

A PRELIMINARY STUDY ON *IN VITRO* TRANSMISSION OF *DIROFILARIA IMMITIS* INFECTIVE STAGE LARVAE BY *AEDES AEGYPTI* (L.) (DIPTERA: CULICIDAE)

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Abstract. This study was performed to study an *in vitro* transmission of infective stage larvae from the mosquito proboscis. There were five experiments with 949 mosquitoes. Liverpool strain of *Aedes aegypti* (L.) were used in this study. They were allowed to feed on *D. immitis* infected dogs with different microfilarial levels which were 1,650, 1,950, 9,000, 9,250, and 11,550 microfilariae per one ml of blood. Mosquitoes were forced to feed on solution (5% sucrose in 5% dog serum) in capillary tubes for 20 minutes at 7-34 days post-blood feeding. Solutions in capillary tubes then were examined and mosquitoes were dissected and examined for *D. immitis* larvae under a light microscope. Second stage larvae could be found in the abdomen and malpighian tubules of mosquitoes and third stage larvae can be found in the abdomen, malpighian tubules, thorax, and proboscis of mosquitoes with different levels of infection. No larvae were detected in the solution in capillary tubes of all experiments.

INTRODUCTION

Filarial worm infection is an infectious disease that can be found in both humans and animals. In Thailand, filarial worm infection in humans is caused by *Brugia malayi* and *Wuchereria bancrofti* and infection in domestic animals by *Brugia pahangi*, *Dirofilaria repens*, and *Dirofilaria immitis* (Gupbavanij *et al*, 1971; Suvannadabha, 1993). All of these filariae are nematodes and members of the family Filariidae.

D. immitis infection or dirofilariasis is an important disease in dogs which can be found globally. The adult stage of *D. immitis* normally resides in the right ventricle and pulmonary artery of the dog. Infected dogs may show clinical signs such as cough, apnea, and weight loss. In some cases, there is a reaction between antigen released from adult worms and the dog's immune system that can cause filarial glomerulonephritis. *D. immitis* infection in humans is rare but can occur; lesions that can be found include pulmonary nodules, pulmonary granulomas, and subcutaneous nodules (Bailey *et al*, 1990; Monchy *et al*, 1993; Mumtaz *et al*, 2004).

There were few surveillance studies on *D. immitis* infection in animals, humans, and mosquitoes in Thailand (Choochote *et al*, 1987a). Mosquitoes are vectors for *D. immitis*; however, vector competency

of each mosquito species for *D. immitis* may be different and there were few studies on vector competency in Thailand (Choochote *et al*, 1987b). Mosquitoes are important in the *D. immitis* life cycle because first stage larvae (microfilariae) released into the dog's blood stream by female *D. immitis* have to develop into second and third stage (infective stage) larvae in mosquitoes. Dogs could then be infected with *D. immitis* if they are bitten by mosquitoes that harbor infective larvae in their proboscis. Infective larvae in the proboscis of the mosquito will be released with its saliva into small capillaries during the feeding process. This study was performed to test the ability of mosquitoes to release infective stage larva of *D. immitis* by using the *in vitro* method.

MATERIALS AND METHODS

Mosquitoes and mosquito rearing

Mosquito strain. The Liverpool strain of *Aedes aegypti* (Linnaeus) was used in this study. These mosquitoes were originally supported by Department of Parasitology, University of Georgia, USA and they were maintained in mosquito laboratory, Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand. All mosquitoes were maintained in controlled environmental conditions ($28 \pm 2^\circ \text{C}$ and $80 \pm 10\% \text{RH}$).

Hatching eggs. Eggs were first immersed in deoxygenated water in a 500-ml jar at room temperature for 2 hours. Larvae were removed from the deoxygenated water and transferred to a mild nutrient broth.

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Larval rearing. Larvae were reared in lots of 200 in chlorinated-free water in 20 x 30 x 4-cm aluminum pans. Larvae were fed with slurry of finely ground fish food (Tetramin®) and ground mouse chow (1:1). The amount of slurry was not more than larvae could consume in 24 to 36 hours.

Pupal rearing. Pupae were picked up manually from the larval rearing pan. They were transferred into a plastic cup and covered with nylon mesh (50 pupae per cup).

Adult rearing. Adult mosquitoes were hatched within 2-3 days after the pupal stage. Mosquitoes were maintained on a 10% sucrose solution.

Experimental dogs

Two mixed-breed dogs that were naturally infected with *D. immitis* were used in this study. They were kept in laboratory animal housing, Faculty of Veterinary Science, Chalalongkorn University, Thailand.

Microfilaria counting

Infected dog blood. One ml of blood was collected from the cephalic vein during the mosquito feeding period. A three-line smear was made from 20 µl of blood on a glass slide. This smear was allowed to air dry, hemolyzed in distilled water, fixed in absolute methanol, and stained with 10% giemsa stain. The stained slide was then examined and microfilariae were counted under a light microscope.

Blood-fed mosquitoes. Blood-fed mosquitoes were randomly selected after feeding. The mosquito was dissected individually and the blood meal was removed from the mosquito's midgut. It was then mixed with 0.85 % NaCl and smeared on a glass slide. The slide was allowed to air dry, hemolyzed in distilled water, fixed in absolute methanol, and stained with 10% Giemsa stain. The stained slide was then examined and microfilariae were counted under a light microscope.

Vector competence for *Dirofilaria immitis*

Mosquito blood feeding. Two- to 3-day-old mosquitoes were used in this study. Mosquitoes were deprived of sucrose for 24 hours before blood-feeding on an infected dog or imbibing a 5% (w/v) sucrose solution with 5% dog serum in phosphate buffer saline (PBS) contained in capillary tubes (0.1 x 4 cm). The dog was sedated, then placed inside a mosquito cage. Mosquitoes were allowed to feed on the dog for about 30 minutes. Fifty blood-fed mosquitoes were then transferred into each plastic cup and maintained in the mosquito laboratory.

Mosquito testing. Each day, mosquitoes were randomly selected from mosquito cups. Wings and legs were removed, and then they were allowed to feed for about 20 minutes on a 5% (w/v) sucrose solution with 5% dog serum in PBS contained in capillary tubes. Thereafter the mosquitoes were dissected. Each organ of the mosquito and the capillary tube solution were examined for *D. immitis* larvae under a light microscope.

RESULTS

Mosquitoes were allowed to feed on different levels of microfilaria in infected dogs which were 1,650, 1,950, 9,000, 9,250, and 11,550 microfilariae per ml of blood (Table 1). The microfilaria level in the mosquito's midgut after blood feeding was correlated with the microfilaria level in the dog's blood stream (Fig 1).

In this study, second stage larvae were found in the abdomen and malpighian tubules of mosquitoes while third stage larvae were found in the abdomen, malpighian tubules, thorax, and proboscis of mosquitoes with different levels of infection. However no larvae were detected in the solution in capillary tubes of all experiments (Table 1).

DISCUSSION

Aedes aegypti mosquitoes can be found worldwide, including Thailand (Jatanasen, 1967). They are

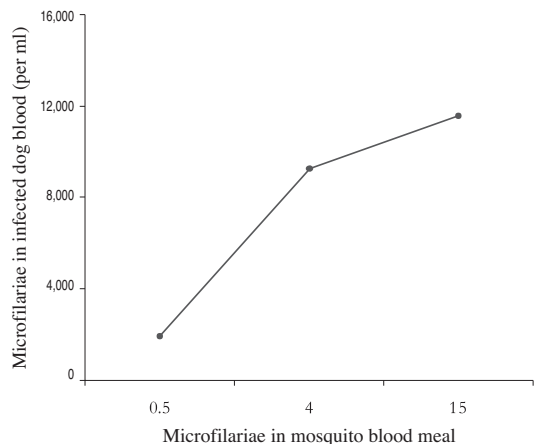


Fig 1- Correlation between microfilaria level in infected dogs' blood stream (per ml of blood) which was the source of blood meal for mosquitoes and mean of microfilaria level in mosquitoes' midgut (per mosquito) after blood feeding.

potential vectors for many pathogens, including viruses, protozoa, and nematodes, especially filarial worms. The Liverpool strain of *Aedes aegypti* was used in this preliminary study on *in vitro* transmission of *Dirofilaria immitis* infective stage larvae because our previous study showed that this mosquito strain is a competent vector for *D. immitis*.

Dissection and examination for the third stage (infective) larvae of *D. immitis* is a routine method that is used for determining the vector competency of mosquitoes for *D. immitis*. However, third stage larvae found in the mosquito will not necessarily be released during the feeding process. Our specific aim in this study was to find another option for determining mosquito vector competency for filarial worms. Infective stage larva released during the feeding process will indicate a vector competency of the mosquito. *D. immitis* was used as a model since it is quite easy to work with and is found in dogs world-wide.

This study was designed by adapting the method that worked very well for studying mosquito transmission of arbovirus, for example, West Nile virus (Goddard *et al*, 2002; Tiawsirisup *et al*, 2004). However no larvae were detected after the feeding process of the mosquito on solution in capillary tubes even though the mosquito had more than 10 infective stage larvae of *D. immitis* in its proboscis. This may suggest that the feeding process for solution in capillary tubes may be different from that in nature, so no larvae were released from the mosquito's proboscis. This method also needs to be confirmed by using other mosquito species.

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Table 1
Percent infection with second and third stage larvae of *Dirofilaris immitis* in the abdomen, malpighian tubules, thorax, proboscis, and saliva of blood-fed *Aedes aegypti* (Liverpool strain) after taking blood meal from infected dogs with different levels of microfilaria (mf).

Exp no.	Blood meal mf (per ml)	Midgut mf	Tested day (PBF)	No. of tested mosquitoes	Percent infection														
					Abdomen			Malpighian tubules			Thorax			Proboscis			Saliva		
					L2 ^b	L3 ^c	L3 ^c	L2	L3	L3	L2	L3	L3	L2	L3	L2	L3	L2	L3
1	9,250	4	14-28	129	1	2	1	0	0	0	0	2	0	0	13	0	0		
2	1,650	ND ^a	14-34	278	0.4	1	0	0	0	0	0	1	0	0	9	0	0		
3	11,550	15	7-17	174	0	0	0	2	0	4	0	4	0	12	0	0	0		
4	1,950	0.5	15-21	185	0	1	0	0	0	1	0	1	0	9	0	0	0		
5	9,000	ND	13-19	177	0	4	1	1	1	0	2	0	0	12	0	0	0		

^aND = Not done; ^bL2 = Second stage larva; ^cL3 = Third stage larva.

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