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A Study of Precipitin and Soluble Antigen Flourescent Antibody Techniques in the Detection of *Dirofilaria immitis* in Canines

by
John H. Ellsworth

Thesis submitted to the faculty of the graduate school of Old Dominion University in partial fultillment of the requirements for the degree of Master of Science.

APPROVAL SHEET

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INTRODUCTION

The difficulty of prompt detection of Dirofilaria immitis is that early clinical signs are lacking[10]. Further, since the canines are mainly infested during the summer months, a considerable amount of time may elapse before enough worms accumulate by seasonal reinfestation to give clinical manifestations. By that time, the dog may be too old or too weak to tolerate the relatively debilating treatment for the disease[12]. The usual technique for diagnosis is the examination of the blood for the presence of microfilaria[11]. However, because of the periodicity of the microfilaria in the circulation, the times when a diagnosis may be made by blood smear are often limited. Therefore, an effective serological technique for diagnosis has often been sought.

Several Different serological techniques have been utilized in the detection of D. immitis. Yet none have received extensive study. These include a tertiary test (a skin test) and secondary tests such as hemagglutination, complement fixation and precipitin tests[13].

A more recent primary method to be considered is the indirect fluorescent antibody technique in which fragments of D. immitis were subjected to canine antisera. The positive antisera and conjugate attached to the internal antigens in broken fragments of D. immitis microfilariae were detected by fluorescent microscopy[16]. Also, a soluble antigen fluorescent antibody (SAFA) technique has been developed by Toussaint,

et al[21,22]. This technique permits the objective screening of sera and has been used in the experimental diagnosis of a number of infections[1,5,8,9,17,18,19]. However, all of the reports were the results of tests with human infections. The veterinary aspects of the SAFA technique have yet to be examined.

The work of Gonzales and Bercovitz has raised suspicion as to the value of the precipitin test as a diagnostic tool[7]. These investigators found only 5 positive reactions in 40 patients with Wucheria brancrofti. Franks and Stoll also reported a relatively low reactivity[6]. Though Swada et al[20] reported good sensitivity with a fractionated antigen, only approximately 13 sera were tested.

It is the purpose of this research to compare the relative sensitivities of the precipitin test and the indirect fluorescent antibody test in the detection of D. immitis. The secondary precipitin test was chosen because of its ease of execution and the use of a soluble antigen. The primary indirect fluorescent antibody test was chosen because of its sensitivity.

MATERIALS AND METHODS

Crude Antigen Preparation - The crude D. immitis antigen was prepared from adult worms according to the method of Chaffee et al[2]. Adult worms, obtained at autopsy from dogs after experimental surgery, were lyophilized in tubes on a Virtis Freeze Mobile (Model 10-140). A 100 mgm quantity of lyophilized D. immitis was transferred to a tissue grinder immersed in a sodium chloride ice water bath (0°C.). Ten milliliters of cold

anhydrous ether were added and the mixture ground for 10 minutes. The homogenate was then poured into a centrifuge tube, another 5 ml of ether was added to the residue in the grinder and grinding was resumed for 2 minutes. The second suspension was combined with the first and spun for 30 minutes at 800xG in a refrigerated (4°C.) centrifuge. The ether was immediately decanted, ether traces were removed by aspiration, and the pellet returned to the tissue grinder. Ten milliliters of Tris (2-amino-2-(hydroxymethyl)-1,3-propanodiol) buffered (0.05M-pH 8) saline (TBS) was added to the grinder and the extraction procedure described above was repeated using TBS as the solvent. The TBS homogenates were pooled and transferred to a refrigerator (4°C.) where extraction was allowed to continue for 4 hours. The suspension was then centrifuged as before. The supernatant (crude antigen) was then transferred to a test tube and stored at -60°C.

Source of Antibody - The sera and plasma tested were obtained from canines brought into the Brentwood Veterinary Hospital, Chesapeake, Virginia. Serum samples were recovered from blood that had been collected in capillary tubes (1.1 mm i.d.) and centrifuged at room temperature. The serum was inactivated at 56°C. for 30 minutes. Plasma was obtained from blood samples which were collected in heparinized capillary tubes (1.1 mm i.d.) and centrifuged at room temperature. Pos. control sera were obtained at the autopsy of canines infested with adult D. immitis.

Test Procedure - The crude D. immitis antigen was applied to the top of the plasma. The tubes were sealed at both ends with clay and allowed to incubate for 4 hours at 37°C. The inactivated sera were drawn into capillary tubes (1.1 mm i.d.) followed by crude antigen. The tubes were sealed and incubated as above. The tubes were then examined for a precipitate.

The results of the precipitin test were compared with the medical records of each canine from which the sample had been taken. The veterinarians' diagnosis of the canine utilized examination of a blood smear for the presence of microfilaria or determining the dog to be infected by means of electrocardiogram and chest x-ray. Those dogs not examined for D. immitis by the veterinarians, but showing a positive precipitin test were counted as uncertain (i.e. unable to compare diagnoses) and were eliminated from further consideration.

Precipitin Test Using Fractionated Antigen - The crude D. immitis antigen was fractionated by the technique of Kent[14]. Aproximately 10 ml of the crude antigen was placed on a DEAE Sephedex A-50 (Pharmacia Fine Chemicals, Inc.) column (1x30 cm). Sodium phosphate buffer solutions (0.01M-pH 7.5, 0.04M-pH 7.5, 0.08M-pH 7.5, 0.1M-pH 6.5)[19] were then sequentially used to elute the antigen fractions. Each buffer was run through the column until minimum absorbance (at 280mμ) of the eluant was reached. The first (0.01M) fraction was rechromatographed to remove any crude antigen that had come through with the first fraction as the column became saturated in the initial sample application.

The fractions were then dialyzed against 0.01M-pH 7.5 sodium phosphate buffer. The entire fractionation and dialysis was carried out at 4°C. The dialyzed fractions were then concentrated on a Roto-Vac (Labline, Inc.) at room temperature. The absorptivity of each fraction was then determined by dividing the absorbance ($\propto$) (at 280mμ) by the product of the concentration of the fraction in gm/l (b) and the sample path length in centimeters (c), $A = \frac{\propto}{bc}$. The fractions were then lyophilized and reconstituted to absorptivities equal to the absorptivity of the crude antigen.

Precipitin tests were repeated using the antigen fractions on selected sera that had been subjected to the crude antigen.

Soluble Antigen Fluorescent Antibody (SAFA) Test - The tests were conducted according to the method of Toussaint[22]. The antigen and sera used were the same as those in the precipitin technique.

Cellulose acetate filter paper discs (Millipore, white type HA-0.45 porosity) were soaked in the antigen solution, at room temperature, for approximately one minute, then dried on bibulous paper for 2 hours. The discs were then stored under a vacuum at room temperature.

Discs (1/4 inch diam.) were punched from the above filters and placed in the wells of plastic Disposotrays (Model 96, Limbro Chemical Co., Inc.). Sera were diluted with TBS containing 2% Tween 80 (TBS-Tween) and 0. ml of diluted serum was added to each disc. The discs were mechanically agi at approximately 80 rpm for 45 minutes at room temperature. Following the incubation, the sera were removed by aspiration and 1.0 ml of TBS was added to each disc. The tray was agitated as before for 10 minutes and the TBS

then removed by aspiration. This washing procedure was repeated twice. A 0.2 ml volume of fluoroscein conjugated (rabbit anti-canine IgC) globulin (Microbiological Associates) diluted 1:20 with TBS-Tween was added to each disc and the tray agitated as before for 30 minutes. The fluoroscein globulin was then removed by aspiration and the 3 washes with TBS were repeated.

The discs were finally placed on bibulous paper to remove excess moisture and mounted at one centimeter intervals on the adhesive side of black plastic electric tape. The control discs were mounted first, followed by the test discs. The discs were then allowed to dry several hours before being read in a fluorometer (Model 111, G.K. Turner Associates) equipped with a chromatogram door, a 254-420mμ primary filter, a sharp cut secondary filter passing $>$ 520mμ and a 10% neutral density filter. The fluorometer dial was adjusted to zero on a control disc (antigen disc reacted with D. immitis negative control serum). The fluorometer readings were then recorded for each disc. A fluorometer dial reading of 20 was considered a positive reaction[5].

Initially, a titration of antibody levels was performed by serum serial dilutions using known D. immitis positive and negative sera. These dilution ranges were tested by the SAFA technique. For screening the test sera, a single dilution was used.

RESULTS

The data in Table 1 were derived from subjecting undiluted sera to varying concentrations of crude antigen. The concentration of the crude antigen resulting from the extraction described in Materials and

Methods is referred to as concentration 1 in the table. This concentration seemed to be the optimal antigen level as dilution of the antigen to 1:2 resulted in no precipitate in nearly half the D. immitis positive sera tested. Further dilutions resulted in even greater loss of reactivity. In addition, concentrating the crude antigen 2x and 4x did not increase the precipitate in the presence of reactive antisera.

When the sera were diluted, they produced precipitin results similar to those of the crude antigen dilutions. No precipitate was observed in all cases at a serum dilution of 1:8. In no dilution was the precipitate larger than that found with undiluted serum. The precipitin screening was executed with crude antigen concentration 1 and undiluted sera.

Table 2 shows the fluorometer dial readings of positive D. immitis sera at various dilutions tested by the SAFA technique. A pre-zone phenomenon was expressed in a number of sera since 1:2 and 1:10 dilutions showed lower dial readings than a 1:20 dilution. The decrease in fluorescence found in dilutions 1:40 and 1:80 reflected comparatively low titers in antisera included in this study. Of the dilutions on either side of 1:20, there were some exceptions showing higher dial readings than those shown at 1:20. The optimum dilution of sera (1:20) for the SAFA test in this work was used for screening a larger group of canines.

The results of 70 canines tested, comparing the precipitin and safa techniques, are summarized on Tables 3 and 4. The precipitin test showed an accuracy of 72%, while the SAFA test showed an 80% accuracy. The difference in accuracy arise from a greater number

of false positive diagnoses in the precipitin technique than in the SAFA technique. As shown in Table 4, the precipitin technique gave 12 false positive results, the SAFA technique gave 6. The breakdown indicates that most of the false positives in both techniques arose from reactions with hookworm and whipworm infested canines. There was some precipitin cross reaction with sera from canines with certain bacterial infections (urinary and lung). Of the 8 canines not showing a positive SAFA reaction, having a known D. immitis infection, 7 also failed to show a positive precipitin reaction. These specimens were all indicated by the veterinarians to have advanced cases of D. immitis.

In an attempt to reproduce the results of the initial precipitin test, a second group of 116 canines, tested by the plasma precipitin technique, were compared with the precipitin results of the 70 canines in Table 3. The comparison showed an increase in diagnostic accuracy of only 1%.

Table 5 indicates that the antigen fractions show no greater specificity or sensitivity than the crude antigen in the precipitin technique. The first fraction (0.01M) was totally unreactive, while the second and third fractions (0.04M and 0.08M) showed variable sensitivity. The last fraction (0.1M) duplicated the results of the crude antigen. No single fraction gave results more accurate than the results with the crude antigen.

DISCUSSION

The results presented above indicate that both the plasma precipitin and the SAFA techniques have diagnostic merit. A chi-square test indicates that the two tests are of a similar accuracy (x^2=0.97, P=0.05, df= 1). However, there are differences in specificity worthy of note.

The sensitivity of the SAFA test in the detection of D. immitis in canines seems comparable to the sensitivity achieved in the detection of human filariasis by the SAFA technique[5] (Canine filariasis detection - 80% accuracy; Human filariasis detection - 84% accuracy). In contrast to the precipitin technique, there were no SAFA cross reactions to canines with bacterial infections.

Though the fractionated antigens used here did not increase the accuracy of the precipitin test, some work with antigen fractions of D. immitis (in intradermal testing) has been successful in isolating a more specific antigen[15]. Further work, using this antigen, may prove its use in the SAFA technique and the precipitin technique.

The cause of the pre-zone phenomenon commonly observed in determining an optimum serum dilution for SAFA is uncertain. It may be that there is some inhibitory factor preventing interaction between canine antibody and the rabbit anti-canine antibody. This inhibitory factor has no apparen effect on the precipitin reaction.

In each technique, cross reactions between other parasitic infections or diseases and the specific antibody occurred. In the

precipitn technique, cross reactions yielding false positives were noted in 13% of the healthy controls, 83% of the hookworm infestations and in four other infections or disease symptoms. Cross reactions were also noted in the SAFA technique, but were limited to hookworm and whipworm infested dogs (33% and 80% respectively). Both techniques show false positives. However, since the SAFA technique showed only intestinal nematode cross reactions, a fecal examination might be valuable in determining the diagnosis of the canine. In areas where other filarids are endemic, actual classification of the filarid may be necessary. Recent work has shown that it is possible to differentiate D. immitis from D. repens by histochemical means[3].

There was a lack of positive diagnoses by both techniques on 7 canines infested with D. immitis. This could be postulated to be caused by a collapse of the antibody response as has been found to occur in other diseases in terminal cases[4]. This problem was also observed in a previous work with precipitin diagnoses of D. immitis[6]. A negative serological reaction, in an otherwise determined D. immitis positive canine, could perhaps be an indication of the canine being in terminal stages of the disease.

The precipitin test shows cross reactivity with certain nematode and non-nematode diseases while the SAFA test shows only cross reactions with the nematode infections. The principle advantage in the precipitin technique is its ease of execution. The SAFA technique requires more sophisticated apparatus. However, the SAFA technique's minute serum requirements and good ilution sensitivity warrant its further study. Though this research is only preliminary, the results suggest that further research in this area could lead to a long sought after early detection of D. immitis.

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Table 1 - Preliminary Serum Precipitin Test for *D. immitis* in Canines

	Crude Antigen Concentration or Dilution						
Canine	4x	2x	1	1:2	1:4	1:8	1:16
D. immitis							
Negative:							
Control	-	-	-	-	-	-	-
D. immitis							
Positive:							
318	+	+	+	-	-	-	-
322	+	+	+	+	+	-	-
327	+	+	+	+	-	-	-
333	+	+	+	-	-	-	-
A4948	+	+	+	+	-	-	-
3085	+	+	+	+	-	-	-
C1932	+	+	+	-	-	-	-

+ = precipitate formed

- = no precipitate formed

Table 2 - Preliminary SAFA Test for *D. immitis* in Canines

Canine	Fluorometer Dial Readings for Serum Dilutions				
	1	1:10	1:20	1:40	1:80
D. immitis					
Negative:					
Control	0	0	0	0	0
D. immitis					
Positive:					
318	29	38	53	32	43
322	0	16	68	23	47
327	0	0	60	23	28
333	43	0	38	35	42
8853	0	15	40	14	17
A4948	0	15	40	2	15
C2809	0	22	72	54	95
C1932	18	65	52	25	30
B4031	26	30	62	30	18

Fluorometer scale: 0-100

TABLE 3

Summary of Results of Precipitin and SAFA Tests on 70 Canines from Brentwood Veterinary Hospital

	Number	Percent of Total
Correctly diagnosed by plasma precipitin technique -	50	72
Incorrectly diagnosed by plasma precipitin technique -	20	28
Correctly diagnosed by SAFA technique -	56	80
Incorrectly diagnosed by SAFA technique -	14	20

TABLE 4

Comparison of Plasma Precipitin and SAFA Tests for *D. immitis* on 70 Canines from Brentwood Veterinary Hospital

		Precipitin Test		SAFA Test	
Diagnostic Status	No. Tested	No. Reactive	%	No. Reactive	%
D. immitis	25	17	68	17	68
Healthy controls	16	2	13	0	0
Other Diseases:					
Hookworm	6	5	83	2	33
Whipworm	5	0	0	4	80
Urinary infection	4	1	25	0	0
Lung infection	4	2	50	0	0
Gastroenteritis	3	0	0	0	0
Orthopedic fracture and infection	1	0	0	0	0
Diabetes	1	1	100	0	0
Hyperthyroidism	1	0	0	0	0
Lymphoma	1	0	0	0	0
Pyometra	1	0	0	0	0
Liver Disease	1	0	0	0	0
Distemper	1	1	100	0	0
Total tested	70				

TABLE 5

Fractionated *D. immitis* Antigen Precipitin Test on Sera of Canines from Brentwood Veterinary Hospital

Canine	Condition of Canine	Crude Antigen (Ag)	0.01M Ag	0.04M Ag	0.08M Ag	0.1M Ag
	D. immitis Positive:					
322	6 adult worms	+	-	trace	+	+
327	3 adult worms	+	-	trace	+	+
3085	heavy infestation	+	-	trace	trace	+
333	4 adult worms	+	-	trace	+	+
K.G.	heavy infestation	+	-	trace	trace	+
	D. immitis Negative:					
Blood Donor	Healthy control	-	-	-	-	-
C4331	hookworm	+	-	-	+	+
B8159	hookworm	+	-	-	+	+
B339	uremic poisoning	+	-	-	-	+
C4538	leg infection	+	-	trace	-	+
A9630	diabetes	+	-	+	+	+
C5045	chronic interstitial nephritis	+	-	trace	trace	trace