[Jpn. J. Parasitol., Vol. 39, No. 5, 425–430, October, 1990]

Occult Infection of *Dirofilaria immitis* in Stray Dogs Captured in Hyogo Prefecture, Japan

SHOJI UGA[1], TAKEO MATSUMURA[1], KAZUMI ISHIBASHI[2], KENJI YATOMI[2], YUKIYOSI YODA[3] AND NOBUMASA KATAOKA[4]

(Accepted for publication; August 17, 1989)

Abstract

A study on occult *Dirofilaria immitis* infection was conducted with a total of 310 stray dogs captured in Hyogo Prefecture. It was found that 182 out of 310 (58.7%) dogs were positive for *D. immitis* adult worms. These positive dogs formed the basis for a detailed study to examine the number and sex of the adult worms in the heart and to determine the presence of mf in the blood.

The cases of occult infection, that is, dogs with adult worms in the heart but without circulating mf accounted for 24.7% of the positive dogs. These are 14.8% of unisexual infection, 1.6% of sterility resulting from immaturity, aging, or drugs, and 8.2% of immune-mediated infections (complete). In 6 cases (3.3%), mf were found in the blood but in extremely small numbers compared with other dogs infected with roughly the same number of adult *D.immitis*. For these cases, examination of the levels of anti-cuticular antibody to mf were detected so that these 6 cases should also be considered as a type of immune-mediated occult infection. These are probably instances of a incomplete or transitional occult infection. The total incidence of an immune-mediated occult infection thus amounted to 11.5% (complete and incomplete) of the positive dogs.

Key words: *Dirofilaria immitis,* heartworm, occult infection, dog, IFA, epidemiology

Introduction

Dirofilaria immitis, known as canine heartworm, is a mosquito-borne filarial nematode most commonly found in dogs. Studies conducted in Japan have revealed that *D. immitis* is the most prevalent species among helminths in dogs and is considered one of the particularly important species of canine parasites (Ohishi, 1986; Kagei, 1988).

When a dog is infected with *D. immitis,* microfilariae (mf) usually appear in the vascular system. However, some dogs do not become positive for mf in blood even though they were parasitized by adult worms. This phenomenon is known as occult or amicrofilaremic infection. Two distinct mechanisms are responsible: 1) lack of mf production, and 2) prompt destruction of mf (Gillis *et al.*, 1984). Occult infection of *D. immitis* has been reported to vary from 10% to 67% in dogs (Otto, 1978; Grieve *et al.*, 1986; Thilsted *et al.*, 1987). Grieve *et al.* (1986) have pointed out that this frequent occurrence of occult infection leads to a low estimation of the incidence of *D. immitis* infection in hyperenzootic areas. All areas in Japan except for certain parts of Hokkaido and Okinawa, are hyperenzootic areas of the heartworm with infection rates of 20–60% (Ohishi, 1986).

However, no precise studies have yet been

[1] *Department of Medical Zoology, Kobe University School of Medicine, Kobe 650, Japan.*
[2] *Animal Administration Office of Hyogo Prefecture, Hyogo Prefecture 673-14, Japan.*
[3] *Itami Health Center of Hyogo Prefecture, Hyogo Prefecture 664, Japan.*
[4] *Kobe University School of Allied Medical Sciences, Kobe 654-01, Japan.*
宇賀昭二　松村武男（神戸大学医学部医動物学教室）
石橋三美　矢冨謙治（兵庫県動物管理事務所）
依田行能（伊丹保健所）
片岡陳正（神戸大学医療技術短期大学部）

made to determine the actual state of occult infection. Therefore, the authors have conducted a detailed survey of occult infection by *D. immitis* in stray dogs in Hyogo Prefecture, Japan and analyzed the cause of the occult infection.

Materials and Methods

The study was conducted over a total of 11 months from May, 1989 through March, 1990. Of the dogs committed to the Animal Administration Office of Hyogo Prefecture, 310 dogs with estimated ages of over 1 year were examined for infection with *D. immitis*. Ages were estimated from their appearance as well as the degree of dental development. The study was commenced by taking approximately 2ml of peripheral blood from the dogs without anesthesia. One-ml blood was used for an indirect fluorescent antibody test (IFA), and the remaining 1ml was used for examination for mf. The dogs were killed by euthanasia, examined for sex and body weight and the heart and lungs were removed and examined for the presence of *D. immitis* adult worms in the right ventricle and/or pulmonary arteries (in the heart).

Mf examination

Since mf are frequently present in lungs, when no mf is found in the peripheral blood, two different methods were used for mf detection in the present study. These were the acetone concentration method and the stamp method. For the former method, peripheral blood was collected from the forelegs of dogs by vein puncture. The latter method, peace of lung (2cm × 2cm) obtained from dogs immediately after sacrificed was used. The density of mf in the lungs was recorded semiquantitatively. It was graded as follows; −: absence of mf; +: 1–2 mf in the specimen; ++: 3–200 mf and +++: more than 200 mf. Some of the adult females recovered from the dogs were examined for mf development in the distal part of the uterus.

Antibody examination

The IFA technique was used to assay the levels of antibody to the mf surface (cuticular) antigen. In the present study, the mf recovered from the uterus of the adult female were used as antigen. The mf were embedded in an investment compound JB-4 (Polysciences Inc., U.S.A.), sectioned with a microtome, and placed on a slide glass. The first reaction was allowed to take place by being exposed to 2-fold stepwise diluted (1:4–1:512) serum at 37°C for 60 min under a humidified condition. After repeated washing in PBS, the second reaction was conducted at a 50-fold diluted fluorescein-conjugated rabbit anti-dog IgG (Cappel Laboratories Inc., U.S.A.). Conditions for the second reaction were identical to those of the first reaction. The specimens were examined for specific fluorescence on mf surface under a fluorescent light microscope.

Results

Table 1 shows the prevalence of *D. immitis* infection among stray dogs in Hyogo Prefecture. Of the 310 dogs, 182 (58.7%) were infected with *D. immitis* adult worms. Infection rates for male and the female dogs were 63.3% and 54.4%, respectively which was not statistically significantly different ($P > 0.05$). The highest count, 117 adult worms (51 males and 66 females), was obtained from a dog that weighed 15 kg and was 10 years old.

The 182 *D. immitis*-positive dogs were then examined for the presence of mf and both sexes of adult worms (Table 2). The results showed that 75.3% (group 1) of the positive dogs were found to have both sexes of *D. immitis* in the heart and mf in the blood. The remainder of 24.7% (groups 2–5) had adult worms in the heart but no mf in the blood and were therefore occult infection. Occult infection cases consisted of 14.8% (groups 2–3) which were infected with only one sex of *D. immitis*. So that these two groups were not attributable to immune-mediated occult infection. The proportion of the positive dogs in which mf were totally absent although they were infected with both sexes of adult worms, amounted to 9.8% (groups 4–5). Further examination showed that one dog out of the three of group 4 had immature adult worms, judging from their size (1 male and 3 females). *D. immitis* recovered

Table 1. Infection with *Dirofilaria immitis* in stray dogs in Hyogo Prefecture

Sex	No. of dogs examined	No. of dogs* positive (%)	Number of adult worms / dog	
			min–max	mean
Male	153	97 (63.3)	1 – 87	18.6
Female	157	85 (54.4)	1 –117	14.2
Total	310	182 (58.7)	1 –117	16.5

* Positive for infection by autopsy.

Table 2. Proportion of microfilaremic and amicrofilaremic infections in dogs infected with adult *Dirofilaria immitis*

Group	Adult		mf			No. of dogs (%)**
	M	F	blood	lung	uterus*	
1	+	+	+	+***	ND	137 (75.3)
2	+	–	–	–	ND	17 (9.3)
3	–	+	–	–	–	10 (5.5)
4	+	+	–	–	–	3 (1.6)
5	+	+	–	–	+	15 (8.2)

* Presence of mf in the uterus of female worms.
** In all, 182 dogs were examined.
*** Three out of 137 dogs were negative by the pulmonary stamp method.

from the remaining two dogs (13 males, 3 females; 2 males, 1 female) were considered fully developed based on size. Although the parasites were fully developed, mf were not found in the uteri of the adult females (Fig. 1b). This gave an 8.2% incidence of dogs infected with male and female *D. immitis* and with evidence of intrauterine mf formation, but without any evidence of mf in the blood (group 5).

The 137 dogs from group 1 were checked for mf using both the acetone concentration method and the pulmonary stamp method (Table 2). Sensitivities (% of *D. immitis* positive cases which were also positive by the test) of the acetone concentration method and stamp method were 100% and 98%, respectively.

Figure 1a shows intrauterine mf of an adult worm recovered from an occult infection dog (group 5 of Table 2). Though most of these were the embryonated egg stage, mf at distal part of the uterus were detected. Figure 1b shows a case (group 4 of Table 2) in which no uterine mf development was observed.

Table 3 compares number of adult worms, with number of mf in blood and lung, and IFA (Fig. 2) titer. Group 0 were control dogs with no

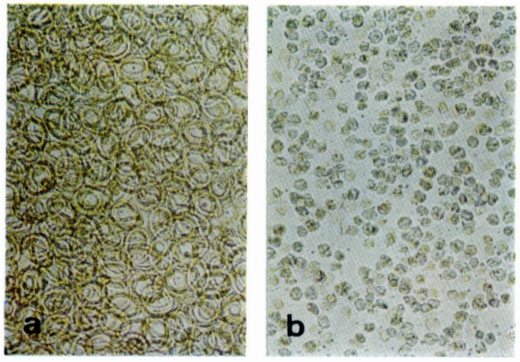

Fig. 1 Specimens from adult female worm of *D. immitis*. Development of embryonated eggs at the terminal end of the uterus were observed in (a) and not in (b).

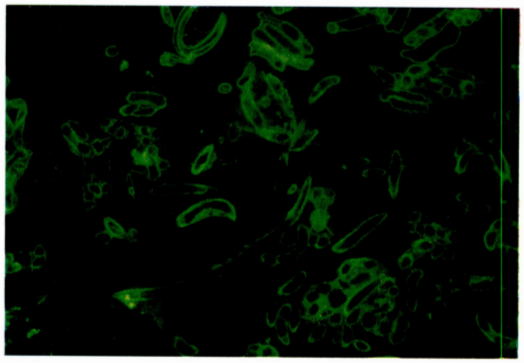

Fig. 2 IFA-positive example. Specific reactions were observed on the surface of mf.

Table 3. Results of IFA test for antibody to *Dirofilaria immitis* microfilariae

Group	Dog number	No. of adults M	No. of adults F	No. of mf blood/10 μl	No. of mf lung	IFA titer
	9	0	0	0	−	1: 8
0	99	0	0	0	−	1: 16
	309	0	0	0	−	1: 16
	30	6	12	440	+++	1: 16
1a	140	14	12	254	+++	1: 32
	252	18	13	396	+++	1:128
	16	2	3	5.2	+	1:128
	21	4	2	0.2	+	>1:256
1b	46	1	5	0.2	+	1:256
	126	1	2	0.3	+	1:256
	23	51	66	0	−	>1:256
	48	8	2	0	−	>1:256
6	213	7	9	0	−	1:256
	276	11	8	0	−	1:256

adult worms, no mf and a low titer. Group 1a dogs had adult worm infection, high levels of mf and low titer. Group 1b dogs had both sexes of adult worm infection, low levels of mf and titers $\geqq$1:128. Group 6 dogs had adult worm infections no mf detected and titers of $\geqq$1:256.

Discussion

The aims of the present study were to determine the incidence and causes of occult *D. immitis* infection in stray dogs. Of 310 dogs examined, 182 (58.7%) were positive for *D. immitis* adult worms by autopsy. Among the 182 positive dogs, 75.3% showed mf in the blood. Ohishi (1986) has demonstrated that *D. immitis* mf accumulates in the lung. In our experiments, both the peripheral blood and the lung were examined for mf. The results showed that while the blood assays had a 100% sensitivity, that of

the lung was 98%. It is therefore concluded that for future studies, it will be possible to obtain adequately reliable results by determining the mf only by the acetone concentration method which is easier than the pulmonary stamp method. Twenty-four point seven percent of the positive dogs were found to have occult infection, that is, *D. immitis* infection without circulating mf. This phenomenon was reported by Webber and Hawking (1955), Otto (1978), Grieve *et al.* (1986) and Thilsted *et al.* (1987).

Rawlings *et al.* (1982) have classified occult infection into the following four types: unisexual heartworm infection (type 1), drug-induced sterility of adult heartworm (type 2), prepatent infection (type 3) and immune-mediated infection (type 4). These four types are now compared with our results shown in Table 2.

Type 1 corresponds to the groups 2 and 3, amounting to a 14.8% share of the positive dogs. Two dogs from group 4 (1.1%) corresponded to type 2 from the view point of sterility. Though Rawlings *et al.* (1982) referred only to the effects of drugs to account for type 2, sterility due to aging is also a possibility. Type 3 corresponds to the remaining dog of group 4, representing an incidence of 0.5%. In Japan transmission of *D. immitis* is restricted to a given period from June to October (Ohishi, 1986, Konishi, 1989). When we consider the prepatent period of the heartworm, occult infections of type 3 can be observed from November through to March. In the present study, young adults were recovered from one dog which was sacrificed on December. Prior to November, it is very difficult to detect immature worms, because the larvae migrate throughout the body of the dog.

A number of immune tests have been conducted to establish an immunological involvement in occult infection (Wong and Suter, 1979; Gillis *et al.*, 1984; Tamashiro *et al.*, 1985; Grieve *et al.*, 1986; Thilsted *et al.*, 1987). If the immune-mediated occult infection is defined as "infection with both sexes of adult *D. immitis* in the total absent of circulating mf despite the presence of mf in the uterus", it corresponds to group 5 and represents 8.2% of the positive dogs. From the mf count of the peripheral blood and from the results of the IFA analysis, group 1b is incomplete occult infection and is probably a transition from full infection to immune-mediated occult infection and presents 3.3% of infected dogs. Future studies will have to clarify whether such transitional occult infection will progress to a complete occult infection or whether it will run its course without further change.

Thus, in Hyogo Prefecture 58.7% of stray dogs were infected with *D. immitis* and 24.7% of positive dogs had occult infection. The main causes of occult infection were immune-mediated, representing 11.5% of the infected dogs (both complete and incomplete) and unisexual infection (14.8%).

Acknowledgments

The authors thank Prof. Isamu Ohishi, Faculty of Agriculture, Tokyo University of Agriculture and Technology, for his helpful advice and encouragement.

References

1) Gillis, J. M., Smith, R. D. and Todd, K. S. (1984): Diagnostic criteria for an enzyme-linked immunosorbent assay for occult heartworm disease: Standardization of the test system in naturally exposed dogs. Am. J. Vet. Res., 45, 2289–2292.
2) Grieve, R. B., Glickman, I. T., Bater, A. K., Grieve, M. M., Thomas, C. B. and Patronek, G. J. (1986): Canine *Dirofilaria immitis* infection in a hyperenzootic area: Examination by parasitologic findings at necropsy and by two serodiagnostic methods. Am. J. Vet. Res., 47, 329–332.
3) Kagei, N. (1988): Human dirofilariasis and its occurrence in Japan. Jpn. J. Vet. Med., 41, 621–629 (in Japanese).
4) Konishi, E. (1989): *Culex tritaeniorhynchus* and *Aedes albopictus* (Diptera: Culicidae) as natural vectors of *Dirofilaria immitis* (Spirurida: Filariidae) in Miki City, Japan. J. Med. Entomol., 26, 294–300.
5) Ohishi, I. (1986): *Dirofilaria immitis*. From the view point of parasitology. Buneido, Tokyo, 27–139 (in Japanese).
6) Otto, G. F. (1978): The significance of microfilaremia in the diagnosis of heartworm infection. Proceedings Heartworm Symposium. Edwardsville, Kan: Vet. Med. Pub. Co., 22–30.
7) Rawlings, C. A., Dawe, D. L., McCall, J. W., Keith, J. C. and Prestwood, A. K. (1982): Four types of occult *Dirofilaria immitis* infection in dogs.

JAVMA, 180, 1323–1326.
8) Tamashiro, W. K., Powers, K. G., Levy, D. A. and Scott, A. L. (1985): Quantitative and qualitative changes in the humoral response of dogs through the course of infection with *Dirofilaria immitis*. Am. J. Trop. Med. Hyg., 34, 292–301.
9) Thilsted, J. P., Whorton, J., Hibbs, C. M., Jillson, G. P., Steece, R. and Stromei, M. (1987): Comparison of four serotests for the detection of *Dirofilaria immitis* infection in dogs. Am. J. Vet. Res., 48, 837–841.
10) Webber, W. A. and Hawking, F. (1955): Experimental maintenance of *Dirofilaria repens* and *D. immitis* in dogs. Exp. Parasitol., 4, 143–164.
11) Wong, M. M. and Suter, P. F. (1979): Indirect fluorescent antibody test in occult dirofilariasis. Am. J. Vet. Res., 40, 414–420.