma for the presence of microfilarial activity (Hendrix et al., 1985), or using the buffy coat smear technique according Murray (1977). The haematocrit capillary tube test is quick and inexpensive but frequently it provides some falsely negative diagnosis results (Rawlings, 1986), but the buffy coat smear techniques exhibit a higher sensitivity.

Modified Knott's Technique

One milliliter of venous blood was mixed with 10-ml of 2 % buffered formaline solution and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the sediment was suspended in 1 or 2 drops of 0.1% new methylene blue. The stained sediment was place on microscope slide, a 22-mm square coverslip was added, and the smear was examined under the microscope (Acevedo and Theis, 1982).

Filter technique

One mL of venous blood (with an anticoagulant of either EDTA or heparin) is added to approximately 10 ml of lysate (0.1% sodium chloride solution). This mixture is passed through a filter chamber, placed on a glass microscope slide, stained, and examined under the microscope (Rawlings, 1986).

Serological test

In dog, many cases (5-67%) of heartworm infections could not be detected by microfilaraemia (Brawlings and Calvert, 1995; Barriga, 1997). For the "occult" heart worm infection serological tests are useful for diagnosis. Occult infection in dogs can be caused within the predating period, from unisexual heartworm infections, from drug-induced sterility of adult heartworms and an immune-mediated infection (Rawling et al., 1982). Heartworm serological tests would be important in dogs, that are candidates starting a preventative program and dogs just completing adulticide and microfilaricide treatment should be determined as free of infection prior to the start of a treatment program. In veterinary practice, indirect fluorescent antibody test (IFA), enzyme-linked immunosorbent assay (ELISA), haemagglutination test and immunochromatographic techniques are used to detect dirofilaria antibody or antigen. Antibody detections are affected with several variables and do not provide the current infection, so antibody tests are not accepted as diagnostic method for heartworm infection. In the diagnosis of canine heartworm disease, antigen testing is preferred to the antibody tests due to their higher sensitivity. The heartworm antigen test detects a series of released proteoglycans produced by the uterus of adult female heartworm.

Generally, there are 3 types of commercially heartworm antigen test kits: Immunochromatographic technique, ELISA and haemaglutination techniques.

For the ELISA technique, specific antibodies are attached to the surface of microwell plates. Target antigen binds to the attached antibodies. After washing, a solution containing antigen- specific antibody conjugated to an enzyme is added. This antibody will bind to antigen if present and colorimetric change (Goodwin, 1998).

The other type of antigen test uses antibodies attached to red blood cells. When exposed to the target antibody a visible agglutination of the red blood cells takes place. A recent technique of heartworm antigen test is the immunochromatographic technique. This test uses specific antibodies absorbed on an immunochromatographic strip. Patient serum samples and reagent are added to the strip followed by colloid gold conjugated anti dog antibodies. In positive cases the reaction takes place.

Table1: Interpretation of Knott's technique and antigen test results for *Dirofilaria immitis* (Henry and Dillon, 1994)

Knott's test	Antigen test results	
	Positive	Negative
Positive	*Patents with heartworm infections,	*False positive test result * Low worm burden * Prepatent infection (within the first 6 months) * Immune response removing specific antigen from circulation *Prenatally transmitted microfilariae (no adult worms) * Microfilariae of other filarial worms
Negative	*False positive results, technique error (no heartworm infection) * Mono-gender-specific infection *Prepatent infection *Occult infection *Seasonal and circadial changes in microfilariae number *Drug induced embryostasis of heartworms	* True negative, no adult heartworms * Low worm burden *Only adult male parasites (low number) * Prepatent infection, * Immune mediated spontaneous clearance of antigen

Fecal examination (Sloss et al., 1994; Kaufmann, 1996; Urquart et al., 1996)

Flotation technique

Fecal flotation is the most commonly technique used in veterinary medicine to detect parasitic material of the bowel. The flotation technique is based on the principle that parasite eggs are less dense than the fluid flotation medium. When feces are emulsified in liquids of high specific gravity and either centrifuged or allowed to stand, the worm eggs and many protozonal cysts are floating to the top while the heavy coarse debris settle to the bottom. The top film can be collected for microscopic evaluation. Many different salt substances can be used to make flotation solutions. The saturated sodium chloride solution (specific gravity 1.20) is the most popular used in veterinary practices because it is inexpensive, easy to prepare, and effective in floating common parasite eggs. However, saturated sodium chloride solution cannot float trematode and some tapeworm eggs, and they will destroy delicate protozoan structures.

Direct smear technique

The direct smear is used to identify protozoal trophozoites or other structures that float poorly or readily distorted by flotation solutions. It is possible to demonstrate the presence of parasite stages (egg or larvae of helminths, oocysts of coccidia) by the examination of a thin smear of emulsified feces. Since the sensitivity of this test is very low, it is not recommended for routine fecal examination but primarily used for diagnosis of *Giardia*. A very small amount of feces is mixed with a drop of saline on the glass slide and examined under the microscope.

Sedimentation technique

Sedimentation is used to isolate the operculated eggs of mainly flukes and some other tapeworms and nematodes whose eggs do not float readily in common flotation solutions. In simple sedimentation, tap water is mixed with about 5 – 10 g of feces, pour it through a strainer in a beaker, and fill up water to a level of about 10 – 12 cm. After 4 - 5 min pour off the content up to a level of 2 cm and refill again with water. Repeat the procedure 2 or 3 times until the solution is clear. Mix the sediment with some drops of methylene blue solution, pour it in a petridish and exam it under the microscope or a stereomicroscope by 40 – 80 fold magnification.

PURPOSE OF THE STUDY

The main objectives of this study are as following:

1. To determine the prevalence and incidence density of canine dirofilariosis in Chiang Mai province.

2. To find out the seasonality pattern and other risk factors of the disease in dog population.

The results of those two studies are needed, if the relevant strategic control of *Dirofilaria immitis* in dog population is to be developed.

In addition the blood picture of canine dirofilariosis cases in the study is investigated.