STUDIES ON THE DIAGNOSIS OF CANINE FILARIASIS WITH SPECIAL REFERENCE TO THE COMPARISON OF HEMAGGLUTINATION TEST AND SKIN TEST

Kazuhiro SHICHINOHE*, Masahiro TAGAWA and Kazuo KUROKAWA

Department of Veterinary Surgery, Nippon Veterinary and Zootechnical College, Tokyo

Koichiro FUJITA** and Hiroshi TANAKA

Department of Parasitology, Institute of Medical Science, University of Tokyo, Tokyo

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Methods used for the diagnosis of canine filariasis are the detection of microfilariae (mf), immunological test (skin test), angiocardigraphy, clinical-hematological test, and electrocardiogram (ECG). Although the detection of microfilaria is most commonly used of them, mf are not always demonstrable by available methods. Under such circumstances, the establishment of an easy method for the diagnosis has been earnestly desired. Immunological studies for the diagnosis of filariasis have been carried out by many investigators. The skin test has been dealt with in most of them, but its standardization has not completely been established as yet. Except for a few instances, the skin test antigens used for the diagnosis of human filariasis have been prepared from animal filariae.

More than 10 years have passed since the hemagglutination (HA) test was first applied to the diagnosis of filariasis. The first report on this test was made in 1960 by JUNG and HARRIS⁴⁾ who used the HA test for the diagnosis of *Dirofilaria immitis* infection in man. In 1961, PACHECO⁶⁾ carried out studies on the specificity and sensitivity of *D. immitis* antigen to canine serum. He examined cross-reactions between *D. immitis* antigen and sera infected with other parasites. In 1968, TANAKA et al. showed that both HA test¹¹⁾ and complement fixation (CF) test¹²⁾ were useful for the diagnosis of cotton rat filariasis, so long as a given animal was free from any other parasite and that homologous antigen was used. The most recent report was made in 1970 by FUJITA et al.³⁾ who had applied the HA test to the diagnosis of *D. immitis* infection in dogs. The demonstration of HA titer and the presence of mf, however, were not always comparable.

The present studies were designed (1) to know a main reason for the incompatibility of the demonstration of HA titer and the presence of mf in canine filariasis, (2) to estimate the degrees of cross-reaction between canine filariae and other intestinal helminths, and (3) to compare results between the HA test and the skin test.

MATERIALS AND METHODS

Experimental animals

A total of 111 dogs (61 male and 50 female) with or without *D. immitis* were collected from areas in and around Tokyo. They were one to two years old and weighed

* Present address: Department of Pharmacology, Nippon Medical School, Tokyo.

** Present address: Department of Hygiene, School of Medicine, Juntendo University, Tokyo.

10 to 15 kg each.

Investigation protocol

Stool specimens were examined first for the presence or absence of helminthic eggs. Blood was collected from the radial vein for serum samples. The number of mf contained in 0.02 ml of blood was counted. The sera were inactivated at 56°C for 30 minutes and stored in a deep freezer at -20°C. Before subjected to the HA test, all the sera were absorbed with sheep erythrocytes for 30 minutes at room temperature. Immunological examination was carried out by two methods, skin test and HA test. The dogs were autopsied after the tests and examined for adult worms of *D. immitis* in the heart and pulmonary arteries, and helminths in the intestine. The adult worms of *D. immitis* were stored at -20°C and used as HA antigen.

Skin test

Two antigens were used for the skin test. One was canine filariasis antigen (C.F.A.) supplied by courtesy of the Takeda Chemical Industries, Ltd. The other was Sawada's purified antigen, FST3⁸⁾, which was presented by Dr. T. SAWADA, professor of Parasitology, School of Medicine, Gunma University.

One-tenth ml of C.F.A. solution containing 150 µg⁵⁾ of C.F.A. was injected intradermally in the groin on one side of the dog and 0.02 ml of FST3 containing 0.05 µg of antigen in the groin on the other side. The dog was examined for reaction 15 minutes after the injection. The positive reaction by C.F.A. was identified by a distinct swelling at the site of injection reaching more than 12.5 mm in diameter (12.5~13.5 mm as false-positive), and the one by FST3 by a distinct swelling more than 9.0 mm in diameter.

Hemagglutination test

This test was done by the method of FUJITA and KOBAYASHI²⁾ devised for the diagnosis of cotton rat filariasis. Sera were collected for the test from the dogs mentioned above. Two standard positive sera were made from rabbits immunized with homogenized adults of *D. immitis* or *Ascaris suilla*.

Two antigens were also used. One was the phosphate buffered saline (PBS) extract of adult *D. immitis* at pH 7.2 prepared by the method of TANAKA et al.¹¹⁾, and the other that of adult *A. suilla*.

Both positive and negative serum and diluent (0.6% normal rabbit serum) were tested as controls. The reaction of tannic acid-treated cells without sensitization was examined at the initial dilution of each serum.

Gel filtration on Sephadex G-200

Two ml of dog serum was dialyzed overnight against PBS at 4°C and transferred to a column of Sephadex G-200 (Pharmacia, Uppsala) 45×2.2 cm in size, which had been equilibrated with PBS and then elutriated with the same buffer at a flow rate of 23 ml per hour. As a result, 2.5 ml of each fraction was collected. After the spectrophotometric examination at 280 mµ, all the fractions were examined for HA activity.

RESULTS

Infection with intestinal helminths

Stool specimens were examined for the eggs of helminths. At autopsy, the presence of worms was ascertained. Although there was a small difference in infection rate between the two methods, intestinal helminths were found at autopsy in the following percentages: *Ancylostoma caninum* 49.5%, *Trichuris vulpis* 65.4%, *Dipylidium caninum* 24.2%, and *Toxocara canis* 0.9% (Table 1).

Infection of *D. immitis*

Table 1. Infection of Helminths in Dogs Examined

		Examination at autopsy							
		<i>Ancy. cani.</i>		<i>Tric. vulp.</i>		<i>Dipy. cani.</i>		<i>Toxo. canis</i>	
		+	—	+	—	+	—	+	—
Stool examination	+	41	6	49	3	6	3	0	0
	—	12	48	21	34	20	78	1	106
Positive rate		53	54	70	37	26	81	1	106
		49.5%		65.4%		24.2%		0.90%	

Total number of dogs: 107.

Table 2. Infection of *D. immitis* in Dogs Examined

		Adult worm	
		+	—
Microfilariae	+	44 (42%)	0
	—	14* (12%)	49 (46%)
		58 (54%)	49 (46%)

Number of dogs: 107.

* Single-sex infection was found in 8 of 14 dogs.

Table 3. Results of Skin Test of Dogs with and without adult *D. immitis*

Antigen		<i>D. immitis</i>	
		with	without
C. F. A.	+	56 (95%)	38 (76%)
	—	3 (5%)	12 (24%)
		59	50
		59	50
FST3		<i>D. immitis</i>	
		with	without
FST3	+	31 (53%)	8 (16%)
	—	28 (47%)	42 (84%)
		59	50

A total of 107 dogs were examined for infection with *D. immitis*. Of them, 44 dogs (42%) were positive for mf in the peripheral blood. Adult worms were recovered in 58 dogs (54%) at autopsy (Table 2). In other words, 14 dogs (12%) were negative for mf but positive for adult worms. Of them, 8 dogs, or 7% of the 107 dogs, were involved in a single-sex infection. Therefore, the mf test was not always sufficient to detect the real infection of dogs with *D. immitis*.

Results of the skin test

In this study, the two kinds of antigens were used. As shown in Table 3, a positive reaction with C.F.A. was presented by 95% of 59 dogs (44 positive and 12 false-positive dogs infected with adult *D. immitis*, and 76% of 50 dogs (38 positive and 8 false-positive dogs) free from adult *D. immitis*. A positive reaction with FST3 was exhibited by 31

Fig. 1. Distribution of Diameter of Distinctly Swollen Areas in Skin Tests by Two Antigens.

(53%) of the 59 dogs with adult *D. immitis*, and 8 (16%) of the 50 dogs free from adult *D. immitis*.

Fig. 1 shows the distribution of the diameters of swollen areas by the two skin-test antigens. The diameters were mostly below 9.0 mm by FST3 in the dogs free from filarial worms. On the other hand, they ranged from 10 to 20 mm by C.F.A. in these dogs and could not be distinguished from those in the dogs with adult *D. immitis*. There was no statistically significant correlation in results between the two skin tests. The correlation in the rate of infection with intestinal helminths between the two skin tests was also calculated, as shown in Table 4. No significant correlation, however, was found with regard to these four helminthic species.

Results of the hemagglutination test

Ninety-three dogs were tested with PBS *D. immitis* antigen. A serum was determined as positive when it showed three-plus agglutination at 1:32 dilution or over. The HA test was positive in 86% (42 head) of 49 dogs with adult *D. immitis* and in 50% (22 head) of 44 dogs free from adult *D. immitis* (Table 5). The HA test was negative in sera from dogs over 4 years old even when the dogs were infected with adult *D. immitis*. So, sera from dogs over 4 years old and sera showing a positive reaction in tannic acid-treated control cells were excluded from the results of the HA test.

The distribution of positive titers among the infected dogs is presented in Table 6. The positive titers were distributed mostly within a range from 1:32 to 1:8192.

The HA titers of the dogs with adult *D. immitis* showed no correlation to the number of adult worms, that of females, density of mf in the blood, or the rate of infection

Table 4. Correlation between Skin Test and Intestinal Helminths

		Ancy. cani.		Tric. vulp.		Dipy. cani.		Toxo. canis	
		+	—	+	—	+	—	+	—
C. F. A.	+	35	58	50	43	17	76	1	92
	—	6	9	9	6	2	13	0	15
χ^2		0.03069		0.20269		0.21797		/	
P		0.9 > P > 0.8		0.7 > P > 0.5		0.7 > P > 0.5		0.86111	

		Ancy. cani.		Tric. vulp.		Dipy. cani.		Toxo. canis	
		+	—	+	—	+	—	+	—
FST3	+	14	25	17	22	8	31	1	38
	—	27	42	42	27	12	57	0	69
χ^2		0.12932		3.0017		0.16089		/	
P		0.9 > P > 0.5		0.1 > P > 0.05		0.7 > P > 0.5		0.38713	

$$\chi^2_{.95}(1) = 3.841$$

Table 5. Results of HA Test of Dogs with and without Adult *D. immitis*

		<i>D. immitis</i>	
		with	without
HA test	+	42 (86%)	22 (50%)
	—	7 (14%)	22 (50%)
		49	44

From this test were excluded sera from dogs over 4 years old and sera showing positive reaction in tannic acid-treated control cells.

Table 6. Distribution of Positive Titer in Dogs Positive for *D. immitis*

HA Titer 1:	Number of dogs
32 > (Negative)	7
32	4
64	0
128	4
256	3
512	8
1024	6
2048	5
4096	1
8192	6
8192 <	5
Total	49

with four species of intestinal helminths (Table 7).

Canine sera were also examined by the HA test using antigen derived from *A. suilla*. As shown in Table 8, the HA test was positive in 88% (50 head) of 57 dogs with adult *D. immitis* and 47% (20 head) of 43 dogs free from adult *D. immitis*. These results were almost the same as those of the HA test using antigen derived from *D. immitis*. This finding shows the existence of common antibody reacting to both *D. immitis* and *A. suilla*.

Table 7. Correlation between HA Test and Intestinal Helminths

		<i>Ancy. cani.</i>		<i>Tric. vulp.</i>		<i>Dipy. cani.</i>		<i>Toxo. canis</i>	
		+	—	+	—	+	—	+	—
HA test	+	27	36	34	29	15	48	0	63
	—	13	25	22	16	4	34	1	37
χ^2		0.74088		0.14792		2.73832		/	
P		0.5 > P > 0.3		0.9 > P > 0.7		0.1 > P > 0.05		0.37623	

$$\chi^2_{.95}(1) = 3.841$$

Table 8. HA Test with Antigen from *A. suilla*

HA test		<i>D. immitis</i>	
		with	without
+		50 (88%)	20 (47%)
—		7 (12%)	23 (53%)
		57	43

Table 9. Single Sex Infection

Dog No.	Adult worm		Skin test		HA test
	Male	Female	C. F. A.	FST3	
23	2	0	+	—	4096
42	0	1	+	—	2048
45	1	0	+	—	512
55	1	0	+	—	TC
62	0	1	(+)	—	8192
74	0	2	+	+	512
88	0	3	+	+	TC
101	2	0	+	—	256

(+): false-positive, TC: positive for tannic acid-treated cells.

Table 10. HA and Skin Tests of Dogs Over 4 Years Old

Dog No.	Sex	Age	Microfilariae	Adult worms	Skin test		HA test
					C. F. A.	FST3	
1	female	6.5	10	9	+	+	0
2	male	7	672	26	+	—	0
3	male	4	324	14	+	—	0
38	female	4	0	0	+	—	0
61	male	4	1046	7	(+)	—	0
79	female	4	3095	+	+	—	0
81	male	4	0	36	(+)	+	128
90	female	7	481	2	+	—	256

(+): false-positive

antigen in the sera of dogs infected with adult *D. immitis*.

Single-sex infection

Eight dogs were suffering from single-sex infection. As shown in Table 9, HA test and skin test by C.F.A. were positive in all the dogs, except a few. The skin test by

FST3 was positive in 2 dogs and negative in 6 dogs.

Results from dogs over 4 years old

The 8 dogs used were over 4 years old, but one of them was free from adult *D. immitis*. As shown in Table 10, the skin test by C.F.A. was positive in all the dogs, but HA test and skin test by FST3 were negative in 5 dogs with adult worms.

Detection of blocking factor of hemagglutination test

As mentioned above, the HA test was negative even in some of the dogs infected with adult *D. immitis*. Three sera and the fractions of 5 sera fractionated with Sephadex G-200 were tested in order to detect any substance obstructing the HA reaction. No blocking factor of the HA reaction, however, was detected from any serum or fraction.

DISCUSSION

In 1970, FUJITA et al. carried out studies on the diagnosis of canine filariasis by the HA test. PBS antigen was found the best for the diagnosis, because it was the most satisfactory in specificity to canine serum. The HA test was carried out on 69 dogs infected with *D. immitis*. The correlation between the HA titer and the presence of microfilaria was not always comparable. Especially among mf-negative adult dogs, a positive HA reaction was observed quite frequently (65.2%).

In the present study, the cause of this discrepancy was investigated first. HA-positive dogs with no filarial worms in the heart decreased to 50% in this experiment. At the beginning, about 15% of the experimental dogs was considered to be negative for mf in the blood positive for adult worms in the heart. This figure was in accord with that of the present investigation in which 12% of the experimental dogs was found to be negative for mf but harbor adult worms in the heart. In this experiment, however, still 50% of the dogs harboring filarial worms in the heart remained to be HA-positive. The reason for this result is unknown, but there is a possibility that filarial worms may also be present in subcutaneous tissue, body cavity¹⁾, and peripheral blood vessels⁹⁾. There is another possibility that the HA reaction may become positive in a dog which has some immature worms but no adult worms at all^{7,10)}. These problems remain to be solved in future.

Conversely, 12 dogs were HA-negative in spite of their having worms in the heart. Eight of them were over 4 years old. Six of the dogs, over 4 years old were HA-negative. This result shows a possibility that the HA reaction may be negative in some dogs over 4 years old in the field. Seven dogs below 4 years old were investigated for blocking factors against the HA reaction, but such factors were not found in the serum or fractionated serum of any dog.

The HA test was also carried out with antigen derived from *A. suilla* and the same kind of dog serum. The results obtained were almost the same as those of the HA test with antigen from *D. immitis*, indicating that there might be some antigens common to *D. immitis* and *A. suilla*. From this standpoint, the cross-reaction with *D. immitis* and other intestinal helminths was investigated in the experimental dogs. There was, however, no correlation between the HA reaction and the infection of any of the four species of helminths.

From the skin test, some interesting results were obtained. The skin test by C.F.A. was considered to be of little value for the diagnosis of canine filariasis, because the distribution of the diameters of swollen areas in the group of infected dogs was in the same range as that in the group of dogs free from worms, as shown in Fig. 1. On the other hand, the distribution of the reactions by FST3 showed such tendency that dogs

free from adult *D. immitis* could be differentiated from dogs with adult *D. immitis*, although a false-negative reaction was observed in many of the dogs with adult *D. immitis*. So, the skin test by FST3 will be useful for the diagnosis of canine filariasis, if it is improved in some points in future. Anyway, standardization of the skin test and investigation of antigen, especially purification of antigen, must be studied further before discussion is made on the merits and demerits of the skin test reaction.

SUMMARY

1. Intestinal helminths were found among 111 dogs tested at the following percentages: *Ancylostoma caninum*, 49.5%; *Trichuris vulpis*, 65.4%; *Dipylidium caninum*, 24.2%, and *Toxocara canis*, 0.9%.

2. Adult worms of *Dirofilaria immitis* were harbored by 54% of the dogs. The presence of adult *D. immitis* was not always comparable with that of microfilariae.

3. The skin test by canine filariasis antigen (C.F.A.) was positive in 95% (including false-positive) of dogs with adult *D. immitis* and 76% (including false-positive) of dogs free from adult *D. immitis*. The skin test by FST3 antigen was positive in 53% of dogs with adult *D. immitis* and 16% of dogs free from adult *D. immitis*. There was no correlation between the positive reaction of either skin test and the infection with any of the four species of intestinal helminths.

4. The hemagglutination (HA) test was positive in 86% of dogs with adult *D. immitis* and 50% of dogs free from adult *D. immitis*. There was no correlation between the HA titers of infected dogs and the number of adult worms, that of female worms, or density of microfilariae in the blood. There was no correlation either between the HA titer and the infection with any of the four species of intestinal helminths.

5. The HA test was negative in sera from dogs over 4 years old, even if these dogs were parasitized with adult *D. immitis*.

6. Eight dogs were found to be involved in single-sex infection. Both HA test and skin test by C.F.A. were positive in these dogs, except a few. The skin test by FST3 was positive in 2 dogs and negative in 6 dogs.

7. No blocking factor of the HA reaction was detected from the serum or any fraction of the serum of dogs with adult *D. immitis*.

8. Canine sera were also examined by the HA test using antigen derived from *Ascaris suilla*. The HA test was positive in 88% of dogs with adult *D. immitis* and 47% of dogs free from adult *D. immitis*.

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Dirofilaria immitis 寄生犬の診断に関する研究 赤血球凝集反応の検討および皮内反応との比較について

七戸和博・多川政弘・黒川和雄
日本獣医畜産大学家畜外科学教室

藤田紘一郎・田 中 寛
東京大学医科学研究所寄生虫研究部

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犬糸状虫症の診断法としては、microfilaria(mf)検査法、皮内反応法が現在広く知られているが、いずれも不備な点を持っている。

今回著者らは、犬糸状虫症の診断における赤血球凝集(HA)反応を検討し、mfの検査成績*Dirofilaria immitis*成虫の寄生状況、他の蠕虫類の検査成績などとの相関を調べ、さらに皮内反応の成績と比較検討した。

HA反応では、フィラリア感染犬の86%、未感染犬の50%が陽性であった。HA titerとフィラリア寄生数、雌虫数、mf数との相関は認められなかった。また内部寄生虫(犬鉤虫、犬鞭虫、犬条虫、犬回虫)との相関は、いずれも5%の有意差で認められなかった。感染犬において14%のfalse-negativeの反応がみられたが、4才以上の犬では、フィラリア感染があるにもかかわらず、HA反応がほとんど陰性になることが判明した。しかし、それらの犬の血清中、あるいは血清分画中には、HA反応を阻害する物質は検出できなかった。

またmfが血液中に検出されずに、成虫が心臓

に寄生していたものが14例あったが、そのうち8例は単性寄生であった。このうち6例は、HA反応陽性を示した。さらに、*Ascaris suilla*抗原を用いHA反応を行なったところ感染犬の88%、未感染犬の47%が陽性を示した。これは、*D. immitis*を抗原とした場合の成績と非常に類似し、両者の間に共通抗原が存在することが示唆された。

皮内反応では、犬フィラリアアンチゲンを使用した場合は、フィラリア感染犬の95%、未感染犬の76%が陽性であった。また反応の分布を調べたところ、感染犬と未感染犬の分布は、ほとんど一致して、区別することができなかった。沢田精製抗原(FST3)では、感染犬の53%、未感染犬の16%が陽性であった。このようにFST3による皮内反応では、感染犬に比較的多くのfalse-negativeが見いだされているが、反応の分布上、感染犬は、未感染犬群に対して高度に反応する傾向が同われ、将来の改善によっては、犬糸状虫症の診断に有望であると思われる。