COMPARATIVE ANALYSIS OF DNA-BASED ASSAYS AND ANTIGEN ELISA FOR DETECTION OF DIROFILARIA INMITIS-HEARTWORM DISEASE

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COMPARATIVE ANALYSIS OF DNA-BASED ASSAYS AND ANTIGEN ELISA FOR DETECTION OF *DIROFILARIA INMITIS* – HEARTWORM DISEASE

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Master's Degree

Jarillys M. Marin

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To the graduate council:

I am submitting herewith a thesis written by Jarillys M. Marin entitled "COMPARATIVE ANALYSIS OF DNA-BASED ASSAYS AND ANTIGEN ELISA FOR DETECTION OF *DIROFILARIA INMITIS* – HEARTWORM DISEASE". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Dr. Chad S. Brooks, Major Professor

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Abstract

Commercial enzyme-linked immunosorbant assay (ELISA) specific for detecting *Dirofilaria immitis*, heartworm, were compared to specific primers used in polymerase chain reactions (PCR) to evaluate a better detection method for heartworm infected dogs found in middle Tennessee. Adding to the novelty of the PCR assays were the utilization of specific *Wolbachia* primers. Similar to the *Wolbachia* bacteria found in *Wuchereria bancrofti* which causes human lymphatic filariasis, these related bacteria are found in a symbiotic relationship with *D. immitis. A total of 78 canine blood samples were collected and tested over a six month period.* PCR using specific *D. immitis* and *Wolbachia* primers revealed heart worm infection could be detected using PCR-based assays in more cases than ELISA. A statistical difference at $p < 0.001$, was observed between the techniques. However, there was no evidence to suggest that PCR assays could detect HW infection to a greater magnitude than ELISA assays.

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Introduction

What is Heartworm?

Heartworm (HW) infections are transmitted by mosquitoes and caused by the filarial nematode *Dirofilaria immitis*. There are at least 70 species of mosquitoes that can serve as intermediate host with *Aedes*, *Anopheles* and *Culex* being the most common genera to transmit this pathogen (4). Patent infections are when the adult HW are actively reproducing inside their host and occur in a wide range of wild and domesticated canine species. The list of wild animals that serve as reservoirs of *D. immitis* is almost as extensive as those who vector it, with wolves, coyotes, foxes, California gray seals, sea lions and raccoons being the most common. With regards to domesticated animals, HW infection occurs primarily in dogs (*Canis familiaris*) and less commonly in cats and ferrets. Dogs are considered to be the definitive host of *D. immitis* and are the focus of this study.

Epidemiology.

The incidence of HW disease is widely distributed among countries with temperate, semitropical, or tropical climates including the United States, Canada, and southern Europe. Recently, HW disease has even reached parts of South Korea and Turkey which were thought to be HW-free (12, 22). In companion animals, the main population at risk is the canine population especially those

housed outdoors due to their constant exposure to mosquito bites. Moreover, most HW disease cases reported yearly are in dogs between the ages of three and eight years old that fall in the medium to large body size range (4).

***D. immitis* Life Cycle.**

The life cycle of *D. immitis* is initiated when a mosquito ingest a blood meal from an infected host taking in the first stage larvae (L_1 microfilaria) along with the blood (Figure 1). Once inside the mosquito, these L_1 migrate to the malpighian tubule system where they will continue to develop and mature into the L_2 and L_3 stages (11). The length of time necessary for maturation is temperature dependent, with an optimal temperature of 86ºF or greater and ranges from 1-4 weeks. Infective L_3 stage microfilaria migrate to the mosquito's salivary glands where they will remain until the next blood meal. Once the mosquito bites a mammalian host, L_3 stage microfilaria are injected into the dog's skin. In 2-3, days the L_3 stage microfilaria molts into L_4 and remain in the subcutaneous layer for two months. Following this incubation period, L_4 molt into an immature adult stage known as L_5; it is this larval form that allows for migration through the mammalian tissues. After about 50 days, L_5 microfilaria arrive in the pulmonary arteries where the will undergo their final molt into the mature adult stage. In the pulmonary arteries, adults will continue to grow for a period of 2-3 months. Mature adults are dimorphic, with males reaching lengths of 15 cm and females

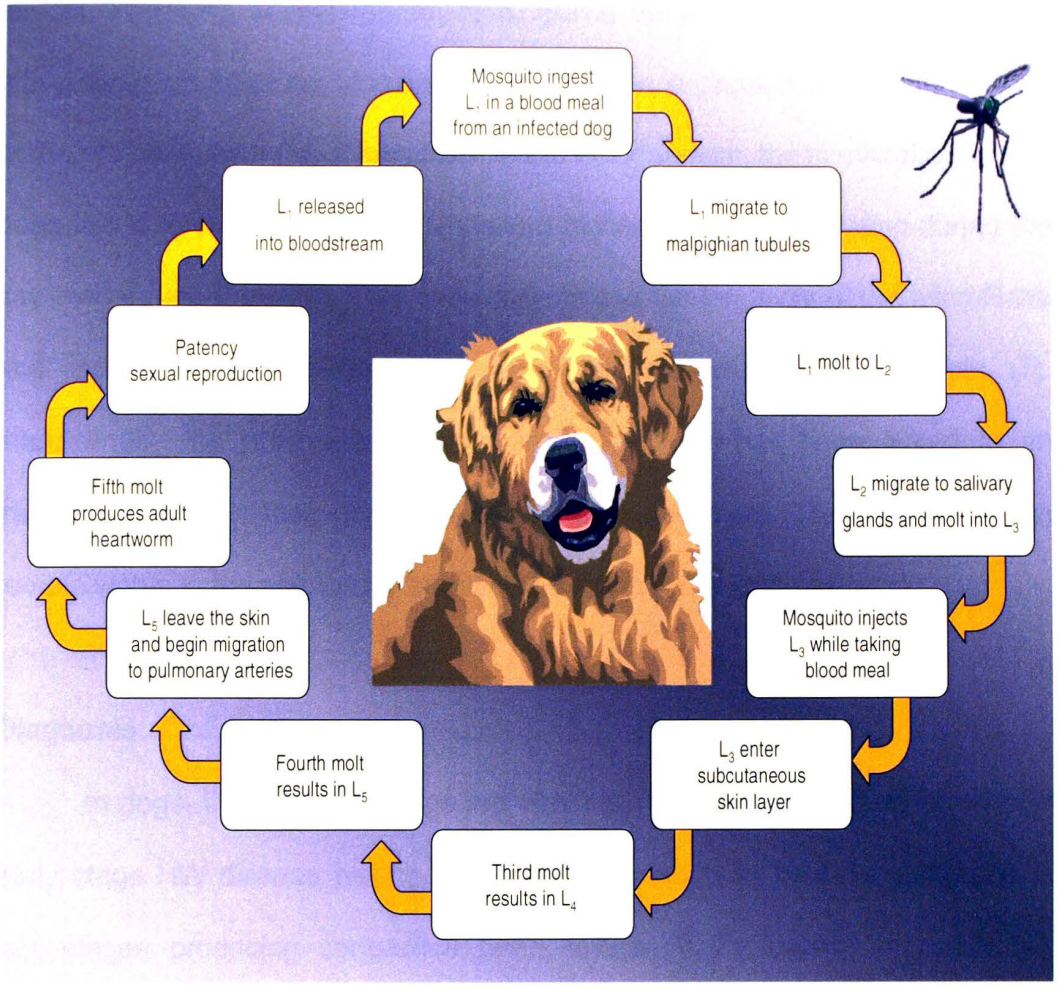

Figure 1. The life cycle of *Dirofilaria immitis*

25 cm. Following sexual reproduction, gravid females produce new L_1 stage microfilaria that enter the bloodstream and remain in circulation for up to 2 years waiting to be ingested by a mosquito. In the blood stream, these microfilaria have been found to exhibit periodicity; meaning higher numbers circulating during the day than during the night. This behavior ensures the longevity of the microfilaria and increases the likelihood that they will achieve a full life cycle (7). Interestingly, the presence of circulating microfilaria has been linked to an increase in the life span of the adults that produced them (16). Adult HWs can remain viable in the pulmonary arteries of the dog for up to 5 years (11).

Diagnosis.

In dogs, the clinical findings are very much tied to the disease onset with early stage HW disease having subjective signs such as light coughing and in late stages producing congestive heart failure (CHF) leading to death (4). Therefore, veterinarians have established a ranking method to measure the verminous burden. Ranks are divided into classes with class I being the least severe stage of infection and class IV being the most severe stage. The clinical signs displayed by a class I (also known as asymptomatic) dog are light coughing, some loss of body condition and slight exercise intolerance (4). A class II (moderately infected) dog will display similar but more pronounced symptoms as seen in class I and it will also have serologically measurable symptoms such as anemia and proteinuria (4). In class III (severe disease), dogs will display all

clinical symptoms previously mentioned in addition to dyspnea, hemoptysis, syncope (loss of consciousness) and ascites (fluid accumulation in the abdomen) (4). When a dog is in Class IV (caval syndrome) it will require immediate surgery to save the dog's life. The main clinical symptoms are collapse and severe respiratory distress (4). Following proper assessment of the disease stage, the treatment administered will vary depending on the severity of disease/class.

Treatment.

Due to *D. immitis* ability to re-infect its mammalian host and increase the verminous burden on an already debilitated animal, diagnostic timing becomes a crucial aspect of successful treatment (4). For several years, researchers have been trying to improve ways to diagnose HW disease especially in occult infections, where dog are amicrofilaremic (20). In the early 1980s with the development of antigen/antibody enzyme-linked immunosorbant assay (ELISA) for the detection of heartworm infection, this problem seemed to be under control. Unfortunately, the current ELISA tests are now recognized as not perfect and do produce false-positive and false-negative results (13, 17, 21). Thus, the search for better ways to diagnose HW infection continues. There have been several research experiments in an attempt to use PCR probes for the accurate diagnosis of HW infections and some have been successful on microfilaremic dogs (13, 17, 21). However, these studies have not taken into account occult infections and amicrofilaremic host. Also, none of these studies have explored

the possibility of using DNA-based assays to screen for *Wolbachia endosymbiont of D. immitis (WeDi).*

Wolbachia are a genus of bacteria that live as endoparasites in many arthropods and filarial nematode hosts (2). The *Wolbachia* bacterium that lives inside heartworms is called, *Wolbachia endosymbiont of D. immitis (WeDi).* These bacteria have been found to have an obligate endosymbiotic relationship with *D. immitis;* meaning that one cannot survive without the other (1, 2). It is interesting to note that *Wolbachia* produces the antigens that cause severe inflammatory response characteristic with HW disease onset (3,18). Moreover, the eradication of *WeDi* bacteria from *D. immitis* could potentially serve as a treatment for HW infection. Current research on this subject is rapidly increasing (1,2). Techniques on the staining, detection, distribution and doxycycline susceptibility assays for *WeDi* are now available (5, 8, 9,10). In fact, there has been research on how to grow the L_3 stage microfilaria *in-vitro* to further study the endosymbiotic parasite-bacterial relationship and antibiotic susceptibility (19).

Part of the reason behind the recent emphasis on *Wolbachia* is because there are very few treatments available for HW disease patients and those that work can be dangerous to the pet. Currently, the only drug available for all HW stages is melarsomine dihydrochloride (4). The dosage and time lapse between injections of melarsomine dihydrochloride vary depending on the class of disease

(e.g. I,II,III,IV) and the size of the dog. For dogs in classes I and II, the prognosis is good. However, the animal must be completely restrained from exercise for up to 6 weeks post treatment (4). Conversely, dogs that fall under classes III and IV have a higher chance of death from the treatment due to postadulticide thromboembolic complications (4). Even with such harsh medicine and decreased chance of survival if the dog makes it through the treatment it is estimated that only 75% of the parasites will die (4). Thus, many times it is necessary to repeat the treatment to ensure the dog is parasite free. Immunization strategies against HW infection have failed to achieve protection for dogs (14). Unfortunately, the only way to fight this disease currently is to prevent it. Although, there are successful prevention methods commercially available, they require monthly dosages and can become increasingly expensive for pet owners with more than one pet (6, 15).

In this study, blood samples from dogs were screened for heartworm disease using polymerase chain reaction (PCR) techniques with primers specific to *D. immitis* and *WeDi*. Also, blood samples were tested with the commonly used enzyme-linked immunosorbant assay (ELISA) in an effort to compare the efficacy of ELISA and PCR diagnostic tools. A diagnostic tool with greater specificity for the detection of HW disease could lead to more successful treatment of infected individuals, specifically those in classes III and IV. Moreover, the ramifications of using *Wolbachia* as a target for diagnosis could

pave the way for other similar nematode infections such as *Wuchereria bancrofti*, the cause of human lymphatic filariasis. The objectives of this study were to (i) determine the competence of PCR as a diagnostic tool for HW disease when using primers specific to *D. immitis*, (ii) investigate the advantage of primers specific to *WeDi* for the detection of HW infection, and (iii) establishing if PCR could detect HW to a greater magnitude than ELISA assays. This study hypothesized that PCR using primers specific to *D. immitis* and *WeDi* is a more sensitive diagnostic tool than ELISA.

Methods and Materials

Sample collection.

For the time period beginning in October 2007 and ending on March 2008, blood samples were collected from the dog population at the Montgomery County Animal Control located at 1231 Highway Drive in accordance with its director David Shelby. Approximately, 1.5 milliliters of blood were collected using a 22G needle and 3mL syringe via cephalic venipuncture on the left forelimb of dogs above two years of age. Collected blood was placed in an EDTA vacu-tube and the animals were cleaned from any residual blood around the puncture wound then disinfected with alcohol. [Note: I have been a veterinary technician for over three years and I am properly trained in canine phlebotomy and restraint techniques. All blood collecting was done solely by me to ensure proper aseptic technique and animal safety. Prior to animal work, animal protocol approval and training was received through APSU-IUCAC (#08-001).] In addition to samples collected from the Montgomery County Animal Control, neighboring clinics submitted samples collected by their veterinarians and following the guidelines herein. The clinics that participated in this study were: All God's Creatures Veterinary Clinic Inc. (114 Kraft St.), Animal Hospital (11 McClure St.), and Oak Grove Animal Hospital (4953 Ft. Campbell Blvd).

Test subject specifications.

Dogs were selected for this study based on their age, gross anatomy and behavioral compliance with the following specifications:

1. **AGE**. If the age information of the animal was available, any dog having two years of age or more was considered for this study. In the cases where age information was not available it was determined by approximation after examining the teeth, paying special attention to decay and plaque build-up. The reason for choosing animals that are two years of age or more is that the period of time from infection to HW disease onset is about seven months (4). By choosing animals that are over two years of age it was hoped to increase our chances of finding HW positive animals.
2. **GROSS ANATOMY**. Special attention was given to the animals overall appearance. Specifically, any signs of emaciation and swelling around the ribcage which are indicative of HW disease (4). Also, any signs of good health were noted for example the animals coat and nails, weight and mouth mucosal tissue.
3. **BEHAVIOR**. Coughing and wheezing, lethargy and difficulty breathing were noted as they are signs common to HW disease onset. These factors helped establish if the animal was displaying clinical signs and were useful in the ELISA versus PCR comparison.

Symptomology assessment.

All dogs were grouped in one of two categories based on the symptoms observed while extracting their blood samples (Table 1). The early symptoms of HW disease onset that we looked for are also symptoms of other canine pathogens that are endemic in the Clarksville, TN area. For example, coughing, wheezing and labored breathing are also symptoms of a disease known as "Kennel Cough" caused by the bacteria *Bordatella bronchiseptica*. Also, some intestinal parasites, like hookworms (*Ancylostoma caninum),* can cause emaciation and lethargy. Therefore, rather than ranking individual symptoms, rank was based on presence or absence of an array of symptoms. Thus, animals displaying at least three of the clinical symptoms for HW disease were placed in group "S", in the cases where less than three symptoms were present the animal was grouped as not displaying clinical symptoms with the letter "H". If an animal was displaying all the symptoms consistent with good health it was grouped with the letter "H". Even seasoned veterinarians sometimes have trouble discerning when it comes to behavioral and gross anatomy observations of these types of symptoms. Therefore, this simple grouping was an effort to decrease the margin of error in this study.

Sample processing.

Once collected, the blood samples were placed in labeled EDTA tubes for transport in order to prevent clotting of the blood. Tubes were labeled with the

Table 1. Grouping of dogs according to the clinical symptoms observed

Category	Symptomology	Group
Not displaying clinical symptoms	Breathing normally Active Healthy coat and nails Good body condition Healthy mouth mucosa	H
Displaying clinical symptoms	Coughing / Wheezing Labored breathing Lethargy Fluid accumulation Emaciated	S

H. Seem healthy

S. Symptomatic

blood collection date and the Montgomery County Animal Control number as well as an assigned laboratory number. Additionally, the animal's symptomology and overall health were recorded. Transport to the laboratory took no longer than 20 minutes. Upon arrival at the laboratory, blood samples were transferred to serum separator tubes and centrifuged at maximum speed for 15 minutes. An average of 650 µL of serum was extracted from the 1.5 to 2.0 mL of blood collected from each dog. The serum portion was then removed and quantified using a micropipette. Half of the extracted sera was placed in sterile microcentrifuge tube labeled with the patient number and were used to run the PCR portion of the experiment. The remaining serum was placed in a tube labeled with the patient number were used to run the ELISA portion of the experiment. This method of extracting HW microfilaria DNA was troubleshooted using HW positive blood obtained from Dr. Rivera-Walker at Animal Clinic of North Clarksville.

Obtaining the positive control.

The positive control was DNA extracted from adult heartworms provided by Dr. Louis Pittman, Head of Pathology at Breathitt Veterinary Center located in Hopkinsville, KY. Approximately 0.5 g of tissue from the adult heartworms were manually diced and digested with 500 µL of proteinase K solution. This digestion was incubated at 37°C for 16 hours. Following incubation the digested tissue was triturated and an equal volume of a 1:1 ratio of phenol:chloroform was added. This mixture was then vertexed for 30 seconds and then centrifuged at 14,000 x

g for 5 minutes. The top, aqueous layer containing DNA was moved to a new tube and the addition of phenol:chloroform followed by vertexing and centrifugation was repeated twice. Once the sample was protein free, it was treated with twice the volume of isopropanol and 100 µl of 3M sodium acetate then placed in -80°C freezer for 5 minutes to allow the DNA to precipitate. The next step was centrifugation at 14,000 x g for 10 minutes at 4°C to pellet the DNA. The aqueous layer was then removed and the pellet was gently washed with 500 µL of 80% ethanol so as not to disturb the DNA. The tube was then centrifuged again at 14,000 x g for 5 minutes and then the aqueous layer was removed. Pelleted DNA was allowed to dry for 25 minutes and then resuspended in 100 µL of molecular grade water.

Primer design.

The *D. immitis* primer set was found in a journal article by Watts and colleagues where they designed a DNA probe to identify *D. immitis* DNA within its mosquito vector (21). The primer set for *Wolbachia endosymbiont of D. immitis* (*WeDi*) was designed using NCBI website tools. First, an rRNA sequence unique to *WeDi* was identified and then nucleotide sequences within the gene consisting of about 25 nucleotides was selected and compared to the entire HW and canine genome. Once a sequence with no matches other than *WeDi* was found, it was selected as a forward primer to ensure specificity to only *WeDi*. Next, sequences down stream on the same gene were selected that consisted of

about 25 nucleotides in length. With the same NCBI nucleotide blast tools, the primers were searched till no matches other than *WeDi* was found and selected as the reverse primer. All primers were PCR-tested to confirm their precision using HW extracted DNA (Table 2).

Polymerase chain reaction (PCR).

For the PCR portion of the experiment, 300 µL of the serum was placed in a sterile microcentrifuge tube and centrifuged at maximum speed for 10 minutes. This helped purify any microfilaria in the sample into a pellet. The supernatant was removed and the pellet was washed twice by adding 500 µL of 80% ethanol and placing samples in -80°C for 10 minutes. This wash assured that the pellet would be free of any serum proteins that could inhibit the PCR reaction. Following refrigeration, samples were vertexed for 30 seconds, centrifuged for 5 minutes at 4°C, the supernatant was removed and the pellet was allowed to dry. The pellet was resuspended in 80 µL of molecular grade water and boiled for 3 minutes to lyse any microfilaria in the pellet. PCR reactions were carried out in 30 µL total volume using GoTaq (Promega) polymerase. A total of four PCR reactions were done for each sample, each reaction was designated a letter (from A to D) for ease in labeling and clarity (Table 3). Samples labeled A screened for *D. immitis* DNA and those labeled B were experimental samples spiked with *D. immitis* DNA to ensure that the absence of a "positive" result in samples "A", was not due to an inhibitory agent. Samples labeled C screened for

Table 2. List of primers used in this study

Species Targeted	Primer Direction	Region	Amplicon Length	Primer
Dirofilaria immitis	Forward	16S	431 bp	GCATCTTAGAACTTGGTCCATCC
	Reverse	16S	431 bp	CAAAGGCGTATTTACCGCCAC
Wolbachia endosymbiont of Dirofilaria immitis	Forward	rRNA	743 bp	GTGAGGAAGATAATGACGGTACTCA
	Reverse	rRNA	743 bp	GGCCATGATGACTTGACGTCATCC

Table 3. Table of Reaction Mixtures for PCR Analysis

Label	Content	Total Reaction Volume
A	15µL of Go taq polymerase 2µL of *D. immitis* primer 8µL of molecular grade water 5µL of Sample DNA	30 µL
B	15µL of Go taq polymerase 2µL of *D. immitis* primer 7µL of molecular grade water 5µL of Sample DNA 1µL of positive control	30 µL
C	15µL of Go taq polymerase 2µL of *Wolbachia endosymbiont of D. immitis* primer 8µL of molecular grade water 5µL of Sample DNA	30 µL
D	15µL of Go taq polymerase 2µL of *Wolbachia endosymbiont of D. immitis* primer 7µL of molecular grade water 5µL of Sample DNA 1µL of positive control	30 µL

WeDi DNA and those labeled D were spiked screens similar to "B". To ensure proper functioning of our PCR equipment every run also included positive and negative controls.

Gel electrophoresis.

PCR samples were viewed using gel electrophoresis consisting of 0.8g of agarose, 1X TAE buffer solution. As with the PCR, a positive and negative control are ran along with the samples to confirm the results in addition to a ladder to establish band length.

Enzyme-linked immunosorbant assay (ELISA).

There were three brands of ELISA used for this study, the Witness®HW manufactured by Symbiotics Corp., the Solo Step®CH by Heska, and the CHAT™ by SA Scientific. Serum samples were centrifuged at >10,000 x g for 10 minutes then used for ELISA following the instructions in the package insert. Positive and negative ELISA results were evaluated based on the Witness®HW ELISA protocol, the Solo Step®CH ELISA protocol, and the CHAT™ ELISA protocol respectively. Despite the difference in brands these ELISA test all assayed for *D. immitis* antigen produced by the gravid female heartworms. The reason for using different brands of ELISA was not for comparison but rather for budget constraints. Samples 1-24 were tested using the Witness®HW ELISA,

25-48 were tested with the CHAT™ ELISA, and samples 49-78 were tested using Solo Step®CH ELISA.

Serial Dilutions.

In order to establish if PCR could detect HW to a greater magnitude than ELISA assays, serial dilutions of a positive sample were tested using PCR and ELISA. Sample 10 and sample 27 were chosen for these dilutions because they were strong positives for PCR and ELISA. Also, there was no samples that resulted positive for all three test. First, 10 µL of the reconstituted pellet DNA of sample #10 were placed in a sterile microcentrifuge tube containing 90µL of molecular grade water. Then 10µL from this, 10^{-1}, dilution were placed in another sterile tube containing 90µL of molecular grade water. This process was repeated until the concentration was 10^{-5}. The five serial dilutions were tested using PCR described above. For the ELISA dilutions sample #27 was chosen because it was a strong positive, the serum was serially diluted in sterile saline until the concentration of 10^{-5} was reached. Sterile saline does not affect the results of the Solo Step®CH ELISA test kit used for this process.

Results

Dr. Louis Pittman was able to obtain adult male and female heartworms from a dog necropsied at Breathitt Veterinary Center located in Hopkinsville, KY. The DNA extracted from these HW adults was used to test the primers and troubleshoot the optimal quantity of control DNA needed to obtain an accurate positive control. The optimal quantity of adult heartworm DNA needed to run a positive control (C+) along with the samples was found to be 1µL of a 10^{-1} dilution of the original DNA extraction from adult heartworms. Therefore, to test the *D. immitis* and *WeDi* primers just 1µL of a 10^{-1} dilution was necessary to obtain the desired bands. The *D. immitis* primers produce an amplicon of 431 nucleotides long (Figure 2). The *WeDi* primer set produced an amplicon that was about 743 nucleotides long (Figure 3). The HW positive blood provided by Dr. Rivera-Walker was used to test the microfilaria DNA extraction procedure. The optimal quantity of DNA extracted from blood needed for a successful PCR for both *D. immitis* and *WeDi* primers was found to be 5 µL, when the concentration of extracted DNA is at least 0.03 µg/µL

Beginning in October 2007 and ending on March 2008 a total of 78 blood samples were collected for this study (Table 4). The majority of the samples, 71 in total, were collected at Montgomery County Animal Control. Veterinarians at

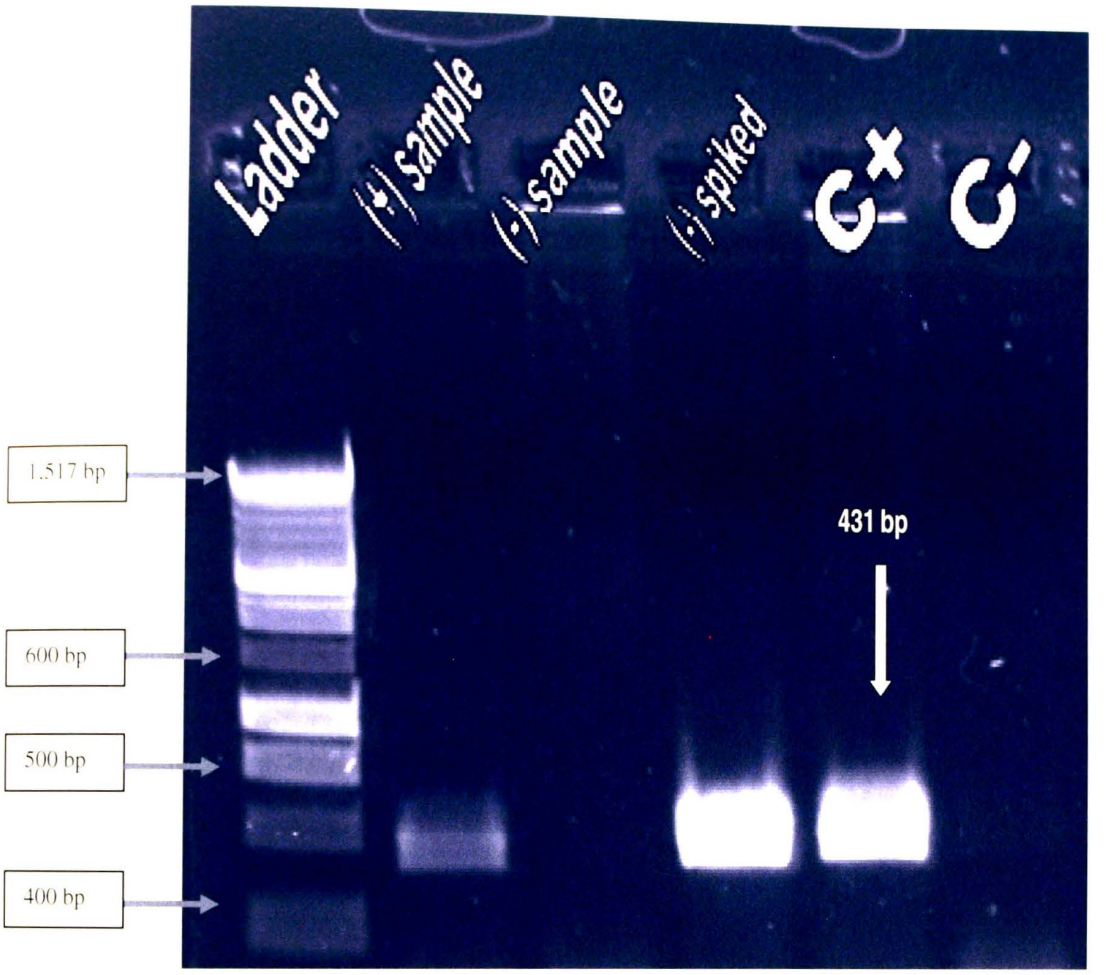

Figure 2. The expected bands seen on a *Dirofilaria immitis* positive animal are shown. From left to right; a DNA ladder, DNA extracted and amplified from *D. immitis* positive blood, DNA extracted from *D. immitis* negative blood, a spiked control of *D. immitis* negative blood, positive control and negative control. Amplicon length is approximated at 431 base pairs (bp).

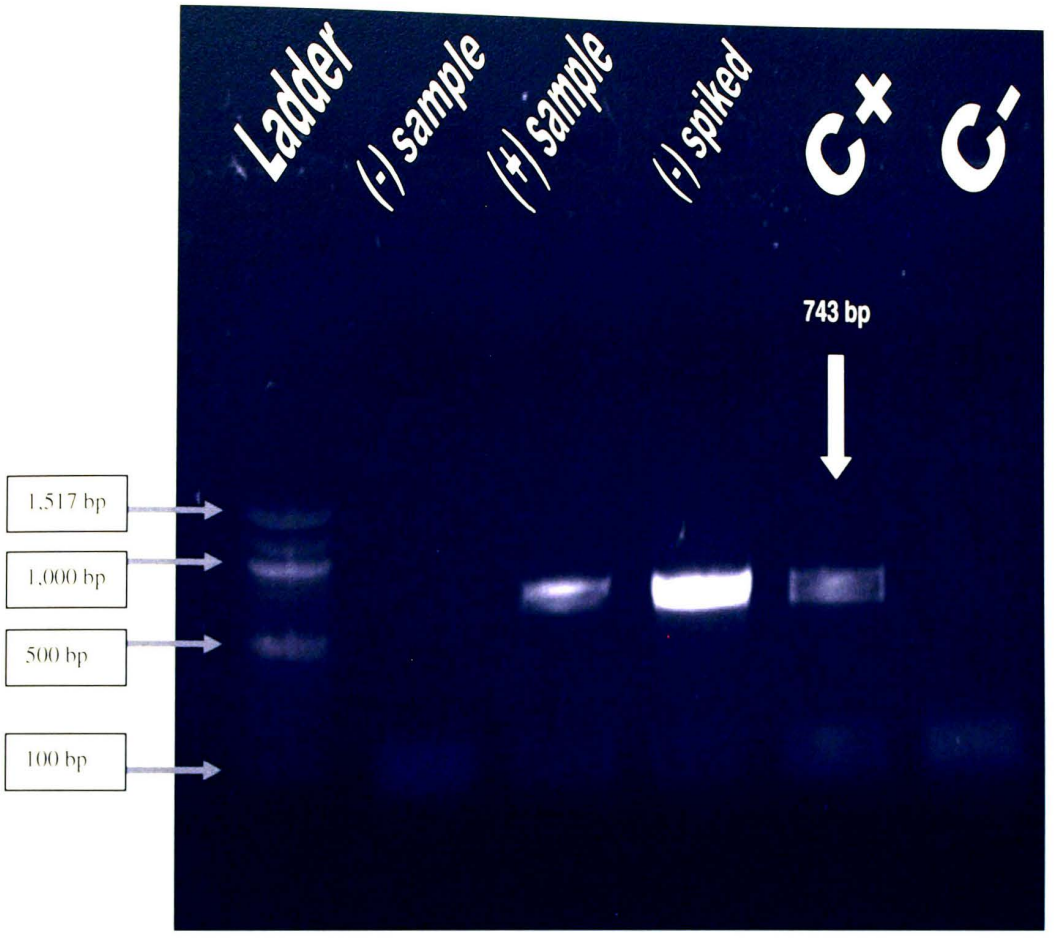

Figure 3. The expected bands seen on a WeDi positive animal are shown. From left to right; a DNA ladder, DNA extracted and amplified from WeDi negative blood, DNA extracted from WeDi positive blood, a spiked control of WeDi negative blood, positive control and negative control. Amplicon length is approximated at 743 base pairs (bp).

Table 4. All data collected for this study

Sample #	Location	A	B	C	D	Symptoms	ELISA
1	MCAC	-	+	-	+	S	-
2	MCAC	-	+	-	+	H	-
3	MCAC	+	+	-	+	S	-
4	MCAC	+	+	-	+	H	-
5	MCAC	+	+	-	+	S	-
6	MCAC	+	+	+	+	H	-
7	MCAC	-	+	-	+	H	-
8	MCAC	-	+	-	+	H	-
9	MCAC	-	+	-	+	H	-
10	MCAC	+	+	+	+	H	-
11	MCAC	-	+	-	+	S	-
12	AH	-	+	-	+	S	-/-
13	AH	-	+	-	+	S	-/-
14	AH	-	+	-	+	S	-/-
15	MCAC	-	+	-	+	H	-
16	MCAC	-	+	-	+	H	-
17	MCAC	-	+	-	+	H	-
18	MCAC	-	+	-	+	H	-
19	MCAC	-	+	-	+	H	-
20	MCAC	-	+	-	+	H	-
21	MCAC	-	+	-	+	H	-
22	MCAC	-	+	-	+	H	-
23	MCAC	-	+	-	+	H	-
24	MCAC	-	+	-	+	H	-
25	MCAC	-	+	-	+	H	-
26	MCAC	-	+	-	+	H	-

A. Result of the PCR done with *D. immitis* primer set
B. Result of the PCR done with *D. immitis* primer set spiked with positive control
C. Result of the PCR done with *Wolbachia endosymbiont of D. immitis* primer set
D. Result of the PCR done with *Wolbachia endosymbiont of D. immitis* primer set spiked with positive control
MCAC. Montgomery County Animal Control
AH. Animal Hospital
AGC. All God's Creatures
OGAH. Oak Grove Animal Hospital
ELISA. Left (test performed in the animal hospital) / Right (test performed at APSU laboratory)

Table 4. All data collected for this study. Continued.

Sample #	Location	A	B	C	D	Symptoms	ELISA
27	MCAC	+	+	-	+	H	+
28	MCAC	-	+	-	+	H	-
29	MCAC	-	+	-	+	H	-
30	MCAC	-	+	-	+	H	-
31	MCAC	-	+	-	+	H	-
32	MCAC	-	+	-	+	H	-
33	MCAC	-	+	-	+	H	-
34	MCAC	-	+	-	+	S	-
35	MCAC	-	+	-	+	H	-
36	MCAC	-	+	-	+	H	-
37	MCAC	-	+	-	+	H	-
38	MCAC	-	+	-	+	H	-
39	MCAC	-	+	-	+	H	-
40	AH	-	+	-	+	S	-/-
41	AGC	+	+	-	+	S	+/+
42	AGC	+	+	-	+	S	+/-
43	MCAC	-	+	-	+	H	-
44	MCAC	-	+	-	+	H	-
45	MCAC	-	+	-	+	H	-
46	MCAC	-	+	-	+	H	-
47	MCAC	-	+	-	+	H	-
48	MCAC	-	+	-	+	H	-
49	MCAC	-	+	-	+	H	-
50	MCAC	-	+	-	+	H	-
51	MCAC	-	+	-	+	H	-
52	MCAC	-	+	-	+	H	-

A. Result of the PCR done with *D. immitis* primer set
B. Result of the PCR done with *D. immitis* primer set spiked with positive control
C. Result of the PCR done with *Wolbachia endosymbiont of D. immitis* primer set
D. Result of the PCR done with *Wolbachia endosymbiont of D. immitis* primer set spiked with positive control
MCAC. Montgomery County Animal Control
AH. Animal Hospital
AGC. All God's Creatures
OGAH. Oak Grove Animal Hospital
ELISA. Left (test performed in the animal hospital) / Right (test performed at APSU laboratory)

Table 4. All data collected for this study. Continued.

Sample #	Location	A	B	C	D	Symptoms	ELISA
53	MCAC	-	+	-	+	H	-
54	MCAC	-	+	-	+	H	-
55	OGAH	-	+	-	+	S	+/-
56	MCAC	-	+	+	+	H	-
57	MCAC	-	+	-	+	H	-
58	MCAC	-	+	-	+	H	-
59	MCAC	-	+	-	+	H	-
60	MCAC	-	+	-	+	H	-
61	MCAC	-	+	-	+	S	-
62	MCAC	-	+	-	+	S	-
63	MCAC	-	+	-	+	H	-
64	MCAC	-	+	-	+	H	-
65	MCAC	-	+	-	+	S	-
66	MCAC	-	+	-	+	H	-
67	MCAC	-	+	-	+	H	-
68	MCAC	-	+	-	+	H	-
69	MCAC	-	+	-	+	H	-
70	MCAC	-	+	-	+	H	-
71	MCAC	-	+	-	+	H	-
72	MCAC	-	+	-	+	H	-
73	MCAC	-	+	-	+	S	-
74	MCAC	-	+	-	+	H	-
75	MCAC	-	+	-	+	H	-
76	MCAC	-	+	-	+	H	-
77	MCAC	-	+	-	+	H	-
78	MCAC	-	+	-	+	H	-

A. Result of the PCR done with *D. immitis* primer set
B. Result of the PCR done with *D. immitis* primer set spiked with positive control
C. Result of the PCR done with *Wolbachia endosymbiont of D. immitis* primer set
D. Result of the PCR done with *Wolbachia endosymbiont of D. immitis* primer set spiked with positive control

MCAC. Montgomery County Animal Control
AH. Animal Hospital
AGC. All God's Creatures
OGAH. Oak Grove Animal Hospital
ELISA. Left (test performed in the animal hospital) / Right (test performed at APSU laboratory)

Animal Hospital provided a total of 4 samples, the staff at All God's Creatures collected 2 samples and Oak Grove Animal Hospital collected 1 sample for this study (Figure 4). From these samples, a total of 69 were found to be negative for HW and 9 were positive for one or more of the variables assayed in this study (Figure 5). All samples were collected from dogs found to be consistent with the test subject specifications listed in the Methods and Materials section. Also, samples submitted by the neighboring clinics that contributed to this study were tested for heartworm using ELISA antigen tests on location and the results were provided (Figure 4). However, after receiving the blood samples, they were re-tested using the ELISA antigen tests noted in the Methods and Materials section for consistency purposes. Thus, the results for the ELISA performed at the clinics are shown on the right side of the slash sign (/) and the results of the ELISA tests performed at the APSU laboratory are shown on the left side in table 4. It is important to note that two of these donated samples tested ELISA-positive for HW on location and ELISA-negative for HW in our laboratory indicating some level of inaccuracy of ELISA assays. Therefore these samples (42 and 55) were not considered to be truly ELISA-positive for our statistical analysis. Only samples 27 and 41 which make up 2.6% of the total population for this study had ELISA-positive antigen results.

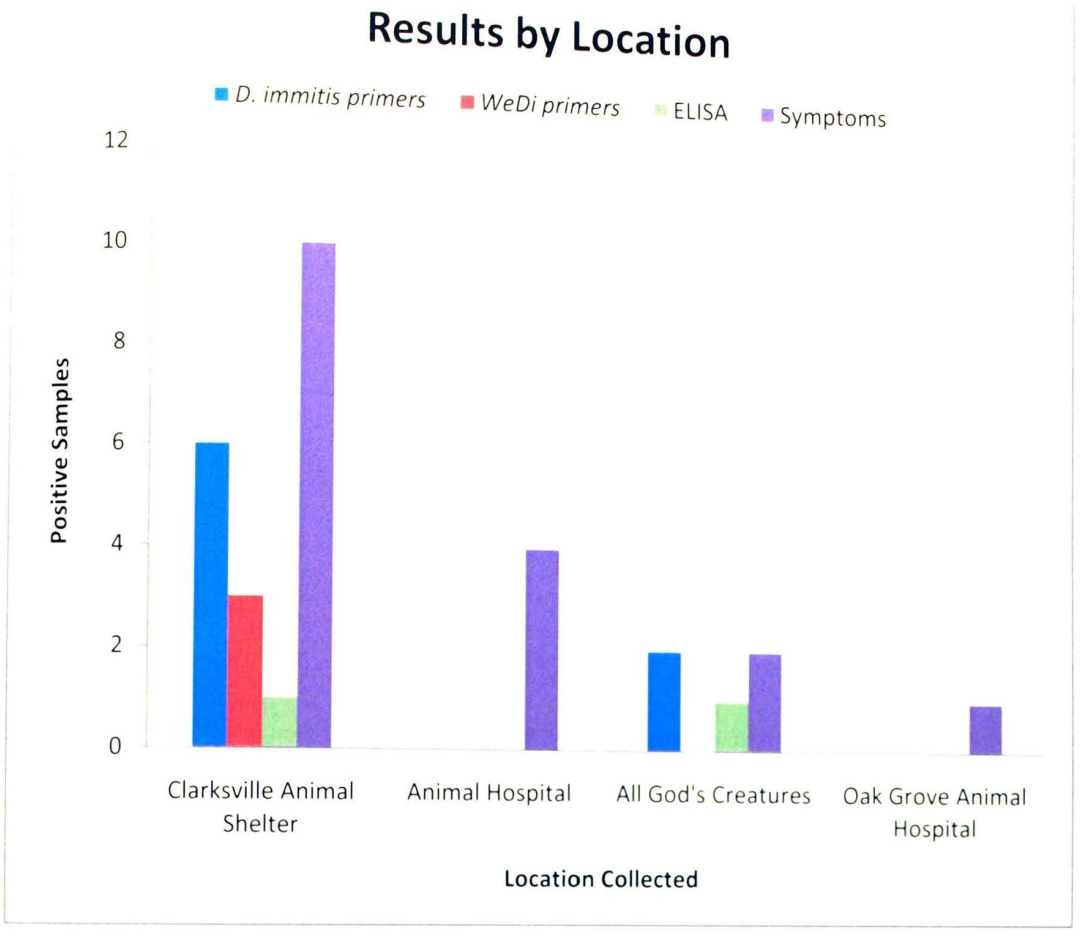

Figure 4. Summary of all positive results for *D. immitis* primers, *WeDi* primers, ELISA antigen test and Symptoms assessed in this study, grouped according to their collection site.

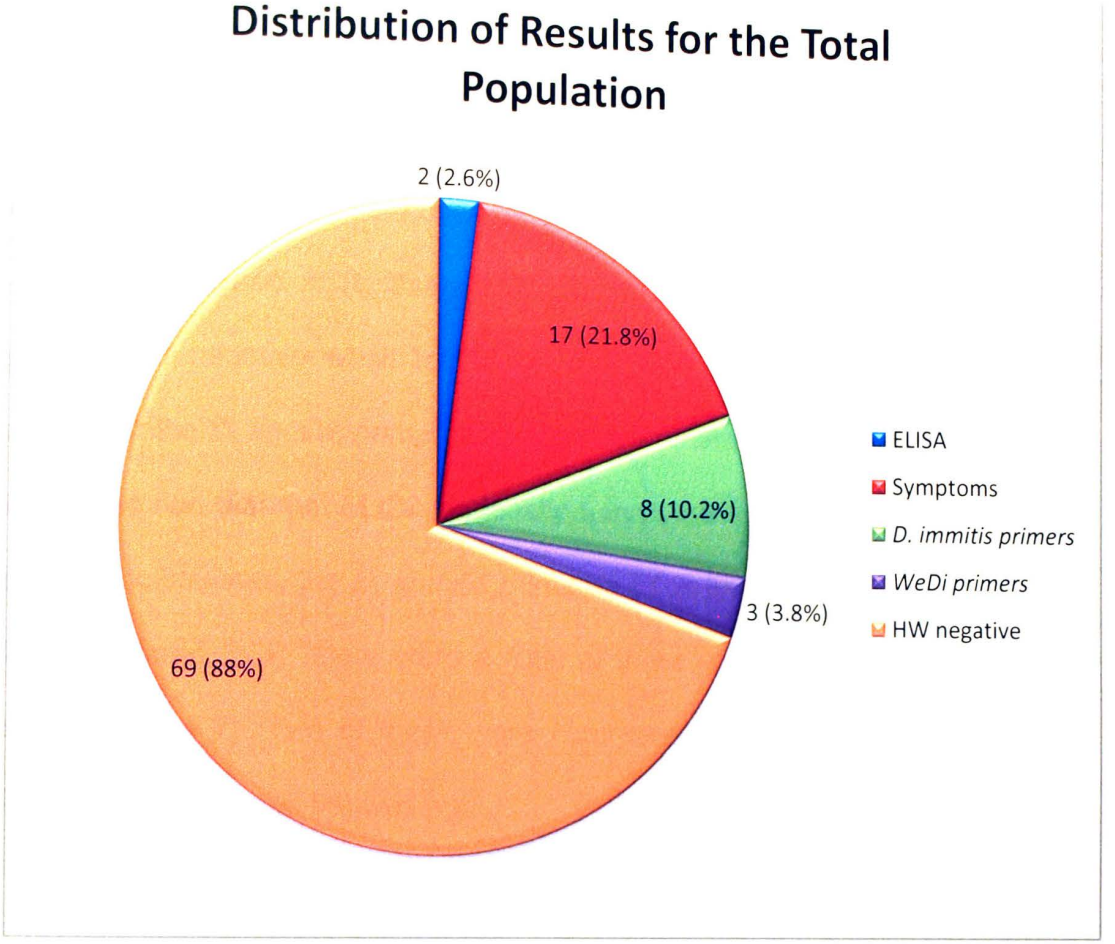

Figure 5. Distribution of negative 88% (69/78) and positive results for *D. immitis* primers 10.2% (8/78), *WeDi* primers 3.8% (3/78), ELISA antigen test 2.6% (2/78) and Symptoms 21.8% (17/78) assessed in this study.

The PCR results indicated an entirely different story from those detected using ELISA antigen test (Table 4). There were a total of eight samples (10.3%) positive for the PCR using *D. immitis* primers (Figure 6). More importantly, samples 27 and 41 which had been positive for ELISA were also positive for the *D. immitis* primered PCR. Sample 42 which had tested positive for ELISA on location and negative when tested in the APSU laboratory was found to be positive for the *D. immitis* primered PCR. A similar situation arose with sample 55 which also had different ELISA on location and laboratory results. In the case of sample 55, however, the *D. immitis* primered PCR had negative results. For the *WeDi* primered PCR, there were a total of three samples (3.8%) with positive results (Figure 7). Two of these were consistent with the *D. immitis* primered PCR and one (sample 56) was not.

The serial dilutions for the ELISA antigen test were done using sample 27 because it showed a stronger initial positive result than all other positive samples. The 10^{-1} dilution of this sample tested positive using the Solo Step®CH by Heska. When the dilution was increased to 10^{-2} however, the Solo Step®CH by Heska was not able to detect the antigen and the result was negative (Figure 8). For the serial dilutions using PCR, sample 10 was used because it had strong positive results with both the *D. immitis* and the *WeDi* primers (Figure 9). The results for both primers used were concurrent with the ELISA detection limit of the 10^{-1} dilution.

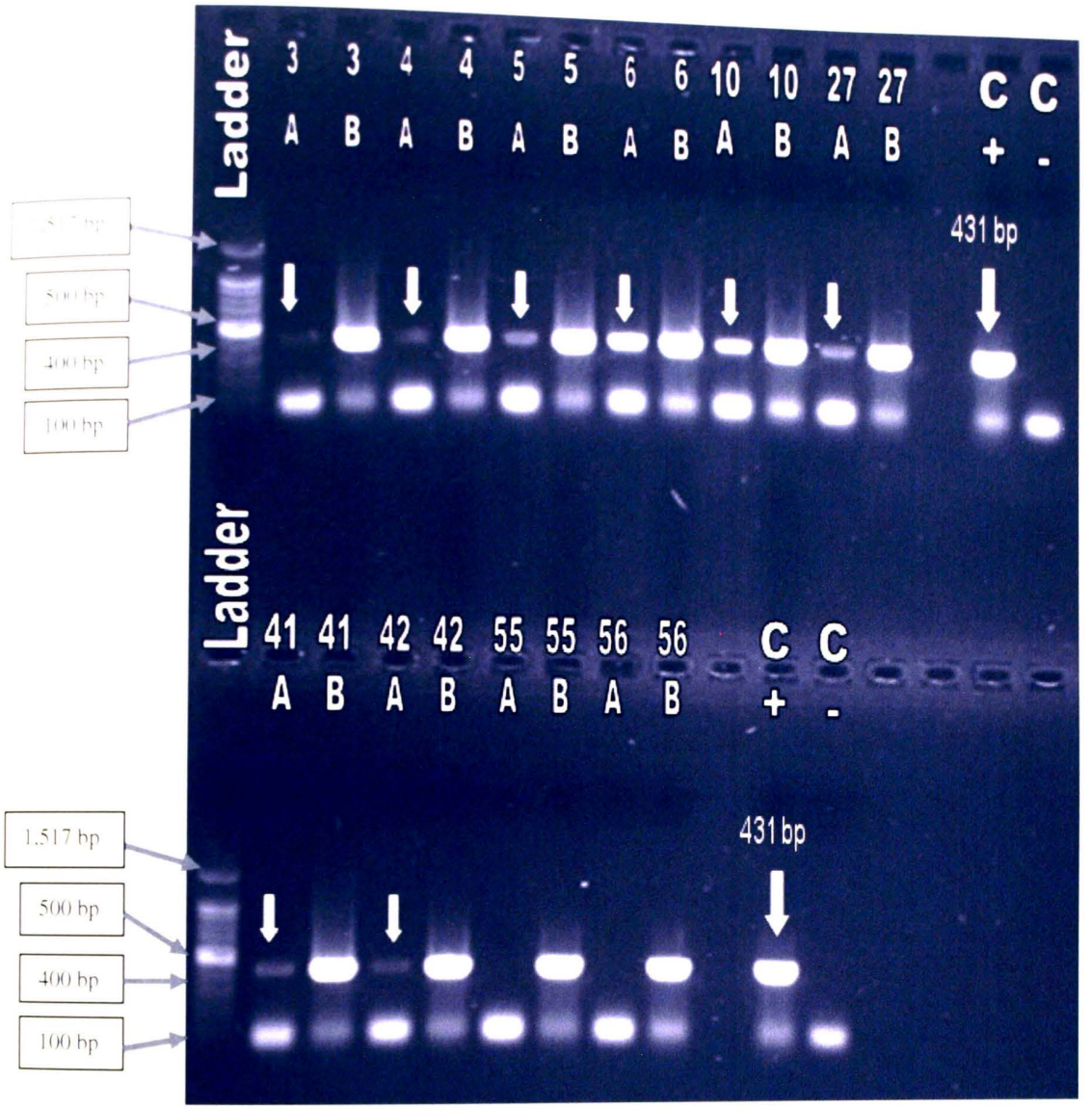

Figure 6. Gel showing the resulting *D. immitis* primered PCR products for all samples appearing positive for any and all test in this study. Letters A and B refer to sample and spiked sample respectively. Number corresponds to sample and letters correspond to sample (A) and spiked sample (B).

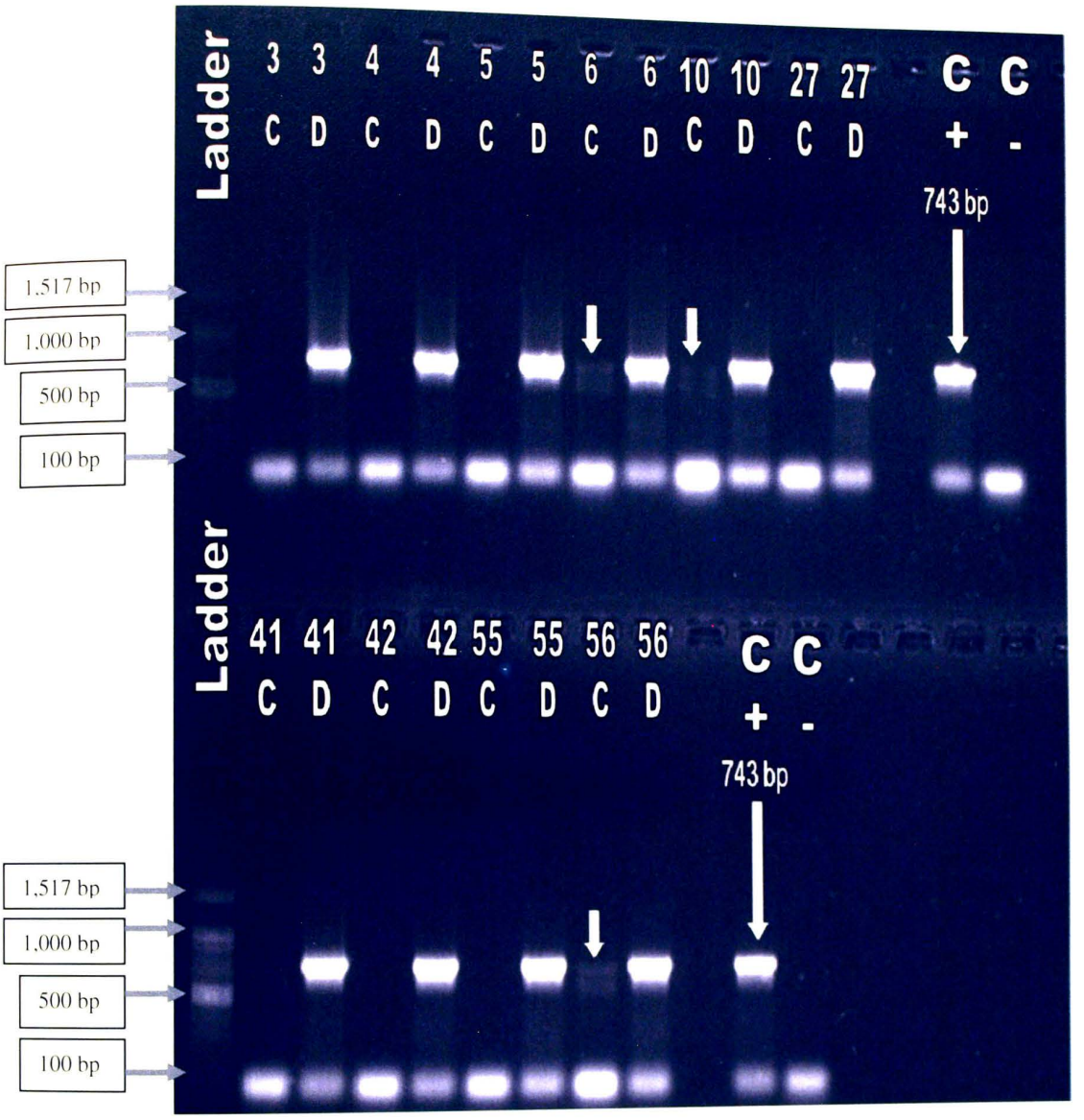

Figure 7. Gel showing the resulting *WeDi* primered PCR products for all samples appearing positive for any and all test in this study. Letters C and D refer to sample and spiked sample respectively. Number corresponds to sample and letters correspond to sample (C) and spiked sample (D).

Figure 8. Serial dilution of sample 27 using ELISA Solo Step®CH by Heska.

On left is the 10^{-1} dilution and on the right is the 10^{-2} dilution results.

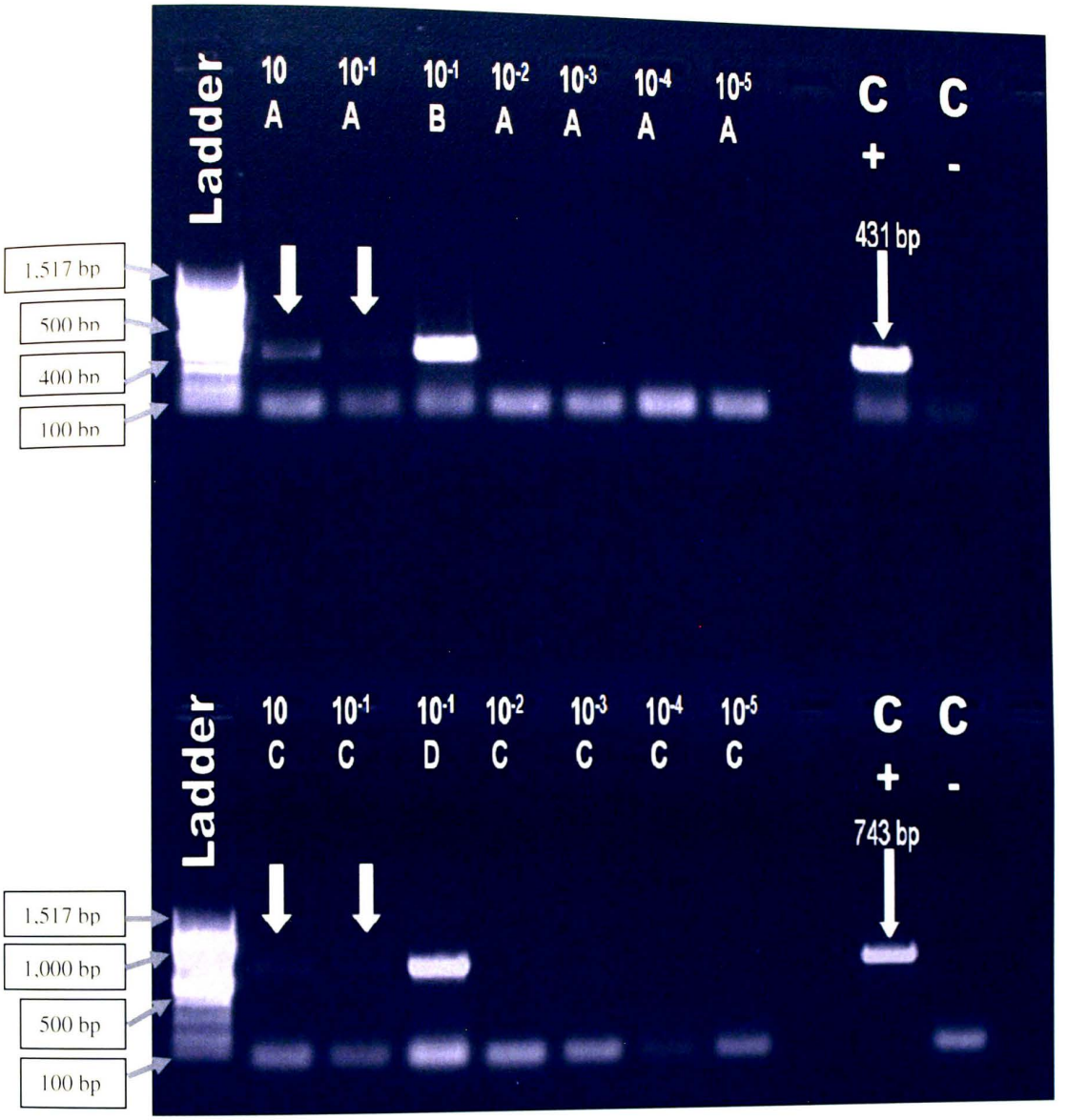

Figure 9. Serial dilutions of sample 10 PCR results. The top view is the original sample followed by the 10^{-1} dilution and spiked 10^{-1} dilution and subsequent dilutions for the *D. immitis* primered PCR. The bottom is are the original sample followed by the 10^{-1} dilution and spiked 10^{-1} dilution and subsequent dilutions for the *WeDi* primered PCR.

Clinical Symptomology was found in 17 (21.8%) patients prior to blood sample collection. A total of 13 patients (dogs) were categorized as symptomatic (S) and were negative for all PCR and ELISA test performed. In five cases were samples resulted positive for either ELISA or PCR, the patient was found not to be displaying any clinical symptoms consistent with the onset of HW disease. Only in four cases did the symptomology assessment coincide with positive test results.

The Chi squared (X^2) test for independence was used to measure the significance of the findings. When comparing the total samples positive for PCR (n=9) with the total samples positive for ELISA antigen test (n=2), significant difference was found for a p value < 0.001. When comparing the total ELISA positives (n=2) to the total *WeDi* primered positives (n=3) the expected value clause is 2.5. For a successful Chi square test, the expected value clause cannot be bellow five. Thus, for the premise that PCR using *WeDi* primers would increase the likelihood of diagnosing HW infection, a statistical analysis could not be performed because the data was insufficient. As for the symptomology assessment and the premise that PCR would detect HW infection better than ELISA, PCR detected HW infections in five animals that did not display clinical symptoms and four animals that displayed clinical symptoms. ELISA detected HW infection in one animal that displayed clinical symptoms and one that did not. Despite the difference in numbers, the values are close enough that half of the

positives for each test were either symptomatic or asymptomatic. Therefore, there is no difference between the likelihood that either test will detect HW infection in asymptomatic animals.

Discussion

During the six month span in which this investigation took place a total of 78 blood samples were collected from four locations in middle Tennessee. It was hoped that collecting most of the samples at Montgomery County Animal Control (MCAC) would increase the likelihood of obtaining HW positive blood. Unfortunately, the majority (69 or 88%) of the samples were found to be negative for heartworm infection by the ELISA antigen test and the PCR assays. The PCR assays used in this study detected nine (11.5%) positive samples in the total population. Conversely, ELISA antigen test detected only two positive samples and had conflicting results on two occasions. Both, the inability of ELISA antigen test to detect a total of seven positive samples and the conflicting results of samples 42 and 55 illustrates the eminent need for a better diagnostic tool for HW infection. Moreover, the PCR results found in this study imply that PCR is a competent diagnostic tool for HW infection and that it is more sensitive than ELISA antigen test.

In any veterinary practice, it is considered good medicine to screen dogs once a year for HW infection and encourage prevention if the animal tests negative. In order to purchase HW prevention, an owner must show proof that their animal has been tested for HW and found negative within that year. If more than seven months have passed since the test date, owners must also show

proof that their animal has been on monthly prevention. Currently, the ELISA antigen test is the most widely used and in many cases, preferred diagnostic tool for HW disease in dogs. When a dog has a false-negative result and is placed on prevention for HW, it can die. If the owner decides not to use prevention then their pet could become a HW reservoir. On the other hand, if a dog that is HW free has a false-positive result it will be placed on expensive treatment unnecessarily. Moreover, the mental stress of the pet owner and the many hours that the veterinary staff will endure taking all the necessary precautions on an animal that is a false positive are priceless.

ELISA antigen test is currently the most widely used diagnostic tool for HW infection mainly because it is practical. The ease with which ELISA is administered and the quick (less than 10 minutes) results are some of its strongest attributes. However, an accurate diagnosis in the clinical setting is crucial for effective treatment. As already discussed, there are an array of canine diseases that have similar symptomology and veterinarians need to be able to trust the current diagnostic methods. This is especially true for HW disease given the severity of its treatment and the implications for all parties involved in the case of a false-positive or a false-negative ELISA result. In this study, the four samples that tested ELISA-positive at some point, only two (27 and 41) had consistent ELISA positive results (Table 4). Samples 42 and 55 tested positive for the ELISA antigen test in the veterinary clinics where they were collected and

when the test was repeated in the APSU laboratory they both tested negative. It appears that sample 42 was truly positive give the confirmatory positive PCR result which indicates that the ELISA performed at the APSU laboratory may have been a false negative. On the other hand, sample 55 tested negative for all PCRs performed and for the APSU laboratory ELISA. Therefore, in the case of sample 55, the on-location ELISA was a false-positive and the APSU laboratory ELISA was a true negative. Also there were seven samples positive for PCR that ELISA failed to detect, two of which were positive for both *D. immitis* and *WeDi* primers.

In regards to the PCR analysis investigated, there were some inconsistencies between the *D. immitis* primered test and those using *WeDi* primers. Only two samples (6 and 10) were positive for both primer sets and seven were positive for one of the two primer sets (Table 4). This could be attributed to the DNA extraction procedure, given their size in relation to that of *D. immitis*, WeDi DNA might be present in smaller numbers and therefore, harder to detect using conventional PCR techniques. Sample 56 was the exception to this, it was positive for *WeDi* and negative for *D. immitis* DNA. The immune system of the dog might be responsible for this irregularity, upon initial infection or during the early stages, the host mounts an immune response against the nematodes (18). During this response, some microfilaria are lysed and their contents spills into the bloodstream, *WeDi* are part of these contents. Thus, it is

possible that sample 56 was collected during this period of initial immune response which would occur before any symptoms and the gravid female antigen could be detected by ELISA. Other than sample 56 there were no irregularities with the PCR analysis. The fact that the *D. immitis* primers produced more positive results than the WeDi might also be attributed to any antibiotics administered to the dogs prior to collecting blood. As previously mentioned some antibiotics such as doxycycline will rid any microfilaria present of their *Wolbachia* endosymbionts and render the adult HWs sterile (1, 5).

Symptomology was observed in 17 animals prior to blood collection and was found consistent with PCR and ELISA results in for 4 samples. As previously mentioned, the early symptoms of HW disease onset are also symptoms of other canine pathogens that are endemic to the area. Therefore, the inaccuracy of the symptomology assessment can be attributed to the similarity between HW disease onset and other canine diseases. It was hoped that establishing the presence or absence of clinical symptoms would shed light on the detection capabilities of PCR and ELISA, unfortunately that was not the case.

The serial dilutions of the strong positive samples showed that ELISA antigen test detected HW to the same magnitude as compared to PCR. The maximum dilution detectable by both methods was 10^{-1}. It was expected that PCR would detect HW to a greater magnitude than ELISA, unfortunately that was

not the case. Perhaps the DNA extraction techniques were not efficient enough. Further research on how to extract the microfilaria DNA from the blood is encouraged.

The Chi squared (X^2) test for independence was used to measure the significance of the findings and to give insight in the comparison of ELISA and PCR. When comparing the total samples positive for PCR (n=9) with the total samples positive for ELISA antigen test (n=2), significant differences was detected between PCR and ELISA results at a $p < 0.001$. This indicated that PCR is not only a competent diagnostic tool for the detection of HW infection but also it is more sensitive than ELISA.

Future focus should be placed on several aspects including sample size, improving microfilaria DNA extraction techniques from blood samples and improving PCR techniques to make them more practical for the veterinary clinical setting. The Montgomery County Animal Control was in the process of moving to a new location during the last two months of this study which prevented the collection of a greater amount of samples. Therefore, a larger population size might reveal more significant results in terms of the symptomology, detection limitations and overall advantage of PCR as a diagnostic tool for HW infection. The DNA extraction techniques developed and tested herein could be improved; perhaps by increasing the amount of blood serum processed initially one would

have a better yield of DNA. Furthermore, employing PCR techniques that are less elaborate and produce faster results might make it more appealing for veterinarians.

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Vita

Jarillys M. Marin graduated from Dr. Santiago Veve Calzada High School in the town of Fajardo Puerto Rico on May 1999. She started her undergraduate degree in the Universidad de Puerto Rico Recinto de Humacao on August 1999 were she attended classes for two years. Later in the fall of 2001 she moved to Miami Florida and began classes at Barry University where she would complete her bachelor's degree in Pre-vet with a minor in Chemistry and graduate on the spring of 2005. During her studies she worked as a veterinary technician in Island Veterinary Hospital under one of the most talented veterinary surgeons in Miami, Dr. Steve W. Eastman. In the fall of 2005 she moved to Tennessee and began pursuit of a Masters degree in Biology at Austin Peay State University. During her Master's she worked at Breathitt Veterinary Center in Hopkinsville Kentucky and acquired experience in the veterinary microbiology diagnostic field. She was the recipient of a Graduate Assistantship and a Minority Scholarship at APSU for the last year of her graduate coursework. After graduating with her Master's in Biology, Jarillys will begin working towards her Doctorate of Veterinary Medicine degree at Tuskegee University in Alabama.