

SUSCEPTIBILITY OF *MANSONIA UNIFORMIS* TO *BRUGIA MALAYI* MICROFILARIAE FROM INFECTED DOMESTIC CAT

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Abstract. Microfilariae of *Brugia malayi* is transmitted to man and other susceptible hosts via mosquito. The transmission of *B. malayi* from cat to man by *Ma. uniformis* bite has never been reported. The *Ma. uniformis* mosquito is the normal vector for *Wuchereria bancrofti* but has never been reported as a vector for *B. malayi*, or a susceptible host for the growth and development of the microfilariae of *B. malayi*. The purpose of this study was to examine the development of *B. malayi* in *Mansonia uniformis* after feeding on the blood of an infected cat in the laboratory. The *B. malayi* infected cat was identified using PCR with the primers *Bm-1/Bm-2* on DNA (at 10 ng/50 µl) extracted from the WBC of the cat. *W. bancrofti* was employed as a negative control. The sensitivity of the *B. malayi* DNA detection by PCR was 0.0001 ng. Adult *Ma. uniformis* mosquitos at the ages of 5, 10, and 15 days, 100 mosquitos in each group, were fed on the infected cat blood. Recovery of third stage microfilariae was found to be the highest in the 5-day old mosquito group (48%), followed by the 10- and 15-day old mosquito groups (32% and 18 %, respectively). The mean number of *B. malayi* microfilariae found in thorax, head, and abdomen of the mosquitos were composed. The 5-day old (40.3%) and 10-day old (41.9%) mosquitos were significantly more susceptible to microfilariae than the 15-day old mosquitos (17.8%) (p-values using the Scheffe method: 0.027 and 0.039, respectively). There was no significant difference in the mean number of microfilariae in the thorax (p=0.482) by age, but the mean numbers of microfilariae in the heads, and abdomens were significantly different by age between the 5- and 10-, and the 15-day old mosquitos (p<0.001 and p=0.004, respectively).

INTRODUCTION

Lymphatic filariasis has a broad geographic distribution. *Brugia malayi*, zoonotic infection is endemic in Asia, especially in the southern part of Thailand, where the microfilariae are transmitted to man and susceptible hosts via mosquito (mainly *Mansonia bonneae*). The preferred habitat for the *Mansonia* spp is in Southern Thailand, specifically the species which are the vectors for *B. malayi*, such as *Ma. bonneae*, *Ma. dives*, *Ma. uniformis*, *Ma. indiana*, *Ma. annulata*, and *Ma. annulifera*. The 1993 microfilarial blood survey was carried out widely among Thai and Myanmar laborers in the border areas of Rayong

Province, by the Ministry of Public Health. The subjects were mostly laborers in the fisheries, illegal floating houses, rubber plantations, and industries and house keepers. Sixty-six subjects were found to be infected with nocturnally periodic *Wuchereria bancrofti* (Sasa, 1976; WHO, 1995). This same survey was carried out in Phang-Nga, Phuket, and Surat Thani. The results showed that the rate of infection had increased from 1% to 1.13-4.34%. *Brugia* and *Wuchereria* microfilariae usually show nocturnal periodicity in the host peripheral blood, which is an adaptation to the vectors that feed only at night. There are some situations where subperiodic strains of these parasites have adapted to diurnally active vectors (some *Mansonia* and *Aedes* species). Periodic strains of *Brugia* spp and *Wuchereria bancrofti* almost exclusively infect man. Subperiodic strains of *B. malayi* commonly infect domestic cats and various wild carnivores, from which transmission to man is not unusual via diurnally active *Mansonia*

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spp (WHO, 1984).

B. malayi occurs only in South and South-east Asia, where its distribution and prevalence have been reduced by control of the vector, *Mansonia* spp, by the simple method of removal of the host plants from their breeding places. Subperiodic strains occur in swamp and forest habitats, transmitted mainly by *Ma. bonneae* in a zoonotic cycle. Periodic strains of *B. malayi* are also transmitted by *Anopheles* spp, but are usually not zoonotic (Wharton, 1962). Iyengar (1953) first surveyed for filariasis, and found it in Nakhon Si Thammarat, Phatthalung, Pattani, and Surat Thani, provinces in the southern region of Thailand. He found *B. malayi* microfilariae infected 5.2% of human, 21% of whom were symptomatic. Harinnasuta *et al* (1970a,b) and Guptavanij *et al* (1971a,b) found nocturnally periodic and nocturnally sub-periodic microfilaria in human blood. The nocturnally sub-periodic form of *B. malayi* can infect both domestic and wild animals (Buckley and Edeson, 1956; Phantana *et al*, 1978), causing zoonotic disease (Dondero *et al*, 1972). *B. malayi* can infect both humans and animals but *B. pahangi* can only infect animals, especially domestic cats (Thammapaolo *et al*, 1993). The past 25 years of the filariasis control program successful reduced the mosquito vectors. In 1987, 1988, and 1989, 4,745, 3,082, and 3,404 cats were surveyed, respectively. The results showed that 115, (2.42%), 119 (3.86%), and 140 (3.86%) cats, respectively, were infected with microfilariae of *Brugia* species. Iyengar (1953) discovered that *Mansonia* spp were the vectors for Malayan filariasis. A prevalence rate of 3-17% was found by Harinnasuta *et al* (1970b) in Chumphon Province. They found the 0.12% of *Ma. uniformis* and 0.04% of *Ma. annulifera* were vectors for filariasis. In Thailand, during the years 1993-1994, Malayan filariasis cases were found in Surat Thani Province (Division of Filariasis, 1996). The appearance of *B. malayi* in cats was monitored every 2 hours, for a total of 24 hours. The highest peak in parasitemia (149 microfilaria/20 μ l) was found at 12.00 hrs and the lowest (23.9 microfilaria/20 ml) at 14.00 hrs. These findings indicate that transmission is mainly at night, which is the best time for an epidemiologist to study

the parasite and its vectors (Lek-Uthai *et al*, 2004). Our study divided the *Ma. uniformis* into 3 age groups (5 days, 10 days, and 15 days) according to their susceptibility to infective larva (L3) of *B. malayi* in infected cats.

MATERIALS AND METHODS

Mosquitos

Female *Ma. uniformis* mosquitos from the field (Lat Krabang, Bangkok) aged 5, 10, and 15 days were prepared in a keeping room. Each group comprised 100 mosquitos. The mosquitos were reared and maintained at the Faculty of Tropical Medicine, Mahidol University with a 6% fructose and vitamin B complex solution absorbed onto filter paper, and kept at 27°C, 80% relative humidity, with a photo period of 12:12 (light:dark) hours.

Extraction of microfilarial DNA from blood samples

Microfilariae were detected by filtration of 5 ml venous cat blood, along with 15 ml of phosphate buffer saline (PBS), through a polycarbonate membrane millipore (0.5 μ m pore size); 10 of the 65 subjects had microfilaremia (3-68 mf/60 μ l). One ml of their blood was then thoroughly mixed with 800 μ l of phosphate buffer saline (PBS) and centrifuged to pellet red cells and microfilariae. The pellets were centrifuged at 5,000 rpm for 10 minutes. After centrifugation, the pellet was washed in 800 ml of PBS and then carefully resuspended in 200 μ l of DSP buffer (20 mM Tris-HCl, pH 7.6, 2.5 mM MgCl₂, 50 mM KCl, 0.01% proteinase K, 0.5% Tween20®). Incubation in DSP was performed at 42°C for 14 hours to lyse the microfilariae and release the DNA. The proteinase K was then inactivated by incubating the samples at 90°C for 10 minutes. Following brief centrifugation to pellet debris, the supernatant was kept for PCR analysis. Aliquots of each blood sample were kept frozen at -20°C in 0.1 M EDTA until they were processed for PCR analysis. In addition, *W. bancrofti*, *D. immitis*, and *D. repens* were used to confirm the species-specificity of the PCR assay. Blood was also obtained from a human volunteer living in a non-endemic area. These additional blood samples were processed

and amplified by PCR exactly as described for the cat blood samples.

PCR screening for *B. malayi* from infected domestic cat

Infected domestic cats were obtained from the Center for Vector-borne Diseases Control 45, Surat Thani Province, Thailand. The cats were tested to have *Brugia* spp at a concentration of at least 5-microfilariae/60 μ l of blood. They were maintained for 15 days in the laboratory. They were tested by polymerase chain reaction (PCR) to differentiate between *Brugia malayi* and *B. pahangi*.

Polymerase chain reaction conditions

Forward and reverse PCR primers for *Bm-1* and *Bm-2* were designed, the sequences of these primers, which allow amplification of a 280 bp DNA fragment from *B. malayi*, were as follows: primer *Bm-1* (sense strand) (5'-GCG CAT AAA TTC ATC AGC AA-3') and primer *Bm-2* (anti-sense strand) (5'-ATG ACA ACA CAA TAC ACG AC-3'). Reagents for the PCR were obtained from the Perkin-Elmer thermal cycler. The PCR reactions were performed using 50 μ l of the DNA extracts prepared from blood samples as described above. The reagents were used at the following concentrations in a 50 μ l total reaction volume: 10 mM Tris-HCl, pH 9.2, 50 mM MgCl₂ 2 μ l, 10 mM deoxynucleotide triphosphate (dNTPs) 8 μ l, 5 μ l of each primer, and 2 units of *Taq* polymerase. The temperature program for the PCR was 30 cycles of 1 minute at 95°C, 1 minute at 95°C, 1 minute at 72°C, and a final extension of 10 minutes at 72°C. Twenty μ l of the PCR product was loaded onto a 1.5% agarose gel and the unique band of 280 bp was visualized with ethidium bromide stain. There was no correlation between the PCR/hybridization signal and the serum antibody levels. Blood from the *B. malayi* positive cats was again collected for thick blood film with 2 x 3 oval smears and stained with Giemsa. Then the filarial worms were examined, counted and recorded at a magnification of 400 then 1,000 times magnification.

One hundred μ l of blood was drawn from each of the 10 cats and placed into EDTA tubes, and examined for *B. malayi* and *B. pahangi*. The *Bm-1/Bm-2* primer concentrations used to de-

tect *B. malayi* DNA 10, 1.0, 0.1, 0.01, 0.001, and 0.0001 ng. This was when less than 1 microfilariae was detected (Fig 1a). The specificity of *Bm-1/Bm-2* primers for *B. malayi* (Fig 1b), was calculated. The PCR products were examined for 280 bp by electrophoresis with agarose gel and ethidium bromide stain. The results were read under ultraviolet light. The *B. malayi* infected cats were confirmed to have no more than 20 microfilariae/60 μ l, which is the appropriate density for feeding. The cats were then sedated with nembutal sodium solution (50 mg/ml) 1 ml/kg body weight in the abdominal muscle.

Blood feeding

Fifteen hours before feeding on the cats, the fructose solution was replaced with water. One hundred female mosquitos from each group, age 5, 10 and 15 days were then allowed to feed on the cats until they were engorged (20-30 minutes). Fifteen to 20 mosquitos fed separately. The mosquitos were then kept at room temperature (23-29°C) and high humidity (78-80%), and fed on a 6-10% glucose solution with vitamin B complex supplementation.

Dissection of mosquitos and analysis of microfilariae

Fourteen days after infection, the infected

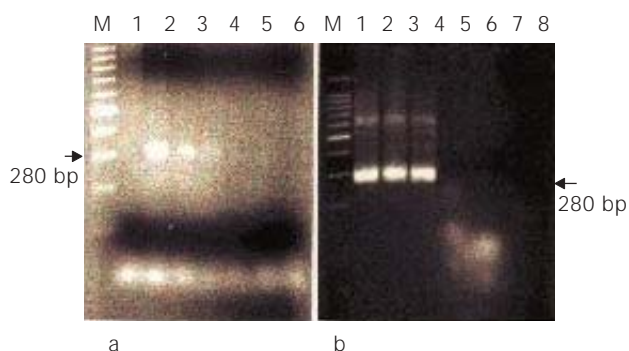


Fig 1-PCR screening for *Brugia malayi* on 1.5% agarose gel by electrophoresis of the PCR products. A result of 280 bp from the *Bm-1/Bm-2* primers was found, giving a sensitivity of less than 1 microfilaria (a) per sample, and a the specificity for (b) *B. malayi* only (lanes 1-3). *Wuchereria bancrofti* (lane 4), *Dirofilaria repens* (lane 5), *Dirofilaria immitis* (lane 6), human DNA (lane 7), negative control (lane 8); M, molecular weight standard ladder.

mosquitos were dissected into 3 parts: head, thorax, and abdomen. Bress' fluid was added to a slide with the dissected parts and microfilariae were identified. Permanent preservation was prepared using mounting media. The number of microfilariae in the head, thorax, and abdominal part were recorded, and then the slides were stained with Giemsa and observed under stereo microscopy.

RESULTS

Collection of adult mosquitos

The microfilariae of *B. malayi* in domestic cats were detected and confirmed by PCR. One hundred and twenty *Ma. uniformis* mosquitos were collected from swamps around Lat Krabang district, Bangkok (Table 1) at 18.00-21.00 hrs. The peak biting time was between 18.00-19.00 hrs (17.4±1.85, $\bar{X} \pm SD$). The lowest biting time was between 20.00-21.00 hrs (0.6±0.8).

Rearing and maintainance of mosquitos

All 120 *Ma. uniformis* mosquitos were fed with 0.2 ml blood after starvation for 12 hours (21.00-9.00 hrs). The blood was from hamsters

sedated with nembotal sodium solution 50 mg/ml. Four to 7 days after the feed the mosquitos laid eggs. When the eggs hatched, the larvae were fed a daily meal of bread yeast, and developed to pupa and adults in 9 days.

Mosquitos population

Of 372, 572, and 588 adult *Ma. uniformis* mosquitos raised in three groups in 12 rearing jars, 268, 196, and 405 female mosquitos were selected, respectively. The female mosquitos were raised until days 5, 10, and 15, respectively, then 100 mosquitos were selected from each of three study groups. The rearing room temperature was kept at 26±3°C with a humidity level of 70-80%.

Microfilariae density

The blood microfilariae of the infected cats were counted. The average number of microfilariae in 60 µl blood was 12.3±1.8. The averages in the 3 groups at 5, 10, and 15 days were 12.7±0.9, 10.7±3.2, and 12.3±2.5 ($\bar{X} \pm SD$), respectively. Table 2 shows the death rates for each of the groups at 14 days after infected blood meal. The percentages of dead mosqui-

Table 1
Collected *Ma. uniformis* mosquitos-human caught.

Man No.	Collected time			Total	X±SD
	18.00-19.00	19.00-20.00	20.00-21.00		
1	19	6	1	26	8.7±7.6
2	20	8	0	28	9.3±8.2
3	16	5	0	21	7±6.7
4	17	6	2	25	8.3±6.3
5	15	5	0	20	6.7±6.2
Total	87	30	3	120	
$\bar{X} \pm SD$	17.4±1.85	6±1.1	0.6±0.8		

Table 2
Death rates of *Ma. uniformis* mosquitos after infected blood meal.

Mosquito age (days)	Microfilariae in cat/60 µl	No. of mosquitos	No. of dead mosquitos	Percent dead
5	12.7±0.9	100	52	52
10	10.7±3.2	100	48	48
15	12.3±2.5	100	51	51
Total	12.3±2.5	300	151	
$\bar{X} \pm SD$	12.3±1.83		50.3±1.7	50.3±1.7

tos in the 3 groups at 14 days after infected blood meal were 52, 48, and 51%, respectively.

Microfilaria development

Table 3 presents the microfilaria densities and infection rates in *Ma. uniformis*. These results show that 5-day old mosquitos were the most susceptible to infection with third stage larvae of microfilaria (Fig 2).

Table 4 shows the numbers and percentages of *B. malayi* third stage microfilariae in *Ma.*



Fig 2—Sheathed microfilariae (arrow) of *Brugia malayi* in dissected mosquitos Giemsa stained at 1,000x.

uniformis mosquitos. The 5- and 15-day old mosquitos had a higher density of *B. malayi* microfilariae in the thoraxes (44% and 79% in 5- and 15-day old mosquitos, respectively) than the heads (32% and 9%, respectively), and abdomens (24% and 12%, respectively). The 10-day old mosquitos were more likely to have the microfilariae in the head (46%) than in the thorax (31%) or abdomen (23%).

Means and standard deviations of *B. malayi* third stage larvae in infected mosquitos among the three age groups dissected into heads, thoraxes, and abdomens were compared. There were significant differences by age between heads and abdomens of the mosquitos ($p=0.0001$, $p=0.004$, respectively), while there were no differences between the thoraxes ($p=0.482$) (Table 5).

There were significant differences between the means for *B. malayi* microfilariae in *Ma. uniformis* mosquitos by head, thorax, and abdomen. *B. malayi* microfilariae developed differently in 5-day old mosquitos than in 15-day old mosquitos in the head parts and the abdominal parts. There were also significant differences between the means for the number of *B. malayi*

Table 3

B. malayi microfilaria densities and infection rates in *Ma. uniformis* for the three groups.

Mosquito age (days)	Dissected mosquitos	Positive mosquitos (3 rd stage larva)	Negative mosquitos	Infective rate (%)
5	48	34	14	70.8
10	52	32	20	61.5
15	49	18	31	36.7

Table 4

Numbers and percentages of *Ma. uniformis* mosquitos infected with *B. malayi* microfilariae by age group and bodily location.

Mosquito age (days)	Infected 3 rd stage larva mosquitos	Dissected part with microfilariae			Total (%)
		Head (%)	Thorax (%)	Abdomen (%)	
5	48	58 (32)	79 (44)	44 (24)	181 (100)
10	32	87 (46)	58 (31)	43 (23)	188 (100)
15	18	7 (9)	63 (79)	10 (12)	80 (100)

Table 5

Comparison of the means and standard deviations of *B. malayi* third stage larvae in infected mosquitos among three aged groups by dissected into heads, thoraxes, and abdomens of mosquitos.

Mosquito age (days)	No.	$\bar{X} \pm SD$ of microfilariae		
		Head ^a	Thorax ^b	Abdomen ^c
5	48	1.2±1.4	1.6±1.9	0.9±1.3
10	52	1.7±1.7	1.1±2.1	0.8±1.4
15	49	0.1±0.6	1.3±2.6	0.2±0.5

^aSignificant difference at F=10.139, df=148, p=0.0001; ^bSignificant difference at F=0.733, df=148, p=0.482;

^cSignificant difference at F=5.769, df=148, p=0.004

Table 6

Comparison of third stage *B. malayi* microfilariae among the three groups of mosquitos by head, thorax, and abdomen.

Mosquito age (days)	Aged group (days)	p-value (Scheffe' method)		
		Head	Thorax	Abdomen
5	10	0.414	0.493	0.925
	15	0.012 ^a	0.728	0.010 ^a
10	5	0.414	0.493	0.925
	15	<0.0001 ^a	0.929	<0.0001 ^a
15	5	0.012 ^a	0.728	0.012 ^a
	10	<0.0001 ^a	1.929	0.024 ^a

a=significant difference

microfilariae in the 3 different age groups between the heads and abdomens.

DISCUSSION

Our results show that *Ma. uniformis* mosquitos are susceptible to infection with *B. malayi* microfilariae. Guptavanij *et al* (1978) found that *Ma. uniformis*, with an infection rate of 0.02, was a vector of periodic *B. malayi* in Pattani, a southern province of Thailand. They found that *Ma. bonneae* and *Ma dives*, with infection rates of 0.18 and 0.20, respectively, were the vectors of *B. malayi* in Narathiwat Province, where microfilarial periodicity is in the subperiodic form. The Institute for Medical Research (1983), Malaysia (Wharton, 1962) and the WHO (1984) reported that nocturnally subperiodic *B. malayi* is transmitted from man to dogs, cats, and monkeys. Guptavanij *et al* (1971a,b) revealed that *B. malayi* in infected man could be transmitted to cats and

probably from infected cats to man (Lek-Uthai, 2004), but the periodicity changed from nocturnally periodic to nocturnally subperiodic. In 1989, Chiang *et al* (1991) compared five strains of *Ma. uniformis* in Malaysia. They found that susceptibility to infection with subperiodic *B. malayi* ranged from 62% to 100%, and there were no significant differences between the means in the five strains of mosquitos. The susceptibility rates were directly related to the microfilarial densities of the cats at the time of feeding. Saratapeian *et al* (2002) studied *Ma. indiana*, which were collected from a non-endemic area for human lymphatic filariasis, and tested for their susceptibility to infection using nocturnally subperiodic *B. malayi*. Three naturally infected cats were used for the experiments. The data revealed that the susceptibilities of the mosquitos ranged from 30% to 70%. The results also showed that the susceptibility rates were not linearly correlated with the microfilarial densities in the cat at the

time of feeding. The microfilarial densities in cats ranged from 15 to 27 per 10 μ l of blood whereas the mean number of third stage larvae in the infective mosquitos ranged from 21.6 to 26.8. This indicates that *Ma. indiana*, collected from non-endemic areas is capable of transmitting nocturnally subperiodic *B. malayi*.

We found the highest numbers of third stage microfilariae in the 10-day-old mosquito group, 41.9% of dissected mosquitos. There were 40.3% and 17.8% in the 5- and 15-day old mosquitos, respectively. The susceptibilities of the *Ma. uniformis* mosquitos to *B. malayi* varied by age group. *B. malayi* microfilariae preferred to develop in the thorax more than the head and abdomen in the 5- and 15-day old mosquitos. Whereas they preferred the head in the 10-day old mosquitos. Chiang *et al* (1991) studied the susceptibility of *Culex tritaeniorhynchus*, *Cx. vishnui*, and *Cx. gelidus* mosquitos to *B. malayi* in Malaysia. They found that the control mosquitos, which were *Ma. uniformis* and *Aedes togoi*, were highly susceptible to subperiodic *B. malayi*, with infection rates of 86.4-100% and 80-89.2%, respectively. Large numbers of infective third stage larvae (>98% of the total developing larvae) were also recovered. They observed that the average number of larvae, in both *Ma. uniformis* and *Ae. togoi*, increased as the microfilarial density of the host increased. There were active microfilariae in the gut of the *Culex* mosquitos but these all died. Trpis (1981) studied the susceptibilities of 4 different species of mosquitos to *B. malayi* and *B. pahangi*. These mosquitos belong to the Tonga group of *Ae. scutellaris*. He found that all tested strains were fully (100%) susceptible to infection with both parasites. He found that when the development of the third stage larvae in the thoracic muscles was complete, the larvae migrated predominantly to the abdomen. Kumar *et al* (1998) studied *Ma. annulifera* and found that this *Mansonia* species is highly susceptible to *B. malayi* parasites. The experimental infection index of this species was relatively high (2.74). The extrinsic incubation period was estimated to be 8 days. Chansiri *et al* (2002) surveyed 53 feline blood samples from the endemic area of Surat Thani and Narathiwat, southern provinces of Thailand; they found that 15 of the domestic cats

were infected with *B. malayi*. They also examined human blood using Giemsa and acid phosphatase stained blood films, PCR - the RFLP profile of the *Hha I* repeat gene and PCR amplification of the Trans-Spliced Leader Exon I (SLX). They revealed that domestic cats play an important role as animal reservoir for *B. malayi* in endemic areas of Thailand.

Our study revealed that *B. malayi* can infect vector mosquitos in any area. We found significant differences between the different age mosquito groups. Filariasis infection can be transmitted via *Ma. uniformis* mosquito. Based on our results, we conclude that *Ma. uniformis* is important for *B. malayi* transmission in the southern provinces of Thailand. To incriminate a species of mosquito as a vector for *B. malayi*, it is necessary to show that naturally caught specimens contain infective microfilariae. For further study, we propose the study of the high percentage of mosquito deaths after blood feeding on *B. malayi* infected domestic cats, in spite of the proper density of microfilariae in the cat blood.

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