

## Quantitative Fluorescent Immunoassay for Measurement of Antibody to *Dirofilaria immitis* in Dogs

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An automated fluorescent immunoassay technique (FIAX; International Diagnostic Technology, Santa Clara, Calif.) has been developed for the quantitation of circulating antibodies in dogs infected with *Dirofilaria immitis*. Two groups of sera, group I consisting of 77 samples and group II consisting of 126 samples, were obtained from experimentally infected microfilaremic dogs, known negative controls, and clinically diagnosed cases of occult dirofilariasis. Antibody against a partially purified trichloroacetic acid-soluble extract of a soluble somatic extract of *D. immitis* was measured by FIAX and by an enzyme-linked immunosorbent assay (ELISA). A standard curve was drawn from four samples with known FIAX titers and fluorescent signal unit values. The standard curve was used to determine titers of unknown samples. The correlation coefficients determined in the analysis of  $\log_{10}$  ELISA and  $\log_{10}$  FIAX values were  $r = 0.9057$  and  $r = 0.8976$  in groups I and II, respectively. Eighty-three percent of the titer values calculated by FIAX in group I were within one dilution, and 95% were within two dilutions, of those titers obtained by ELISA. In group II, 79 and 96% of the values obtained by FIAX were within one and two dilutions, respectively, of those obtained by ELISA. FIAX proved to be a reproducible and convenient assay for the measurement of serum antibody in dogs experimentally infected with *Dirofilaria*.

*Dirofilaria immitis* is a filarial nematode parasite which, as an adult, typically, inhabits the right ventricle, pulmonary artery, and adjacent pulmonary vasculature of dogs. The parasite is widely distributed in warmer climates throughout the world and may cause serious disease and death.

The infection in dogs inhabiting endemic areas can be prevented by daily administration of diethylcarbamazine (3, 4). However, the cost or inconvenience of this treatment may be prohibitive in some instances. Patent infections can be treated by killing the adult parasite with thiacetarsamide (2). The toxicity of the arsenical and the consequences due to emboli of dead parasites make thiacetarsamide treatment relatively dangerous.

Definitive diagnosis of this infection can be made only in those cases where microfilariae (embryos) of *D. immitis* are recovered from peripheral blood. In many cases, however, infected dogs have no circulating microfilariae, yet are still subject to damage by the adult parasite. An immune-mediated phenomenon may precipitate the amicrofilaremic state (9); in these instances there is an immune response directed at the microfilarial stage but not at the adult. An indirect fluorescent-antibody test using intact mi-

crofilariae has been used successfully to detect anti-microfilarial antibody in those dogs with this stage-specific immune response (8, 9). Amicrofilaremic infections may result from the presence of only one *D. immitis* sex in the dog or from an effete infection. Optimally, a serodiagnostic test would detect amicrofilaremic infections regardless of the etiology and would also detect the immature, precardiic stages during which treatment may be instituted without the danger of worm embolism.

In this study we were interested in using a partially purified, adult *D. immitis*-derived antigen in a quantitative indirect fluorescent-antibody test (FIAX; International Diagnostic Technology, Santa Clara, Calif.), for the serodiagnosis of *D. immitis* infections. This assay was compared with a manual, semiquantitative, enzyme-linked immunosorbent assay (ELISA), using the same antigen.

### MATERIALS AND METHODS

**Serum samples.** Two groups of sera were assayed for anti-*D. immitis* antibody by FIAX, and the results were compared with those obtained by ELISA, employing the same serosystem. Groups I and II consisted of 77 and 126 samples, respectively. Serum samples were obtained, as described previously (6), from ex-

perimentally infected microfilaremic dogs and their sibling controls, which were maintained in arthropod-free conditions. Sera from dogs with clinically diagnosed cases of occult dirofilariasis were also examined. The samples selected were evenly distributed across ELISA titers of 1:2 through 1:1,024.

**ELISA procedure.** All titers were determined by using an ELISA, as described previously (1a). Polystyrene Microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) with round-bottom wells were used as the support matrix. The antigen was identical to that used in FIAX. Twelve wells were coated with 50  $\mu$ l of antigen per well at a concentration of 5  $\mu$ g/ml. Samples were serially diluted 12 times with a 50- $\mu$ l Microtiter diluter (Dynatech Laboratories, Inc.), and the endpoints were determined by visual inspection.

**FIAX assay.** The support matrix used in the FIAX assay was a key-shaped plastic stick (STIQ; International Diagnostic Technology) with a cellulose acetate-nitrate polymer-coated disk fixed to both sides of the terminal portion. The antigen used in both FIAX and ELISA was a trichloroacetic acid-soluble extract of a soluble somatic extract of adult *D. immitis* which was purified by gel filtration and cation-exchange chromatography (1). Optimum antigen concentrations were determined in separate experiments by block titration. The antigen was applied at a final concentration of 20  $\mu$ g/ml of 0.1 M carbonate buffer (pH 9.6). Approximately 0.5  $\mu$ g of antigen was evenly distributed over disk surface no. 1 and allowed to air dry for 2 h. Surface 2 was untreated and used as a nonspecific sample control. Antigen-coated STIQs were stored in a storage cassette at 4°C for up to 1 month.

All wash steps and dilutions were made with 0.01 M phosphate-buffered saline with 0.15% (vol/vol) Tween 20, pH 7.4. The buffer was refrigerated and either used or discarded within 7 days of preparation.

Fluorescein isothiocyanate-conjugated anticanine immunoglobulin G (lot S598, Miles Laboratories, Elkhart, Ind.) was diluted 1:150 with phosphate-buffered saline-Tween buffer. This conjugate concentration was judged to be optimum in separate, standard titration experiments.

Four test tube racks, each with four rows of 12- by 75-mm tubes containing the necessary reactants, were placed on a horizontal shaker and maintained at a 45° incline for the duration of the assay. Agitation at a 45° incline ensured uniform application of reagent over the disk surface during the incubation periods and wash steps. Serum samples were diluted 1:41 (15  $\mu$ l of serum plus 600  $\mu$ l of buffer) using the IDT automatic pipettor (International Diagnostic Technology). Samples of known FIAX titer (calibrators) were prepared identically but were run in duplicate. One STIQ per sample was incubated at room temperature in the serum dilution on the shaker for 40 min, with the antigen side down. A 600- $\mu$ l amount of buffer was dispensed into each tube of racks 2 and 4 for the wash steps, and 500  $\mu$ l of diluted conjugate was placed into the tubes of rack 3. Upon completion of the incubation in diluted sera, the STIQs were transferred to the first wash and agitated for 10 min. STIQs were then incubated in conjugate for 30 min at room temperature, followed by a wash in buffer for 10 min. Fluorescence

was measured within 10 min after completion of the final wash.

A fluorimeter (International Diagnostic Technology, Santa Clara, Calif.) was used to measure the fluorescence of the STIQ surface. First, the base line of the fluorimeter was adjusted to zero, then the highest titer sample was inserted antigen side up, and the gain was set so that the fluorimeter display read 160. The fluorescent signal units (FSUs) indicated on the display were recorded. The nonspecific fluorescence of each sample was determined by measuring the fluorescence on the side of the STIQ without the antigen.  $\Delta$ FSUs were obtained by subtraction of FSUs due to nonspecific fluorescence from FSUs obtained from measurement of the antigen-coated surface. The fluorescence measured from each sample ( $\Delta$ FSUs) was proportional to the quantity of anti-*D. immitis* antibody in that sample.

**Analysis of data.** The data from group I were graphed, with  $\log_{10}$   $\Delta$ FSU plotted on the ordinate and  $\log_{10}$  of reciprocal ELISA titer plotted on the abscissa. A line of best fit was determined by least-squares linear regression analysis and was drawn through the plotted values for all positive samples (>1:64); sample titers were calculated by interpolation. Titrers were corrected for nonspecific fluorescence by using  $\Delta$ FSU values when interpolating from a standard curve or best-fit line.

Samples from group I with  $\log_{10}$  ELISA- $\log_{10}$  FIAX coordinates which fell on the line of best fit were chosen as calibrators. Their titers were measured repeatedly to determine variability between assays, the mean titer, and standard deviation. A standard curve was calculated with the data obtained for four of the calibrators, with  $\log_{10}$  of reciprocal FIAX titer plotted on the abscissa and  $\log_{10}$   $\Delta$ FSU plotted on the ordinate. Titrers of the remaining samples in group I were determined by reference to the standard curve. Titrers were determined for the 126 samples in group II, and the data were analyzed to verify the results obtained from group I.

Two samples were chosen randomly from group I to determine variability within each assay. Twelve determinations of sample 1 and 11 of sample 2 were made, and their titers were determined in the same assay. The coefficient of variation was calculated for samples 1 and 2, using the data obtained.

## RESULTS

Classification of samples as negative or positive was based on the distribution of  $\Delta$ FSUs illustrated in Fig. 1. Samples with FIAX titers of >1:20 had  $\Delta$ FSUs proportional to ELISA titers. However, all sera with titers of <1:20 had equivalent  $\Delta$ FSUs. Thus, a FIAX titer of 1:20 was judged to be the cut-off value for a positive test. A horizontal line was drawn through the  $\Delta$ FSU value of 29, which corresponded to a 1:20 FIAX titer (Fig. 1). Ninety-five percent (56 of 59) of the samples in group I with titers of  $\geq$ 1:64 were above this line, 91% (10 of 11) with titers of <1:32 fell below the line, and 1:32 samples were evenly distributed above and below the line.

Thus, half of the 1:32 ELISA samples were positive and half were negative when assayed by FIAX. All positive samples had ELISA titers of  $\geq 1:64$ .

The calculated FIAX titers were compared with the values obtained by ELISA. Of the 77 samples run in group I, 83% were within one dilution and 95% were within two dilutions of the titers obtained by ELISA. A correlation coefficient of  $r = 0.9057$  was calculated when  $\log_{10}$  FIAX and  $\log_{10}$  ELISA titers were compared.

Calibrator samples were selected from the best-fit line drawn for the samples in group I. To assign a reliable FIAX titer to a given calibrator, the samples were assayed repeatedly; mean titer and standard deviation were calculated based on these data (Table 1). Four calibrators were chosen evenly distributed across a titer range of 1:12 through 1:2,710, and the  $\log_{10}$   $\Delta$ FSU and  $\log_{10}$  ELISA values of the four calibrators were plotted on the ordinate and abscissa, respectively. A best-fit line drawn through these points served as a standard line. The data for a typical curve are given in Table 2, and the calculated curve is illustrated in Fig. 2. The regression coefficient for the standard curve illustrated in Fig. 2 was  $r = 0.987$ .

The data obtained for calibrator samples were used to determine variability between assays. All samples had a percent coefficient of variation of  $<35$  (Table 1). Within-run variability was calculated based on data from two samples. Sample 1 had a mean FIAX titer of 181.45 with

TABLE 1. Mean ( $\bar{x}$ ) FIAX titer, standard deviation (SD), and percent coefficient of variation (CV) for calibrator samples

Sample no.	n	FIAX titer	
		$\bar{x} \pm SD$	%CV
1	14	2,210 $\pm$ 253	11
2	10	160 $\pm$ 23	14
3	17	55 $\pm$ 11	20
4	17	16 $\pm$ 2.6	16
5	5	37 $\pm$ 1.5	4
6	5	1,190 $\pm$ 216	18
7	5	523 $\pm$ 53	10
8	13	2,710 $\pm$ 552	20
9	12	551 $\pm$ 192	35
10	13	218 $\pm$ 64	29
11	12	10 $\pm$ 3	33

TABLE 2. Fluorimeter readout for standard-curve calibrator samples ( $n = 2$ ;  $r = 0.9870$ )<sup>a</sup>

FIAX titer	Antigen side (surface 1)	Control side (surface 2)	$\Delta$ FSU
2,710	160	13	147
	152	14	138
551	87	13	74
	87	13	74
200	69	12	57
	67	12	55
12	30	11	19
	37	11	26

<sup>a</sup> Surface 1 - surface 2 =  $\Delta$ FSU.

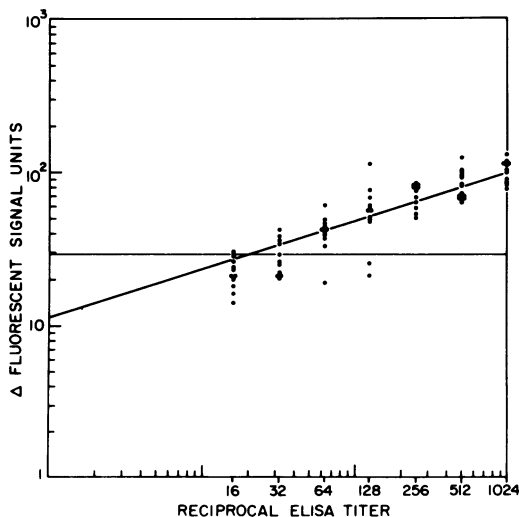


FIG. 1. Comparison of  $\Delta$ FSUs and ELISA titers measured for serum samples in group I. A  $\Delta$ FSU above the horizontal line represents a positive test with FIAX.

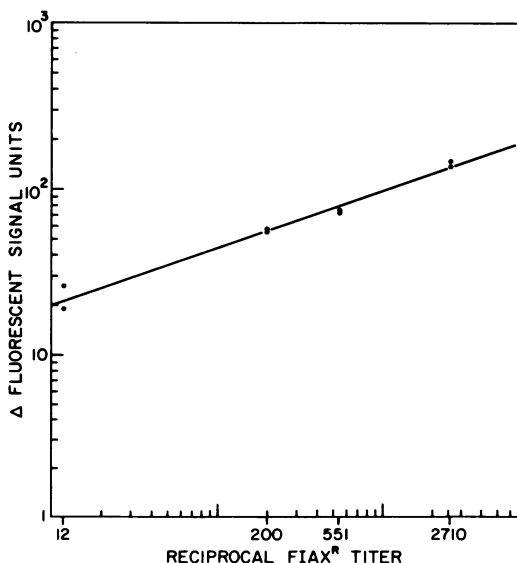


FIG. 2. Representative FIAX curve for four selected calibrator samples chosen from group I.

a percent coefficient of variation of 16.64, and sample 2 had a mean FIAX titer of 239.87 with a percent coefficient of variation of 14.39.

In group II, 126 samples were evaluated by FIAX and ELISA to verify the results obtained in group I. Seventy-nine percent of the FIAX titers were within one dilution and 96% were within two dilutions of the titers determined by ELISA. Of the 49 negative ELISA samples in group II assayed, 6.3% had calculated FIAX titers of  $>1:20$ . The correlation coefficient between  $\log_{10}$  FIAX titer and  $\log_{10}$  ELISA titer was  $r = 0.8976$ .

## DISCUSSION

The purpose of this study was to develop a semiautomated quantitative immunoassay for the serodiagnosis of dirofilariasis. The results indicated that FIAX is comparable to the manual ELISA for the detection and quantitation of serum antibody against a semipurified soluble somatic extract of adult *D. immitis*. This antigen had been reported to be specific for *D. immitis* (5) and more specific than crude preparations derived from the same source (1).

The value of ELISA in diagnosing clinical cases of occult dirofilariasis has not been tested adequately under field conditions. Results based on serum antibody titers measured in experimentally infected dogs suggest that the ELISA system used in our laboratory is sensitive and demonstrates some evidence of specificity (1a).

Both FIAX and ELISA measure serum antibody bound to antigen affixed to an inert support matrix. Conjugated anti-species immunoglobulin G is used for indirect measurement of antibody bound in both assays. The similarities between both assays support the conclusion that ELISA and FIAX demonstrate comparable sensitivity in quantitating anti-*D. immitis* antibody. FIAX does offer several advantages over the manual ELISA. FIAX is semiautomated, can be easily modified for quantitation of antibodies in other serosystems (6, 7), and allows the processing of large numbers of samples with little increase in assay time. FIAX is a single-dilution assay which measures serum antibody as a continuous variable. ELISA, however, uses a doubling dilution technique, and the titers are therefore expressed less precisely as discrete values. Thus, FIAX may allow us to discriminate titers between the dilutions measured with ELISA. Quantitation of antibody titers may also be more precise with FIAX because of the narrow confidence interval established with repeated calibration of standard samples. It is expected that an automated single-dilution ELISA system would eliminate many of the problems inherent in the manual system and would produce results similar to FIAX in sensitivity and specificity since

both tests are indirect quantitative assays for antibody against the same antigen.

An indirect fluorescent-antibody test using intact microfilariae has been used for the serodiagnosis of occult dirofilariasis (8, 9). However, amicrofilaremic infections due to parasitism by single sex, immature, or effete worms may not be detected by indirect fluorescent-antibody assay. In addition, the need for expensive fluorescent microscopic equipment limits the use of an indirect fluorescent-antibody test in veterinary practice.

FIAX was demonstrated to be as precise as ELISA in the detection of anti-*D. immitis* antibody present in the sera of experimentally infected microfilaremic and amicrofilaremic dogs. Thus, FIAX may be an aid in the serodiagnosis of occult dirofilariasis. In our laboratory, seroepidemiological studies are in progress to determine the clinical usefulness of FIAX and ELISA as serodiagnostic tests for occult dirofilariasis.

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