5-2018

Genetic Markers Involved in Macrocyclic Lactone Resistance in Dirofilaria immitis and Estimation of Heartworm Prevalence in Resident Canine Populations of the Cumberland Gap Region of Kentucky, Tennessee, and Virginia.

Hailey Watlington

Charles T. Faulkner

GENETIC MARKERS INVOLVED IN MACROCYCLIC LACTONE RESISTANCE IN *DIROFILARIA IMMITIS* AND ESTIMATION OF HEARTWORM PREVALENCE IN RESIDENT CANINE POPULATIONS OF THE CUMBERLAND GAP REGION OF KENTUCKY, TENNESSEE, AND VIRGINIA

HAILEY WATLINGTON, BS

A thesis submitted to the faculty of Lincoln Memorial University
In partial fulfillment of the requirements for the degree of MASTER OF SCIENCE
May 1, 2018

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BIOGRAPHIC SKETCH OF THE AUTHOR

Hailey Watlington was born in Seattle, Washington, and raised in Waterford, Virginia. She pursued her undergraduate degree at James Madison University in Harrisonburg, Virginia, where she obtained her Bachelor of Science degree in Biology.

Following graduation, Hailey moved to Gulu, Uganda to the position of Country Director for a non-governmental organization called Zion Project, where she oversaw all aspects of the organization including an employment program for formerly trafficked women, a community outreach, and a home for girls who had been trafficked and exploited. Hailey moved to Seattle after her time in Uganda where she worked as a veterinary assistant in a general practice and emergency and referral center and took classes to fulfill veterinary school pre-requisites.

In 2015, Hailey came to Lincoln Memorial University to pursue her Master of Science degree in Life Science Research. During her first year, she was mentored by Dr. Melissa Henderson working on *Caenorhabditis elegans* transgenics. She then moved to Dr. Charles Faulkner's parasitology laboratory to begin work on her master's thesis project.

Now, Hailey is a second-year veterinary student at Lincoln Memorial University-College of Veterinary Medicine. She has professional interests in global public health, policy, and rural mixed animal practice.

Outside of veterinary school, Hailey enjoys kayaking, backpacking with her husband and two dogs, cycling, and going to breweries.

DEDICATION

To my husband Greg, for your unwavering support and love during the completion of this thesis.

ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Charles Faulkner for his encouragement and guidance, and my committee members Dr. Melissa Henderson, Dr. Vina Faulkner, and Dr. Ashutosh Verma for their help in developing and editing my project and thesis. Dr. Henderson's guidance on the molecular portion of this thesis project was instrumental to my success (and my sanity).

I would also like to thank Dr. Jason Johnson and the Lincoln Memorial University-College of Veterinary Medicine for their support and funding for my work over the summers.

I would also like to show my appreciation for the Master of Science graduate research program, Dr. Adam Rollins and Dr. Amiel Jarstfer for supporting and funding my project.

I would also like to thank my people – Ana, Andrea, Melissa, Lauren – for reminding me of class times and Vet Path journals, sending me study guides, and providing me with comic relief and Mexican food during this process. It takes a village. You are the reason I haven't failed out of vet school.

Lastly, I would like to thank my husband Greg and my family for their constant support and encouragement throughout this journey.

ABSTRACT

GENETIC MARKERS INVOLVED IN MACROCYCLIC LACTONE RESISTANCE IN *DIROFILARIA IMMITIS* AND ESTIMATION OF HEARTWORM PREVALENCE IN RESIDENT CANINE POPULATIONS OF THE CUMBERLAND GAP REGION OF KENTUCKY, TENNESSEE, AND VIRGINIA

Infection with heartworm (*Dirofilaria immitis*), is a significant cause of disease in companion animals worldwide. Macrocyclic lactones (MLs) have been the standard treatment for heartworm prevention for the last 25 years. Although widely used and highly effective, recent studies have shown a potential loss of efficacy. Investigations suggest the involvement of a single-nucleotide polymorphisms (SNPs) of a gene encoding a fragment of P-glycoprotein (P-gp) within the *Dirofilaria. immitis* genome. This reveals a potential relationship between SNP frequencies within the P-gp gene and ML resistance. The prevalence of heartworm infection in the Cumberland Gap Region (CGR) is reported to be 2.73% based on dogs tested at veterinary clinics. This may underrepresent the true prevalence of infection because up to 55% of pet dogs in the area do not receive regular veterinary care. The goal of this study was to obtain a more accurate estimate of the true prevalence of heartworm infection and the microfilaremic status of infected canine hosts and survey the population of *D. immitis* infecting dogs in the CGR for the existence of the single nucleotide polymorphism within P-glycoprotein and to. We found an overall prevalence in the CGR of 5.04%, higher than what was reported. Additionally, we did not find the GG-GG genotype that is suspected to be involved with ML resistance, but we did find a SNP of GA-GG at the loci of interest.

KEYWORD LIST

Heartworm, *Dirofilaria immitis*, macrocyclic lactone, loss of efficacy, resistance, single nucleotide polymorphism, prevalence, antigen, microfilaria, Cumberland Gap Region

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CHAPTER 1

INTRODUCTION

Dirofilaria immitis, a filarial nematode, is the causative agent of heartworm disease in pet dogs and cats worldwide. The disease is more prevalent in areas of the world where there are temperate or tropic climates. Heartworm disease is insidious in its onset, associated with significant cardiovascular disease and may result in premature death. Cases of "lack of efficacy" (LOE) or failure to prevent heartworm infection in monthly treated dogs were first reported in 1998 and increased incrementally to over 1500 reports by 2003 (Pulaski et al, 2014). Hypothesized factors associated with the phenomena included (1) the emergence of true genetically based drug resistance, (2) increased FDA drug surveillance for LOE, and (3) climate mediated extension and availability of heartworm susceptible mosquito vector populations (Pulaski et al, 2014).

In 2011, the genomic basis of canine heartworm resistance to macrocyclic lactone drugs was described in a series of papers (Bourguinat et al, 2011a; Bourguinat et al, 2011b; Geary et al, 2011). These papers demonstrated the correlation of LOE in the macrocyclic lactone drugs with the presence of a GG-GG genotype within the P-glycoprotein gene in *D. immitis*. Subsequent studies confirmed the importance of this SNP and its possible role in the interaction between macrocyclic lactones and the efflux of macrocyclic lactone drugs to potentially sub-therapeutic concentrations in individual worms possessing the characteristic. Further studies have demonstrated variation in susceptibility of the worms to formulations of particular macrocyclic lactone compounds leading to claims of increased efficacy or superior protection coverage by

pharmaceutical manufacturers. For the pet owning consumer making informed choices among competing heartworm prophylactic products with varied degrees of efficacy has become increasingly difficult.

Developing an estimate of the natural occurrence of canine heartworm in the dog population of the Cumberland Gap Region (CGR) is an important pre-requisite to any effort to identify the presence of the genetic trait conferring macrocyclic lactone resistance in the heartworm population in the region. Current estimates of canine heartworm for the CGR are largely anecdotal and based on sporadic sampling of cases identified at local veterinary practices for statistical mapping on the website of the Companion Animal Parasite Council and other reporting bodies. This approximation of regional canine heartworm prevalence is based on pet dogs that benefit from veterinary care as well as dogs in animal shelters and rescues is an important foundation for assessing the significance of the presence or absence of the genetic trait associated with heartworm susceptibility to monthly preventive medications.

This thesis describes the results of a two-fold study to estimate the serological prevalence of heartworm infection in dogs residing in the CGR, and the effort to determine the presence of the presence of a GG-GG genotype or other single nucleotide polymorphisms within the P-glycoprotein gene that are potentially associated with LOE in macrocyclic lactone drugs used for prevention *D. immitis* infection. The objectives of this study are to:

1. Estimate the prevalence of canine heartworm infection in CGR dogs by serologic detection of antigen produced by adult *D. immitis* worms.

2. Identify the presence of the GG-GG single nucleotide polymorphism (SNP) or other SNPs and their gene frequencies in the gene that encodes P-glycoprotein for drug efflux in adult *D. immitis* worms recovered by necropsy of canines euthanized at regional animal shelters.

CHAPTER 2

LITERATURE REVIEW

Introduction

Dirofilaria immitis is a nematode parasite classified in the superfamily Filaroidea. It is the causative agent of heartworm disease in dogs, cats, and ferrets world-wide (Bowman et al, 2009). The parasite also infects wild canidae including red and gray fox, coyotes, raccoon dogs, and crab-eating zooro (fox), sea lions, penguins, and humans (Bowman et al, 2009). The pathogenesis of the disease includes an infected host, a mosquito vector, and a definitive host (domestic or wild canids). In order to develop a full understanding of the scope of the disease, it is necessary to examine each element as a piece of the puzzle. This includes mosquito biology and transmission, pathogenesis, diagnosis, treatment, prevention, epizoology of dog heartworm in domestic and wild canids, and the molecular basics of the relationship between *Dirofilaria immitis* and macrocyclic lactones.

Life cycle biology and mosquito vector transmission of *Dirofilaria immitis*

The life cycle of *D. immitis* includes developmental stages within the vertebrate and invertebrate hosts. Dogs become infected when third-stage larvae (L_3) exit the labium of an infected mosquito during a blood meal. The L_3 then enter through the wound created by the feeding mosquito, at which point development from L3 to L4 occurs in musculature and submuscular layers after approximately 3 days. Between 50 to 58 days after the initial infection, larvae develop to the young adult stage, where they enter the bloodstream through the surrounding vasculature and migrate to the pulmonary arteries. The parasite then completes its maturation in the hosts'

cardiac and pulmonary tissue. Mature parasites reproduce, and gravid females release microfilariae in to the host's bloodstream, which are detectable 6 to 9 months after infection. The microfilariae circulate in the host's bloodstream where they can be taken up by a mosquito feeding during a blood meal (Ledesma et al, 2011).

Because *D. immitis* is transmitted by the bite of an infected mosquito, the simultaneous presence of both infected dogs and the appropriate vector species may pose an increased risk of transmission among dogs in a certain area (Tzipory et al, 2010). The prevalence of heartworm disease is dependent on the existence and distribution of competent vectors. Likewise, the infected competent vector population (in this case, mosquitoes) is dependent on the population of infected animals present (Fryxell et al, 2014).

Over 60 species of mosquitoes have been shown to be competent vectors (Lok et al, 1988). A study from 1987 (McLaren et al) describes 24 species of mosquitoes found to be natural infected with *D. immitis*. The strongest evidence for vector incrimination in a mosquito is the detection of infective-stage *D. immitis* in wild-caught specimens (Bowman et al, 2009). This particular finding demonstrates the mosquito is capable of feeding on infected hosts, becoming infected, accomplishing necessary life stages within the mosquito, and transmitting infectious heartworm to another host (Ledesma et al, 2011). If a species of mosquito is found in a high endemic area, it is likely there is transmission occurring via mosquito vector. A study in Tennessee trapping and testing mosquitoes for *D. immitis* detected *D.* immitis genomic DNA in 1.3% of samples in eastern Tennessee, and detected *D.* i*mmitis* genomic DNA in 8% of samples in western Tennessee (Fryxell et al, 2014). This study implicated multiple species of mosquito, including

Culex nigripalpus, Culex pipiens, Aedes vexans, Anopheles quadrimaculatus, and *Aedes japonicus. Culex* mosquitoes were significantly more likely to be polymerase chain reaction positive with *Dirofilaria immitis* than *Aedes* mosquito during the trapping period (Fryxell et al, 2014). However, a 1998 study (Scoles et al) described *Aedes vexans* mosquitoes to have the highest rate of natural infection with *D. immitis*.

There appears to be a seasonal variation in the number of microfilariae found in the blood of dogs. In most geographic areas, the maximum number of microfilariae in circulation tends to be during the late afternoon and early evening during the spring and summer, compared to the fall and winter (Newton and Wright, 1998). This is logical, due to the higher presence of the mosquito vector during warmer months. Additionally, larval development within the mosquito is affected depending on temperature (Bowman et al, 1990). It takes approximately 10-14 days of temperatures of around 80°F for the larvae to reach the infective stage (Slocombe et al, 1989). In the United States, most geographic regions do not maintain a temperature of 80°F year-round, so the larval development within the mosquito is affected. This trend in development has been used to determine periods of expected transmission within the United States and Canada (Lok et al, 1998). These transmission maps are based on Heartworm Development Units (HDUs). HDUs define the number of Degree Days that the larval stage of *D. immitis* is above 57°F. The larvae accumulate a specified number of HDUs (approximately 130) to be defined as infective (Lok et al, 1998). These predictions only serve as a guideline for mosquito activity.

There is no doubt that the majority of heartworm transmission occurs in the seasons predicted by HDUs, but there is good reason to suspect that transmission can occur year-round in certain

places in the United States (Bowman et al, 2009). It has been shown that despite cooler weather's detrimental effect on mosquito survival, they can enter a period of quiescence, where they can rest and survive until warmer weather, where they resume activity (Lok et al, 1998). Additionally, it has been shown that *D. immitis* larvae are able to pause development when cooled and resume once the appropriate temperature for development has been reached (Lok et al, 1998). Moreover, cooling mosquitoes to 53°F did not affect the viability of L_3 larvae after the temperature was increased (Ernst et al, 1983). Additional studies on the effects of *D. immitis* infection on the overwintering mosquito are needed to fully understand this effect. It is essential to consider the preference of mosquito vectors for breeding sites and geographic areas when discussing the epidemiology of dog heartworm (Ledesma et al, 2011). In summary, in addition to a competent vector having access to a susceptible host, it is necessary to consider average temperature, rainfall, humidity, and the availability of viable breeding sites in a geographical area (Ledesma et al, 2011).

Pathogenesis and treatment

The spectrum of disease produced by *D. immitis* is associated with significant immune-mediated inflammatory damage to the pulmonary arteries and lung. The severity of the damage is dependent on the number of worms, exercise, and duration of infection. Many pathophysiological changes occur as a result of infection with *D. immitis*, but the most notable issue is the proliferation of the villi associated with the inner lining of the arteries in which the worms reside. The pulmonary arteries become increasingly thrombosed and noncompliant, becoming obstructive to blood flow and unable to respond during increased oxygen demand, leading to diminished exercise capacity (Bowman et al, 2009). Contributing to the pathogenesis

is hypoxia produced by ventilation-perfusion mismatch caused by the thrombosis within the vessels. The end result of the prolonged vasoconstriction is pulmonary hypertension, decreased cardiac output, and overall difficulty of the heart to respond to demands placed upon it. In response to the increased stress, the heart can develop secondary tricuspid insufficiency and perivascular edema as a result of increased pulmonary vascular permeability. Additionally, the host can develop eosinophilic pneumonitis as an inflammatory response to the presence of the pulmonary microfilariae (Bowman et al, 2009).

Infection with heartworm is classified by severity in to four stages. Stage 1 includes asymptomatic to mild heartworm disease. Some fatigue and a mild cough may be present, but radiographs and physical examination are normal. Inflammation begins as soon as the worms present within the vasculature. Stage 2 is characterized as moderate heartworm disease. Anemia may be present, as well as abnormal radiographs indicating right ventricular enlargement. Physical exam may reveal loss of condition, occasional cough, and fatigue. Stage 3 heartworm disease earns a guarded prognosis and may require carefully managed chemotherapeutic intervention. Physical exam reveals constant fatigue, difficulty breathing, and persistent cough, indicating severe heartworm disease. Moderate anemia may be present. Radiographs may reveal signs of right-sided heart failure. Stage 4 heartworm disease is the most severe. The organism *D. immitis* is found in the vena cava and right atrium, often referred to as caval syndrome (American Heartworm Society, 2007). Animals with this severe of infection cannot be treated with melarsomine and the adult worms must be removed surgically. Caval syndrome is a result of the accumulation of large worm burdens and their displacement from the pulmonary artery in to the right ventricle and atrium, which produces obstruction to the right heart and produces

tricuspid insufficiency. In addition to the murmur produces as a result of the large number of worms residing in the heart, the dogs also have heartworm-induced pulmonary hypertension and cardiac arrhythmias that further complicate their disease process. Hemoglobinuria is considered pathognomonic for caval syndrome and is the result of lysis to the red blood cells passing through the collections of heartworms now in the right atrium and vena cava. This intravascular hemolysis as well as the resulting hepatic dysfunction induce an impaired ability of normal coagulants to circulate through the body. This, in turn, leads to disseminated intravascular coagulation, which is fatal within 24 to 72 hours without treatment (Bowman et al, 2009).

Additionally, *D. immitis* harbors an obligate, intracellular, endosymbiotic bacteria of the genus *Wolbachia*. It has been found that *D. immitis* relies on their endosymbionts for a many biological processes, including the encoding of enzymes for anabolic pathways that are lacking in the worm, such as the synthesis of heme, purine, and pyrimidines (Godel et al, 2012). Similar to other filarial species, elimination of *Wolbachia* is detrimental to *D. immitis* (Kramer et al, 2018). When *Wolbachia* was removed from *O. volvulus*, through antibiotic treatment of the infected host, the inflammatory response was diminished considerably (Bazzochi et al, 2003). Similarly, studies have shown that dogs naturally infected with *D. immitis* mount a humoral response to a protein derived from *Wolbachia* (Kramer et al, 2005). This could contribute to the inflammatory response of the host to treatment and subsequent thromboembolic complications.

Cats can also be infected with *D. immitis,* however they are not competent hosts. Larvae can mature to adult worms but are rarely able to reproduce and do not produce enough microfilaria to infect mosquitoes. Heartworm disease in cats tends to be less severe than that in the dog host and

manifests as mild respiratory disease, called heartworm-associated respiratory disease (HARD), characterized by an inflammatory response to the migrating larvae. The resulting disease resembles feline asthma. Infection in both cats and dogs is ultimately fatal (Bowman et al, 2009).

Although rare, humans are also able to be infected with *D. immitis*. It has been shown that human incidents of dirofilariasis are higher in areas where there is high prevalence in the dog and cat population, but can occur anywhere the parasite is found (Theis, 2005). In 50-80% of human cases, the disease is non-patent, making humans a dead-end host for the parasite (Lee, 2010). Infective larvae follow a similar path to those in canid hosts, traveling through the vasculature before arresting in the pulmonary arteries, where the adult worms form granulomas called "coin lesions" (Theis, 2005).

Diagnosis

There are several tests that can be performed to determine an animal's heartworm status. The most common method for determining heartworm status is the enzyme-linked immunoassay (ELISA) and immunochromatographic antigen test. These tests detect *D. immitis* antigen primarily associated with sexually mature female worms. These assays demonstrate good ability to detect heartworm positive animals (sensitivity) and very good specificity, or ability to identify animals that are not infected with heartworm (Henry et al, 2018).

Prior to the widespread commercial availability of heartworm antigen detection assays, diagnosis of *D. immitis* infection was based on identification of the circulating microfilarial stage recovered from the peripheral blood (the modified Knott's test). *D. immitis* microfilariae are

distinguished from other filarial species circulating in the blood by diagnostic measurement (Newton and Wright, 1956). Although heartworm diagnosis by microfilariae detection is likewise susceptible to false negative results, it has relevance for the epidemiologic survey portion of this research because it is direct evidence that active transmission is occurring between the infected host population and the susceptible vector population of mosquitoes.

Post-mortem recovery of adult *D. immitis* is the only way to unequivocally confirm the infection status of the host. Adult worms residing in the pulmonary vasculature or right ventricle of the heart are removed for determination of sex and measured for estimation of their age.

In dogs with heartworms, radiographic abnormalities are present in approximately 85% of cases (Bowman et al, 2009). Radiography provides the most objective method to assess the severity of cardiopulmonary abnormalities as a result of heartworm infection. However, thoracic radiography is an effective method for determining the severity of the disease and cannot be used alone diagnostically. Additionally, serial radiography can assist in tracking changes during and after treatment.

There are limitations with each of the tests listed above. In the Modified Knott's test, occult infections where there are adult worms present in the heart, but no circulating microfilaria will lead to a false negative. Additionally, it takes approximately 6-9 months for an infection to produce circulating microfilaria, as opposed to the 4-5 months for the worms to mature in the host. Because it only uses 1ml of blood, the Modified Knott's test also has a low sensitivity compared to other methods. Radiographs tend to be expensive and cannot be used as the primary

source of determining a heartworm infection due to the possibility of other cardiopulmonary pathology. Lastly, antigen tests detect a female reproductive tract, so the female worms must be matured before the test will display a positive result. Antigen tests can also display false negative tests due to the timing of the development of the worms within the host. If the dog is tested within the 4- to 6-month window after being infected, there is a chance they would have a negative test despite their developing infection. At this point in the infection, the worms are not mature enough and therefore the host has not developed a sufficient antigen response to turn the test positive. With cardiac dissection in a dead animal, the person performing the procedure visually assesses the heart and pulmonary vasculature for adult worms. Worms may be overlooked or difficult to identify in decayed cadavers if the person conducting the necropsy is inexperienced. Worms may also be sequestered in occluded branches of the pulmonary artery and difficult to find. These sources of investigator or bias or human error are important points to recognize.

Treatment options vary depending on the clinical status of the patient. A complete history must be taken, including the activity level of the dog. High activity levels are one of the most significant factors contributing to complications after treatment (Dillon et al, 1995). The American Heartworm Society recommends a monthly macrolide drug regimen to prevent further infection and reduce circulating microfilariae (American Heartworm Society, 2014). Melarsomine dihydrochloride injected into the epaxial lumbar muscles is the only adulticidal drug approved by the FDA. The two-dose protocol (2.5mg/kg body weight 24 hours apart) kills 90-95% of adult worms. The three-dose protocol (one injection of 2.5mg/kg body weight dose followed by two injections 24 hours apart at least one month later) kills 98-99% of adult worms.

The American Heartworm Society recommends the three-dose protocol due to the added advantage of decreased complication rates. In a highly microfilaremic patient, corticosteroids or antihistamines can be administered to reduce the potential for adverse reaction (Bowman et al, 2009). The administration of oral Doxycycline prior to beginning adulticide treatment has been shown to suppress microfilaremia burden (Bazzocchi et al, 2008). Studies have shown that experimentally infected dogs treated with doxycycline before being treated with melarsomine had less pulmonary damage associated with the death of the heartworms (Kramer et al, 2011). A heartworm positive dog can be the host to worms of varying age. It is possible that the incomplete efficacy of melarsomine is a result from its inability to be effective against worms less than 4 months of age (Dzimianski et al, 1990). Administering a macrocyclic lactone preventative for two months prior to melarsomine can minimize microfilaria load and eliminate existing susceptible larvae. It is recommended to give a macrocyclic lactone preventative at the time of diagnosis in order to eliminate the treatment and susceptibility gap. In doing so, worms that have infected the animal in the last 1-2 months are killed before reaching infective stage, and allows worms infected in the last 2-4 months to mature to the stage of development that they can be killed using melarsomine (Wolstenholme et al, 2015).

Prevention and molecular basis of drug resistance

Macrocyclic lactones (MLs) have been widely utilized for the last 25 years as heartworm prophylaxis. The Companion Animal Parasite Council (CAPC) and the American Heartworm Society (AHS) endorse year-round monthly heartworm preventative use for companion animals (Hampshire, 2005). All macrocyclic lactone drugs available on the market have been approved by the Food and Drug Administration as 100% effective against heartworm disease after field

and laboratory testing (McCall et al, 2005). These macrocyclic lactones include ivermectin (HeartGard™), milbemycin oxime (Interceptor®), selamectin (Revolution) and moxidectin (ProHeart6®, Advantage Multi®), and are derived from *Streptomyces* microorganisms (Martin et al, 2002). Macrocyclic lactones (MLs) are large hydrophobic molecules with a macrocyclic lactone ring structure (Lespine et al, 2006). MLs prevent the development of infective L_3 *D. immitis* larvae to adulthood, and diminish the likelihood of a patent infection (Wolstenholme et al, 2016). Most chemoprophylaxis is designed to be given monthly, so it effectively removes circulating L_3 and L_4 stage parasites that have infected the host since the previous dose. Though the mechanism of macrocyclic lactones against filarial nematodes is not fully understood, it is widely accepted that the glutamate-gated chloride (GluCl) channels are the most important and biologically relevant target, leading to paralysis of the pharynx and/or body muscles, rendering the parasite unable to eat or move (Wolstenholme et al, 2016). However, this interaction has not been sufficiently studied in the filarial nematode *D. immitis*. In *Caenorhabditis elegans* and *Haemonchus contortus*, high-affinity binding of ivermectin to GluCl channels has been shown (Wolstenholme et al, 2016). Additionally, the effects of ivermectin, which include paralysis and the inhibition of the pharyngeal pump, were found in many nematode species (Wolstenholme et al, 2016). It is also worth noting that the mode of action of MLs against gastrointestinal parasites has been proven to act on the GluCl channels of adult worms (as opposed to larvae), leading to paralysis of feeding and subsequent death of the parasite (Geary et al, 1993). It has also been suggested that an action of the MLs is to lower the immune response of the host to enhance survival of the parasite (Wolstenholme et al, 2015).

As previously mentioned, to obtain regulatory approval from the Food and Drug Administration, MLs must demonstrate 100% efficacy against *D.* immitis in both laboratory and field experiments (Hampshire, 2005). Resistance to macrocyclic lactones in *D.* immitis was considered an unlikely occurrence (Prichard, 2005), though ML resistance in nematodes of livestock is a common problem among livestock breeders and herdsmen (Kaplan et al, 2004). More recently, and perhaps more notably, ivermectin resistance has been reported in a filarial nematode found humans, *Onchocerca volvulus*, which is closely related to *D. immitis* (Osei-Atweneboana et al, 2007). Previous to this particular study, an investigation of genetic polymorphisms in populations of *O. volvulus* from untreated and ivemerctin-treated patients discovered significant differences in allelic frequencies between the two groups in P-glycoprotein and beta-tubulin genes (Eng et al, 2004).

With this information in hand, there are two possibilities for the source of the loss of efficacy (LOE) of MLs against *D. immitis.* The first includes animals that have been inconsistently treated with either a missed or a late dose, or the animal has obtained a lower-than-effective dose due to incorrect dose for the animal's weight, vomiting or diarrhea. These animals have not been exposed to an adequate level of the drug to be effective against the parasite. The second alternative is that despite appropriate levels of drug in the animal's system, there are parasites that are genetically distinct from "wild-type" parasites (Geary et al, 2011). This would theoretically result from selection for alleles that allow this mutation to a drug resistant phenotype, displaying a heritable change in an individual parasite that would enable it to survive against drug dosages that would be effective in a normal parasite.

Potential emergence of ML loss of efficacy in *D. immitis* was first brought to light by Hampshire in 2005, in which reports of LOE to the FDA sparked questions about the reported 100% efficacy of MLs. A study in 2010 described two dogs experimentally infected with infective third stage larvae of the Georgia MP3 *D. immitis* strain and subsequently treated with ivermectin and milbemycin oxime, respectively. The MP3 strain was originally isolated from a naturally-infected dog from Georgia with no history of heartworm prophylaxis (Snyder et al, 2011a). Each dog contained a single adult female at necropsy, though this would not be considered a patent infection (Snyder et al, 2011a). It is important to note that while significant, these findings do not in themselves mean a lack of ML efficacy, because MLs are designed to be given for successive months to be completely preventative against the disease (Snyder et al, 2011a). However, the presence of these female worms suggests that effectiveness of an ML treatment could be affected by the isolate the animal is infected with, regardless of an owner's compliance to consistent chemoprophylaxis (Snyder et al, 2011a). Another study from 2011 inoculated beagle dogs with infective third-stage larvae of the MP3 *D. immitis* strain, aiming to investigate the effectiveness of four preventatives (ivermectin, moxidectin, selmectin, and milbemycin oxime) against the MP3 isolate. The dogs in the study were split in to four groups, each group receiving a single dose of 1 of 4 heartworm preventative. One or more adult male and female *D. immitis* were recovered in 7 out of 8 dogs in each group except that treated with moxidectin plus imidacloprid (Blagburn et al, 2011). A study completed later in 2011 inoculated dogs with two different strains of *D. immitis* (the Michigan strain and MP3 strain), which showed that 3 doses of milbemycin were completely effective at preventing the establishment of a patent infection with the MP3 isolate (Snyder et al, 2011b). Though these findings are substantial, it is important to

remember that macrocyclic lactones are approved with the belief that they can arrest development and prevent infection following a single dose.

Bourguinat, et. al also reported on ML LOE in a natural infection, describing a dog from Hurricane Katrina that was transported to Canada for adoption. The animal received 5 doses of melarsomine to kill adult worms and a total of 17 doses of macrocyclic lactones in an attempt to kill the microfilariae. The dog continued to be microfilaremic for 18 months despite a negative antigen test, indicating the adult worms had been successfully killed (Bourguinat et al, 2011a). The same authors published a study in the same year in which three isolates from LOE cases were compared to control isolates. They also performed a microfilaria *in vitro* sensitivity assay, where the number of motile worms in a concentration of ivermectin was recorded (Bourguinat et al, 2011b). In response to this information, a study from 2013 developed in *in vitro* bioassay for measuring the susceptibility of *D. immitis* to macrocyclic lactones (Evans et al, 2013).

Yet another, more recent, study used two "high index of suspicion of ML resistance" dogs from Louisiana to inoculate mosquito cultures, then used the resulting L_3 to experimentally infect two dogs each. One dog from each group was treated monthly with ivermectin subcutaneously, and one dog received a propylene glycol sham treatment for a total of 6 monthly treatments. Despite the monthly treatments, all dogs in the study developed patent infections, which demonstrated that the parasites used to inoculate the dogs were truly resistant to standard macrocyclic lactone treatment and are circulating in Louisiana (Pulaski et al, 2014). Similarly, a study infected two groups of beagle dogs with resistant isolates of infective L_3 larvae. Those two groups of dogs were treated with ivermectin for 9 months and 5 months, respectively. A third group was treated

with a long-acting moxidectin, then inoculated with infective L_3. Interestingly, the researchers discovered a 23.8% efficacy of ivermectin in the group treated for 9 months, and 71.3% in the group treated for 5 months. Lastly, in the group treated prophylactically with long-acting moxidectin, they discovered efficacy was 21.6% (Bourguinat et al, 2015).

Pulaski, et al described a four point "high index of suspicion" criteria in order to identify and isolate resistant strains of *D. immitis*: a) a clinical history of failure of efficacy and full monetary compensation by a commercial pharmaceutical firm; b) residence in an area identified as a "hot spot" of suspected drug resistance in the 2009 statewide veterinary practitioner survey of LOE cases; c) persistence of circulating microfilariae seven days after an acceptable microfilaricidal dose of macrocyclic lactone; d) high frequency of a genotype marker previously reported to be correlated with potential ML resistance, single nucleotide polymorphism at sites 11 and 618 (GG-GG) of a gene encoding for P-glycoprotein (Pulaski et al, 2014).

P-glycoprotein (Pgp), a gene product of *MDR1*, is a membrane protein that belongs to the family of ATP-binding cassette (*ABC*) transporters (Lespine et al, 2007). It is located in the intestine, liver, kidney and in blood-tissue barriers, and acts as a defensive barrier, limiting drug bioavailability and also potentially reducing drug efficacy (Bodo et al, 2003). Pgp has also been shown to play a clinically relevant role in drug-drug interactions (Ho et al, 2005). Pouliot, et al. demonstrated the role that Pgp plays as the *ABC* transporter in ivermectin transport (Pouliot et al, 1997). Pgp is located on the blood-brain barrier and protects mammals against ivermectin penetrating in to the brain and resulting in neurotoxicity (Lespine et al, 2007). Despite the

extensive research that has gone in to macrocyclic lactone resistance in gastrointestinal parasitic nematodes, no mechanism for resistance has been proven.

A study investigating polymorphisms within the *D. immitis* genome focused on single-nucleotide polymorphisms (SNPs) within the gene encoding Pgp by performing an *in* vitro sensitivity assay on 67 microfilariae obtained from 3 "low-responder" dogs originating from Arkansas and Louisiana, and molecular analysis on adult worms extracted from dogs experimentally infected, as well as worms from naturally infected dogs (Bourguinat et al, 2011b). Microfilariae from the low responder groups used for sensitivity assays showed reduced sensitivity to ivermectin. The study also discovered two common SNPs found in the 620-bp Pgp fragment, located at position 11 and position 618. The genotype GG-GG was found to be significantly higher in their low-responder samples, correlating with the reduced activity of ivermectin against the microfilaria in these groups, suggesting that particular SNP confers a low response or resistance to MLs. This study supports the suspicion that potential ML resistance could be due to genetic selection on *ABC* transporter genes with repeated ML treatment, similar to what has previously been published (Eng et al, 2005; Bourguinat et al, 2011a,b),. Additionally, results from the study revealed a loss of heterozygosity within the low-responder group, a common effect in loci when drug selection is occurring (Bourguinat et al, 2011b). Another study from 2011 from some of the same authors revealed a dog from Canada who remained microfilaremic despite repeated attempts to dose with macrocyclic lactones. The dog had been adopted from the southern United States from an area where it is becoming more common to see cases of loss of efficacy. A sample of microfilaria was taken from the dog for molecular analysis and revealed a very high frequency of the GG-GG genotype within Pgp. That being said, it is not possible to definitively

rule in or out the hypothesis that the GG-GG genotype is causative of macrocyclic lactone resistance, and further work is needed to determine the connection.

Another possibility for the mechanism of resistance is the use of macrocyclic lactones as a "slow-kill" method that administers suboptimal dosing, potentially leading to an ideal environment of resistance. Ivermectin has limited effect against adult nematodes, instead targeting the L_3 and L_4 stages of the parasite. Adult, breeding parasites that are unaffected by microfilaremic doses of macrocyclic lactones would be at a great advantage in this situation. Companion Animal Parasite Council has revised their treatment guidelines to include recommendations for "immediate and aggressive" treatment to minimize this risk (CAPC, 2013).

Epizoology of heartworm in domestic and wild canids

Heartworm continues to be a risk to the canine population throughout the United States. A 2009 survey showed a nationwide distribution of *D. immitis*, with the highest prevalence being in the southern states (Bowman et al, 2009). The survey described a regional mean prevalence of 0.6% in the Northeast, 0.8% in the Midwest, 1.2% in the West, 3.9% in the Southeast, with a national overall mean of 1.4%. The study reports a prevalence of 3.6% in Tennessee, 1.1% in Virginia, and 1.1% in Kentucky, with the highest prevalence in Mississippi at 7.4%. It is worth noting that despite the large sample size (over 3 million dogs), samples were submitted by practices offered a rebate from IDEXX (Westbrook, ME) for providing the samples, which may not be a completely representative sample of the canine population. When prevalence of infection was considered at a county level, a pattern of endemic foci within areas of relatively lower prevalence was discovered (Bowman et al, 2009). For example, in California, Tehema and Lake Counties

had significantly higher prevalence reported (10.1% and 12.5%, respectively) compared to the reported prevalence for the rest of the state (1.2%). This could be due to multiple factors, including an increase in population, leading to dogs moving in to the area from "more endemic" regions. This is supported by findings following the exodus of dogs and cats from Louisiana, Mississippi, and Texas in the aftermath of Hurricanes Katrina and Rita in 2005 to northern, less endemic areas. Dogs from the hurricanes had a 48.8% positive rate, leading to an increase in prevalence in the areas they were sent to (Levy et al, 2007). Additionally, veterinarians may be more likely to encourage heartworm tests for dogs that have travelled from areas more endemic for heartworm disease (Bowman et al, 2009).

A recent survey from the American Heartworm Society on the incidence of adult heartworm infections showed there has been a 21.7% increase in the average cases per veterinary clinic from 2013 to 2016 (Drake and Wiseman, 2018). The study reported an overall decrease in proportion of dogs receiving heartworm preventative (36.68% in 2013 to 35.69%), indicating less dogs per year are receiving the necessary protection against contracting and transmitting *D. immitis*. In addition, the study found that incidence in the southeastern USA increased by 17.9% while the rest of the United States incidence increased by 11.4%, with the states of Texas, Arkanasas, Louisiana, Alabama and Georgia significantly exceeding the national rate (Drake and Wiseman, 2018). The reasons for this increase are multifactorial, including decreased usage of prophylaxis, decreased compliance when administering the medications, or increased resistance to preventative medications, or some combination of all three.

In Tennessee, Kentucky and southwestern Virginia, current data is sorely lacking. A study from 1991 describes a prevalence of 8.21% in stray dogs from random sources, and 5.08% in "clinic dogs" (dogs that had been seen at the small animal clinic at the University of Tennessee College of Veterinary Medicine) (Patton et al, 1991). Current research supports a higher prevalence in shelter animals compared to dogs seen regularly by a veterinarian. A study from Florida comparing prevalence among racing greyhounds, shelter dogs, and pet dogs found a prevalence of less than 2%, while it was diagnosed in 14.6% of shelter dogs (Tzipory et al, 2010). Shelter dogs are commonly relinquished as unwanted pets by their owners, free-roaming strays that are collected by animal control, or animals seized by law enforcement for neglect or abuse cases. It is likely that these animals have not been treated with heartworm prophylaxis at any point in their life, nor have seen a veterinarian for preventative care. Many animal shelters do not have the funds to provide consistent heartworm prophylaxis for animals under their care, due to the cost associated with providing the care (Colby et al, 2011). Failure to provide treatment for heartworm-positive dogs combined with a lack of prophylactic care to susceptible dogs could create a greater risk for transmission (Tzipory et al, 2010). Therefore, it is logical that the prevalence of infection in shelter dogs may be more than 10 times the prevalence in locally owned pet dogs. In the Southeast, infection rates have been reported to range from 10% to 50% of adult dogs in animal sheltering agencies (Levy et al, 2007; Tzipory et al, 2010) compared with rates of 1–7% in owned pet dogs seen in private veterinary clinics (Bowman et al, 2009).

The median income across the Cumberland Gap region is $29,000 and more than 30% of the residents live below the poverty line. Residents are in the lowest quartiles for overall health and wellness indicators (Census Data), and there is a 25% high school dropout rate. Approximately

78,000 households (50%) in the Cumberland Gap region have a pet (AVMA, 2017). Residents living below the poverty line are less likely to be administering heartworm prophylaxis (Nolan and Gates, 2010).

As mentioned previously, the Cumberland Gap region surrounds the intersection of Tennessee, Virginia, and Kentucky. During the summer (June through August), temperatures range from 90-92°F on average, falling during the winter (December to February) to 50-55°F (US Climate Data). The average rainfall in the area is approximately 2-5 inches, with humidity ranging from 50-100%, highest during June and July. It is known that the heartworm does have seasonal trends and develops above 80°F, and studies performed on mosquitoes found an increase in pools of mosquitoes positive for *D. immitis* during the summer months of June and July, when temperature and humidity are at an all-time high for the region (Fryxell et al, 2014; Bowman et al, 2009).

Though domestic dogs are the main focus of heartworm research in contemporary veterinary medicine, wild canids play a very important role as reservoirs for maintaining the parasite and infecting mosquitoes for transmission to pet dogs (Brown et al, 2012). Prevalence in coyotes and wild canids appears to depend on location. A study completed in the Gulf Coast in Texas and Louisiana, 71% of coyotes tested were positive for heartworm disease (Custer and Pence, 1981). In eastern Washington state, necropsies were performed on 556 coyotes and none were infected (Foreyt et al, 2008). The authors suggested the climate in Washington state may not be suitable to support development of heartworm larvae within the mosquito due to the state's temperate climate. In California, coyotes sampled at the county level described a wide range of prevalence

of 0-25%, the highest being in northern California counties (Sacks et al, 2004). Interestingly, a 2009 serologic survey found high prevalence of infected domestic dogs in northern California counties as well (Bowman et al, 2009). Foster, et al. (2003) found 43% of coyotes collected were heartworm positive. A study in coyotes in South Carolina reported 40% of adult coyotes were positive for microfilaria (Miller et al, 2009). In central Georgia, 52% of coyotes tested were heartworm positive (Gates et al, 2014). In Tennessee, Van Den Bussche et. al, (1987) reported 38.5% statewide prevalence based on 267 coyotes sampled from 29 counties over 4 years. Faulkner and Donnell (2011) also found 37% of coyotes from Knox County, TN were microfilaremic hosts for *D. immitis* and capable of maintaining the heartworm lifecycle in their local territories. As natural host for parasite populations that are not under drug selection pressure, coyotes may play an important role in the geographic spread of heartworm as they migrate to establish territorial ranges.

There have been no studies performed on the prevalence of heartworm disease in the Cumberland Gap Region, and the data reported by CAPC most likely underrepresents the true risk of infection. Estimates of heartworm prevalence in this area is mostly anecdotal and based on sporadic sampling of resident dogs and cases identified at local veterinary practices. Obtaining a more accurate representation of the risk of the transmission of heartworm disease is essential for the veterinary team to accurately relay the risk to owners. Additionally, identifying the presence or absence of the GG-GG single nucleotide polymorphism would give a more complete picture of possible macrocyclic lactone resistance in the Cumberland Gap Region.

CHAPTER 3

EXPERIMENTAL METHODS

Serology study

Population sampling

Animals older than six months of age were eligible for serology testing. Blood samples were screened as diagnostic procedures in conjunction with health screenings to determine eligibility for placement for adoption or as pre-surgical preparation for sterilization. Samples were taken from two populations. The first population included stray or surrendered dogs from Bell County Animal Shelter in Pineville, Kentucky, and other regional animal shelters (besides Bell County Animal Shelter) that provided animals to the Lincoln Memorial University's DeBusk Veterinary Teaching Center for pre-surgical screening. The second population included pet dogs belonging to owners attending "Pet and People" fairs that were funded by the Human Animal Bond Research Institute (HABRI). Dogs older than six months of age were eligible for the study. Qualitative information regarding each dog's approximate age, sex, and status (owned, stray, or owner surrender) was collected for sample identification. When available, heartworm prophylactic drug status was collected as well.

Blood collection and testing

Approximately 2 mL of blood was taken from the cephalic or jugular vein using a 22-gauge needle and 3 mL syringe while the dog was appropriately restrained. Blood was transferred to a purple-top blood tube containing EDTA and inverted several times to mix. Samples were

transported to the parasitology lab at Lincoln Memorial University (Harrogate, TN) for analysis and stored at 4°C until testing. Assays were conducted with ZippTest Canine Heartworm Antigen tests (SafePath Laboratories, LLC, Carlsbad, California). Using a pipette provided by the kit, one drop of whole blood was added to the testing well, and two drops of provided conjugate buffer solution were added to the well. There was an eight-minute wait time for test results. The ZIPP test has a sensitivity of 100% and specificity of 100% (Barr, 2011). Positive tests were tested an additional time with the ZippTest, and a modified Knott's test (Knott, 1939) was performed to determine the presence of microfilaria. If microfilaria were present, they were measured in order to confirm species, and quantified per high-powered field. Heartworm test result, approximate age, status and source were recorded in an Excel spreadsheet. Age was defined as follows: young was estimated to be between 6 months to 3 years, middle aged between 4 years and 7 years, and aged older than 8 years.

Molecular study

Sampling

Dog cadavers were opportunistically recovered from Bell County Animal Shelter. This included dogs that had been previously euthanized for terminal illness, grave behavior issues, or reasons that would make the dog unadoptable. Additionally, we were able to examine the hearts of dogs that were being used in other research studies at the university. These animals had already been euthanized and appropriately stored in the freezer, so some animals were used for blind necropsies (meaning we did not know their heartworm status). Only animals from the Cumberland Gap Region were used.

Removal of adult *Dirofilaria immitis* worms from canine cadavers

If available, sex, approximate age, breed, cause of death and general observations was recorded prior to beginning necropsy. Animals that had been donated for other studies were used for blind necropsies (we did not know their heartworm status). A ventral midline incision with animal in right lateral recumbency was performed. Axillary muscles of left forelimb, and skin and muscle over thoracic cavity were reflected. Ribs were cut at costochondral joint and reflected dorsally. A cut was made through the pericardium, revealing the thoracic structures. Effort was made to obtain 5-10 milliliters of blood was collected in ethylenediaminetetraacetic acid (EDTA) purple-top tubes. The cranial and caudal vena cavas, pulmonary veins and arteries and ascending aorta were dissected, with care taken to observe for any heartworms in the pulmonary vasculature. A vertical, shallow incision was made in the right ventricle of the heart. The cardiac cavities and pulmonary vasculature were fully explored for presence of adult heartworms. If heartworms were present, they were placed in 70% ethanol solution in 6oz polypropylene histology container for transport. It was later determined that the ethanol solution was most likely drying the heartworm samples and negatively affecting the quality of DNA that was present for extraction, so transporting directly to the lab without ethanol was preferred. Samples were transported directly to the parasitology lab, where they were determined to be male or female, stored individually in 1.5 milliliter eppendorf tubes with no storage solution, and placed in a -20°C freezer until DNA extraction.

Genomic DNA Extraction from adult heartworms

Following the protocol used by Bourguinat, et. al (2011a,b), genomic DNA from individual adult worms was extracted using the DNeasy™ Blood and Tissue kit (Qiagen Inc., Mississauga,

Canada). The manufacturer's protocol was utilized, with some modification for poor DNA quality as a result of drying out from being transported in ethanol. The worms were removed from the -20°C freezer and allowed to thaw for 5-10 minutes on ice after removal from the freezer. When poor DNA quality became a concern for worms transported in ethanol, we added a step to the protocol including transferring samples to a heat block at 56°C for 10-15 minutes with the top of the microcentrifuge tube open to allow for evaporation of any remaining ethanol. After allowing the ethanol to evaporate, 200-300µL of phosphate-buffered saline (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM $KHPO_4$) was pipetted in to the microcentrifuge tube on top of the worm and placed in a heat block at 56°C for 12-24 hours to rehydrate.

Each worm was then cut in to 2-4 pieces, each segment weighed (to ensure no sample over 25mg by weight per the manufacturer's recommendation) and placed in a 1.5mL microcentrifuge tube with the appropriate identifying data. Then 180µL Buffer ATL and 20µL proteinase K were added to the tube. Again, when poor DNA quality was of concern, a step including a sterile pestle was introduced to the protocol to further lyse the sample. The sample was mixed thoroughly by vortexing and incubated at 56°C overnight in a shaking incubator at 140rpm. For some samples, 4µL RNase was added, but it was determined later that the RNase was degrading the quality of the sample, and the RNase step was removed.

After overnight incubation, the samples were removed and vortexed for 15 seconds. In to a 0.5mL microcentrifuge tube, 200µL Buffer AL and 200µL 100% ethanol were added together and vortexed, then added to each sample and vortexed thoroughly. The mixture was then pipetted in to the DNeasy mini spin column and placed in a 2mL collection tube (provided by kit) and

centrifuged at 8000 rpm for 1 minute. Flow through was discarded with collection tube. The DNeasy Mini spin column was placed in a new 2mL collection tube (provided), and 500uL Buffer AW1 was added and placed in the centrifuge at 8,000 rpm for 1 minute. The flow through and collection tube were discarded, and the DNeasy Mini spin column was placed in a new 2mL collection tube (provided), and 500µL Buffer AW2 was added and placed in the centrifuge at 14,000 rpm for 3 minutes to dry the DNeasy membrane. The flow through and collection tube were discarded. The DNeasy Mini spin column was placed in a clean 1.5mL microcentrifuge, and 200µL Buffer AE was pipetted directly on to the membrane. When DNA quality became an issue, the protocol was modified to pipet 50µL Buffer AE on to the membrane for this step. The sample was incubated at room temperature for 1 minute and centrifuged at 8,000 rpm for 1 minute to elute. The manufacturer's protocol recommended a second elution step, but in favor of higher DNA concentration, that step was omitted as a part of our protocol.

The DNA concentration of the sample was then quantified using a NanoDrop 8000 Spectrophotometer (Thermo Scientific) according to the manufacturer's protocol. The "nucleic acid" and "single sample" settings at 260nm/280nm were selected, and sample IDs manually entered. After the water sample step and blanking with buffer AE, 1 µL of sample was placed in the sample well and measured. The results were recorded in a report, both graphically and numerically, and printed for the laboratory notebook.

Polymerase chain reaction and gel electrophoresis

Polymerase chain reaction (PCR) amplification of a 620-bp segment of a *Dirofilaria immitis* P-glycoprotein (Pgp) gene was completed, using primers published by Bourguinat, et. al. (2011b)

as follows: Pgp-1-sense 5'gga caa tta tcc ggt ggt ca3' and Pgp-1-antisense 5'tcg caa att tcc ttc cac tt3'. Denaturation was performed at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extension at 68°C for 2 minutes for 35 cycles. Phusion High Fidelity DNA Polymerase (New England BioLabs, Inc., Ipswich, MA) was used to avoid introducing errors during amplification. Template DNA was added to a concentration of approximately 200 ng per sample. If the concentration of template DNA was greater than 200ng, a 10-fold dilution was completed. A master mix was prepared including all of the ingredients listed in Table 1 except template DNA and Nuclease-Free water.

PCR amplification was confirmed using 1.2% agarose gel electrophoresis. First, 5 μL SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) was added to the agarose/TAE solution (40mM Tris, 20mM acetic acid, 1mM ethylenediaminetetraacetic acid). and mixed before allowing the gel to solidify. PCR product in the amount of 5 μL was mixed with 2 μL of 6X gel loading dye (New England Biolabs, Ipswich, MA) and pipetted in to the appropriate lane. The last lane was occupied by an exACTGene Low Range Plus DNA Ladder (Fisher BioReagents, Hampton, NH). Standard electrophoresis was conducted with 100V for 1 hour and 15 minutes, using a 9.5cm 1.2% agarose gel in 1x TAE buffer. The gel was taken to a UV transilluminator equipped with a digital camera where pictures were taken. The bands were compared to the ladder to determine the size of the PCR product and subsequent success of the PCR.

DNA purification

The QIAquick Spin Kit (Qiagen) was used to purify PCR products of dNTPs and excess primers in preparation for sequencing. The manufacturer's protocol was followed. First, 225 μL of Buffer

PB was added to the 45 µL PCR sample and mixed (the PCR sample was originally 50 µL, with 5 µL removed for agarose gel electrophoresis; kit protocol states 5 volumes of Buffer PB to 1 volume of PCR sample). A QIAquick spin column was provided in the 2 ml collection tube. The sample was applied to the QIAquick column and centrigued for 30-60 seconds to bind the DNA. The flow-through was discarded and collection tube was re-used, adding 0.75 ml Buffer PE to the QIAquick column and centrifuged for 30-60 seconds to wash. The flow-through was discarded, the QIAquick column placed back in the same tube, and the column centrifuged for an additional 1 minute. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. 50 µL warmed nuclease-free water was added to the center of the QIAquick membrane and allowed to incubate at room temperature for 5 minutes. The tube and column were then centrifuged for 1 minute to elute the DNA. The DNA concentration was then quantified using the NanoDrop 8000 Spectrophotometer, using the protocol outlined above.

Sequencing

After purification, PCR products were sent to the University of Tennessee Genomics Core for sequencing. Sanger Sequencing was performed on an Applied Biosystems 3730 Genetic Analyzer using Applied Biosystem's BigDye version 3.1. For the DNA template, 100ng of template with 2µL primers (Pgp-1-sense and Pgp-1-antisense) at 10µM were utilized. Electronic sequencing results were sent via email as .ab1 files and viewed using 4Peaks (Nucleobytes B.V., The Netherlands).

Sequence alignment

The reverse complement sequence was elucidated from the reverse sequence file using the open-source website : http://www.bioinformatics.org/sms/rev_comp.html. The forward and reverse sequences were combined in to a contig using the assembly site http://doua.prabi.fr/software/cap3. The sequence alignment was accomplished by entering the query sequence in to NCBI Blast Alignment Tool. The resulting alignment was visually assessed for polymorphisms at any point, with special attention paid to the loci of interest (11 and 618). SNPs at any loci were recorded. Ambiguous reads were confirmed using 4Peaks viewer.

Data analysis

The frequency was calculated for any SNP that was discovered and compared to the published GenBank sequence (accession number: HM596853). Distribution of genotypes was analyzed for each worm.

CHAPTER 4

PREVALENCE OF DOG HEARTWORM, *DIROFILARIA IMMITIS*, IN THE RESIDENT DOG POPULATION IN THE CUMBERLAND GAP REGION

Introduction

In 2017, the Companion Animal Parasite Council (CAPC) reported that approximately 2.37% of animals tested positive for heartworm disease in Kentucky, Tennessee, and Virginia **(Table 4.4).** However, anecdotal results indicate a greater prevalence. This would indicate a grossly underestimated prevalence of heartworm disease in the area, affecting the accuracy of information disseminated to local pet owners by veterinary health professionals. Additionally, domestic dogs that present to veterinary offices are likely to represent a biased sample of the population of domestic dogs.

The purpose of this research was to gain a more complete picture of the true prevalence of heartworm disease in the Cumberland Gap Region, consisting of Southeastern Kentucky, Southwestern Virginia, and Northeastern Tennessee **(Figure 4.1)**. Obtaining more accurate and thorough data for prevalence and incidence of heartworm disease in the CGR would allow more accurate information to be circulated to pet owners, and most importantly, provide an informed estimate of risk for their pet to become infected with heartworm.

Results

A total of 753 dogs were sampled, 653 dogs from animal shelters and 98 from were pet dogs sampled from HABRI-funded “Pet and People” fairs **(Table 4.2)**. Shelter dogs were 56% male

and 44% female, and 50% were classified as young, 19% as middle aged, and 29% as aged. Pet dogs were 36% male and 64% female, and 43% were aged as young, 40% middle aged, and 17% aged. 55% of owners reported no or inconsistent use of heartworm prophylaxis, and 45% of owners were currently using heartworm preventatives.

35 of 753 shelter animals tested positive. Of the positive dogs, 54% were male and 46% were female, 36% were classified as young, 29% as middle aged, and 35% as aged. There were no pet dogs that tested positive **(Table 4.1)**.

At Bell County Animal Shelter, 10 out of the 14 dogs that tested positive were subsequently microfilaremic **(Table 4.3).** For the animals tested as a part of the pre-surgical screening at Lincoln Memorial University, microfilaria status is unknown. All of the dogs that tested positive from Bell County Animal Shelter were classified as strays.

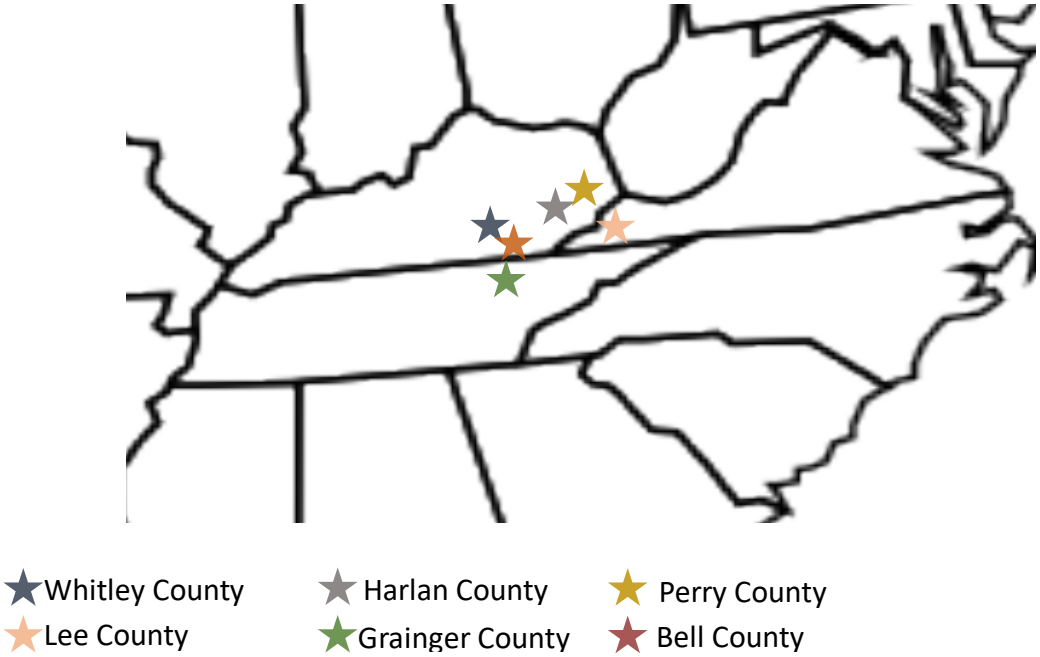

Figure 4.1. A map of shelter locations that provide animals for pre-surgical assessment at LMU.

Table 4.1 Age and sex (%) for shelter animals and pet dogs sampled from the Cumberland Gap Region.

Population	Male	Female	Young	Middle Aged	Aged
Shelter Animals	332/753 (56)	421/753 (44)	376/753 (50)	143/753 (19)	218/753 (29)
HABRI Pet and People Fairs	35/98 (36)	62/98 (64)	42/98 (43)	39/98 (40)	16/98 (17)

Table 4.2 Summary of overall heartworm prevalence in the CGR from independently sampled animals from Bell County Animal Shelter, HABRI Pet and People Fairs, and pre-surgical candidates from Lincoln Memorial University (LMU).

Population	Positive	Total Tested	Percent Positive
Bell County Animal Shelter	14	120	11.67%
LMU Pre-Surgical Candidates			
Whitley County, KY	8	157	5.09%
Perry County, KY	8	218	3.66%
Harlan County, KY	1	35	2.85%
Bell County, KY	3	51	5.88%
Lee County, VA	1	74	1.35%
Pet dogs from HABRI Pet and People Fairs	0	98	0%
TOTAL	**35**	**753**	**5.04%**

Table 4.3 Assessment of age, sex and microfilaremic status of heartworm positive animals sampled from Bell County Animal Shelter. These animals were sampled independently and separately from the animals included in the population of pre-surgical animals. FI = female intact, MI = male intact.

Dog	Age	Sex	Microfilaria status
1	Young	FI	+
2	Young	FI	+
3	Aged	MI	+
4	Young	FI	+
5	Middle aged	MI	+
6	Young	MI	-
7	Middle aged	MI	+
8	Young	MI	-
9	Aged	MI	-
10	Aged	MI	-
11	Aged	MI	+
12	Aged	FI	+
13	Middle aged	FI	+
14	Middle aged	MI	+

Table 4.4 Tested heartworm prevalence in the CGR from animals presenting to Lincoln Memorial University for pre-surgical assessment compared to CAPC reported data from 2017.

County	**Tested**	**CAPC reported (2017)**
Whitely County, KY	8/157 (5.09%)	5/94 (5.92%)
Perry County, KY	8/218 (3.67%)	3/470 (0.64%)
Harlan County, KY	1/35 (2.86%)	4/217 (1.84%)
Bell County, KY*	17/171 (9.94%)	12/1220 (0.98%)
Lee County, VA	1/74 (1.35%)	6/242 (2.48%)
Average prevalence	**35/655 (4.58%)**	**34/2317 (2.37%)**

*Combined data from pre-surgical candidates and animals residing at the shelter.

Discussion

The Cumberland Gap region (CGR) is a primarily low-income area that is comprised of Claiborne and Grainger counties in Tennessee; Lee County, Virginia; and Bell, Whitley, Harlan, and Perry Counties in Kentucky.

Across Tennessee alone, there is an estimated $24 million dollars spent on heartworm disease treatment. Something to consider is the cost of prevention – a mere $3 million, approximately 12% of treatment cost (AVMA). Not only is this significant in the landscape of the economic status of the Cumberland Gap region, it is also a source of emotional anguish for pet owners, and potentially a cause for pet surrender. In the CGR, using the 2.37% positive tests reported would result in approximately over $1 million of lost household income for pet owners.

As mentioned above, CAPC reports that 2.37% of dogs in the CGR are heartworm positive **(Table 4.4)**. The maps are presented as prevalence maps, but most likely more accurately represent incidence, which is the number of new cases coming in to the population. Additionally, because veterinarians report the number above, it represents a small portion of the entire population of dogs – the dogs that are receiving veterinary care prior to receiving their heartworm prophylaxis, which is likely an underrepresentation of the total canine population in the area, demonstrated by the prevalence in this study calculated between 4.58% and 5.04%.

The prevalence of *D. immitis* in dogs in shelter situations in the CGR is similar to data reported previously (Tzipory et al, 2010, see **Tables 4.2, 4.4)**. Studies investigating heartworm prevalence in animal shelters are common, demonstrating a prevalence range of 1% (Bowman et al, 2009) to

over 15% (Tzipory et al, 2010), with a greater prevalence seen in southern states than in the northern regions of the United States, which supports regional risk of heartworm infection based on HUDs. Higher numbers in shelter animals is likely due to the lack of chemoprophylaxis and subsequent treatment if the animal is found positive. Reasons for lack of appropriate preventative care in shelter animals is similar to that of the population in the CGR, due to lack of funds and other resources that is common in this area. It is also possible that owners are not aware of the true risk of their animal becoming infected.

It does not appear that age ($p = 0.8983$) or sex ($p = 0.6078$) are a factor in an animal testing positive for heartworm infection as there was not a significantly significant difference in heartworm status based on age.

No information on household income was collected for the pet dogs sampled from the HABRI Pet and People fairs, so economic reasons for withholding heartworm prophylaxis could not be quantified. No pet dogs tested positive, despite 55% of owners reporting no or inconsistent use of heartworm prophylaxis, which could be the result of a number of factors. There was no association between owner age ($p = 0.2397$) or gender ($p = 0.3678$) and the use of heartworm prophylaxis for their pet. Likewise, pet gender ($p = 0.5448$) and pet age ($p = 0.6060$) did not have any bearing on whether or not the animal was receiving prophylaxis.

It is unlikely that the pet dogs tested falsely negative, because the pet dogs were tested with the same assay as the shelter dogs. The indoor residence of many pet dogs reduces risk of mosquito exposure and heartworm infection as does residence in neighborhoods with few infected

mosquitos. Research investigating the role of these variables is ongoing and beyond the scope of this study.

Interestingly, not all of the animals that tested positive were also microfilaremic using a modified Knott's test. For the 4 positive animals that were not subsequently microfilaremic, there are several explanations, the first being user error. Secondly, it could be due to a lack of females that are able to reproduce. The antigen tests detect a gravid female, so it is a possibility that the animal lacks a male for the female to sexually reproduce microfilaria with. Additionally, it is also a possibility (and probably most likely) that there are gravid females present within the host, but microfilariae have not begun to circulate. The presence of microfilariae within positive dogs demonstrates that active transmission is occurring in the Cumberland Gap region.

Though predictions can be made on how long an animal is infected based on cardiac changes and clinical presentation, it is impossible to know exactly how long and therefore what month each animal was infected. However, we can make observations based on trends. More animals tested positive in the spring/summer (April-September) than those in the fall, which supports previous research that there is a seasonality to heartworm infection (Lok et al, 1998).

In the Cumberland Gap Region, it is common for dogs to roam freely, likely contributing to the maintenance of heartworm disease. Of the approximately 20,000 pet dogs in the CGR (AVMA), more than 50% of the total pet population lives below poverty. It is likely that these animals do not receive any kind of heartworm prophylaxis. It is also probable that if a dog in one of these

households was to contract heartworm disease, it would not be treated, further contributing to heartworm positive dogs that are acting as vectors for disease transmission.

There are three "groups" that contribute to the population of heartworm positive dogs in this area: those that have never seen a veterinarian before, those that have but have never had an issue in the past with heartworm disease, and those that seek veterinary care for their pets, but are non-compliant in the delivery of the heartworm prophylaxis to their pets. Due to the nature of the cycle of *D. immitis* in the host, any positive dog is acting as a vector for transmission of disease. This is why it is so important to consider the underreporting of the prevalence of heartworm disease. A higher prevalence could indicate a higher rate of transmission and maintenance of the parasite in the area, leading to greater monetary consequences in an area that is already economically compromised.

This data would certainly benefit from further study and continued sample accumulation to continue to improve predictions of incidence and prevalence in the Cumberland Gap Region. This information is vital for all members of the veterinary care team in order to provide the most accurate information to their clients.

CHAPTER 5

GENETIC MARKERS INVOLVED IN MACROCYCLIC LACTONE RESISTANCE IN *DIROFILARIA IMMITIS* IN RESIDENT CANINE POPULATIONS OF THE CUMBERLAND GAP REGION

Introduction

Mounting evidence of resistance to MLs, though previously considered unlikely, has sparked many studies of loss of efficacy both *in vivo* and *in vitro*. Hampshire published the first report of evidence of low ML efficacy in *D. immitis* (Hampshire, 2005). A plethora of studies have explored potential mechanisms and explanations for the loss of efficacy. Ivermectin resistance has been studied in *O. volvulus*, which is a close relative of *D. immitis* (Osei-Atweneboana et al, 2007). This triggered extensive study in to the mechanism of developing ivermectin resistance in *O. volvulus*. No evidence of selection was discovered in GluCl or GABA channels, as was previously thought, but significant selection on β-tubulin and P-glycoprotein (Pgp) was discovered (Eng and Prichard, 2007). Consistent with these findings, a polymorphism in a *D. immitis* P-glycoprotein gene (HM596853) was found in several LOE isolates. The frequency of this polymorphism increased with the proportion of individual microfilariae that showed decreased sensitivity to sensitivity to an increased dose of ivermectin *in vitro* (Bourguinat et al, 2011b,c).

Results

A total of 56 worms were recovered from 8 domestic canine hosts for DNA extraction **(Table 5.1).** Dual sex infections (male and female present) occurred in 5 of 8 dogs, and 3 dogs had

single sex infections. There were 18 male worms, 37 female worms and 1 worm whose sex could not be identified**.** The number of worms per host ranged from 2 to 15. The distribution of worms in each of the hosts and their sex ratio was similar to that described in naturally infected wild canidae hosts by Faulkner and Donnell (2011).

Of the 56 sequences, 41 were able to be aligned. The remaining 15 sequences had too many ambiguous nucleotide reads to assemble an appropriate contig, so were excluded from analysis. The GG-GG genotype was not found in any of the sequences. One SNP (GA-GG) was found in the Pgp fragment at the loci of interest (positions 11 and 618). The GA SNP at position 11 was found in two male worms, both from animal 08. Additionally, a SNP at position 158 was discovered in 2/41 samples (GA instead of AA) **(Figure 5.1, 5.3)**. The GA SNP at position 158 was found in two male worms, one from animal 07 and one from animal 08 **(Table 5.1)**.

The genotypes from the SNPs at positions 11 and 618 were combined for diplotype analysis. For example, the AA-GG genotype corresponds to genotype AA at position 11 and genotype GG at position 618. Of the nine possible combined genotypes, two were found in the sample population (AA-GG and GA-GG). 95% of the population displayed the AA-GG genotype and 5% of the population displayed the GA-GG genotype **(Figure 5.2)**.

Table 5.1 Sex of worms, total number aligned, SNP locations, and sex of worms containing SNPs removed from dogs used for molecular study. Sex was confirmed using microscopy and morphologic analysis.

Animal	Male	Female	Unknown	Total	Total aligned	SNP	Sex of worm containing SNP
01	2	4		6	5		
02	1	4		5	2		
03	1	1		2	2		
04	4	8		12	11		
05	0	2		2	2		
06	0	7	1	8	5		
07	4	11		15	8	GA at 158	Male
08	6	0		6	6	GA at 158	Male
						GA at 11	Male
						GA at 11	Male
Total	**18**	**37**	**1**	**56**	**41**		

Figure 5.1 Alignment result of sample 2Z2, illustrating the GA single nucleotide polymorphism (red box) at position 11 discovered in 2 out of 41 samples. The blue box is located at the second locus of interest (position 618).

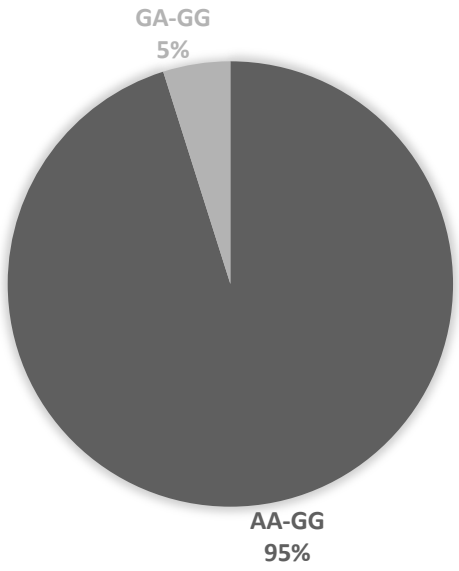

Figure 5.2. Gene frequency of each combined genotype from 41 worms in 8 dog hosts.

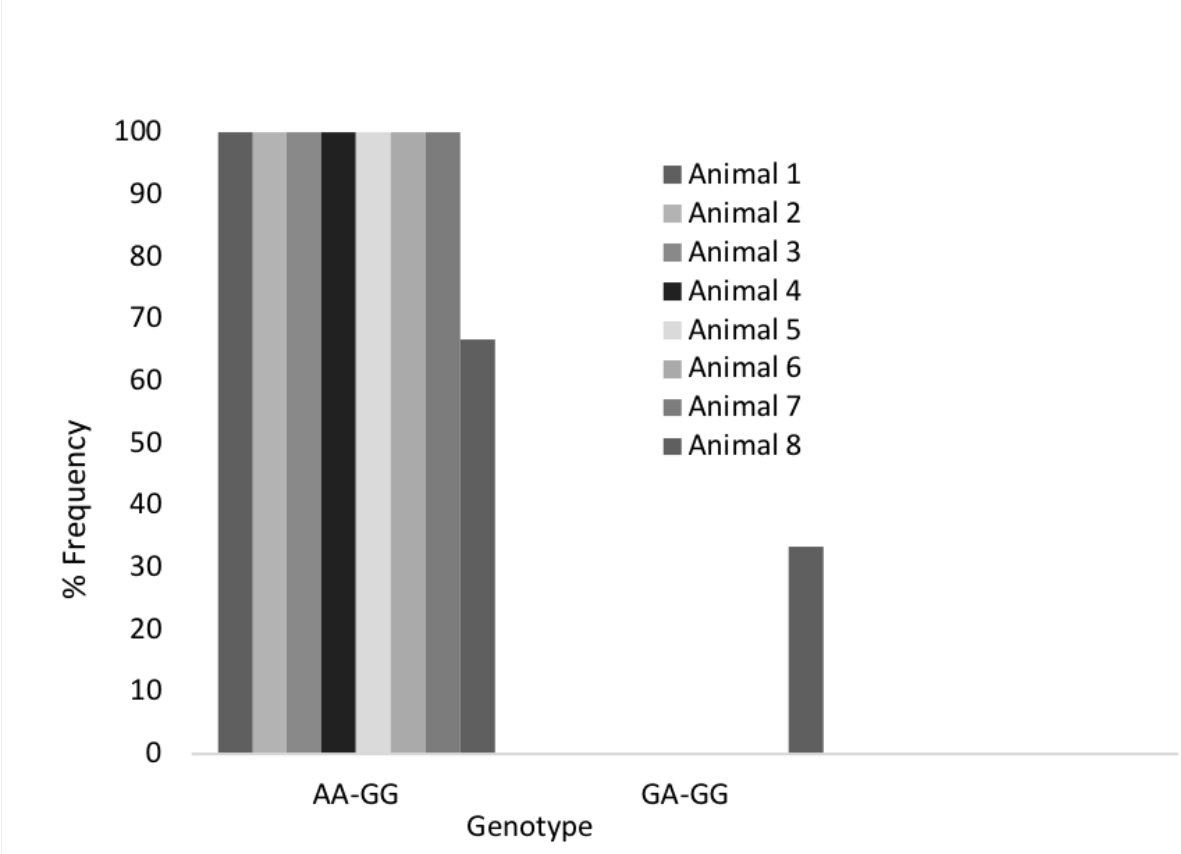

Figure 5.3. Genotype frequency of the combined genotypes and SNP at positions 11 and 618 of P-glycoprotein of individual adult worms from animals sampled.

Discussion

Macrocyclic lactone resistance is well established in nematode parasites of sheep, goats, cattle and horses (Prichard et al, 2012; Wolstenholme et al, 2012) and appears to be surfacing in *O. volvulus*, a nematode closely related to *D. immitis* (Osei-Atweneboana et al, 2007). These results have significant influence on the continued reliance on macrocyclic lactones for control of heartworm disease in veterinary medicine (Bourguinat et al, 2015). It is a strong possibility that this resistance is due to genetic selection on *ABC* transporter genes with repeated macrocyclic lactone treatment (Ardelli et al, 2004). Bourguinat et al (2011a, b, c, 2015) were the first to identify a single nucleotide polymorphism (GG-GG) within the *ABC* transporter P-glycoprotein gene in several LOE isolates. They reported a loss of heterozygosity within their LOE isolates, an effect commonly seen in populations that have experienced selection.

This study aimed to locate the polymorphism (GG-GG) at positions 11 and 618 in a fragment of the gene encoding P-glycoprotein within heartworms found in adult dogs necropsied from regional animal shelters. We did not discover any samples that contained the GG-GG genotypic polymorphism. However, we found an GA-GG SNP **(Figure 5.1)** at the loci of interest in two samples, as well as a second GA SNP at position 158 in two samples. The AA-GG genotype was found in approximately 55% of 54 microfilariae sampled from "Buster", a dog who had been experimentally infected with the susceptible Missouri isolate, in the Bourguinat 2011(a) study. Microfilariae associated with these samples showed a higher susceptibility to ivermectin than the low responder samples in the study. It is possible that the AA-GG genotype confers a susceptible phenotype. There have been no reports of a SNP at position 158 having any contribution to loss

of efficacy. It is likely this was a spontaneous point mutation that has no bearing on the parasite's viability.

Despite the evidence of the polymorphism as a very important factor in loss of efficacy, more studies would need to be performed before declaring the Cumberland Gap Region to be free of resistant organisms. It is likely that multiple genes, including those involved with structure and sensory aspects of the nematode, are part of the resistance mechanism (Freeman et al, 2003). Other possible mechanisms include changes to the glutamate-gated chloride channels (McCavera et al, 2007), other ligand-gated channels involved in drug efflux (Njue et al, 2004), or modulating the immune response of the host to the parasite.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The serologic prevalence of heartworm disease in the Cumberland Gap region was found to be 5.04%, higher than the number reported by CAPC of 2.37%. The higher prevalence observed in shelter animals in the CGR is consistent with other studies investigating the discrepancy between the prevalence in shelter animals and in pet dogs (Tzipory et al, 2007). The cost of heartworm treatment and maintenance of infected dogs combined with a lack of technical expertise and time most likely contributes greatly to this phenomenon. It is important to remember that positive animals serve as a reservoir of possible infection. As previously mentioned, 55% of owners from HABRI-funded "Pet and People" fairs indicated they were not maintaining their pets on heartworm prophylaxis, despite no positive samples from that population. This could be due to a number of factors. Animals in this population could have previously received doses of heartworm preventative. It is also possible that the animals have less exposure to mosquitoes because they reside mostly within the residence. Additionally, this data could potentially indicate a lower risk of transmission in the Cumberland Gap region as compared to an area such as the Mississippi Delta.

It is likely that the discrepancy seen between owners' perception of heartworm disease risk and the veterinary team's recommendations is due to communication difficulties, as well as a failure to accurately relay the risk of heartworm infection and cost of subsequent treatment. It is imperative that the veterinary team communicate effectively with owners, as it is probably that owners would take the risks of heartworm disease more seriously if they fully understood the

financial burden and emotional toll of treatment of heartworm infection. The implication for this study is to emphasize to local practitioners the importance of explaining the true risk of heartworm disease in the Cumberland Gap region and encouraging year-round chemoprophylaxis.

There has been some anecdotal clinical evidence of loss of efficacy of macrocylic lactones in the region, but published studies are lacking in data specific to the Cumberland Gap Region. Despite the absence of the GG-GG genotype in the adult worm samples collected in the Cumberland Gap region for this study, it is still possible that there are resistant organisms in the area. The GA-GG genotype was found in suspected LOE samples (Bourguinat et al, 2011a, c), but was not correlated with further resistance to macrocyclic lactone treatment. Future studies should consider investigating pooled microfilaria for the GG-GG polymorphism to determine if it is present in microfilaria populations. Circulating microfilaria containing the SNP would be available for continued life cycle development and subsequent transmission via the mosquito vector, potentiating possible ML LOE in the CGR. It would also be prudent to consider continuing adult worm sampling as well, most notably performing ivermectin susceptibility assays on microfilariae while concurrently analyzing p-glycoprotein for the GG-GG polymorphism in adult worms.

CHAPTER 7

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Master of Science Thesis Defense Form

Master of Science Thesis title: Genetic Markers Involved in Macrocyclic Lactone Resistance in Dir ofilaria immitis and Estimation of Heartworm Prevalence in Resident Canine Populations in the Cumberland Gap Region of Kentucky, Tennessee, and Virginia

Student Hailey Watlington

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