Laboratory and field studies of some entomological aspects of the canine dirofilariasis problem in Japan¹⁾

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INTRODUCTION

In Japan, as in many areas where canine dirofilariasis occurs, little has been done to determine the true importance as vectors of indigenous mosquito species assumed to be involved in the epidemiology of this disease. The studies reported in this paper were conducted to obtain at least a small amount of basic entomological information on dirofilariasis in Japan. Specific objectives were to determine: (1) *Dirofilaria immitis* infection rates among individuals of seven species of culicine mosquitoes, and one anopheline species allowed to feed on infected dogs in the laboratory; (2) Whether there was a correlation between age of the host mosquito and either infectivity rate with *D. immitis* or development of the parasite in the intermediate host; (3) Effects, if any, of treatment of an infected dog with a filaricidal drug on infectivity of microfilariae in the dogs' blood for mosquitoes, or on subsequent development of *D. immitis* larvae in the mosquito host; (4) Correlation of experimental infection rate data found in laboratory studies with *D. immitis* infection rates

in mosquitoes captured and dissected in the field in a region of high endemicity for dirofilariasis.

MATERIALS AND METHODS

All mosquitoes used in feeding experiments were from colonies maintained at the 406th Medical Laboratory. The eight species involved were: *Aedes aegypti*, *Aedes albopictus*, *Aedes togoi*, *Anopheles sinensis*, *Armigeres subalbatus*, *Culex fatigans*, *Culex pipiens pallens*, and *Culex tritaeniorhynchus*. The *Aedes aegypti* were of the "Denken" strain obtained from the Institute for Infectious Diseases, University of Tokyo and had originally been established with specimens from the United States. The *Culex fatigans* colony had been established with specimens collected at Naha, Okinawa. The remainder of the colonies had been established with material from Japan. One of these, the *C. tritaeniorhynchus* colony, had been maintained continuously at the 406th Medical Laboratory since 1956.

Adult mosquitoes used in feeding experiments were held in 18 inch square screened cages. The single experimental feeding on a dog was the only blood meal for each mosquito. Additional nourishment was provided by sliced apples suspended on a wire, and by sugar water in a soaked cotton pad placed on top of each cage. Temperature in the insectary varied from 75-80 degrees F. Humidity fluctuated from 70-80%.

In most cases an 18 inch square cage was used for feeding mosquitoes on dogs. The top and three of the sides of the cage were screened. The bottom was of plywood, and the front of the cage was covered with a stockinet sleeve. During feeding periods the shaven hind quarters of the dog were placed in the cage. Feeding periods for each of the three species of *Aedes*, *Armigeres subalbatus*, and *Culex tritaeniorhynchus* commenced at 1330 hours and were of

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30–60 minutes duration. Specimens of *Culex fatigans*, *Culex pipiens pallens*, and *Anopheles sinensis*, which showed a marked reluctance to feed during the daytime, were given overnight feedings. This was done by holding the dog in a small animal cage which was placed inside a larger screened cage. Overnight feedings commenced at 1630 hours and terminated at 0800 the next morning. Starting and finishing hours were chosen only because they coincided with duty hours of insectary personnel. At the end of the feeding period engorged mosquitoes were removed and held in the 18 inch square cages previously described for 12 days. At the end of this period the mosquitoes were dissected and examined for presence of *Dirofilaria immitis* larvae. Dissection technique was as described by Kartman (1953) except that the saline used was lightly tinted with methylene blue. Records were kept of the number and location of *D. immitis* larvae in each mosquito.

The single treated dog used in feeding experiments was a mongrel male obtained from the Yokohama dog pound. The animal was approximately three years of age, and weighed 51 pounds at the beginning of the study. In spite of a rather high microfilarial count the dog was in good physical condition.

Blood for microfilarial counts was drawn from the radial vein of the forelimb between 1300–1330 hours on the day of examination. Counts were made of microfilariae in each of two wet preparations of 20 cmm. Microfilarial counts for this animal prior to treatment varied between 50–75 mf/20 cmm of blood. Counts were made on the day the blood was drawn, and on the day mosquitoes were fed on the animal.

The filaricide used in treatment of this dog was a commercially produced preparation containing diethylcarbamazine as the active ingredient. The minimum effective dose recommended by the manufacturer was administered. This was 30 mg/lb body weight daily for 21 days. The daily medication was given in three equal doses orally at 0800, 1200, and 1600 hours. The microfilarial count recorded each day was the mean of the counts from the two 20 cmm preparations.

Specimens of *Aedes togoi* of 5–10 postemergence days in age were allowed to feed on the treated dog in the manner previously described.

Aedes togoi was chosen for this test because of high infectivity rates obtained in earlier studies. This mosquito is found in Japan, Korea, Northern China and Siberia and in some areas have been suspected as a vector of human filariasis.

During April and May of 1966 a field survey of incidence of *D. immitis* microfilaremia in dogs, and of *D. immitis* larvae in wild-caught mosquitoes was carried out at Kochi City, Shikoku and adjacent areas. This region was chosen because preliminary studies in October, 1965 had shown that *Aedes togoi*, which had been reported by Inoue (1937) and Kume (1958) as a highly receptive host for *D. immitis*, was numerous in the area and because spring weather conditions were more suitable for mosquito surveys in this area than in the Kanto plain.

Dogs examined during the survey were from three main sources: (1) kennel dogs (commercial or fighting); (2) privately owned dogs kept at residences, and (3) dogs in an animal shelter at Kochi. Some of the privately owned animals were from the sea-side area; some from in and around Kochi City, and others from nearby wooded foothills.

Data recorded for each dog included physical description (particularly with respect to hair length) age, sex, past history of infection, and for privately owned animals, information as to whether the dog was allowed to run free at any time of the day or was kept outdoors or inside the house at night.

RESULTS AND DISCUSSION

Results of experimental feedings of mosquitoes on an infected dog are summarized in Table 1. The evident superiority of *Aedes togoi* as a host is not surprising in view of similar results obtained by Inoue (1937). This mosquito was also described as a suitable host for *D. immitis* by Kume (1958), who used it for production of infective *D. immitis* larvae for his studies of prophylactic therapy against *Dirofilaria* infection in dogs. Results obtained by other workers with *Aedes aegypti* and *Culex fatigans* have shown considerable variation. Although Rosaria (1936) found *C. fatigans* in Manila highly susceptible to infection, and Travis (1947) reported an experimental infection rate of 23% in *C. fatigans* on Guam, Rosen (1954) wrote that, although some larvae of *D.*

Table 1 Occurrence of *Dirofilaria immitis* larvae in mosquitoes of eight species dissected 12 days following a blood meal on an infected dog

Species	No. examined	No. negative	No. with non-infective larvae only	No. with both non-infective and infective larvae	No. with infective larvae only
<i>Aedes</i>					
<i>albopictus</i>	64	63	0	0	1
<i>aegypti</i>	92	73	7	3	9
<i>togoi</i>	107	3	1	22	81
<i>Culex</i>					
<i>fatigans</i>	36	35	0	0	1
<i>pipiens pallens</i>	33	32	0	0	0
<i>tritaeniorhynchus</i>	144	139	2	2	1
<i>Armigeres</i>					
<i>subalbatus</i>	12	12	0	0	0
<i>Anopheles</i>					
<i>sinensis</i>	9	0	9	0	0

immitis completed development in *C. fatigans* in the laboratory, only one of more than 1,000 wild caught *fatigans* in a highly endemic area in French Oceania was infected. This single specimen harbored immature larvae. While Rosario (1936) also reported that *Aedes aegypti* in Manila was quite susceptible to infection, Travis (1947) found that only two percent of *aegypti* allowed to feed on an infected dog developed infective stage larvae. Kartmann (1953) infected both *aegypti* and *fatigans* during his studies of factors affecting infection of mosquitoes with *immitis*, and concluded that *aegypti* is a generally refractory host for *D. immitis*, but that in some regions there are specially susceptible individuals from which, through a process of selection, a population may arise that shows a superior host efficiency and a high incidence of infection.

Studies to determine the relative effectiveness as hosts for *D. immitis* of *A. togoi* of different age groups were undertaken because of the work of Duxbury, Moon, and Sadun (1961). These workers found that more larvae of a related species, *Dirofilaria uniformis*, developed in specimens of *Anopheles quadrimaculatus* 12-13 days old than in mosquitoes 4-5 days old. Similarly, Desowitz and Cellapah (1962) maintained that in dealing with *Brugia* sp. in *Culex fatigans*, the older the mosquito the better it was as a host. Jordon, however, (1962) who questioned these previous findings, found no significant differences in numbers of *Wuchereria*

bancrofti larvae in *Culex fatigans* of different age groups, provided that all third-stage larvae present were counted—not just those in the proboscis.

While our results, shown in Table 2, clearly demonstrate high infectivity of *D. immitis* for mosquitoes in each of the four age groups tested, the small numbers of mosquitoes involved necessitate caution in predicting which of the four age groups involved would actually be most effective as hosts for *D. immitis*.

Fluctuations in microfilaria density per 20 cmm of blood of a dog during a 21 day period of treatment with a filaricidal drug are shown in Figure 1. The same figure shows fluctuations in numbers of infective larvae of *D. immitis* developing in mosquitoes, *Aedes togoi*, fed on the dog on ten of the days during the period of treatment. This study was undertaken to determine whether microfilariae in peripheral blood of a dog under treatment with a filaricidal drug would remain infective for mosquitoes, or whether, as reported by Manson (1952) during his work with *Wuchereria bancrofti* in the Fiji Islands, treatment of the host would so modify the microfilariae that they would lose capability of normal development in the mosquito.

Our results were much like those of Rosen (1955) who reported that *W. bancrofti* microfilariae from the blood of persons previously treated with Hetrazan developed as readily in *Aedes polynesiensis* as did microfilariae from blood of untreated individuals.

Table 2 Location and numbers of infective *Dirofilaria immitis* larvae in experimentally infected mosquitoes, *Aedes togoi*, of four age groups*

	Age Groups			
	5-7 days	10-12 days	15-17 days	20-22 days
No. examined	26	55	21	5
No. infected	25	53	21	5
Average No. larvae/mosquito in :				
labium	6.85	8.69	10.60	5.00
Malpighian tubules	0.42	0.27	1.53	0.40
haemocoel	5.38	1.95	5.48	1.00
Total : Average No. larvae/mosquito	12.65	10.91	16.61	6.40

* Days after emergence from pupal stage.

Fig. 1. Fluctuation in *Dirofilaria immitis* microfilaria density in a dog under treatment with a filaricidal drug during a 21 day period, and in numbers of infective *immitis* larvae developing in mosquitoes, *Aedes togoi*, fed on the dog on 10 days during the same period. The microfilaria density is expressed as per cent of the count on the day treatment was started. This was 650 mf/20 cmm. of blood. The infective larva count is expressed as the average number of infective larvae found in mosquitoes fed on the dog on each day indicated and examined 12 days thereafter.

Although the dog at first seemed to tolerate the drug well, it became ill on the third day of treatment and regurgitated the medication. This was responsible for the abrupt rise in microfilaria density shown in Figure 1 for days 4 and 5. Failure of the infective larval count in mosquitoes to decline proportionately with the microfilaria density was not unexpected. Hawking and Worms (1961) reviewed studies of the quantitative relationships between the number of microfilariae in the blood and the number taken up by the vector. They sum-

marized that the actual uptake is extremely variable, and is usually less than might have been expected from the volume of blood ingested, although *Culex fatigans*, feeding on patients with *W. bancrofti* in Malaya seemed to take up three times as many microfilariae as would have been expected from their blood meal. They concluded that there was no evidence of a chemotropic factor which attracts microfilariae to the bite. In instances where mosquitoes ingested less microfilariae than might have been expected, they theorized that this might be caused by a variation in the number of microfilariae present in individual cutaneous capillaries, or to feeding of mosquitoes from a pool of blood which was escaping from capillaries, the erythrocytes escaping more rapidly than microfilariae. After the 13th day of medication it was much easier to demonstrate infection in the mosquitoes than in the dog. The infectivity of very small numbers of *D. immitis* microfilariae for mosquitoes could be of considerable practical importance in control efforts. Hawking and Worms (1961) described a similar situation in which *Aedes polynesiensis* were fed on *W. bancrofti* patients with a microfilaria density of only one microfilaria/50 cmm of blood. About five percent of these mosquitoes became infected, each mosquito containing only a single larva. Similarly, 10 % of specimens of *Mansonia longipalpis* fed on patients with only one microfilariae/40 cmm of blood became infected. Each of these mosquitoes harbored only one infective larva. Rosen (1955) concluded that any density of microfilariae likely to be detected on the examination of a single specimen of 20

cmm of blood was infective for *Aedes polynesiensis*.

Although the dog used in our feeding experiments lost 13 pounds during the first five days of treatment, it showed gradual improvement in its physical condition, and no further weight loss following its illness on the third day of treatment. Survival rates of mosquitoes fed on the dog during treatment days 1-7 varied between 60-80 %. It dropped to 38 % among mosquitoes fed on the dog on the ninth treat-

ment day, but thereafter rose gradually. The survival rate was 100 % among mosquitoes fed on the dog two days following termination of treatment.

Results of microfilaremia surveys among dogs at Kochi, Shikoku and vicinity are shown in Tables 3-6. Although data obtained were grouped according to nature of the "housing" of dogs examined, and according to the degree of restraint to which they were subjected, the only factor, other than age, which showed a

Table 3. *Dirofilaria immitis* microfilaremia in dogs of four age groups in the vicinity of Kochi, Shikoku, Japan

Age in years	No. examined	No. infected	% infected
1	7	0	0.0
1 and 2	69	8	11.6
3, 4, and 5	57	13	22.8
6 and over	29	15	51.8
Totals :	162	36	22.2

Note: Data given above were obtained by questioning owners.

Eight of 41 dogs (19.5 %) of undetermined age and origin from the Kochi dog pound were infected.

Table 4. *Dirofilaria immitis* microfilaremia among 162 dogs from three habitats at Kochi, Shikoku, Japan

Habitats*	Dog examinations		
	No. examined	No. with microfilaremia	% with microfilaremia
Seaside	52	5	9.6
Urban Kochi	56	7	12.5
Wooded suburban foothills	54	24	44.5

Note: The seaside area was 1,000-2,500 meters from urban Kochi. The wooded suburban foothills were 7,000-10,000 meters from urban Kochi. The average age in years of dogs at the seaside in urban Kochi and in the suburban foothill area was 4.2, 3.7, and 3.9 respectively.

Table 5. *Dirofilaria immitis* microfilaremia among 203 dogs of four categories with respect to housing at Kochi, Shikoku, Japan

"Housing" status	Dog examinations		
	No. examined	No. with microfilaremia	% with microfilaremia
Dog house	73	16	21.9
Kennel (commercial or private but at least with several dogs)	53	12	22.2
Owner's home	28	6	21.4
No home (pound dogs) or strays	49	10	20.4

Note: Several of the animals were fighting dogs raised specifically for this sport, which is popular in the Kochi area.

Table 6. Incidence of *Dirofilaria immitis* microfilaremia among dogs subjected to varying degrees of restraint at Kochi, Shikoku, Japan

Degrees of restraint	Results of dog examinations		
	No. examined	No. with microfilaremia	% with microfilaremia
Allowed to run free			
Daytime only	5	0	0.0
Nighttime only	8	2	25.0
Day and night	62	11	17.0
Never	128	31	24.2

high degree of correlation with microfilaremia incidence was habitat. The relatively high incidence (44.5 %) of infected dogs in the wooded foothill area was in marked contrast to the much lower rates found in the two other habitats. This undoubtedly reflects the greater abundance and variety of mosquito vectors in the foothill area. Although Lindsey (1961) found that prevalence of *D. immitis* did not vary according to sex or hair length of dogs examined, Wallenstein and Tibola (1960) had found *immitis* infections in male dogs four times as frequently as in females. In our own survey 27 (28.2 %) of 96 male dogs examined were infected, while only 17 (15.9 %) of 107 female dogs were parasitized. Seven (14.0 %) of 50 long haired dogs were infected, and 37 (24.2 %) of 153 short haired dogs were positive for *immitis* microfilaremia.

Dissection and examination of 1,813 adult mosquitoes of seven species obtained in light traps, and in resting and biting collections in the vicinity of dog kennels was rather unrewarding as only eight infected mosquitoes were found. These results are summarized in Table 7.

The most prevalent mosquito in hand and

light trap collections in the seaside area was *Culex fatigans*. Four (0.62 %) of the 649 specimens dissected in this area contained *D. immitis* larvae. In three of these specimens infective larvae of *immitis* were present in the labium. One mosquito contained four larvae, one two, and the third a single larva. The fourth positive specimen contained two early third stage larvae.

Other adult mosquitoes collected and examined in this area were *Armigeres subalbatus* (84); *Anopheles sinensis* (41); *Anopheles sineroides* (1); and *Aedes togoi* (10). None of these was infected. In the beach area numerous larvae of *Culex fatigans* were found in man-made containers such as tin cans, water storage tanks, and manure pits. *Armigeres subalbatus* larvae were also found in manure pits. A few *Aedes togoi* larvae were found in a water container about 1,000 meters from the shore. A single *C. tritaeniorhynchus* larva was also found in this area.

Light trap, and resting and biting collections of adult mosquitoes in urban Kochi yielded 219 specimens of *Culex fatigans*, 22 of *Anopheles sinensis*, 1 of *Anopheles sineroides* and 31 of

Table 7. Occurrence of third stage larvae of *Dirofilaria immitis* in mosquitoes of seven species at Kochi, Shikoku, Japan

Species	No. examined	No. positive	% positive
<i>Culex fatigans</i>	982	5	0.51
<i>Culex tritaeniorhynchus</i>	43	0	0.00
<i>Armigeres subalbatus</i>	140	0	0.00
<i>Aedes albopictus</i>	99	0	0.00
<i>Aedes togoi</i>	349	3	0.86
<i>Anopheles sinensis</i>	197	0	0.00
<i>Anopheles sineroides</i>	3	0	0.00
Total	1,813	8	

Aedes albopictus. One of the *C. fatigans* examined contained two infective *immitis* larvae in the labium. All other mosquitoes examined were negative. *Culex fatigans* larvae were abundant in a variety of man-made containers in urban Shikoku.

Adults of seven species of mosquitoes were collected in the wooded foothill area 7,000–10,000 meters from urban Kochi. The included *Culex fatigans* (114); *C. tritaeniorhynchus* (43); *Armigeres subalbatus* (56); *Aedes togoi* (339); *Aedes albopictus* (68); *Anopheles sinensis* (134); and *Anopheles sineroides* (1). This was the only area in which *Aedes togoi* was the most abundant species taken. Three of the *A. togoi* contained infective larvae of *D. immitis*. One specimen contained 17 infective larvae in the labium, two in the head capsule, and one early third stage larva in the Malpighian tubules. The second positive mosquito contained a single infective stage larva in the labium. The third contained four infective larvae in the labium. The stomach of one of 24 recently engorged *Culex fatigans* collected in the area contained seven living microfilariae. All other mosquitoes were negative.

Throughout the foothill area *Aedes togoi* larvae were numerous in water containers, together with larvae of *C. fatigans*. Larvae of *Anopheles sinensis* were numerous in rice paddies in the area. *Armigeres subalbatus* larvae were found in polluted water in many places.

Apparently little has been published concerning incidence of *D. immitis* larvae in wild caught mosquitoes. In his surveys in French Oceania Rosen (1954) found infective stage larvae of *immitis* in only two of 2,390 specimens of *Aedes polynesiensis* and eight of his 446 *Culex annulirostris* captured near animal shelters in a region of high (40%) endemicity for dirofilariasis. None of 1,061 *Culex fatigans* examined by him was infected. A somewhat higher percentage of infected wild caught mosquitoes was reported by Phillips (1939) who found four of 21 *Anopheles punctipennis*, 2 of 10 *Anopheles quadrimaculatus*, and a single *Aedes excrucians* taken with sausage-shaped larvae of *D. immitis* in the Malpighian tubules. These mosquitoes were collected in unscreened kennels harboring infected dogs. After the dogs were removed, no further mosquitoes were found.

SUMMARY

1. Tests were conducted to determine susceptibility of eight species of mosquitoes to infection with microfilariae of *Dirofilaria immitis*. Highest infection rates and largest numbers of infective larvae were found in specimens of *Aedes togoi* fed on an infected dog and examined 12 days thereafter. Other species tested were *Culex pipiens fatigans*, *Culex pipiens pallescens*, *Culex tritaeniorhynchus*, *Aedes albopictus*, *Aedes aegypti*, *Armigeres subalbatus*, and *Anopheles sinensis*.

2. There were no outstanding differences in *D. immitis* infection rates among *Aedes togoi* of four age groups allowed to feed on an infected dog.

3. Microfilariae in peripheral blood of a dog under treatment for dirofilariasis during a 21 day period remained infective for mosquitoes, *Aedes togoi*, fed on the dog on 10 days during the course of treatment. Decline in numbers of microfilariae in blood of the dog was much more rapid than decline in numbers of infective *immitis* larvae in mosquitoes which had been fed on the dog and dissected 12 days thereafter.

4. In surveys of microfilaremia among dogs at Kochi, Shikoku the factors which showed most striking correlation with high incidence of infection with *D. immitis* were age and habitat. Dogs from wooded foothills outside Kochi City proper showed a much higher microfilaremia rate than those from urban Kochi and from a seaside area near the city. Short haired dogs showed an infection rate nearly twice that of long haired animals, and males were infected nearly twice as frequently as females.

5. Only eight of 1,813 adult female mosquitoes of seven species obtained in light traps, and in resting and biting collections in the vicinity of dog kennels at the seaside, urban Kochi, and wooded suburban foothills 7,000–10,000 meters from urban Kochi were infected with larvae of *Dirofilaria immitis*. Five of these were specimens of *Culex fatigans*, the remaining three were examples of *Aedes togoi*. Four of the infected *C. fatigans* were collected at the seaside area, and one was found in urban Kochi. The three infected specimens of *Aedes togoi* were found in the wooded foothills, the

only area where this mosquito was most numerous.

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摘 要

日本における犬糸状虫についての
室内実験および野外実験

井上 (1937) は *Dirofilaria immitis* の中間宿主として *Aedes togoi* の重要性を報告した。この研究は彼の結果を追試するために行なわれた。そして、井上が報告する如く *Aedes togoi* が供試した蚊の中では最上位をしめし、この関係は高知における野外試験での結果においても同じである。