USE OF SEROLOGIC TECHNIQUES IN DIAGNOSIS OF HEARTWORM DISEASE

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PREFACE

This study is concerned with the evaluation of Dirofilaria immitis infections in dogs. The primary objective was to evaluate existing criteria for diagnosis of occult heartworm disease. The second objective was to determine the prevalence of occult infections that occured in selective feral dogs from two animal shelters in northcentral Oklahoma.

The author wishes to express her appreciation to her major adviser, Dr. Helen E. Jordan, for her guidance and assistance during this project. A special appreciation is extended to Dr. J. Carl Fox for his valuable assistance in reviewing serological data and guiding me in table and figure preparation. Appreciation is also expressed to the other committee member, Dr. Sidney A. Ewing, for his guidance and cooperation. A special thanks to my fellow graduate student Carole Barnett, for her cooperation in obtaining dogs for the study and sharing her knowledge of breeds and ages. Appreciation is also extended to Tamara George for her technical assistance and performance of some of the indirect fluorescent immunoassay FIAX serology tests.

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Special gratitude is extended to my husband, Charles R. Mullins, and to my children, Saen and Erin, for their love and understanding and encouragement during this period of my life, and to my parents, John and Juanita Tillotson for their patience and love during this time.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Explanation of the Problem

Dirofilaria immitis causes a serious and even fatal disease in dogs, but it may be cured or arrested if properly treated in the early stages. The most widely used method of diagnosis involves demonstration of immature forms called microfilariae in the circulating blood of suspects. However, microfilariae are not always recovered from the peripheral blood and thus there is nothing to alert the veterinarian to needed action. Clinical signs may not be present in many dogs until damage to the animal by the parasite is irreversible. Serum antibody testing is being recommended by parasitologists and veterinary specialists to clinicians for use as an aid in the diagnosis of heartworm. Serum antibody testing at the College of Veterinary Medicine, Oklahoma State University, indicates the possible presence of *D. immitis* in 47 (42%) of the dogs that were tested in 1983, (Fox unpublished). During the same year most of the dogs tested for serum antibody, as well as, additional dogs were checked for circulating

microfilariae. These tests indicated a 10% prevalence of D. immitis (Jordan and Mullins, unpublished, 1983). The difference between the serologic and microfilariae findings would suggest a much higher prevalence of occult heartworm infection in Oklahoma than previously suspected.

A need exists for a rapid laboratory procedure that would enable the veterinarian to diagnose D. immitis before clinical signs develop in an animal without circulating microfilariae. Serologic techniques to aid in confirming occult heartworm infections are available to the veterinary clinician. There were two tests available for use by the veterinarian at the time this research was conducted, DIROTECT manufactured by Malincrodt Laboratories Inc. and TRACK XI produced by Daryl Laboratories Inc. In addition there was a test conducted using the FIAX International Diagnostic Technology, Santa Clara, CA. The availability of more accurate serological tests including FIAX for veterinarians is by submission of specimens to a diagnostic laboratory.

Objectives of the Study

The objectives of this study were: 1. to evaluate three serological methods; two indirect immunofluorescent antibody assays TRACK XI and FIAX, and an enzyme-linked immunosorbent assay (DIROTECT); and 2. to correlate antibody titers to the worms recovered from post mortem examinations.

CHAPTER II

LITERATURE REVIEW

Occult Heartworm Studies

The diagnosis of heartworm disease in dogs is complicated when circulating microfilariae are not present in the peripheral blood of the infected animal. The number of occult infections with Dirofilaria immitis may be as great as those that exhibit circulating microfilariae (Streitel et al., 1977). These researchers examined 500 dogs from a humane shelter in Columbus, Ohio, and found that half of the dogs which harbored adult D. immitis at necropsy were negative for circulating microfilariae. The dogs with occult infection in that study were characterized as having only one sex of worm present in the heart. Welch et al. (1979) conducted a similar study on 1150 dogs from the temperate east coastal zone of Australia. All dogs were checked by three methods [direct smear (wet mounts), centrifuged cell mass examination and immunological tests] and 545 of them were necropsied. Their results indicated a range from 6% in Cherboug, 12% in Brisbane to as great as 88% in the Kowanyama areas.

Causes and Classifications of Occult Infections

The cause of occult heartworm infections was studied on experimental dogs by Wong et al. (1973). Some animals were sensitized with D. immitis microfilariae and then challenged with homologous infective larvae. Patent infections developed without circulating microfilariae as shown by detection of antibodies in an indirect fluorescent antibody (IFA) test and by necropsy. Another group was sensitized with D. immitis microfilariae and then given infective larvae of a heterologous species, Brugia phangi. This group exhibited circulating microfilariae of that species between 2.1 and 2.5 months within the normal period of prepatency. Other dogs were not sensitized but were given infective larvae of D. immitis. All of the dogs in this latter group developed normal infections and D. immitis microfilariae were detected between 6.2 and 6.8 months postinoculation. Wong et al. concluded that, theoretically, dogs might develop occult dirofilariasis as a result of previous sensitization.

Four types of occult heartworm infection (prepatent, one sex, drug-induced sterility, and immune-mediated) were demonstrated and classified by Rawlings et al. (1982). Type 1-prepatent infections were characterized when at six months post-inoculation with infective larvae, microfilariae were not demonstrated in the peripheral blood and the tests were negative but the pulmonary radiographic arteriograms

showed severe alterations. Another dog with naturally occurring D. immitis infection that had three mature non-gravid female heartworms at necropsy was classified as Type 2-one sex infection. Animals that developed patent infections with microfilariae following inoculation with infective larvae were treated with thiacetarsamide, followed six weeks later with dithiazanine. Microfilariae persisted after these treatments, and levamisole hydrochloride was then administered. Adult worms were seen in pulmonary arteriograms after the levaimsole treatment but microfilariae were not detectable in the blood. When the dogs were necropsied one year after the initial treatment, five viable nongravid females were found in each dog. These dogs were classified as Type 3-drug-induced sterility. Other dogs were considered to have immune-mediated occult infections or what was called Type 4-immune-mediated sterile infection. The Type 4 infections were described from naturally infected animals with heartworm-induced bilateral obstruction of the iliac and femoral arteries but without circulating microfilariae. Other dogs became microfilariae-positive after being exposed to infective larvae but later ceased to show evidence of circulating microfilariae between 5 and 6 1/2 years. Two other dogs were positive upon arrival from an animal shelter and were Knott's test-negative several months later. Adult heartworms were seen in pulmonary arteriograms of all of

these dogs and gravid female worms of D. immitis were removed surgically or by necropsy. In addition to establishing a classification for occult heartworm infection, Rawlings et al. (1982) also demonstrated that some treatments may only sexually sterilize adult heartworms and not kill them; these were classified as Type-2 infections.

Serologic Techniques

Wong and Guest (1969) described an indirect fluorescent antibody (IFA) test, one of the first techniques for identification of dogs with circulating antibodies against D. immitis. This test involved the incubation of a suspension of microfilariae in serum, followed by washes with phosphate buffered saline. The microfilarae were then resuspended in rabbit anti-dog IgG serum labeled with fluorescein isothiocyanate. After incubation and another wash, the microfilariae were examined for fluorescent staining patterns. An evaluation of this technique by Dawe et al. (1980) documented it as being 100% sensitive when circulating microfilariae were present. However, the presence of antibodies were not detected when dogs had adult D. immitis without circulating microfilariae. Therefore, some occult infections were not diagnosed by this method. Other serological techniques were investigated that would detect antibody to D. immitis. Grieve et al. (1981)

studied antibody response in experimentally infected dogs using an enzyme-linked immunosorbent assay (ELISA) and adult-derived antigen. Animals were treated with diethylcarbamazine citrate (DEC) daily before inoculation and continued for 29 weeks. Significant antibody levels were demonstrated in the untreated control dogs at 16 weeks post-inoculation. The dogs placed on (DEC) treatment developed significant titers 11 weeks after inoculation. There was no correlation between antibody levels and either adult worm numbers at necropsy or microfilariae counts. Scholtens and Patton (1983) using the ELISA technique and adult D. immitis antigen showed that titers in noninfected dogs and dogs with patent infections differed significantly as did titers for noninfected animals, those that were previously infected, and those that were suspected of having occult infection. Glickman et al. (1984) used ELISA in combination with radiographic examinations and modified Knott's tests to investigate the influence of host factors, environmental exposures, and chemoprophylaxis on serologic patterns in dogs. The result of this study indicated that dogs with definite radiographic evidence of heartworm disease were more likely to be seropositive than dogs with questionable or negative radiographic findings. Antibody was detected before microfilariae were demonstrated in the blood or positive radiographs were seen in this study.

Gittleman et al. (1981) compared a new technique, quantitative indirect fluroescent antibody test (FIAX) and ELISA to evaluate serum antibody levels in 203 dogs. The antigen used was derived from adult D. immitis. A standard curve was drawn from four samples with known titers and their fluorescent signal unit values derived during the test. This curve was then used to extrapolate the titers for the unknown serum samples. Two groups of sera consisting of 77 (Group 1) and 126 (Group 2) samples were tested. In Group 1, the values calculated by FIAX were within one dilution of ELISA for 83% of the sera tested and 95% were within two dilutions. In Group 2, 79% were within one dilution of ELISA and 96% were within two dilutions. The conclusions derived from the results were that FIAX was "a reproducible and convenient assay for the measurement of Dirofilaria antibody in the dogs experimentally infected with Dirofilaria".

Evaluation of Serological Techniques

Several parameteres of the ELISA technique were examined by Bullock and Walls (1977) in an attempt to standardize procedures. Parameters included the type of enzyme tag, appropriate substrates, reproducibility, stability of the reagents, washing reagents, differences in plastic microtiter plates and tubes, time of incubation, pH, stability of color reaction, and accuracy of visual

and spectrophotometric readings. The results of their study indicated that careful control of all components and technical manipulations were necessary for reproducible results. They recommended horse-radish peroxidase as the best enzyme tag. The substrate of choice was 5-aminosalicylic acid, used at pH 6.0 with hydrogen peroxide. A color reaction terminator (1N sodium hydroxide) helped stabilize the endpoint color. Spectrophotometric readout at 450nm was recommended over more subjective visual readings.

An evaluation of established indirect diagnostic techniques to eliminate any false results due to technical errors was undertaken by O'Beirne et al. (1982). They compared ELISA techniques to radioimmunoassay (RIA) and immunofluorescent assay (IFA) for several viral diseases. RIA was the most sensitive but required expensive equipment and reagents and was considered a potential health hazard because of radioactive isotope-exposures and waste-disposal problems. IFA was more cost-effective but was thought to be too subjective an evaluation due to the microscopic interpretations required in most of these methods.

The different ELISA techniques in use for Toxoplasma, Rubella virus, Cytomegalovirus and Herpes virus (TORCH) were also evaluated. These tests were compared to the more traditional methods previously employed for the same series of organisms (TORCH) using a variety of methods. These

traditional methods were immunofluorescent assay (IFA), indirect hemagglutination assay (IHA), hemagglutination inhibition assay (HAI), neutralization titer assay (NT) and sucrose gradient fractionation (SGF). These workers were of the opinion that extrapolation of ELISA values, from standard curves into titers was not a good approach because most ELISA techniques being used involved only one dilution of serum. Within the ELISA methods there are errors which are caused by such factors as incubation temperature, improper timing, reagent concentration, visual versus spectrophotometric readouts and dependability of enzymes and substrates. They indicated that temperature variation was particularly important because the end point indicator, which is a color change produced by an enzyme-substrate reaction, is accelerated as temperature is increased. Sometimes enzymes are denaturated if temperatures are too high. The reaction curve was distorted by high incubation temperatures regardless of the enzyme-label used. O'Beirne et al. (1982) agreed with Bullock and Walls (1977) that quantitative spectrophotometric readings were more dependable than subjective visual readings.

Occurrence of Cross-Reaction in Serology Tests

Cross-reaction of antibodies with D. immitis and other helminth species has been reported, and this may be of importance in the interpretation of heartworm serology. Tizard (1982) described cross-reactions to the presence of identical antigenic determinants found on a number of different molecules, therefore allowing antibody directed against one particular antigen to react unexpectedly with antigen from an apparently unrelated source.

Welch et al. (1979) demonstrated cross-reactions between Toxocara canis and Dirofilaria immitis antigens. They surveyed dogs in various areas of Australia to determine the prevalence of heartworm infection, both patent and occult. They checked the feces of all of the dogs (1150) for Toxocara canis eggs as well as testing for D. immitis microfilariae and serum antibody. The antibody tests used were a cyanogen bromide indirect fluorescent antibody assay and a cell-mediated immunity test using parasite antigen purified by affinity chromatography. Cross-reaction of the two organisms was demonstrated when samples were tested with a 'crude' antigen. When a 'pure' antigen was used to test the same sera they were able to detect true occult D. immitis infections as confirmed by necropsy. Welch et al. (1979)

also concluded that occult infections were related to the immunity of dogs to the microfilarial stage, which agreed with Wong (1964). They concluded that the proportion of occult infections increased with the prevalence of D. immitis in a specific geographic area. Cross-reactivity of Dipetalenema with D. immitis antigen using ELISA was shown in a study done by Glickman et al. (1984). Four of nine dogs with Dipetalonema reconditum microfilariae were ELISA-positive, but occult infections of D. immitis could not be ruled out as necropsies were not performed. Dogs that were experimentally infected with D. reconditum by Glickman et al. (1984) had ELISA titers to D. immitis for 4 to 16 weeks after inoculation but were negative thereafter. They concluded that there may be a cross-reactivity limited to the time span that overlaps the patency period for D. reconditum.

Occurrence of Ectopic Infections of Dirofilaria immitis

The possibility that adult D. immitis may inhabit ectopic sites in dogs may bias the results in investigations where only normal definitive sites are examined for the presence of heartworms during necropsy. Ectopic sites that have been verified are the right femoral artery (Burt et al., 1977), subcutaneous tissue or submuscular membrane (Kotani and Powers, 1982) and the anterior chamber of a

dog's eye (Weiner et al., 1980).

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

A total of 178 dogs were obtained from animal shelters at Ponca City (96) and Stillwater (83), Oklahoma. All available dogs older than six months (determined by examination of teeth), were used in the study. The size, age, sex and breed of each dog was recorded at the time of euthanasia.

Blood Collection

Blood samples were drawn at the animal shelters and brought to Oklahoma State University, College of Veterinary Medicine for conducting laboratory procedures. Ten ml. of blood was drawn from each dog. Five ml. was placed in EDTA anticoagulant and five ml. was allowed to clot. The serum was separated from the clot and frozen immediately for later testing.

Examination for Microfilariae

Blood samples collected in EDTA anticoagulent were

examined for microfilariae using the Modified Knott's technique. The results were correlated with results of necropsy examinations and serological tests for Dirofilaria immitis.

Serology

The sera were tested for anti-Dirofilaria immitis antibodies using the following: 1. FIAX™, an indirect fluorescent antibody test (International Diagnostic Technology, Santa Clara, CA.); 2. DIROTECT™, an enzyme-linked immunosorbent assay (ELISA) (Malincrodt Lab. Inc.); 3. TRACK XI®, an indirect immunofluorescent antibody technique (Daryl Laboratories, Santa Clara, CA).

FIAX Serology

Antigen for the FIAX procedure was prepared from homogenized adult Dirofilaria immitis. The worm was homogenized for three minutes using a Brinkman Polytron. It was then diluted in distilled water and frozen and thawed three times. The antigen was titrated against positive antisera to determine the optimum antigen concentration.

The fluorescein conjugate utilized was fluorescein isothyocyanate (FITC)-conjugated rabbit-antidog immunoglobin G produced by (Cooper Biomedical, Malvern, PA). This conjugate was diluted 1:200 with phosphate buffered saline at pH 7.2.

Three control sera with predetermined FIAX values were

included with each group of unknowns; a negative control, a low-positive control, and a high-positive control. The positive control sera were obtained from dogs with demonstrable circulating microfilariae of D. immitis; these samples were tested several times to determine average FIAX values. The negative serum controls were from a dog in which no microfilariae had been demonstrated and antibody responses were considered negative.

The sera were diluted 1:100 with phosphate buffered saline pH 7.2. A FIAX StiQ™ (Surface technique for immuno-quantitative) coated with antigen was first placed in the sera, diluted 1:100 in buffer with 0.15% Tween 20, and incubated 30 min. with continuous mixing. StiQs were then transferred to a buffer-tween 20 for 10 min. The third step involved incubation of the StiQ in the fluorescein conjugate with continuous mixing for 10 min., followed by another 10 min. wash. The fluorescence was measured using the FIAX 100 Fluorometer. All sera were tested in duplicate and the values recorded as mean values.

DIROTECT Serology

The Dirotect test was performed in duplicate according to the procedure outlined by the manufacturer in the product insert. Sera were adsorbed with Toxocara canis antigen to eliminate cross-reacting antibodies. A microtiter plate with wells previously coated with D. immitis antigen was supplied in the kit. The conjugate

was goat anti-dog antibody to which the enzyme horse-radish peroxidase was conjugated.

The tests were observed both visually for color change as described in the test kit, and with a Bio-Tek Model #EL307 spectrophotometer at a wavelength of 490 nanometers.

TRACK XI

The third technique (TRACK XI) was performed according to the test kit directions outlined by the manufacturer, Daryl Laboratories, Dirofilaria test. The antibody titers were measured using the TRACK XI^{R} system flurometer. Results were given as titers of antibodies to D. immitis. Titers of less than 32 were considered negative. Titers greater than 32 were considered positive values and were indicative either of prior or current infection. Soluble extracts of adult worms were provided as antigens in the commercial test kits. The conjugated antiserum was labeled with fluorescein isothiocyanate (FITC).

Worm Recovery and Characterization

The animals were humanely killed at the animal shelters and immediately transported to the College of Veterinary Medicine and refrigerated. A necropsy was performed within 48 hours of death. The thoracic cavity was opened and the heart and lungs removed. The heart and lungs and blood vessels leading into or leaving these organs were opened

and examined for adult and immature D. immitis. Worms found were placed in glass jars with the proper identification tag attached and no fixative. Worms were fixed in 7% formalin after identification of sex and examination of the females for microfilariae.

The adult worms were counted and categorized as to sex, fertility and location in the host (Levine, 1980).

CHAPTER IV

RESULTS

General Observations

One hundred and seventy eight dogs were necropsied and tested for antibody to Dirofilaria immitis by three serological methods (FIAX, DIROTECT, and TRACK XI). Necropsy revealed a total of 21 dogs (11.7%) harbored D. immitis adults in their hearts or pulmonary vessels. Sixteen were from Stillwater, Payne Co., Oklahoma, and the remaining five were from Ponca City, Kay Co., Oklahoma.

Figure 1 shows that a total of 99 (57%) sera were positive by at least one serological method, but only 27 (15%) were positive by all three techniques. A total of 76 (43%) were negative by all three methods.

Necropsy Results

Table I contains a summary of the worms that were found at necropsy. Of the 21 dogs in which D. immitis were found 15 were males and six were females. The size of the dogs ranged from that of a small terrier-mix (approximate weight 10-15 pounds) to that of a large St. Bernard. The ages ranged from approximately 8 months to 8 years. The

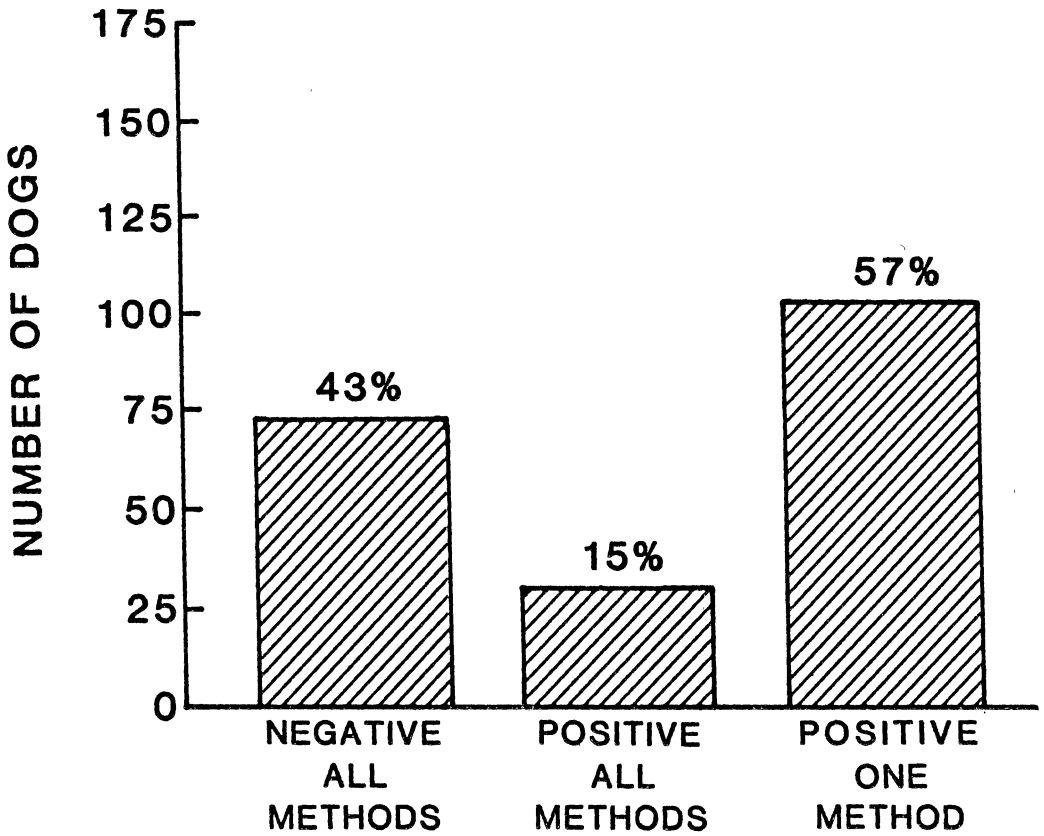

Figure 1. Serological Results of 178 Dogs Collected from the Ponca City and Stillwater, Oklahoma, Animal Shelters and Tested for Dirofilaria immitis Antibody Using (FIAX, DIROTECT and TRACK XI) Testing Methods

TABLE I

SUMMARY OF HEARTWORM EXAMINATION FOR 178 DOGS COLLECTED AT STILLWATER AND PONCA CITY, OKLAHOMA, ANIMAL SHELTERS

Number and (%) Positive	Number of Worms and Characterization			Dogs with Microfilariae	Number Occult Cases
	M	F	I		
21 or (11.7%)	107	133	24	14	7

M = Male
F = Female
I = Immature

numbers and sex of D. immitis found during necropsy varied from 1 immature female to as many worms as 41 (20 males and 21 females); see Table I. Dirofilaria immitis was found in the right ventricle of the heart in 19 of the 21 dogs, and in pulmonary vessels only in two dogs. In both of the latter cases the worms were immature. One animal had adult worms in both the right ventricle and the vena cava. Table II, contains data reflecting the status of fertility for worms recovered from individual dogs found infected at necropsy. Seven (33%) of the 21 dogs positive at necropsy were classified as having occult type infections based on the presence of one sex or nonfertile female D. immitis present. Table III, contains the sex and fertility status of worms recovered from dogs with occult infections and their serological results.

Serology Results

Antibody Detection in All Tests

Figure 1 shows that 57% of the 178 dogs that were necropsied were positive by at least one testing method. The FIAX method (an indirect fluorescent immunoassay) indicated that 54 (30%) of the total number of dogs were seropositive. This number did not include any animals with FIAX values in the suspect range (70-90 FIAX units). Three different samples tested by this method gave below zero readings and these were considered to be seronegative.

TABLE II

NECROPSY AND SEROLOGICAL FINDINGS FOR 21 DOGS FROM WHICH *Dirofilaria immitis* INFECTIONS WERE VERIFIED

Identification Number	Worms Present		Serologic Results		
	Fertile Adults	Sex or Unfertile	FIAX	DIROTECT	TRACK XI
S3	+		+	-/+	-
S12	+		-	+	+
S13		+	+	+	+
S14	+		+	-/+	+
S28	+		+	+	+
S38	+		+	+	-
S53	+		+	-/+	-
S55		+	+	+	+
S60		+	+	+	+
S68	+		+	-/+	-
S71		+	-	-/+	-
S73		+	+	-/+	+
S74	+		+	+	+
S76	+		-	-/+	-
S84	+		?	-	-
BJ	+		+	-/+	+
PC63		+	+	-	-
PC110	+		+	+	+
PC111		+	+	+	+
PC141	+		+	+	+
PC142	+		+	-	+

-/+ = Variable
? = Suspect Range

TABLE III

WORM CHARACTERIZATION AND SEROLOGY RESULTS
FOR SEVEN DOGS WITH VERIFIED
OCCULT INFECTION

Identification Number	Worms Present	Serologic Results		
		FIAX	DIROTECT	TRACK XI
S13	9M	128+	+	526+
S55	1M 1FI	94+	+	107+
S60	4FI	139+	+	148+
S71	3M 4FI	37-	-/+	18-
S73	3FI	118+	-	211+
PC63	2M 2FI	96+	-	9-
PC111	1FI	177+	+	57+

M = Male
FI = Female, immature

Figure 2 is a statistical analysis using a Dice-Leraas diagram to illustrate the results.

The enzyme-linked immunosorbent assay (ELISA) DIROTECT detected 68 (38%) dogs with antibody to D. immitis by at least one of two tests, (Table IV). Only 26 (15%) of the 178 dogs were positive for antibodies in one of the duplicated tests.

TRACK XI (an indirect immunofluorescent antibody technique) detected 56 (31%) seropositive in all animals. Table V shows a comparison of the results of the three methods employed.

Thirty-eight (22%) of the dogs had detectable antibody with both the FIAX and DIROTECT tests. Thirty-five (20%) of the dogs had detectable antibody by FIAX and TRACK XI and 29(16%) by DIROTECT and TRACK XI tests. Table V presents the the comparisons of these results.

Dogs with Adult Dirofilaria immitis

Eleven of the 21 dogs with worms were positive by all three serological methods and two dogs were negative by all three methods. If FIAX values in the suspect range were omitted and the score with varying results (-/+) in the DIROTECT test were considered negative then there were 18 seropositive animals. Only one dog (S-84) harbored gravid females but was negative for antibodies to D. immitis by all serologic methods using duplicate testing for FIAX and DIROTECT. The statistics (Dice-Leraas diagram)

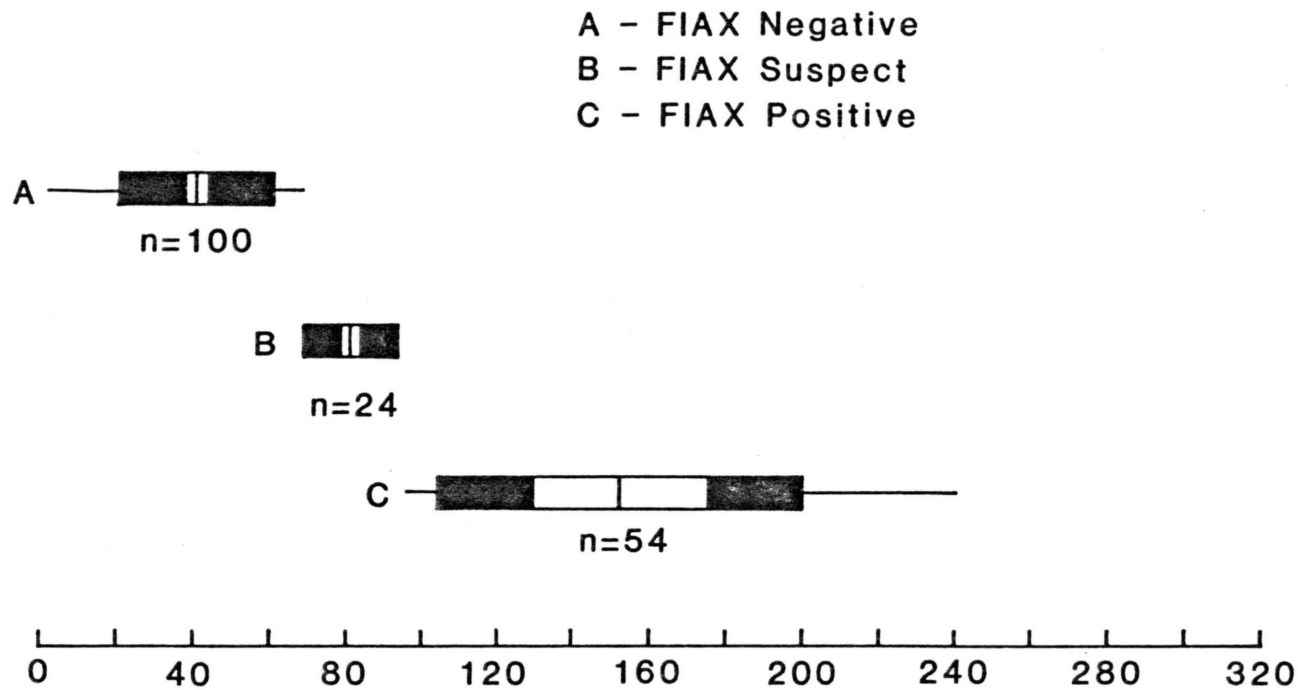

Figure 2. Dice-Leraas Diagram of Mean FIAX Values (in Duplicate) for Heartworm Antibody Levels in 178 Dogs. Verticle Lines of the Diagram Represent the Mean; Black Areas, the Standard Deviation; White Areas, Twice the Standard Error; and Horizontal Lines the Range. Sera Were Tested Against Adult Dirofilaria immitis Antigen.

TABLE IV

COMPARISONS OF THREE SEROLOGICAL METHODS ON 178 ANIMAL SHELTER DOGS OBTAINED FROM PONCA CITY OR STILLWATER, OKLAHOMA, DURING 1983 AND 1984

	Test Method		
	FIAX	DIROTECT	TRACK XI
Number Positive	56	68	56
Percent Positive	31	38	31

TABLE V

NUMBER OF DOGS SERO-POSITIVE BY MORE THAN ONE TESTING METHOD AND SEROLOGIC AGREEMENT BETWEEN TESTS

	Test Method		
	FIAX & DIROTECT	FIAX & TRACK XI	DIROTECT & TRACK XI
Number Positive	38	35	29
Percent Positive	22	20	16
Number Unmatched	52	39	46
Percent Agreement	71	78	74

for these results are presented in Figure 3. A summary of all test results on necropsy-positive dogs are listed in Table VI. Dogs with FIAX values higher than 200 also harbored the largest number of adult D. immitis at necropsy (S-53, S-14, S-74). These three dogs had FIAX values between 223-239 (Table VI). One of these dogs (S-53) also had circulating microfilariae. The other two animals had large numbers of fertile females, but circulating microfilariae were not demonstrated. Similar correlation was not seen in the other tests.

Three of the necropsy-positive dogs had FIAX values in the negative range. However, two of these animals (S-12 and S-76) had circulating microfilariae. One dog (S-71) had no microfilarema, but harbored three males and four immature females and was seronegative by all three serological testing methods.

Microfilariae-Positive Dogs

Fourteen of the 178 dogs necropsied had microfilariae (Barnett, 1985), only 12 had Dirofilaria and two had Dipetalonema, but worms were recovered from only 11 of the 12 dogs with Dirofilaria microfilariae. Table VII presents the serologic values obtained by the three methods (FIAX, DIROTECT and TRACK XI) for the 14 dogs with microfilaremia. Antibodies were detected in 10 (71%) of the animals by FIAX, 5 (36%) by DIROTECT and 8 (57%) by TRACK XI.

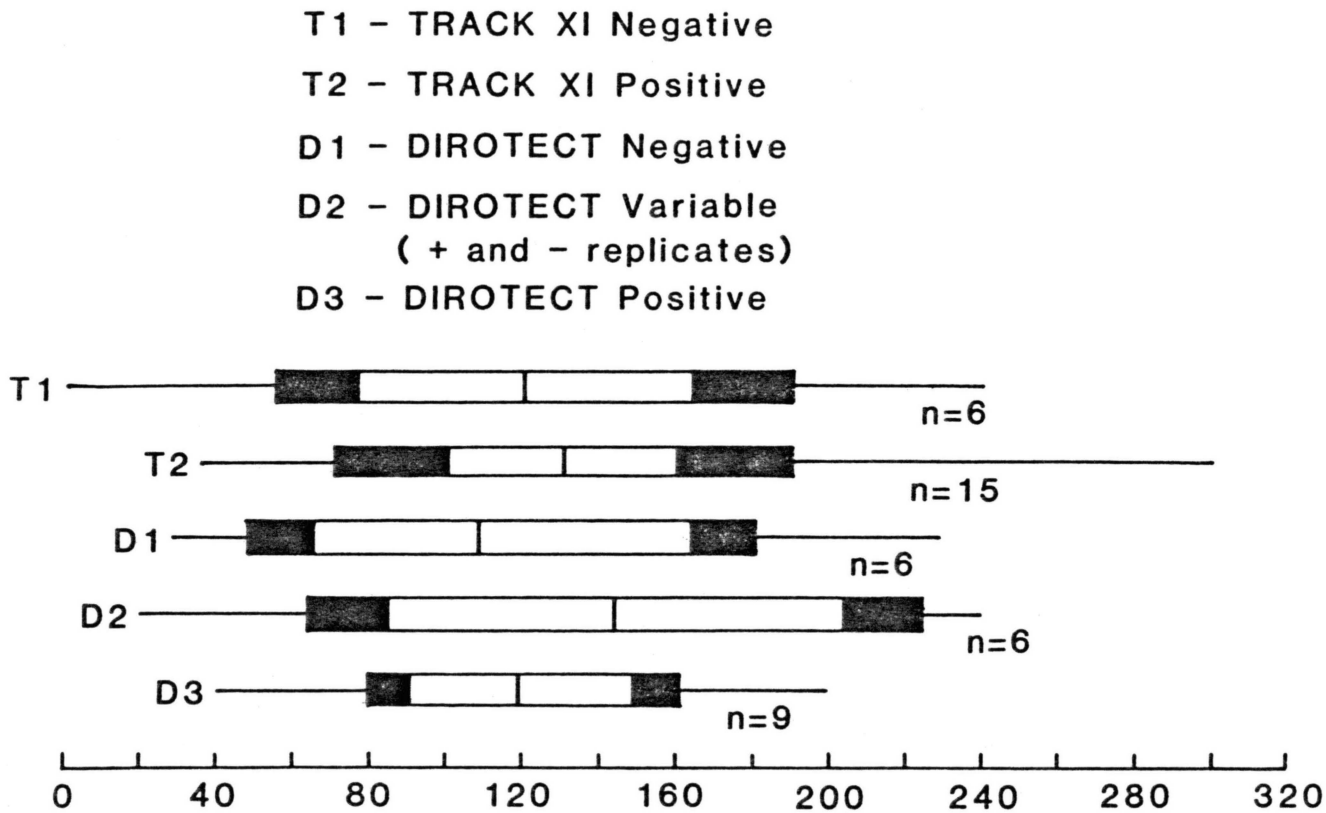

Figure 3. Dice-Leraas Correlation of Serological Results on Necropsy-Positive Dogs Comparing DIROTECT and TRACK XI Techniques to FIAX Values. Verticle Lines of the Diagram Represent the Mean; Black Areas, the Standard Deviation; White Areas, Twice the Standard Error; and Horizontal Lines the Range.

TABLE VI

A SUMMARY OF THE MODIFIED KNOTT'S (MK) TEST, SEROLOGICAL RESULTS AND WORM NUMBERS AND CHARACTERIZATION FOR 21 NECROPSY-POSITIVE DOGS

Identification Number	MK	FIAX	DIROTECT	TRACK XI	Adults Present		
S12	+	44-	+	55+	1M	2F*	
S76	+	53-	-	79+	1M	4F*	
PC142	+	95+	-	38	9M	12F*	1IF
BJ	+	102+	-/+	281+	3M	7F*	
S38	+	104+	+	104+	5M	7F*	
S3	+	104+	-/+	9-	8M	16F*	
PC141	+	112+	+	57+	5M	9F*	5IF
S28	+	119+	+	274+	1M	1F*	
S68	+	159+	-/+	29-	1M	2F*	1IF
PC110	+	179+	+	87+	6M	4F*	
S53	+	232+	-	5-	20M	21F*	
S71	-	37-	-/+	18-	3M		4IF
S84	-	88?	-	3-	2M	3F*	
S73	-	93+	-	211+			3IF
S55	-	94+	+	107+	1M		1IF
PC63	-	96+	-	1-	2M		2IF
S13	-	128+	+	526+	9M		
S60	-	139+	+	148+			4IF
PC111	-	177+	+	57+			1IF
S14	-	223+	-/+	68+	13M	24F*	
S74	-	239+	-/+	317+	15M	24F*	

- = Negative
+ = Positive
* = Gravid females
-/+ = Variable
? = Suspect range
M = Male
I = Immature

TABLE VII

SEROLOGICAL RESULTS ON DOGS WITH CIRCULATING MICROFILARIAE AS DETECTED BY MODIFIED KNOTT'S TEST

Identification Number	Test Method		
	FIAX Value	DIROTECT	TRACK XI Titer
S12	44-	+	55+
S76	53-	-	79+
S86	74?	-	1-
PC142	95+	-	38+
BJ	102+	-/+	281+
S38	104+	+	104+
S3	104+	-/+	9
PC141	112+	+	57+
S28	119+	+	274+
S68	159+	-/+	29-
PC110	179+	+	87+
S53	232+	-	5-
PC65*	72?	-/+	3-
PC116*	94+	-	23-

- = Negative + = Positive
-/+ = Variable ? = Suspect Range
* = Dipetalonema sp. microfilariae

Occult Infections

Seven (33%) of the dogs that harbored adult and immature *D. immitis* at necropsy were catagorized as occult type infections (Table III). The three serological tests varied in their ability to detect antibodies to *D. immitis* in these animals. Using FIAX 6 (86%) of the 7 were positive for antibody. Only one dog (S-71) which harbored three males and four immature females was negative by this method. The DIROTECT assay detected antibody in 4 (57%) of the dogs with occult infections. Two of the three negative dogs (S-84 and PC-63) had males and immature females present, and the other dog (S-73) harbored only immature females. The TRACK XI assay detected antibody in 5 (71%) of the dogs with occult infections. Both of the negative dogs harbored males and immature females at necropsy. Dog S-71 was the only animal of this group that was negative by all three methods. Negative results were obtained with DIROTECT and TRACK XI for dog PC-63, and with DIROTECT alone on dog S-73.

Correlation of Serological Tests

Correlations of serological results for all 178 dogs are shown in Figures 4 and 5. Figure 4 shows FIAX values for samples that were negative by both TRACK XI and DIROTECT. Many of these dogs had FIAX values in the FIAX positive range (90 or above). The greatest number of these

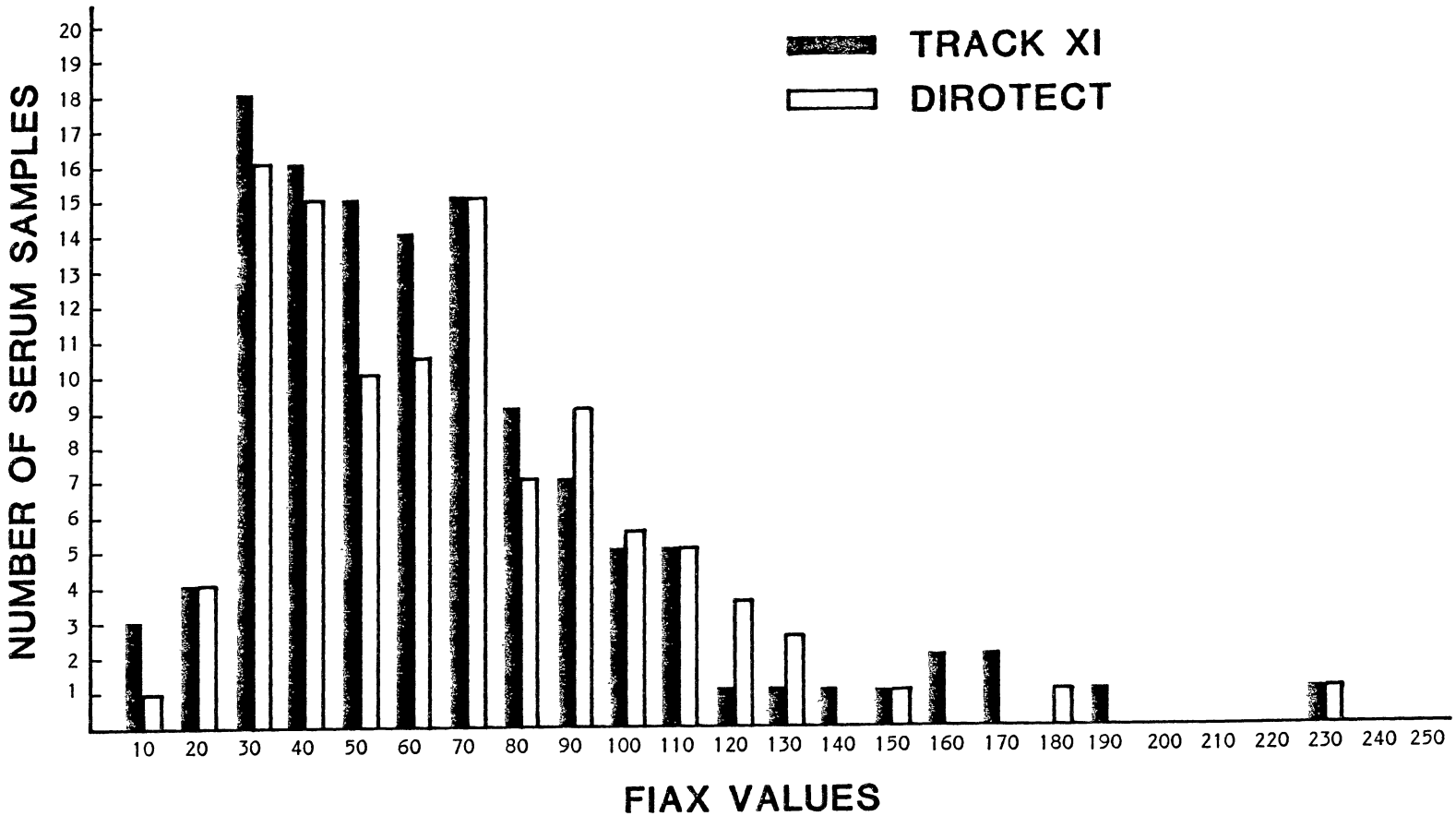

Figure 4. Frequency Distribution of FIAX Values for Canine Sera Found to be Seronegative by TRACK XI and DIROTECT Heartworm Tests. Values Above 90 Are Considered Positive by FIAX Methods.

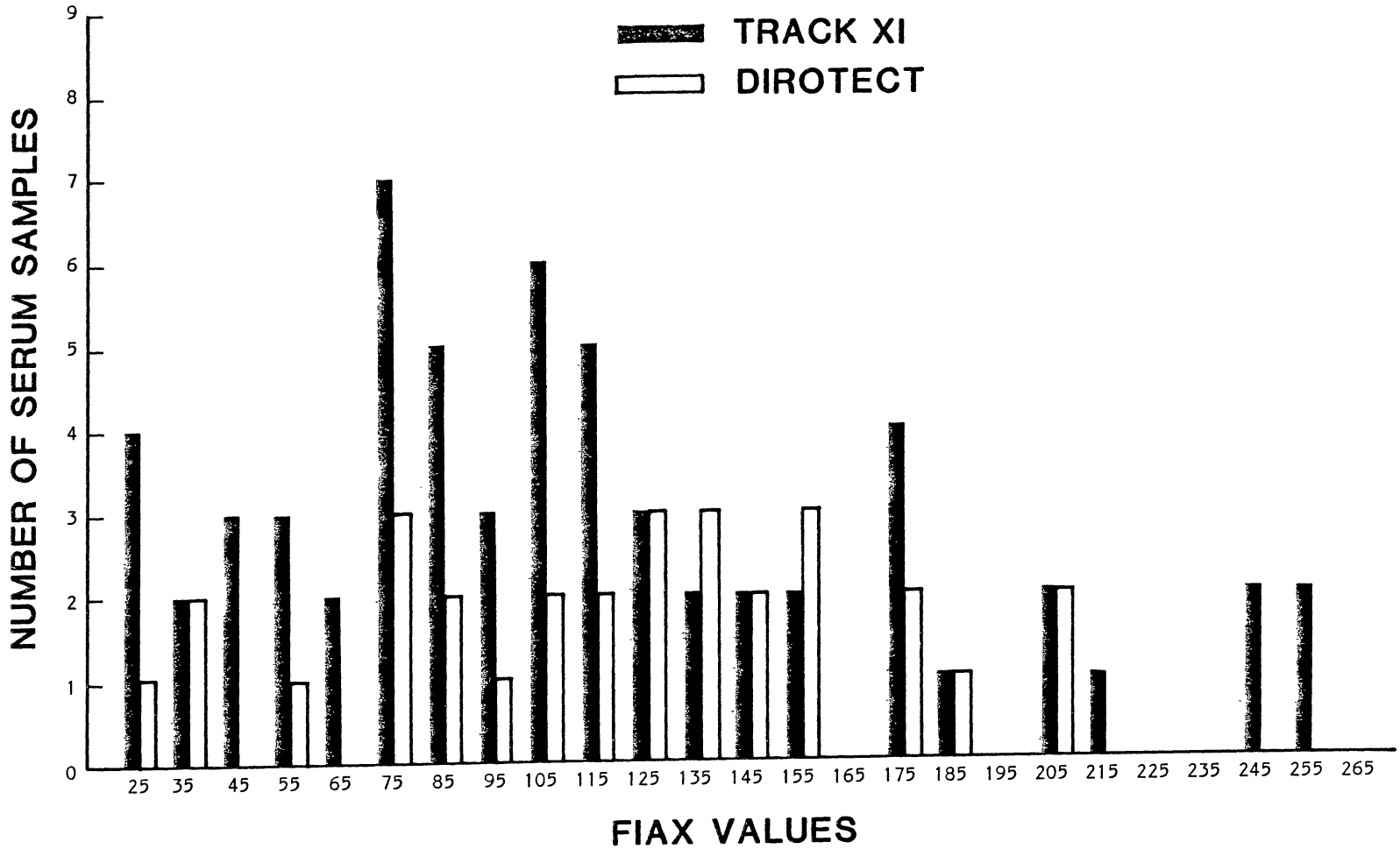

Figure 5. Frequency Distribution of FIAX Values for Canine Sera Found to be Seropositive by TRACK XI and DIROTECT Heartworm Tests. Values Above 90 Are Considered Positive by the FIAX Method.

animals had values between 20 and 60 FIAX units. Those samples above 90 FIAX units that tested negative by DIROTECT and TRACK XI would be considered positive by FIAX. The majority of the TRACK XI and DIROTECT seropositive dogs had FIAX values between 25 and 165 units, see Figure 5. A total of 17 dogs were positive at a higher value range. Several of these animals had FIAX values less than 70. These would be considered seronegative by FIAX.

Figure 6, is a Dice-Leraas representation of FIAX results illustrating serum values for dogs with microfilariae and dogs which were harboring worms at necropsy. The diagram also shows an analysis of dogs with low, intermediate and high FIAX values. The mean FIAX values for the different response ranges were 105, 150, 250, respectively.

The percent agreement between each of the tests and one of the others is shown in Table V. The highest percent of agreement occured between FIAX testing and TRACK XI (78%), leaving 22% unmatched. The next in agreement was DIROTECT and TRACK XI (74%) with 26% unmatched; followed by FIAX and DIROTECT which agreed 71% of the time, leaving 29% unmatched.

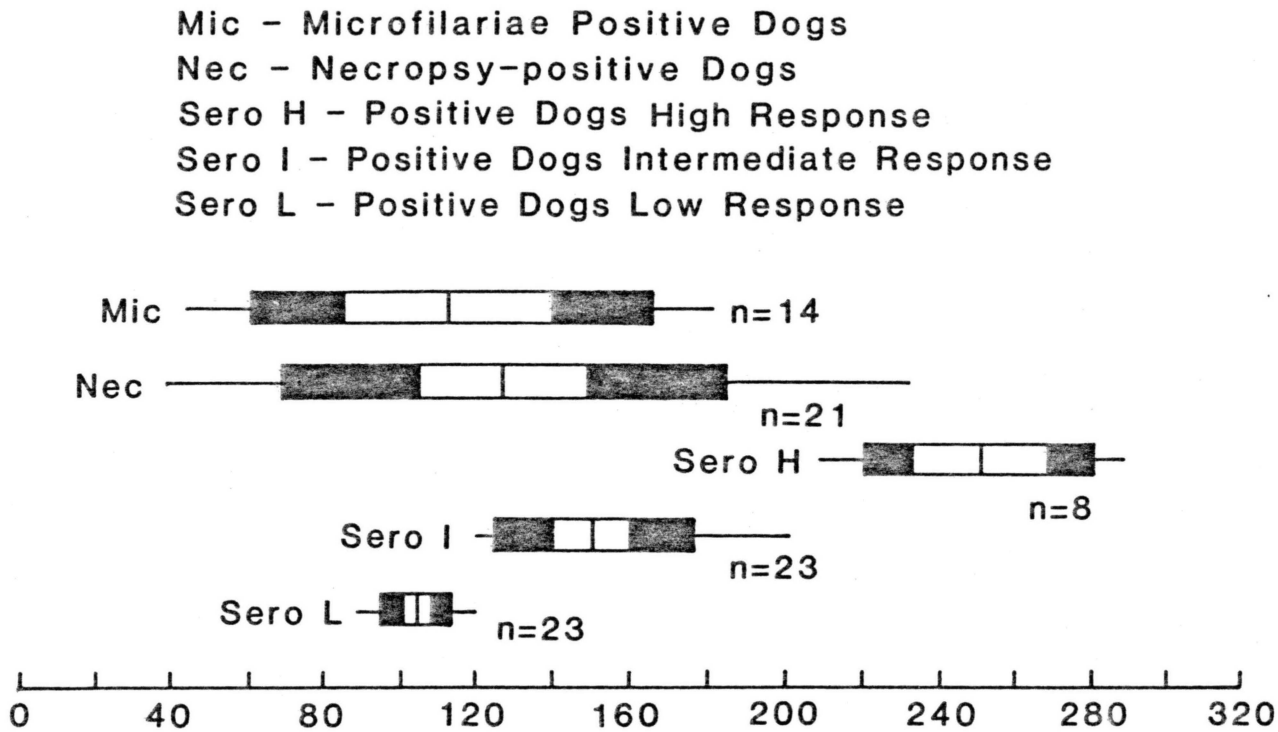

Figure 6. Dice-Leraas Diagram of FIAX Values for Microfilariae-Positive and Necropsy-Positive Dogs and for All Other Seropositive Dogs at the Various Response Levels. Verticle Lines Represent the Mean; Black Areas, the Standard Deviation; White Areas, Twice the Standard Error; and Horizontal Lines the Range.

CHAPTER V

DISCUSSION

General Observations

The 178 dogs used in the study were chosen on the basis of age and availability at the animal shelters. Dogs judged to be under six-months of age were not chosen for examination and only four dogs under one-year of age were selected for the study. This population sampling, therefore, would be classified as stratified.

The seropositive rate in necropsied dogs was 31% by the FIAX method, whereas 111 (42%) of sera from animals tested through the College of Veterinary Medicine, Oklahoma State University, (Fox, 1983) were positive. The latter percentage may be a biased sampling because many of the dogs tested were suspected of having occult D. immitis infections and were showing clinical signs of heartworm disease.

Necropsy Results

The finding of 11.7% D. immitis at necropsy constitutes the first report for north central Oklahoma. The prevalence is similar to that found by necropsy in

coyotes (8%) in Kansas and Colorado (Graham, 1975). Similar percentages have been reported in dogs from North Carolina (10.1%) by Butts (1979), Indiana (15.2%) by Kazacos (1979), and Arkansas (8.9%) Jordan and Mullins (1982). Necropsy findings in this study approximate the prevalence of circulating microfilaria demonstrated in recent years for the same geographic area (Jordan and Mullins, 1983) and Arkansas a nearby region (Jordan and Mullins, 1982). This constitutes a drastic increase in a relatively short time span when microfilariae were not demonstrated in 100 dogs from the same region less than 15 years between the two studies (Pennington, Parish, and Ewing, 1970).

The detection of microfilariae in only 11 of the 21 dogs that harbored adult D. immitis at necropsy was similar to the findings of Streitel et al. (1977) who reported microfilariae in 12 of 24 dogs with heartworms at necropsy.

One-third of the dogs which harbored adult D. immitis at necropsy were classified as occult infections based on the presence of only one sex or nonfertile female D. immitis. Streitel et al. (1977) reported one-half of the dogs in their study had occult infections and Welch et al. (1979) found 12% of the dogs with diagnosed heartworm infections in the Brisbane, Australia area with occult infections. These results indicate that a large proportion of heartworm infections cannot be diagnosed only by testing for circulating microfilariae.

Serologic Results

The number of animals (57%) with antibody detected by at least one serologic test is larger than expected from past experience at the Veterinary Medicine College, Oklahoma State University. A large contributing factor in these results is probably the extreme variability experienced among the different test systems. The percent agreement between the tests is shown in Table V. The greatest agreement occurred between FIAX and TRACK XI, while the poorest occurred between FIAX and DIROTECT. The best agreement left 22% of the seropositive sera unmatched.

The presence of a cross-reacting antibody (*Toxocara canis*) to *D. immitis* antigen was not considered important in the FIAX test or TRACK XI methods. There were not any cases in which cross-reactivity appeared to be an evident cause of positive results, however, this cross-reactivity could account for false positives in FIAX and TRACK XI testing.

Dogs with Adult *Dirofilaria immitis*

The summary in Table VII shows that dogs with the highest serum antibody levels as measured with FIAX, occurred in animals that harbored the largest number of adult *D. immitis*. Antibody levels in TRACK XI and DIROTECT testing showed no correlation with worm numbers. These data suggest that the sensitivity of FIAX is reliable

even in the presence of large numbers of adults which could cause antigen excess in the dog as cited by Tizzard (1977) and Wong and Suter (1979).

One dog (S-84) that harbored adult heartworms was considered negative by all three serologic methods (FIAX, DIROTECT and TRACK XI). It is possible this dog was somehow immunesuppressed or in a state of tolerance.

Data shown in Figure 3 indicates large variation within TRACK XI and DIROTECT testing. The ranges for negative and positives overlapped in TRACK XI testing, when compared to FIAX thus there is not a significant difference in negative and positives in this figure. The standard error overlapped in DIROTECT testing when compared to FIAX.

Statistical comparison of antibody levels for necropsy-positive dogs (Figure 6) illustrates that FIAX values for this group do not differ significantly from dogs found to have circulating microfilariae. The mean FIAX value for microfilariae-positive dogs was 110 as compared to 125 in necropsy-positive dogs.

The possibility exists that adult D. immitis located in ectopic locations were not detected in this study. If adults were present ectopically and antibody to these worms was formed, a possibility according to Weiner et al. (1980), then some of the sero-positive dogs may be attributed to this factor. Burt et al. (1977) reported the presence of D. immitis in the right femoral artery and Kotani and Powers (1982) found adult heartworms in the

subcutaneous tissue or submuscular membranes of two dogs. Rawlings et al. (1982), Streitel et al. (1977), Wong et al. (1973), Wong and Suter (1979), and Grieve et al. (1981) all necropsied dogs used in their studies but did not report whether examination was made of other locations. The few reports mentioning ectopic heartworm infections leads one to believe that only a very small number of animals would be sero-positive because of heartworms in ectopic locations.

Microfilariae-Positive Dogs

The FIAX technique was most sensitive for detecting antibody to D. immitis when circulating microfilariae were present. Ten (71%) of the 14 dogs with circulating microfilaria had positive antibody titers by this method. This finding is in conflict with results of Wong et al. (1973) and Wong and Suter (1979); they reported the absence of antibody titers in all dogs with circulating microfilariae when tested by IFA testing. The FIAX results in the current study agrees with the results of Welch et al. (1979). The FIAX values as illustrated in Figure 6 demonstrates that there was no significant difference in serum antibody levels between the microfilariae positive dogs and the necropsy-positive dogs. Findings in the present study substantiates the theory that antibody can be detected in all types of heartworm infections but it is not always detectable. Serum antibody levels measured by FIAX indicated significant differences between the means

for three response ranges used in this test. The low range had a mean of 105, intermediate 150, and high range 250 respectively.

Five (35%) of 14 dogs with circulating microfilariae were antibody-positive when tested by DIROTECT. Technique errors in the ELISA testing systems as discussed by Bullock and Walls (1977) and O'Beirne et al. (1982) may have contributed to the variability experienced with the DIROTECT test.

Eight (57%) of the 14 dogs with circulating microfilariae were antibody-positive by TRACK XI. This would indicate that this method is comparable to FIAX in accuracy.

Two dogs were positive for circulating microfilariae of Dipetalonema reconditum. Table III demonstrates that cross-reaction may have occurred with the FIAX tests, as one case was in the suspect range (72 FIAX units) and the other was a low positive (94 FIAX units). DIROTECT tests were positive in one animal and negative on the other one. TRACK XI was negative for both dogs. The cross-reactivity of D. reconditum antibody with D. immitis antigen is a definite possibility as demonstrated by Glickman et al. (1984) with animals both experimentally and naturally infected with D. reconditum.

Occult Infections

FIAX detected antibody to D. immitis in six (86%)

of the seven dogs classified as having occult infections which demonstrates the reliability of this test system to detect occult infections. Occult infections were defined as dogs harboring worms without microfilariae present in the uterus. Therefore, the antibody detected is against the adult worm in each of the seropositive dogs.

One occult animal (S-71) had a negative antibody response by FIAX and TRACK XI, and it was negative once and positive once with DIROTECT. This dog harbored three male and four immature female worms in the heart and could be classified as a Type-I prepatent infection by Rawlings et al. (1982).

Dog PC-63 had a low antibody response with FIAX and negative responses with each of the other tests. This dog had two male and three immature female worms present in the heart at necropsy. This animal would appear to be similar to the previous one (S-71) except for the low antibody response detected by FIAX. Dog S-73 showed antibody response by both FIAX and TRACK XI tests and a negative response with DIROTECT. The values for both FIAX (118 units) and TRACK XI (211 units) would be considered low to medium values by those tests. This data suggests that the DIROTECT was less reliable than FIAX for detection of adult D. immitis antigen by DIROTECT.

Correlation of Serological Results

The correlation of serological results in Table IV shows that between 56 and 68 dogs were positive by each FIAX, DIROTECT and TRACK XI methods respectively (Figure 5). The percentage-positive for paired tests was similar in the three comparisons with values of 20, 21 and 22% (Table V).

FIAX methodology was developed as a quantitative serological technique. Gittleman et al. (1981) reported on use of the FIAX system for diagnosis of dirofilariasis. The sera in that study used to establish the control FIAX values came from experimentally infected dogs and their siblings. Based on this report and the availibility of FIAX, this system was selected to use for comparing the other two systems. The FIAX system also measures non specific binding of the fluorescein conjugates or serum factors and makes adjustment for it in calculating the FIAX values.

Only 15% of the 178 total samples were positive by DIROTECT method for antibody both times the serum was tested. However, dogs that were positive once and negative once as well as the duplicates was 68 (39%), including sera that gave variable results (i.e., positive once and negative once). The protocal was followed according to directions in the DIROTECT kit and closely approximated the way in which a veterinarian would probably perform tests (i.e.,

in small groups of 10 sera). The sera were tested twice at different times using the same preadsorbed sera. Results were read independently in each case, both visually and with a spectrophotometer and the results correlated. The color of a positive sample as stated in the kit is green. It was observed that when one well is positive it makes the adjacent well also appear green tinged, making the result difficult to determine. O'Beirne et al (1982) refers to the problem associated with visual reading of colored substrate complexes. The procedure states an incubation time of 10 to 15 minutes before reading the reaction. Users of this kit may try to read the reaction as soon as the positive control becomes green-tinged which is at the minimum incubation time of 10 min. Samples with a lower antibody level may not appear to be positive until the maximum incubation time as stated in the kit is reached. Reading the reaction at the minimum time leads to variability in reading the results and may account for some false negatives. The package insert also states that the negative control may result in blue-tinged substrate, and this could lead to false-positive readings depending upon the ability of the technician to discern blue-green color differences.

Factors that must be controlled when using an ELISA technique include temperature and pH. The DIROTECT kit gives instructions on storage temperature but not for the actual performance of the test. Temperature during the

test performance may be an important contributing factor to result variability as cited for ELISA tests by Wall and Bullock (1977) and O'Bierne (1982).

When all of the samples were read on a spectrophotometer, 78% of the variable (+/-) results were determined to be probable negatives. A wavelength setting that was considered optimal for the color spectrum was used. A negative-positive cut off value was determined based on the mean optical density readings of the negative and positive controls.

The TRACK XI method was simple to use but required special tracks which provide the solid-phase matrix for doing the test, and a special fluorometer was necessary to read the track. This method differs from FIAX by not requiring a serum dilution and also by not providing for correction of non-specific fluoresence in the serums.

Examination of the TRACK XI titers using a frequency distribution indicated a possible break down of values into three result groups a negative range, suspect range and positive range. The TRACK XI test kit instruction states that serum values below 32 were negative and above 32, positive. By comparing TRACK XI titers to FIAX values it would apear that titers of between 32 and 128 TRACK XI should be placed in a suspect range and retested later or by a different method as indicated by DIROTECT instructions for their variable results and as indicated by FIAX testing when sera fall in the suspect range. During the development

of the TRACK XI system, ELISA titers were used as values for their control sera. ELISA methods were reported in titers on a two-fold dilution system; thus a value of 128 on the TRACK XI system is only two dilutions more than the designated cut off of 32. Considering the evaluation of ELISA by O'Beirne et al (1982) differences existing between each testing laboratory using traditional methods, the use of ELISA titers to express TRACK XI results may be incorrect. Therefore, errors may have been compounded by correlating a new method with values from an unrelated test system and other laboratories.

The comparison of positive and negative samples tested by DIROTECT and TRACK XI as illustrated in Figures 5 and 6 show that there were differences in detection of antibody in the three methods. Some of these differences are probably due to errors inherent in performing the techniques as suggested by previous studies by Bullock and Walls (1977) and O'Beirne et al (1982).

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

Heartworm infection was confirmed in 21 (11.7%) of the 178 dogs in this study by microfilaremia (of *Dirofilaria immitis*), or by recovery of adult and/or immature *Dirofilaria immitis* at necropsy. Seven (33%) of the 21 infections were classified as occult based on single sex infections or on fertility status of female worms. Eighteen (86%) of the necropsy-positive dogs were diagnosed by any one of the three serological methods (FIAX, DIROTECT, and TRACK XI) and a concentration procedure for recovery of circulating microfilariae. However, if only the FIAX test and a concentration method for circulating microfilariae was used nine (76%) of these dogs were diagnosed. The highest FIAX values in the necropsy-positive group occurred in dogs that harbored the largest number of adult *D. immitis*.

In twelve of the 178 dogs, circulating microfilariae of *Dirofilaria immitis* were demonstrated by the modified Knott's technique. Two of the total 178 dogs had circulating microfilariae of *Dipetalonema reconditum*. Serum

antibody levels for these two dogs, as determined by FIAX, were in the suspect and low response range. DIROTECT values were positive in one animal and negative in the other one. TRACK XI was negative for both dogs.

The three serological tests were in agreement in detecting antibody for D. immitis in 26 (15%) of the 178 dogs but individually each test detected over 30% (FIAX 31%, DIROTECT 38%, and TRACK XI 31%).

Conclusions

The limited information available on the use of new quantitative serological testing for antibody to D. immitis makes evaluation of the different methods employed in this study difficult. Problems occurred with all three serological test systems; therefore, additional research and continued education is necessary to increase the accuracy of diagnostic tests for the clinician. FIAX appeared to be the most sensitive in the presence of circulating microfilariae and the test detected antibody in the largest number (17) of 21 dogs found by necropsy to harbor adult D. immitis. The commercial kits available (DIROTECT and TRACK XI) both have shortcomings. DIROTECT directions must be carefully followed, and the tests should be read using a spectrophotometer or observed visually by more than one person to insure proper endpoint observation. An evaluation of the test results should be made on the basis of clinical history and symptoms, and

animals should be retested when inconclusive findings occur. TRACK XI has fewer technical problems than DIROTECT in the performance of the test. An additional consideration may be that a specialized instrument is needed to read the tests with TRACK XI, which is not necessary for DIROTECT testing as all supplies are provided.

A test for Toxcara canis should also be conducted on all dogs that are tested serologically to eliminate the possibility of cross-reaction to this parasite, even though the best test method used in this study (FIAX) does not consider heterologous antigens. A diagnosis should not be made on the basis of one serological test alone because antibody may be present in the absence of worms. If antibody levels occur in the suspect or low range of test systems and the dog has no clinical signs or history indicating the source of the antibody, the animal should be evaluated both clinically and serologically, again at an interval of one to four months.

The author is of the opinion that veterinarians should not rely solely on their own expertise in conducting serological tests because of inconsistency in findings with the available test kits. In addition to tests conducted in their clinics, they should take advantage of the regional diagnostic laboratories in their areas for serological testing and for confirming identity of circulating microfilariae. A reliable method for concentration of circulating microfilariae should be employed by all

veterinarians and the procedures for these tests carefully followed to insure proper identification of any microfilariae present.

An interesting aspect of this study is that almost a third of the animals infected, confirmed by necropsy, were classified as having occult infections. The fact that positive serological values are not always indicative of current status infection was also confirmed by comparisons of serum antibody levels to results of necropsies on these 178 dogs.

One of the dogs with occult infection in this study did not have any detectable antibodies for Dirofilaria immitis by any of the methods used. Two dogs had detectable antibodies by only one or two of the methods but not all three. These findings indicate that diagnosis of D. immitis, at times, is missed even when all available laboratory tests are employed.

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