EXPERIMENTAL INFECTION OF SUBPERIODIC *BRUGIA MALAYI* IN LABORATORY RATS WITH EMPHASIS ON EVALUATION BY FOUR TECHNIQUES

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INTRODUCTION

The use of cats, dogs and monkeys for experimental studies on *Brugia malayi* is expensive and the animals are more difficult to maintain and to handle. Attempts to maintain the parasite in small laboratory rodents have had limited success (Edeson *et al.*, 1958, 1962; Laing *et al.*, 1961; Zaini *et al.*, 1962; Ahmed, 1967a, b; and Cheong *et al.*, 1967); until Ash and Riley (1970a) successfully obtained patent infections in mongolian gerbils (*Meriones unguiculatus*). Attempts were made to establish this parasite in a variety of other rodents, but only a limited successful result was obtained (Ash and Riley, 1970b; Sivanandam *et al.*, 1975; Crandall *et al.*, 1981; and Cruickshank *et al.*, 1983). The results of transmission *B. malayi* to laboratory white rats (Sprague-Dawley, SD) and black-white rats (Long-Evans, LE), and evaluation by four experiments including xenodiagnostic test, histochemical staining, and measuring spicules and rectal protuberances in confirming the species are reported herein.

MATERIALS AND METHODS

Experimental animals: Fifteen white rats (Spraque-Dawley strain) and 36 black-white rats (Long-Evans strain) only males were used. The animals were examined repeatly over 4 weeks to ensure that they were negative for microfilaremia.

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Parasite and vector: The subperiodic *B. malayi* used was originally obtained from cats from South Kalimantan (Borneo), Indonesia and maintained in the U.S. Naval Medical Research Unit No. 2 laboratory since 1971 (Cross *et al.*, 1984) transmitted to gerbils (*M. unguiculatus*) and was the source of microfilaria for the study. Three species of laboratory raised *Aedes togoi*, *Ae. aegypti* (Liverpool strain) and *Armigeres subalbatus* mosquitoes were used. The first two species were used for establishing laboratory colony, and the last one for the experimental vector.

Mosquito infections: Infected gerbils with a microfilaria density 50-100/20 c.mm blood were restrained with wire netting and allowed to be bitten by *Ae. togoi* mosquitoes in the cage (28 cm^3) for about 1-2 hours. The exposed mosquitoes were provided with 5% sugar solution in paper cups and placed in screened wooden escape-proof cases, and maintained in an insectary (room temp. 80-88 °F and relative humidity 70-94 %). After 9-14 days, the mosquitoes were crushed with a glass tube under ice anesthesia, and placed in Earle's balance salt solution (pH 6.8-6.9) in a Baermann funnel. The infective larvae (L_3) rapidly migrated out of the fragmented mosquitoes and settled to the bottom of the funnel. They were drawn off and counted for experimental infections by the method described by Ash and Riley (1970a).

Rat infections: Sprague-Dawley (SD) and Long-Evans (LE) rats were syringe-inoculated into the groin areas with 50-100 infective larvae (L_3) of *B. malayi*, according to Ash and

Riley (1970a), and examined for microfilariae (mf) by the membrane filtration technique of 0.8 ml of tail venous blood at weekly intervals starting 10 weeks after inoculation. When an animal was found positive for the mf, 8 quantitative (20 c.mm) thick blood smears were made and stained with Giemsa solution (5%).

Some rats died and others killed by sodium pentobarbital anesthesia at various times. At necropsy, the entire body were examined for worms and mf. The worms recovered were fixed in hot 70% alcohol and placed into 5% glycerine-alcohol, cleared in glycerine evaporation and mounted in glycero-gel medium. The worms and the spicules were measured by the method described by Wang and Fan (1983).

Periodicity studies: Periodicity investigations were based on the examination of 20 c.mm blood smears taken from five rats at 3-hourly intervals for 48 hours. In addition, weekly blood examination after the first appearance of microfilariae in one rat was also taken; 20 c.mm blood smears for determination of mf fluctuation for 49 weeks.

Xenodiagnostic test: In this experiment, three species of *Ar. subalbatus*, *Ae. togoi* and *Ae. aegypti* (Liverpool strain) were used, the first species was for the experimental vector, and the others for the control. They were divided each into two groups, one group in three species of mosquitoes fed on a LE rat infected with *B. malayi* 5 mf/20 c.mm blood); and the another group of three species, fed on a SD control rats infected with *B. pahangi* (50 mf/20 c.mm blood); and dissected individually 9-14 days after exposure for detecting the L_3 and their numbers also counted under stereoscopic microscope for determining their susceptibility. This method was described by Edeson *et al.*, (1960).

Histochemical staining: The thick blood smears were prepared, some were collected from the LE rat infected with *B. malayi*, and the remainder collected from the SD rat infected with *B. pahangi*. These smears air-dried, fixed in absolute acetone, at 4°C for one minute, then stained with naphthol-AS-TR-phosphate according to method of Barka and Anderson (1963), and examined for comparison of the characteristic distribution of acid phosphatase activity in both species of *Brugia* microfilariae.

Measurements of rectal protuberances: *Aedes togoi* female mosquitoes used were divided into two groups, the first group of mosquitoes fed on a LE rat (No.36) infected with *B. malayi*, the second group fed on a SD rat (No. 55) infected with *B. pahangi*. These were dissected and examined for detecting the rectal protuberances from day 1 to day 9 after feeding. The rectal protuberances were found and measured, their length and width as well as the distance from the protuberance to the end of the L_1 and L_2 of both species of *Brugia* mf, was as described by Beckett *et al.*, (1972).

Measurements of spicules: Six adult males of *B. malayi* recovered from the LE rats and 3 adult males of *B. pahangi* collected from the SD rats, were carefully measured by the method of Wang and Fan (1983). The length of both left and right spicules and their ratio (Lt/Rt) between the two species of *Brugia* male worms were compared.

RESULTS

Susceptibility of *B. malayi* in rats: Table 1 shows the results of experimental infections in 51 (36 LE and 15 SD) rats. Microfilaremiae were found in 7 (4 LE and 3 SD) rats, the mf. positive rate was 14% (11% for LE rats and 20% for SD rats). The mean prepatent period was 143 (110-161) days for LE rats and 106 (99-112) days for SD rats.

Twenty six (20 LE and 6 SD) rats were killed, and 18 (12♀ and 6♂) adult worms were

Table 1

Susceptibility of laboratory rats to subperiodic *Brugia malayi* following subcutaneous inoculation.

Rat strain (Sex)	No. of rats inocul.	No. of L_3 inoculated	Rat for microfilariae				Rat for adult worms				Total posit. (%)
			No. exam.	No. pos.	mf/20 c.mm*	Prepat. period (days)	No. exam.	No. pos.	No.& sex	Locality	
SD ♂	6	300	6	1	34	112	5	3	6(3♀, 3♂)	testes	4
SD ♂	9	630	9	2	6, 8	99, 106	1	0			2
Total	15	930	15	3	48	99-112	6	3	6(3♀, 3♂)	,,	6
				(20.0)**	(16)	106		(50.0)			(40.0)
LE ♂	6	300	6	1	24	148	ND				1
LE ♂	13	910	13	2	6,34	110, 161	10	3	9(6♀, 3♂)	,,	4
LE ♂	12	960	12	0			9	1	3(3♀)		1
LE ♂	5	500	5	1	6	153	1	0			1
Total	36	2,670	36	4	70	110-161	20	4	12(9♀, 3♂)	,,	7
				(11.1)	(17.5)	143		(20.0)			(19.4)

SD = Sprague-Dawley rat.
LE = Long-Evans rat.
ND = Not done.

* No. of mf/20 c.mm blood at peak count.
** Mf. positive rate and/or worm positive rate in parenthesis.

Table 2

Microfilariae of subperiodic *B. malayi* at peak and last counts and patent infection in seven infected rats.

Rat strain-No.	Original infection from	No. of L_3 inoculated	Prepatent period in days	Microfilariae/20 c.mm			Days from inoculat. to necropsy
				Peak count (days)	Last count (days)	Known duration (days)	
LE-1	Gerbil	50	148	24(288)	6(378)	378	530*
LE-17	Gerbil-SD-2	70	110	34(414)	15(685)	703***	824
LE-18	Gerbil-SD-2	70	161	6(185)	2(200)	208***	380
LE-27	Gerbil-LE-18	100	153	6(295)	2(425)	425	610*
			(143)	(18)**	(6)(422)	(438)	(597)
SD-2	Gerbil	50	112	34(164)	10(236)	236	377*
SD-9	Gerbil	70	106	6(264)	2(543)	543	749*
SD-10	Gerbil	70	99	8(241)	3(285)	285	418*
			(106)	(16)	(5)(355)	(355)	(515)

* Died naturally.
** Mean number in parenthesis.
*** The L_3 were found in *Aedes togoi* mosquitoes 10 days after feeding on the infected rat (No. SD-2).

Table 3

Measurements of subperiodic *Brugia malayi* recovered from Long-Evas (LE) and Sprague-Dawley (SD) rats.

Rat strain	Sex, No. of worms meas.	Age of worms (days)	Body length (mm)	Body width (micron)		
				Mid-esophagus	Joint of eso. & int.	Mid-intestine
Long-Evans	Male 3	380	13.5-17.2 (14.9)*	68.8-71.3 (70.2)	70.0-73.8 (72.1)	73.8-81.3 (76.7)
	Female 3	365	26.6-28.4 (26.9)	95.0-98.8 (97.1)	97.5-1p1.3 (99.6)	102.5-105.0 (103.8)
Sprague-Dawley	Male 3	343-379 (361)	17.5-19.5 (18.4)	73.8-78.8 (75.8)	77.5-81.3 (78.8)	81.3-90.0 (i85.5)
	Female 3	343	31.0-39.0 (33.6)	87.5-117.5 (105.3)	91.3-120.0 (108.0)	107.5-140.0 (126.3)

* Mean in parenthesis.

recovered only in the testes of 7 (4 LE and 3 SD) rats, giving a worm positive rate of 37% (20% for LE rats and 50% in SD rats).

Prepatent and patent periods: Five (2 LE and 3 SD) rats died and 2 LE rats were killed. The tail blood of 7 rats was examined weekly starting 10 weeks after inoculation. The mean prepatent period in 4 LE rats was 143 (110-161) days, and those in 3 SD rats was 106 (99-112) days. The microfilaremia lasted 422 (200-685) days in LE rats, and 355 (236-543) days in the SD rats; the mean patent period was 438 (208-703) days in former and 355 (236-543) days in the latter (Table 2).

Microfilarial periodicity: Counts of mf in 20 c.mm blood at 3-hourly intervals over a period of 48 hours in 2 LE rats (NO. 17 and 36) and 3 SD rats (No. 2, 9 and 10) were made. Comparatively, the mean number of 30 and 21 mf at peak count per 20 c.mm blood films were detected at 6:00 and 12:00 AM in SD and 2 LE rats respectively.

Weekly microfilarial counts: The LE rat (No. 1) inoculated with 50 L_3, 5 mf were first found in 20 c.mm blood smear on day 148, and counted mf weekly until the rat died for a period of 50 weeks. The mf level was low and varied from 3 to 24 per 20 c.mm blood.

Measurements of *Brugia malayi* adult worms: Table 3 shows that 6 (3♀ and 3♂) adult worms were recovered from 2 LE rats on day 365 and 380 after infection. The mean length and width at mid-esophagus, joint of esophagus and intestine, and mid-intestine of 3 males was 14.9 mm, 70.2 μ, 72.1 μ and 76.7 μ respectively; and those of 3 females were 26.9 mm, 97.1 μ, 99.6 μ and 103.8 μ respectively. While, 6 (3♂ and 3♀) adult worms recovered from 3 SD rats on day 343 and 379, the corresponding figures of the males were 18.4 mm, 75.8 μ, 78.8 μ and 85.5 μ; and those of the females were 33.6 mm, 105.3 μ, 108.0 μ and 126.3 μ respectively. In contrast, both sexes of worms were apparently bigger in the latter than in the former. It also identified that the worm development was better in the SD rats than in the LE rats.

Susceptibility of mosquitoes to *Brugia* sp. by xenodiagnositic test: In these experiments, 365 *Ar. subalbatus*, 124 *Ae. aegypti* and 289

Ae. togoi were used. The first species was employed as the test group, and the other two species as the control. All three species of mosquitoes were divided into two parts, the first part including 229 *Ar. subalbatus*, 69 *Ae. aegypti* and 159 *Ae. togoi* fed on the LE rat (No. 36) infected with *B. malayi* (5 mf/20 c.mm blood), and the second part 136, 55 and 130 mosquitoes fed on SD rat (No. 55) infected with *B. pahangi* (50 mf/20 c.mm blood) respectively. On dissection, all 26 *Ar. subalbatus* mosquitoes exposed to *B. pahangi* became infected (100%), but none of 185 *Ar. subalbatus* mosquitoes exposed to *B. malayi* were infected (0%). In control group, susceptibility was much higher in *Ae. aegypti* (100%) and *Ae. togoi* (87%) exposed to *B. pahangi* than those exposed to *B. malayi* (41% and 57%) respectively.

Comparison of *Brugia* sp. microfilariae by histochemical staining: Forty thick blood smears including 20 from the LE rat (No. 36) infected with *B. malayi*, and 20 from the SD rat (No. 55) infected with *B. pahangi*, were air-dried, fixed in absolute acetone at 4°C, then stained with napthol-AS-TR-phosphate according to method of Barka and Anderson (1963). The characteristic distribution of acid phosphatase acitivity was demonstrated. The excretory and anal pores of *B. malayi* mf were more prominant sites of red azo dye precipitation (stained deep red), and each could be seen easily; the remaining part of the body exhibited practically no enzymatic activity (stained less red). In contrast, *B. pahangi* mf showed heavy diffuse acid phosphatase activity (stained deep red) along the length of the body. The excretory and anal pores, however, still recognizable because of thier more intense staining.

Spicules of *Brugia* sp. male worms: Six subperiodic *B. malayi* and 3 *B. pahangi* male worms recovered from 2 LE rats and 1 SD rat respectively were compared. The length of both left and right spicules was prominantly longer in *B. malayi* (345-384 μ and 116-136 μ with a ratio of 2.9:1) than those in *B. pahangi* (180-220 μ and 72-99 μ with a ratio of 2.3:1) respectively.

Rectal protuberances of *Brugia* sp. larvae: Both species of *Brugia* larvae were collected from *Aedes togoi* mosquitoes from 1-9 days after feeding on infected LE rats, then killed with 5% formalin solution and examined immediately under steroscopic microscope for the features of rectal protuberances. These were found clearly in all 1st- and 2nd-stage larvae (L_1 and L_2) on day 2 to day 8, but none was found either on day 1 and on day 9 or in the 3rd-stage larvae. In contrast, the rectal protuberances was significantly larger in the length and the width of *B. malayi* larvae (5.06-7.93 μ and 3 28-4.87 μ) than those of *B. pahangi* larvae (3.71-5.05 μ and 3.19-3.43 μ) respectively. The distance from the rectal protuberance to the end of the tail was shorter in the former (4.17-8.31 μ) than those in the latter (5.58-12.29 μ).

DISCUSSION

In the present experiments, both LE and SD rats that have been infected with subperiodic *B. malayi* became patent infections. However, the levels of microfilariae were low (6-34 mf/20 c.mm blood) and the prepatent periods were longer (99-153 days) compared to those reported from cats. Unfortunately, a number of rats died of other causes before necropsy, thus necropsy could not be made. All recovered worms were completely mature, but most were single sex infection.

Edeson and Wharton (1958) were the first to attempt to infect rodents with subperiodic *B. malayi*, failing with two white mice and one guinea-pig. Laing *et al.*, (1961) stated occasional successful transmission of subperiodic *B. malayi* to golden hamsters and white rats; although the prepatent periods were over 120 days, the white mice were resistant

to infection. These workers failed to infect white rats, mice and hamsters with the periodic form of *B. malayi*. Zaini *et al.*, (1962) was successful in transmission of subperiodic *B. malayi* to hamsters, but failed to infect guinea-pigs and rabbits. Hamsters were not good laboratory hosts as cats or cotton rats. Edeson *et al.*, (1962) obtained patent infection with this species in hamsters and cotton rats. The prepatent periods ranged from 93 to 124 days in hamsters and from 92 to 98 days in cotton rats. Ahmed (1967a) failed to infect guinea-pigs and rabbits with subperiodic *B. malayi* by removal of the spleen, by irradiation and by treatment with 6-mercaptopurine. In later studies (1967b), he was able to produce patent infections in 2 of 12 splenectomized white rats, the prepatent. period was 180 and 193 days respectively. Cheong *et al.*, (1967) obtained patent infections in white rats infected with subperiodic *B. malayi* with prepatent period over 100 days. Ash and Riley (1970b) reported *Meriones uniguiculatus*, *M. libucus*, *Neotoma lipida* (wood rat), 3 *Dipodomys merriami* (Kangaroo rat) and hamsters infected with L_3, and patent infections were produced in hamsters. *M. unguiculatus* developed patent infections, but the mf density in gerbils remained significantly lower than those reported for *B. pahangi* (Ash and Riley, 1970a).

Sivanandam *et al.*, (1975) reported *Rattus muelleri* (giant wild rat) and *R. sabanus* infected with subperiodic *B. malayi;* although they can develop to adults, they are poor hosts. These rats, therefore, are probably not important in the zoonotic transmission of subperiodic *B. malayi* in Malaysia. Crandall *et al.*, (1981) reported that with inbred strains (CB, LSH, LHC, PD4 and MHA) of hamsters each infected with 200 subperiodic *B. malayi*, patency in all strains was detected between 80-100 days postinfection, adult worms were recovered from all strains of hamsters, but the level of infection was low, only 2.5-6.5% of the infecting dose. Cruickshank *et al.*, (1983) used inbred PVG (-RTIC) rats of different RTI haplotype were also susccessfully infected the human parasite of *B. malayi*, 33% becoming patent infection. It was suggested that the inbred and nude rats may provide a valuable model to study human filariasis.

In the present experiments both the LE and SD rats have been infected with both species of *Brugia;* the mf infection rate and mf density were higher in *B. pahangi* than those in *B. malayi*, which are in accordance with Ash and Riley's report (1970b). The periodic pattern of both species of *Brugia* mf are similar, but, the morphological features of mf infective larvae, and adult worms are difficult for diagnosis and differentation. Definitive identification was confirmed by sensitive and critical experiments, including xenodiagnostic test, histochemical staining, spicule and rectal protuberance measurements.

In the above experiments, all dissected *Ar. subalbatus* mosquitoes were completely refractory to subperiodic *B. malayi*, but another group of *Ar. subalbatus* mosquitoes were highly susceptible to *B. pahangi*. These results are in agreement with previous reports (Edeson *et al*, 1960; Wharton, 1962; Cheong *et al.*, 1965). Thick blood smears of both species of *Brugia* mf stained with naphthol-AS-TR-phosphate, showed that the excretory and anal pores of subperiodic *B. malayi* exhibited acid phosphatase activity, and only a little activity was seen in the remainder of the body. *B. pahangi* mf showed heavy diffuse acid phosphatase activity along the entire length of body as reported by Redington *et al.*, (1975). The mean length of left and right spicules with a ratio, were much longer in subperiodic *B. malayi* mf than those in *B pahangi* mf, as in previous reports (Schacher, 1962; Ash and Riley, 1970a, b; Guptavanij *et al.*, 1971). The rectal protuberances of both 1st-and 2nd-stage larvae (L_1 and L_2) from day 2

to day 8 were significantly larger and also closer to the posterior end of subperiodic *B. malayi* than those of *B. pahangi*. These results conform with those of Beckett and Macdonald's report in 1971.

Based on the above successful experiments and results obtained, it can be concluded that both LE and the SD rats are suitable hosts of subperiodic *B. malayi*.

SUMMARY

Infective larvae of subperiodic *B. malayi* from South Kalimantan (Borneo), Indonesia collected from laboratory-raised *Ae. togoi* mosquitoes after feeding on infected mongolian gerbils (*Meriones unguiculatus*) were inoculated subcutaneously into the groin areas of 15 SD and 36 LE rats. Blood was examined weekly by membrane filtration and thick smears starting 10 weeks post-infection. Microfilariae were found in 3 SD and 4 LE rats, the mf infection rate of 20% and 11% respectively. The prepatent period was significantly shorter in the SD rats (99-112 days) than those in the LE rats (110-153 days). The patent period was longer in the LE rats (208-703 days) than in the SD rats (236-543 days), and the mf density was similar (17.5 mf/20 c.mm blood against 16 mf/20 c.mm blood).

At necropsy, 6 (3♀ and 3♂) adult worms were recovered from 3 of 6 SD rats and 12 (9♀ and 3♂) adult worms from 4 of 20 LE rats; all worms were found in the testes.

The results of xenodiagnostic, histochemical staining and measuring spicules and protuberances, demonstrated clearly the difference between both species of *Brugia*. All dissected *Ar. subalbatus* mosquitoes exposed to *B. pahangi* became infected (100%), but none of those to subperiodic *B. malayi* were infected (0%). The mf of both species of *Brugia* in thick films stained with naphthol-AS-TR-phosphate showed that the excretory and anal pores of subperiodic *B. malayi* mf exhibited acid phosphatase activity and only a little activity was seen in other parts; while *B. pahangi* mf showed heavy diffuse acid phosphatase activity along the entire length of the body. Six subperiodic *B. malayi* and 3 *B. pahangi* male worms recovered and measured showed both left and right spicules were much longer in the former (360 μ and 127 μ with ratio of 2.9 : 1) than those in the latter (207 μ and 91 μ with ratio of 2.3 : 1). The rectal protuberances were found in both species of *Brugia* L_1 and L_2 on day 2 to day 8; the length and width were significantly larger in subperiodic *B. malayi* (5.06-7.93 μ and 3.28-4.87 μ) than those in *B. pahangi* (3.71-5.05 μ and 3.19-3.43 μ); but the distance of protuberance to the tail was longer in *B. pahangi* (5.58-12.29 μ) than in subperiodic *B. malayi* (4.17-8.31 μ).

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