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Indirect Hemagglutination Test for Diagnosis of Canine Filariasis

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Abstract. Four kinds of *Dirofilaria immitis* antigens, prepared from intrauterine microfilariae (I-Mf), circulating microfilariae (C-Mf), migrating larvae and adult worms (A-Di), were assessed by indirect hemagglutination (IHA) test in terms of immunological specificity and sensitivity. The highest specificity and sensitivity were observed in phosphate buffered saline (PBS) extract of I-Mf among these antigens. In contrast, PBS extract of C-Mf was found to have extremely low IHA antigenicity. Cross reactions between *D. immitis* and some intestinal parasites, such as *Toxocara canis, Ancylostoma caninum* and *Trichuris vulpis,* were evaluated by agglutinin absorption assay. It was revealed that no substantial cross reaction was present between I-Mf or A-Di and the above three intestinal parasites. Agglutinin absorption assay also suggested that the antigenicity of A-Di antigen differed from that of I-Mf antigen, although some common antigens were present between them. The present results indicate that I-Mf antigen is useful for IHA test in *D. immitis* infection in dogs.

Indirect hemagglutination (IHA) tests, using crude [1, 2, 6, 12, 16, 19] or purified [8, 20] antigens of adult *Dirofilaria immitis,* have been extensively employed not only for the diagnosis of *D. immitis* infection but for assessing the kinetics of humoral immune responses in the infected dogs. In these studies, however, little attention has been paid to the specificity and sensitivity of antigens to be employed. In regard to the antigens, furthermore, no information is available on the antigenic reactivities of various developmental stages of *D. immitis* worms. It seems, therefore, that the standardization of IHA tests for serological diagnosis of canine filariasis has not been established yet.

From the above, the present study was undertaken to compare IHA antigenic reactivities of *D. immitis* microfilariae (Mf), migrating larvae and adult worms, and also to provide baseline data on the clinical application of IHA test for the diagnosis of canine filariasis.

Materials and Methods

Dog sera: Peripheral Mf positive dogs in Tokyo area were used as infected dogs. *D. immitis* infection in these dogs was confirmed at necropsy. Younger dogs (less than three months old) which had never passed potentially infective season, were used as non-infected controls, all of which were found negative for *D. immitis* at necropsy. These dogs were bled and sera separated and stored at −40°C until use. All serum samples were diluted fourfold with saline, and inactivated at 56°C for 30 min before use.

Antigens: Four antigens were prepared as follows; (1) intrauterine Mf (I-Mf) antigen; intrauterine microfilariae were collected from the uteri of adult female worms and washed several times with phosphate buffered saline (PBS) at pH 6.4 and homogenized with the aid of teflon homogenizer with a

small amount of PBS. The resulting homogenates were frozen and thawed several times, and kept overnight at 4°C. On the following day, they were centrifuged for 60 min at 27,500×g. The resulting supernatant was used as antigen. (2) circulating Mf (C-Mf) antigen; 60 m*l* of whole blood from a dog with microfilaremia were added to 100 m*l* of 0.5% saponin in saline and stirred for 15 min at room temperature. This hemolyzed mixture was centrifuged for 15 min at 225×g and the sediment obtained was washed several times with saline. The viability of Mf separated was almost 100%. This Mf antigen was made by the same procedure as described in I-Mf antigen. (3) larval antigen; developing 4th stage larvae, collected from the subcutaneous tissues of dogs 85 days postinfection were processed in the same manner as described above. (4) adult worm (A-Di) antigen; equal numbers of adult male and female worms, collected from the heart of infected dogs, were used for preparing a homogenate, which was later delipidized by sequential centrifugation with chilled aceton and ether. The supernatant was discarded and the sediment was extracted overnight in PBS at 4°C. This emulsion was then centrifuged for 60 min at 27,500×g and the supernatant obtained was used as antigen.

Protein concentrations of the above described four antigens were determined by the method of Lowry et al. [7].

IHA test: The IHA test was performed by a modification of the method of Sato et al. [15], using microtiter technique. Briefly, formalinized sheep red blood cells (SRBC) were prepared by the method of Csizmas [3]. The SRBC were washed thrice with saline and treated with tannic acid (Junsei chemical Co. Ltd., Tokyo), diluted 1:10,000 with PBS at pH 7.2, for 15 min at 37°C. The tanned SRBC was sensitized with optimal concentration of antigen for 10 min at room temperature. Serum samples were serially diluted with PBS at pH 6.4 containing 1 per cent normal rabbit serum, which had been previously autoclaved for 15 min at 120°C and then kept at 4°C until use. The diluted sera were mixed with the sensitized-tanned cell suspension. The results were read according to the criteria described by Stavitsky [17]. Preliminary studies indicated that serum dilutions of 1:32 or higher could be regarded as positive IHA reaction.

Absorption of sera: The homogenized and lyophilized materials of adult *Toxocara canis, Ancylostoma caninum, Trichuris vulpis* and *D. immitis* worms as well as I-Mf were used as antigens for absorption. The preliminary examinations indicated that the optimum amounts of antigens to be added to 0.4 m*l* of serum sample, diluted 1:4 with saline, were 3 mg for A-Di and *T. canis* antigens, 6 mg for *A. caninum* and *T. vulpis* antigens, and 10 mg for I-Mf antigen, respectively. The mixture was incubated at 37°C for 2 hr and then overnight at 4°C. The serum was then centrifuged for 15 min at 2,000×g and the supernatant was recovered. IHA tests were performed on these absorbed serum samples.

Statistical analysis: The significance of the difference in IHA test results was analyzed by Chi-square test.

Results

1. Specificity and sensitivity of antigens: Optimum concentrations of I-Mf, larval and A-Di antigens for IHA test were found to be 55 μg, 150 μg and 500 μg protein per m*l*, respectively. The results of IHA tests with these antigens are shown in Table 1. In the studies of infected dogs, I-Mf antigen yielded the best results among three anti-

Table 1. Results of indirect hemagglutination tests for sera from infected and non-infected dogs using three different antigens

Antigen	Infected group			Non-infected group		
	No. of dogs tested	No. of positive (%)	Mean titer (min-max.)	No. of dogs tested	No. of positive (%)	Titers of positive cases
I-Mf	74	69 (93) *	1:891 (1:8–1:16,384<)	44	5 (11)	1:32, 1:64, 1:128, 1:512, 1:1,024
Larval	49	44 (89)	1:168 (1:8–1:4,096)	44	1 (2)	1:32
A-Di	74	60 (81) *	1:64 (<1:8–1:2,048)	44	1 (2)	1:32

I-Mf: Intrauterine microfilaria. A-Di: Adult *D. imitis*. * Difference between these values was significant ($p<0.05$).

Table 2. Comparison of antigenic reactivity between two microfilarial antigens by indirect hemagglutination test

Serum	Antigen*	
	I-Mf	C-Mf
1	1:256	<1:8
2	1:4,096	<1:8
3	1:8	<1:8
4	1:128	<1:8

* Protein concentration was 55 $\mu g/ml$ in both antigens. I-Mf: Intrauterine microfilaria. C-Mf: Circulating microfilaria.

gens, in terms of both positive rate (93%) and mean titer (1:891). In contrast, A-Di antigen gave the lowest positive rate (81%) and mean titer (1:64). Larval antigen produced intermediate value for both parameters. Statistically significant difference in positive rate was found only between I-Mf and A-Di antigens ($p<0.05$). For the sera of non-infected controls, both A-Di and larval antigens showed the same degree of false positive (2%), which was lower than the value (11%) from I-Mf antigen, although the difference was not statistically significant. However, the IHA titers of five positive sera from non-infected controls, as assessed by IHA test with I-Mf antigen, decreased to less than 1:8 when being absorbed with 10 mg of lyophilized I-Mf antigen.

Comparison of antigenic reactivity between I-Mf and C-Mf antigens was conducted on four infected dog sera using identical protein concentration (Table 2). The data indicated that all sera exhibited negative IHA reactions against C-Mf whereas these sera, except for serum No. 3, gave higher levels of IHA titer when I-Mf antigen was employed.

2. Cross reactions between *D. immitis* and canine intestinal parasites: The degree of cross reactivity between *D. immitis* and

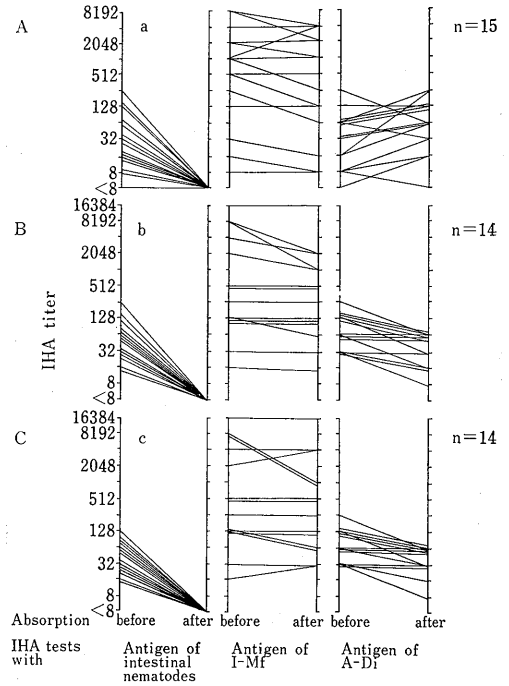

Fig. 1. Cross reactions between *D. immitis* and intestinal nematode parasites, as assessed by indirect hemagglutination test following agglutinin absorption.

intestinal nematodes (*T. canis*, *A. caninum* and *T. vulpis*) was studied by agglutinin absorption assay on sera from infected dogs (Fig. 1). IHA assays with various antigens were made on sera from infected dogs, all of which had been previously absorbed with *T. canis*, *A. caninum* or *T. vulpis* antigens. Consequently, when sera were absorbed with *T. canis* antigen, IHA titers, as detected with *T. canis* antigen, decreased to less than 1:8 (Fig. 1). In contrast, IHA titers detected by IHA test with I-Mf or A-Di antigens were variable; titer decreased in some cases while in other cases, slightly increased or unchanged. Maximum decrease in titer in the above cases was only two logs. Thus, the present results are suggestive of the absence of substantial cross reactions between *T. canis* and *D. immitis* infections. Similarly, no significant

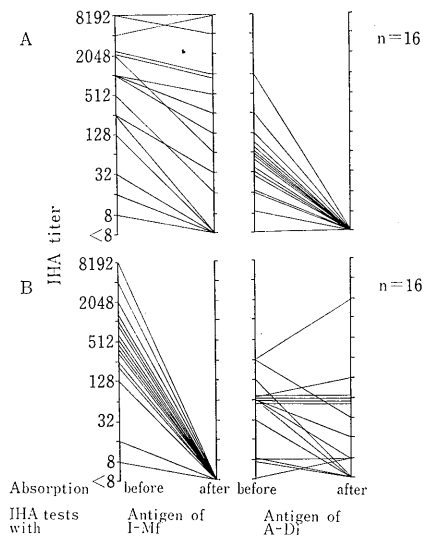

Fig. 2. Cross reactions between intrauterine microfilaria and adult worm of *D. immitis*, as assessed by indirect hemagglutination test following agglutinin absorption.

I-Mf: Intrauterine microfilaria. A-Di: Adult *D. immitis*. IHA: Indirect hemagglutination. A: Absorbed with A-Di antigen. B: Absorbed with I-Mf antigen.

cross reactions were present between *A. caninum* and *D. immitis* and between *T. vulpis* and *D. immitis* infections (Fig. 1).

3. Cross reactions between I-Mf and A-Di antigens: Antigenicity of both I-Mf and A-Di antigens was studied by agglutinin absorption assay (Fig. 2). IHA antibodies against A-Di antigen were completely absorbed by the homologous antigen whereas those against I-Mf antigen were not successfully absorbed by the A-Di antigen. Likewise, when absorption was carried out using I-Mf antigen, IHA titers against A-Di antigen were somewhat variable, but those against I-Mf antigen all decreased to less than 1:8. It seems likely, therefore, that the antigenicity of A-Di antigen differs from that of I-Mf antigen.

Discussion

Immunodiagnostic studies on canine heart worm disease have been conducted by many investigators using complement fixation test [2, 12, 18], precipitin test [4, 5], IHA test [1, 2, 6, 8, 12, 16, 19, 20], fluorescent antibody technique [4, 9], bentonite flocculation test [2] and skin test [1, 8, 10, 16]. All of these studies have concerned with the usefulness of these methods for diagnostic purposes, and some of them have focused the attention on comparison of antigenic reactivity between crude and purified antigens prepared from adult and microfilarial *D. immitis*. However, little study has been done to assess the specificity and sensitivity of these immunodiagnostic tests. With regard to the specificity of the tests, little attention has been paid to cross reactivity between *D. immitis* and other helminth parasites, and also to the occurrence of false positive or false negative reactions. In addition, many serological tests described in these reports are generally less sensitive: In IHA test, for instance, titers reported in previous papers [6, 8, 20] are not necessarily high, since even maximum titer for naturally infected dogs was only 1:4,096 [20].

The current study indicated that the IHA test with I-Mf antigen was the most sensitive among those with three antigens tested, in terms of mean IHA titer and percentage positive for sera from *D. immitis* infected dogs (Table 1). Unfortunately, however, the IHA test with I-Mf antigen yielded the highest false positive rate for non-infected-dog sera (Table 1), although there was no statistically significant difference in the values of false positive rate between these antigens. Our recent finding that the IHA antibody, specific to *D. immitis*, could be passively transferred from maternal dogs to newborn puppies via colostrum (unpublished data), has strongly suggested that the false positive reactions of the sera from

non-infected control dogs would be due to the presence of such an antibody in the sera examined. This supposition may also be supported by the observation that the IHA activity of the non-infected-dog sera against I-Mf antigen was completely absorbed by the addition of homologous antigen. It seems, therefore, that the I-Mf antigen would be the most satisfactory for IHA assay among antigens examined in this study, in terms of both specificity and sensitivity.

It is noteworthy that both A-Di and migrating larval antigens were less sensitive than I-Mf antigen (Table 1). The reason for this remains to be established. The present study is, however, indicative of the distinct difference in IHA antigenicity between A-Di and I-Mf antigens (Fig. 2). It may be conceivable, therefore, that the higher sensitivity of I-Mf antigen is probably associated with the antigenic substance(s) unique to the particular stage of *D. immitis*.

It is of interest to note that the antigenic activity of C-Mf was extremely lower than that of I-Mf (Table 2). Although the reason for this is still unknown, the difference in antigenic activity between these two Mf antigens may be due to a contamination of some unknown inhibitory substances originating from hemolytic erythrocytes, or due to an alteration in microfilarial antigenic activity following entrance into circulation. A weak antigenic activity of *D. immitis* C-Mf antigen has also been observed in precipitin test [5]. Furthermore, less antigenicity of C-Mf antigen has been described in other filarial species, e.g. *Wuchereria bancrofti*, as assessed by precipitin test [11] or fluorescent antibody technique [14], and *Dirofilaria uniformis*, as assessed by fluorescent antibody technique [13].

Finally, agglutinin absorption assays (Fig. 1) suggested that the simultaneous infections of some intestinal nematodes such as *T. canis*, *A. caninum* and *T. vulpis* with *D. immitis* did not affect substantially the IHA data specific to *D. immitis*, when assessed with I-Mf or A-Di antigens. It must be stressed therefore that the I-Mf antigen would be useful not only for diagnostic purposes but for assessing the humoral immune responses of various experimental animals, infected or sensitized with *D. immitis*.

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要　約

犬糸状虫症の診断における間接赤血球凝集反応の検討：早崎峯夫(東京農工大学家畜内科学教室)——犬糸状虫より作製した4種の PBS 抽出抗原について IHA 反応における特異性と鋭敏性を比較検討した．寄生犬血清における陽性率と平均抗体価は成熟雌虫子宮内子虫（子宮内子虫）抗原では 93%，1:891 であり，これは幼虫抗原（89%，1:168）や成虫抗原（81%，1:64）の成績を上まわるものであった．一方，未感染犬血清における false positive は子宮内子虫抗原で 11% であり，これと幼虫抗原(2%)，成虫抗原（2%）との間に推計学的有意差は認められなかった．また末梢血子虫抗原は子宮内子虫抗原に比べて IHA 反応抗原活性が著しく低かった．犬回虫，犬鉤虫および犬鞭虫の各抗原が子宮内子虫抗原あるいは成虫抗原を用いた IHA 反応に及ぼす交叉反応の影響を凝集素吸収試験により検討した結果，腸内寄生虫抗原による影響は極めて弱いものであった．子宮内子虫抗原と成虫抗原の抗原性の差異を凝集素吸収試験により検討した結果，一部共通抗原も存在するが，それぞれに特異な抗原の存在が示唆された．以上のことから子宮内子虫抗原を用いた IHA 反応は種特異性が高く，かつ高抗体価の得られることから鋭敏性も高く，犬糸状虫特異抗体の検出にきわめて有用であると考えられる．

Fig.1 Remarks.

A: Absorbed with T. canis antigen. B: Absorbed with A. caninum antigen. C: Absorbed with T. vulpis antigen. a: IHA test with T. canis antigen. b: IHA test with A. caninum antigen. c: IHA test with T. vulpis antigen. I-Mf: Intrauterine microfilaria. A-Di: Adult D. immitis. IHA: Indirect hemagglutination.