Drug Resistance in the Canine Filarial Parasite, Dirofilaria immitis: Emergence and Clinical Epidemiology in the Lower Mississippi River Alluvial Valley

Cassan Nicole Pulaski
Louisiana State University and Agricultural and Mechanical College

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DRUG RESISTANCE IN THE CANINE FILARIAL PARASITE, DIROFILARIA IMMITIS: EMERGENCE AND CLINICAL EPIDEMIOLOGY IN THE LOWER MISSISSIPPI RIVER ALLUVIAL VALLEY

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Pathobiological Sciences

by

Cassan Nicole Pulaski
B.S., B.A., University of Mississippi, 2008
M.P.H., Louisiana State University, 2012
D.V.M., Louisiana State University, 2013
May 2022
This work is dedicated to my mentor, Dr. Jack Malone. Thank you for taking a chance on a first-year vet student back in 2008, and for your seemingly endless supply of patience, and for allowing me to take risks, make mistakes, and shine. I can never repay everything you’ve done for me and my career, but I promise to pay it forward and model your enthusiasm and kindness with my future students. You were the first to show me the chaotic beauty of these complex yet microscopic creatures (and forever changed my career trajectory). You taught me how far humor, generosity, and a lack of arrogance can go when working overseas or with a diverse team. And, I will never underestimate the unifying power of a lab happy hour. Thank you, Dr. Malone; I am ever grateful.

This dissertation is also dedicated to my unwavering husband, Justin. Over the course of my PhD, we met, fell in love, got yoked, adopted a new pet or two, and started new jobs in a new state (during a global pandemic)… But throughout it all, despite all the set-backs and when I was convinced I’d be ABD forever, you were always firm in your belief that I was going to finish. Thank you for supporting and loving me, imperfections and all. I could not have survived without you.
Acknowledgments

This PhD/residency program has been a challenging (albeit rewarding) journey, and I want to share my gratitude and love for the many people that have supported me and contributed to this accomplishment. This list is by no means exhaustive, as I could fill pages with thanks and adoration; alas, with my whole heart, I declare my dissertation would not have been possible without these precious people:

My family, especially my parents, for their unconditional support and guidance. Always.

My dear Mollie, for never missing an opportunity to uplift and support me, and for making sure every milestone was recognized (and filled with treats!).

My LSU SVM lab family (Jennifer, Brooke, Ryan, and Ms. Pat), for creating a supportive environment, free of toxic competition and selfishness, and teaching me what it really means to be a “work family.”

My brilliant committee members (Drs. Rebecca Christofferson, Kristen Healy, Roger Prichard, and Charles Lee), for their guidance and empathy. For challenging me, without being condescending or cruel, and for helping me reach my own potential.

The LSU Parasitology faculty (Drs. Jim Miller and Thomas Klei), my FDA Supervisor (Dr. Renee Shibukawa), and my favorite practice owner (Dr. Andrea Andersen), for providing mentorship, an open ear, and great conversations over dinner and wine.

The organizations and professional societies (NCVP, AAVP, CAPC, AHS) that have been crucial in my development as a (funded) veterinary parasitologist; my UGA colleagues for welcoming and accepting me and all my chaos; and my first mentor, Dr. Ken Sufka, for teaching me how to be a scientist.
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Abstract

**Background:** Strains of *Dirofilaria immitis* suspected of lack of efficacy (LOE) to macrocyclic lactone (ML) preventive drugs have been increasingly reported in dogs by practicing veterinarians since 2005 in the Lower Mississippi Delta region. If proven, and not controlled in the early stages, the emergence of ML drug resistance threatens to become a widespread problem in the US that may limit the effectiveness of current preventive drug treatment methods.

**Methods:** To validate practice reports, a statewide survey of Louisiana veterinarians was done to define the extent of the problem and identify focal ‘hotspots’ of reported ML LOEs using Geographic Information Systems (GIS) methods. The present study utilized microfilariae (Mf) from two canine field cases from different state locations that fit criteria for a high index of suspicion of LOE against heartworms by ML drugs. Blood containing Mf from the field cases was used to infect and produce infective larvae (L3) in *Aedes aegypti* for experimental infection of two groups of dogs, each of which contained two laboratory dogs, one treated with prophylactic ivermectin (12 μg/kg) monthly for 6 months, and one untreated control.

**Results:** Both treated and untreated dogs from Group I and Group II developed patent *D. immitis* infections by 218 DPI and 189 DPI, respectively, as evidenced by a positive occult heartworm antigen test and Mf by the Knott’s test. Mf counts gradually increased post-patency in test and control dogs. L3 raised from Mf from the treated Group I dog were used to successfully establish a second generation isolate, confirming heritability of resistance in the face of a monthly ivermectin challenge dose of 24 μg/kg, given monthly for 3 months.

**Conclusions:** These experimental infection studies provide *in vivo* evidence of the existence of ML drug resistance in dogs infected by *D. immitis* L3 from suspect field LOE cases in the Lower Mississippi Delta. Results encourage further work on mechanisms underlying the emergence of...
ML resistance in *D. immitis* and development of evidence-based resistance management strategies for heartworm preventives in order to extend the useful life of current drugs.
Chapter 1. Introduction

In 2005, an evaluation of the efficacy of heartworm preventive products was published by the Center for Veterinary Medicine, US Food and Drug Administration (FDA) that reported concerns of an increased number of reports by practicing veterinarians on lack of efficacy (LOE) of macrocyclic lactone (ML) preventive drugs [1]. Eighteen years after the first monthly ML drug formulation was approved in 1987, monthly ML drugs had largely replaced daily diethylcarbamazine, an approved treatment since 1977, to become the dominant means of preventing heartworms in companion animals in the USA. The first ML LOE reports to the FDA, beginning in 1998, had grown in number to several hundred in 1999–2000, nearly 1000 in 2000–2001 and over 1500 in 2002–2003 [1]. It was proposed that either true emerging resistance, enhanced FDA surveillance records systems or climate-based shifts in mosquito vector population species may have had a role.
Chapter 2. Literature Review

In response to this, and anecdotal reports of increasing LOE cases from the veterinary community in heartworm endemic areas, pharmaceutical firms initiated reimbursement programs in 2004 to cover costs of treatment of LOE cases if owners and their veterinarians could document full compliance with recommended preventive treatment practices. Concurrently, several research efforts were initiated, investigating the spatio-temporal scale and dynamics of LOE reports [2], potential in vitro indicators of resistance [3,4], possible genetic markers of resistance via genomic studies [5-10] and *in vivo* experiments, the gold standard, to establish confirmed resistant strains from canine field cases in the laboratory [11,12].
Chapter 3. Establishment of Macrocyclic Lactone Resistant *Dirofilaria immitis* Isolates in Experimentally Infected Laboratory Dogs

3.1. Objectives

The objectives of studies reported here are to record results of 1) a 2009 survey to document the experience of veterinarians in Louisiana on potential emerging ML resistance by *D. immitis*, and 2) establishment of ML resistant isolates of *D. immitis* in experimentally infected laboratory dogs using L3 raised in *Aedes aegypti* fed on microfilaremic blood from 2 dogs with a ‘high index of suspicion of resistance’.

3.2. Methods

**Objective 1: statewide practitioner survey**

In August 2009, a one-page ‘check-off’ questionnaire survey was sent to all Louisiana veterinarians listed in the Louisiana Veterinary Medical Association (LVMA) database and in the 2008 billing records of the Louisiana Animal Disease Diagnostic Laboratory (LADDL). Fifteen survey questions queried whether ML LOE cases had been seen in client dogs, including year of the first ML LOE case, specific drug used on suspected LOE cases, and the number of ML LOE cases reported. A total of 855 surveys were sent. Survey data results were entered into a Microsoft® excel (Microsoft Office, 2007) database and linked to a map of Louisiana within a geographic information system (GIS) according to the longitude/latitude geographic point location and Zip Code of each responding veterinarian’s clinic using ArcGIS 9.3 software (ESRI, Redlands, CA) [2].

----------

This chapter was previously published as Pulaski, C.N., Malone, J.B., Bourguinat, C. et al. Establishment of macrocyclic lactone resistant *Dirofilaria immitis* isolates in experimentally infected laboratory dogs. Parasites Vectors 7, 494 (2014).
Objective 2: experimental infection studies

Field isolates

Microfilariae (Mf) positive blood from canine field cases from different state locations was collected from client-owned dogs for use in the current study. After review and approval of experimental protocols by the Louisiana State University Institutional Animal Care and Use Committee, owner consent forms informing clients of the study purpose and design were agreed upon and signed prior to enrollment in the study. Based on practitioner and client cooperation, two dogs were selected based on the following criteria for a ‘high index of suspicion of ML resistance’ by *D. immitis*:

a) History of failure of efficacy and full monetary compensation by a commercial pharmaceutical firm;

b) Residence in an area identified as a ‘hotspot’ of suspected ML drug resistance in the 2009 statewide veterinary practitioner survey of LOE cases [2];

c) Persistence of circulating Mf following an accepted microfilaricidal dose of ML [8] and;

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 [5].

Dogs

Four male hound-cross dogs of 4–5 months-of-age were purchased from a USDA approved commercial supplier for experimental infections. All dogs were housed strictly in indoor runs in a mosquito-free kennel facility confirmed by periodic overnight sampling using a CDC Miniature Light Trap, Model 512 (John W Hock Co., Gainesville, FL). All dogs tested negative for adult heartworm antigen (DiroCHEK® Canine Heartworm Antigen Test Kit,
Symbiotics Corporation, San Diego, CA, USA) in serum samples collected prior to experimental infection and at 1-, 2-, 3-, and 4-months post-infection. None of the dogs were treated with any ML in the holding period before experimental infection of dogs with *D. immitis* L3 at 12–15 months-of-age.

**Experimental design**

Dogs were randomly assigned to two groups of two dogs for experimental infection by the two field isolates (Group I – “LSU 10” strain; Group II – “LSU 13” strain). At 27–33 days after infection, one dog in each group was treated with 12 μg/kg ivermectin diluted 1:9 in propylene glycol by the subcutaneous (SC) route. The untreated control dog received a similar volume of only propylene glycol by the SC route. At monthly intervals thereafter, dogs received respective ivermectin (12 μg/kg) or propylene glycol sham treatment for a total of 6 monthly treatments.

Although not required to confirm resistance, the ivermectin monthly treatment was repeated six times, at double the recommended dose, in order to ensure the presence of resistance. Ivermectin drug (Merial, Ivomec® 1% Injection, 50 mL) was from batch number BA132/10 (certificate of analysis available).

**D. immitis infection**

Blood containing Mf from the two canine field cases was collected and used to infect laboratory-raised *Aedes aegypti* mosquitoes using a membrane feeding apparatus (Thermo NESLAB, Model RTE-111, Neslab Instruments, Newington, NH, USA). Mosquitoes were infected and held in an insectary for 14–16 days (27°C, 80% RH, 12 hour light cycle) prior to harvesting L3 according to the procedure of the NIAID/NIH Filariasis Research Reagent Repository Center (FR3), Athens, GA, USA [13]. Each dog received a SC inoculation in the
inguinal area containing infective third-stage D immitis. Recovered L3 were held in RPMI tissue culture media at room temperature for up to 3 hours until SC inoculation. Group I (LSU 10) dogs each received 69 L3 (31 on 23rd March 2012 and 38 on 30th March 2012) and Group II (LSU 13) dogs each received 75 L3 (test) or 114 L3 (untreated control) on 15th June 2012. Group I dogs were infected twice, one week apart, because an adequate number of L3 larvae were not obtained following the initial harvest. Whole blood samples were collected from each dog at monthly intervals for four months following experimental infection, and at two week intervals thereafter, to determine D. immitis infection status and the onset of patency. Blood samples were analyzed for adult heartworm antigen (Dirochek® Canine Heartworm Antigen Test Kit, Symbiotics Corporation, San Diego, CA) and the presence of microfilariae was monitored using a 20 μL direct blood smear and the modified Knott’s technique [14]. Results were recorded over time as number of days post-infection (DPI).

**Heritability**

In separate follow-up studies, infective larvae raised from microfilariae from the treated Group I dog (LSU 10 strain) were used to confirm heritability of resistance in the face of a monthly ivermectin challenge dose of 24 μg/kg for 3 monthly treatments. Similar procedures to those previously described were used to infect (45 L3) and monitor the dog used to establish this second generation isolate of the LSU 10 strain (LSU 10-II). An ivermectin treatment dose at 4 times the recommended preventive level was used to further confirm strain resistance, which had previously been assessed at twice the recommended level.

**Ethical approval**

This study was reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (PRN 13–072).
3.3. Results

Statewide survey

Of the 855 one-page mail surveys sent to all Louisiana practitioners in August 2009 regarding their opinions of ML LOEs reported within their clinics, 221 were returned (25.8% response rate). Of the 221 surveys submitted, 70% were from clinics classified as ‘small animal’ and 25% were from ‘mixed animal’ facilities. GIS analyses of survey results indicated there were focal locations with veterinarian perceived high rates of reported LOEs of ML drugs against heartworms and that numbers increased from 2005 to 2008. Pertinent survey questions with results are shown in Figure 1 [2].

Experimental infections

Both treated and untreated dogs from Group I (LSU 10) and Group II (LSU 13) developed patent *D. immitis* infections by 218 DPI and 189 DPI, respectively, as evidenced by a positive occult heartworm antigen test and microfilaremia by the modified Knott’s test. Mf counts gradually increased post-patency in test and control dogs (Table 1)

Heritability of resistance

Microfilaremia in one of the dogs (LSU 10a), initially found in moderate numbers, became sporadic with biweekly counts of 2–8 Mf per ml of blood. Microfilariae from this dog (LSU 10a) were used to 1) maintain an adequate Mf lev generation passage of the to a new laboratory dog (LSU 10-II); 2) to confirm resistance in the face of challenge treatment by a
Figure 1. Pertinent Survey Questions with Results
Table 1. Monthly Parasitological Monitoring

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Bold, italicized values indicate first detection of circulating Mf. DOI, Date of Infection; Occult, Adult Heartworm Antigen Test; DS, Direct Smear using 20 μL of whole blood; Knotts, Modified Knott’s Technique; DPI, Days Post-Infection; ND, No Data.
24 μg/kg dose of ivermectin given monthly for 3 months, and 3) to demonstrate heritability of the LSU 10 resistant strain. Monthly monitoring of the LSU 10-II dog showed it became positive for circulating antigen by 204 DPI and became patent with circulating Mf by 259 DPI. Five laboratory dogs harboring resistant strain *D.immitis* isolates from two separate client-owned dogs (LSU 10, LSU 13) are thus currently available for further study:

**Dog challenge treatment**

- LSU 10a ivermectin (12 μg/kg, monthly for 6 months)
- LSU 10b untreated control
- LSU 10-II ivermectin (24 μg/kg, monthly for 3 months)
- LSU 13a ivermectin (12 μg/kg, monthly for 6 months)
- LSU 13b untreated control

We propose that we have successfully isolated two strains from proven cases of resistance in client-owned dogs in Louisiana that can be used to further study and characterize the emergence of ML resistance by *D. immitis*.

**Genomics studies**

Samples of 20–30 individual Mf, from Group I and Group II *D. immitis* infected dogs, were collected, preserved in isopropyl alcohol, and analyzed blindly in order to genotype the circulating Mf population for two single nucleotide polymorphism loci on a P-glycoprotein gene previously shown to be correlated with a loss of efficacy of macrocyclic lactone heartworm anthelminthics [5-7]. The investigation of percentage frequencies of GG-GG genotype in the different groups was the main interest. DNA extraction of individual Mf had been extracted following the protocol of QIAamp DNA Micro kit from Qiagen® (www.qiagen.com). Also, individual Mf DNA samples had been amplified using Repli-g screening kit from Qiagen®, to
increase DNA concentration of individual samples. Individual genotypes had been identified after PCR, Sanger sequencing, and analysis of chromatograms using Sequencher® (http://genecodes.com/). Table 2 shows results of preliminary studies on Mf from the original two client owned dogs (LSU 10, LSU 13) used to establish resistant strains in the laboratory as compared to Mf from two dogs from a police dog kennel (LSU 11, LSU 14). Results showed a higher percentage frequency of GG-GG genotype in Mf populations from LSU 10 and LSU 13 strain dogs compared to Mf population from LSU 11 and LSU 14.

Table 2. Genomics Studies

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<td>41.4</td>
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<tr>
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<td>60.9</td>
<td>4.4</td>
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<td>17.4</td>
<td>0.0</td>
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<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
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</tr>
</tbody>
</table>

Note: Preliminary study of PCR analysis of 23–30 individual microfilariae showing high frequency of the GG-GG marker from dogs harboring proven resistant strains (indicated in bold) as compared to pooled microfilariae from two infected dogs from a local police dog kennel. Result?? Inconclusive (position 11 undetermined, position 618 determined)
3.4. Discussion

The aim of the current study was to provide evidence to test the following hypothesis – ‘Resistant strain(s) of *D. immitis* exist in the general population of Louisiana dogs, and these resistant strains can be identified by case history, in vivo tests and genetic markers to enable development of effective alternative drug therapies to extend the useful life of current preventive drugs’. Beginning in 2005, LOE reimbursement programs by pharmaceutical firms provided an opportunity to study pre-screened records of possible resistance and made it more likely to identify sporadic cases of resistance, if present, in the general population of dogs in South Louisiana. The ‘microfilariae reduction test’ proposed by Geary et al. [8] provided a simple method for testing potential drug failure that could be used in clinical settings when resistance was suspected. Initial studies on the ‘Katrina dog’ [5] and other suspect cases [6,7] provided a potential new tool to find cases based on genetic markers of ML resistance by *D. immitis*. Finally, a statewide survey was done of Louisiana veterinary practices in 2009 to identify the extent and geospatial distribution of company reimbursed cases (Figures 1 and 2) [2]. Statewide survey results indicated that current drugs were still effective in most practices, but that troubling hot spots of suspected resistance were emerging. Some practices in high prevalence areas had over 50 cases per year, some reported resistance in up to 25% of dogs under their care, and 74% of practices indicated they had seen at least one LOE case in the past year.

Faced with the uncertainty presented by apparent frequent drug failure, especially in large breed outside dogs subjected to bites from large mosquito populations, practices in some areas abandoned the commonly used monthly preventive drugs and began searching for alternative preventive protocols, including use of monthly preventive drugs administered at higher doses, long-acting injectable or topical ML drugs that maintain high continuous blood levels [8], or
strategic use of doxycycline in combination with ML drugs [15-17]. Some of the long-acting ML drugs have additional label claims of efficacy against other helminths in which ML is given at elevated doses above that needed for HW prevention alone [9]. Controversies continued, as explanations, including environmental changes, vector population shifts, and owner compliance issues, for the growing number of LOE cases multiplied. There was a clear need to establish the ‘gold standard’ of experimental isolation and characterization of suspect cases of canine heartworm resistance in controlled experiments.

Figure 2. Survey Question 2 - with field isolate residences noted as ‘LSU 10’ and ‘LSU 13’
We adopted a four point ‘high index of suspicion’ criteria to enable identification and isolation of resistant strains of D. immitis: a) clinical history of failure of efficacy and full monetary compensation by a commercial pharmaceutical firm; b) residence in an area identified as a ‘hotspot’ of suspected drug resistance in the 2009 statewide veterinary practitioner survey of LOE cases [2]; c) persistence of circulating Mf seven days after an accepted microfilaricidal dose of ML [8] and 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 [5].

Using these criteria for high index of suspicion, LADDL began searching for cases in high prevalence foci of infection in Louisiana by retrospective study of positive confirmatory antigen-based tests and Knott’s examination done on LOE cases referred to LADDL by veterinary practitioners in order to qualify for LOE case reimbursement by pharmaceutical firms. Two candidate dogs (LSU 10 and LSU 13) were identified in separate geographic locations (Figure 2) that fit the four criteria and had sufficient circulating microfilariae for successful experimental infection of laboratory dogs. Dogs were infected using L3 raised in A. aegypti from circulating microfilariae in blood from candidate dogs [13]. For each pair, one dog was treated with monthly subcutaneous doses of ivermectin of 12 μg/kg (twice the recommended preventive dose for 6 months) and one dog served as an untreated control given a sham subcutaneous dose of propylene glycol at the same monthly interval. Both treated and untreated dogs infected with the LSU10 isolate and the LSU 13 isolate developed patent D. immitis infections by 218 DPI and 189 DPI, respectively, as evidenced by a positive occult heartworm antigen test and detection of microfilariae by the Knott’s test. Mf counts gradually increased in number of Mf after patency in experimentally infected dogs (Table 1), although microfilaremia in one dog (LSU 10a), initially
found in low to moderate numbers, became sporadic with biweekly counts of 2–8 Mf per ml of blood. It is known that up to 25% of naturally infected dogs develop ‘occult’ infections (presence of adult heartworms without circulating Mf). This is thought to occur in some dogs due to development of immunity-mediated removal of circulating Mf [15].

Microfilariae sent to McGill University for genomic studies showed higher genotype frequency of the GG-GG marker [5] loci on P-glycoprotein gene from Mf population from LSU 10 and LSU 13 strain dogs compared to Mf population from LSU 11 and LSU 14 (Table 2). In a separate follow-up study, infective larvae raised from Mf from the treated Group I dog (LSU 10a) were successfully established as a second generation isolate (LSU 10-II), confirming heritability of resistance in the face of a monthly ivermectin challenge dose of 24 μg/kg, 4 times the recommended preventive level for a total of 3 monthly treatments.

We propose that we have successfully isolated two strains from proven cases of resistance that can be used to further study and characterize ML resistance by *D. immitis*. In a concurrent, related study reported elsewhere, Kaminsky et al. [12] reported identification of ML resistant strains using the same animals used for genomic and Mf motility assay studies previously reported [3], providing independent evidence of resistance in three dogs that also originated in LA or AR. Our study and that study are mutually confirmatory and together provide strong evidence of the existence of ML resistant strains of *D. immitis* in the Lower Mississippi Delta.

Resistance to ML and other drugs is well known for nematodes in other host species [18] and the question may be posed as to why it took so long for resistance to emerge as a problem with heartworm ML preventive drugs. The long, essentially annual life cycle of *D. immitis* [19], a large refugia of ‘wild type’ populations in both domestic dogs and wildlife, environmental
perturbations of mosquito vectors [20-23], and selection pressure by ‘slow-kill’ adulticide protocols have been proposed as having a role [10,24]. Evidence of resistance to ML has been reported for *Onchocerca volvulus* in long-term preventive ML chemotherapy programs in endemic areas of Africa. It is interesting that it took a similar time frame for *O. volvulus*, approximately 20 years, for documented evidence of ML resistance to emerge in the similarly long-life cycle of this filarid species, the cause of human ‘river blindness’ [25].

The role of practicing veterinarians as the first line of defense for detection of adverse drug effects, with FDA, has apparently been effective as a surveillance mechanism in current heartworm control strategies. Moreover, clinical experience gained from practitioners willing to try elective off label use of drugs and new resistance management strategies promises to lead to new approaches to therapy that can then be proven in controlled published experiments [18,24]. For example, diethylcarbamazine was used for many years for daily use in preventing heartworms but was then replaced by ML drugs after practitioners, kennel owners, and sporting dog groups found that off-label use of recently released ML drugs for livestock, protected against heartworms, intestinal helminthes and other internal parasites with very striking benefits for dogs [26,27]. This was soon followed by controlled pharmaceutical company experimentation leading to FDA approval and marketing in the late 1980’s of current ML drug formulations at the very low dose levels now used for prevention of canine heartworms [26,27].
Chapter 4. Conclusions

These experimental infection studies provide in vivo evidence of the existence of ML drug resistance in dogs infected by *D. immitis* L3 from suspect field LOE cases in the Lower Mississippi Delta. The emergence of ML resistance by *D. immitis*, if not controlled in the early stages, threatens to become a widespread problem in the US that limits the effectiveness of current preventive drug treatment methods. There is a need to develop and implement evidence based resistance management strategies to prevent widespread selection of resistant strains where it has been shown to exist and to suppress potential spread to other areas of the country. There is current evidence to support the value of potential measures that may be included as part of a resistance management strategy if confirmed by additional research and adopted by the veterinary community. The aim is to suppress spread from dogs harboring resistant *D. immitis* strains and to extend the useful life of currently available drugs: 1) Wider use of the Mf reduction test in veterinary practices to identify suspect resistant cases [8]; 2) Development of laboratory tests to identify resistance markers in suspect cases by microfilariae or adult worms submitted by veterinarians; 3) Confirm that doxycycline/tetracycline therapy can be used to suppress viability of infective larvae that develop in mosquitoes from microfilariae of resistant dogs [17] as a way to prevent infection of other dogs where strong evidence of resistance exists (e.g. resistant dogs in the same household or neighborhood or where greatly elevated prevalence of *D. immitis* in mosquitoes is likely after feeding on circulating microfilariae in a resistant dog) [20]; 4) Use of American Heartworm Society recommended adulticide/microfilaricide treatment protocols and avoidance of slow-kill treatment methods in which repeated treatment of infected dogs may potentiate selection of resistance [10]; and 5) Investigate the value of alternative preventative treatment protocols where resistant cases are found, including use of drugs that
maintain high, sustained ML blood levels [28-30] or selective return to use of older alternative classes of preventive drugs (e.g. daily diethylcarbamazine) [15] pending future development of new alternative drugs.

Additionally, a time-space surveillance system may be warranted to alert the veterinary community on the need for intervention by mapping the location of reported LOE cases and ‘high index of suspicion’ LOE cases that can be confidently attributed to true drug resistance, such as by future genetic marker testing. Regular updates by the FDA on the status of required LOE reports, with other records collected by pharmaceutical industry partners, may serve this purpose. The value of an effective surveillance system may be further enhanced by development of biology-based predictive models on the potential for spread of ML resistance based on climate/environmental suitability determinants, and use of ecological niche models for surveillance using geographic information systems (GIS) risk analysis methods [31-34].
Chapter 5. Epilogue

The study, and subsequent publication described was crucial to validate the existence of macrocyclic lactone resistant strains of *Dirofilaria immitis* (“it is also now indisputable that genuinely ML-resistant heartworms are circulating, at least in the Mississippi delta region of the USA (Pulaski et al. 2014)” (Wolstenholme et al., 2015), and the 2014 paper has now been cited in over 45 independent publications (CrossRef: 48, Springer 96th percentile rank). Once the presence of drug resistant heartworm infections was confirmed, the need to understand the emergence and prevalence of these strains became apparent. The following studies are examples of just a few of the approaches taken to further investigate these drug resistant strains.


As previously described, in 2005, Hampshire published the first peer-reviewed article on the potential emergence of macrocyclic lactone resistance in *Dirofilaria immitis* based on lack of effectiveness (LOE) claims submitted to the Center for Veterinary Medicine (CVM) within the U.S. Food and Drug Administration (FDA). At the time, the macrocyclic lactone LOE reports received by CVM had dramatically increased by 57% between 2000 and 2003 (reports appeared to be decreasing between 2003 and 2004), with the majority of failures reported from heartworm endemic states, specifically in the southeastern USA. Over the next 10 years, the veterinary community would continue to see increasing numbers of suspect ML LOE cases, predominantly focused in the Lower Mississippi River Alluvial Valley, and eventually a laboratory-verified macrocyclic lactone resistant *D. immitis* strain would be found circulating in the area by Pulaski and others in 2014 [35]. However, the extent of drug resistant heartworms is unknown, with a great deal of information lacking on the prevalence and geographic range of such cases. The objective of this study is to analyze macrocyclic lactone LOE reports submitted to CVM since
the 2005 Hampshire paper, including the years 2004 to 2015, in order to identify possible changes, patterns, and interactions found between and within cases.

Between the years 2004 to 2015, approximately 45,000 ML LOE claims have been reported to CVM, with the largest numbers being reported between 2008 and 2010. Each report is received and assigned a ‘causality score’, ranking the probability of true macrocyclic lactone drug ineffectiveness, and scores are based primarily on timing, owner compliance, region of incidence, and HW testing history (Figure 3). However, the assignment of a proper causality score for each report is essential for the accuracy of these analyses, and unfortunately the majority of reports (approx. 38,000) had not received an actual CVM causality score at the start of this project in 2016. A comprehensive analysis is on-going, searching for epidemiologic trends and geospatial patterns among the reports, with a focus on animal signalment, concomitant medications, space-time relationships, and the identification of possible ‘hot spots’ around the country.

Using a data sampling approach, a statistically significant, random sample of reports was selected for review and case series analyses, combined with reports that had previously been reviewed.
Ineffect Score of 0 (Possible)
1. Previous use of HW prevention, switched to preventive being scored: either no test pre-adm, or NEG test pre-adm of first drug, and no subsequent NEG tests
2. No previous known HW prevention, NEG test pre-adm, no 2nd test 6 mos later
3. NEG Ag test post HW treatment performed less than 6 months after treatment was administered, now POS (only one test post-treatment performed)
4. POS MF test, NEG Ag test
5. Started on HW preventive after 8 wks of age but less than 7 mos of age, no 6-7 mos HW test, 1st Ag test POS
6. Seasonal adm, then POS in spring (consider location and time between last dose and POS test)

Ineffect Score of 6 (Probable, Likely)
1. On HW preventive being scored for one or more years with NEG test after first 7 months of admin, with good compliance in administration or purchase history
2. Post-adulticide treatment, 2 Ag tests completed 6 mos apart, with the first test performed 6 or more months after Inmiticide. 1st Ag test is NEG, 2nd Ag test POS
3. Puppies started before 8 weeks of age: Good compliance in administration or purchase history (missed no more than 1 dose in a row over many months), now POS
4. Puppies on preventive being scored: NEG on 6-7 month Ag test, subsequently POS with good compliance in administration or purchase history
5. Ag NEG but persistent microfilariae (<99% reduction) 28 days after administration of topical moxidectin (Advantage Multi), starting with reports received after Nov 2013 (NADA 141-251, suppl.)

Ineffect Score of -8 (Unlikely, Information lacking)
At least 7 months old, no previous HW prevention, started on HW preventive being scored without a HW test

Ineffect Score of -9
- Not applicable: eg. if not an ineffect (Ag test is POS, confirmation test is NEG)
- Blatant non-compliance, including large gaps (>3m) in purchase history
- Inmiticide “failures” (HW preventive being scored administered before/during/after adulticide with no NEG test following HW treatment)

Figure 3. Scoring System Used by FDA to Evaluate Validity of Practitioner Reports of Lack of Efficacy (LOE)
Figure 4. Individual Lack of Efficacy (LOE) Reports (2004-2015) by Clinic Zip Code - point-located reports that scored either “0” (colored) or “6” (bullet)

5.2. Mississippi River Delta Veterinary Practitioners’ Knowledge, Attitudes, and Practices Regarding Macro cyclic Lactone ‘Lack of Effectiveness’ Against *Dirofilaria immitis* Infection, Then and Now

As illustrated in previous work [35], practitioner surveys can provide insight on complex biological questions for limited costs, and were utilized in the present study for investigating clinic-level perceptions and management approaches for suspected drug resistant heartworm infections. Furthermore, as previously discussed, reports of suspected macro cyclic lactone resistant heartworm infection reached a peak in 2007-2010, as noted in FDA LOE reports, but these appear to be decreasing. Many have hypothesized as to the reasons for the decline in LOE reports, although little work has been done to determine the factors which may have contributed to the perceived variation in case numbers and/or locations. One way to examine these trends is through veterinary practitioner surveys, which the authors have found to be useful in the past for
investigating drug resistance in heartworms, and in 2009, the authors conducted a statewide survey of veterinarians in Louisiana. The results of the previous survey helped to define the extent of macrocyclic lactone resistance in the state and to identify focal ‘hot spots’ of reported macrocyclic lactone LOEs. The objective of this study is to evaluate current trends in heartworm disease management as reported by veterinarians in the Lower Mississippi River Alluvial Valley, with a specific focus on changes in suspect LOE case numbers and heartworm disease prevention and treatment protocols.

During the summer of 2016, approximately 6000 questionnaire surveys were sent out to veterinary practitioners in the 5 Lower MS River Delta states (AR, LA, MS, MO, TN). Surveys were mailed to every licensed veterinarian in each state, with directory information obtained from each state’s licensing Board of Veterinary Medicine. Each practitioner received a one page ‘check-off’ questionnaire (Figure 5), with the option to complete via Qualtrics online survey service or return via fax or pre-addressed mailer. Survey questions pertained to suspected macrocyclic lactone LOE cases encountered in client dogs, including number of cases and potential trends (i.e., increasing or decreasing cases). Questions also assessed each practitioner’s currently used heartworm prevention and treatment protocols, as well as other related information. The goal was to compare these survey results with those obtained from a similar 2009 project, with a focus on investigating possible changes or trends in HW disease management and LOE case reporting. Survey data results were linked to a map of each state according to the zip code of responding clinics using ArcGIS 10.5 Software, and then further analyzed.
Figure 5. Results For Selected Questions - Survey of veterinarians on changes in knowledge, attitude and practices from 2005-2010 in relation to emergence of macrocyclic lactone drug resistance in a five-state area in the southeastern United States
A total of 789 of the 5931 questionnaires distributed were returned (13.30% response rate). Maps and detailed statistics of each survey question are available, including individual state results and overall totals. Major highlights include:

- 53.37% of practitioners had seen at least one highly suspected ML LOE case within the past 5 years; More than half of MO and TN practitioners reported not seeing a ML LOE case within the past 5 years
- 31.85% reported increasing numbers of ML LOE cases since 2010, 24.28% reported decreasing numbers, and 43.88% reported that case numbers were about the same
- 71.59% reported seeing less than 5 ML LOE cases within the past year
- 64.05% reported that they elected to treat less than 10% of their HW+ patients with a ‘slow-kill’ method (vs the recommended melarsomine protocol) within the past year: 11.34% reported treating >75% of their HW+ patients using ‘slow-kill’
- Most practitioners (83.87%) administer a microfilaricidal drug, as part of their HW treatment protocol: 76.70% report that this protocol has not changed within the past years
- 93.22% administer doxycycline/minocycline as part of their HW treatment protocol: 55.39% have started including this step within the past 5 years

Based on our survey results, we were able to identify regions within the 5 states (AR, LA, MO, MS, TN), wherein practitioners reported lower or higher numbers of suspected macrocyclic lactone LOE cases compared to 2009. Overall, most clinics reported decreasing numbers of suspected macrocyclic lactone LOE cases. Most clinics also reported using doxycycline or minocycline (as part of their heartworm treatment protocols) more frequently over the past 5 years, which may help explain why several areas are seeing fewer LOE cases (when compared to
those cases from 2005-2010). This is particularly interesting when considering that over 10% of clinics reported treating >75% of their HW+ patients using a ‘slow-kill’ technique, and these clinics also reported decreasing macrocyclic lactone LOE case numbers and increased use of doxycycline/minocycline.

5.3. The Potential Use of Levamisole in a Macro cyclic Lactone Resistance Management Strategy in Dirofilaria immitis Infections

Prior to the introduction and widespread use of macrocyclic lactone drugs in Dirofilaria immitis infections, few options were available to prevent heartworm disease and/or eliminate circulating microfilariae. At that time, diethylcarbamazine citrate (DEC) was the drug of choice, although administration to microfilaremic dogs frequently resulted in severe anaphylaxis-like reactions. Another option, levamisole hydrochloride (LEV), a synthetic imidazothiazole, showed efficacy against D. immitis, but its use was limited. The existence of strains of D. immitis resistant to currently available macrocyclic lactone preventive drugs has now been demonstrated on multiple occasions, in multiple regions of the USA. Anecdotal reports in Louisiana now indicate the number of heartworm preventive drug ‘lack of effectiveness’ (LOE) claims are decreasing, nevertheless veterinary practitioners in some regions around the state continue to see suspect LOE cases. Traditionally, a dog was suspected as a LOE case and thought to be infected with resistant strains of DI when the animal developed heartworm disease, despite proper administration of ML preventive drugs. Another approach to identify a suspect LOE case requires the monitoring of Mf counts following the administration of macrocyclic lactones at a dosage known to be microfilaricidal. This approach, better known as the ‘microfilariae reduction assay’ has been shown to be useful in clinical veterinary practice situations [9].
Case Series

History

In the summer of 2015, LSU was contacted by a veterinarian in the state regarding suspect cases of ML resistant DI infections. The practitioner was actively involved with an animal rescue group that specializes in the transport of shelter animals from Louisiana to various states in the northeast USA, particularly in the New England area. Prior to transport, all adult animals were required to be sterilized, up-to-date on vaccines, and negative for HW disease, including both negative antigen and Mf tests. At the time, several adult dogs, including those with adopters already arranged, were being held at the practitioner’s clinic because they continued to have circulating Mf (detectable on blood smears), despite receiving *D. immitis* adulticidal and microfilaricidal treatments. Many of these dogs were *D. immitis* antigen negative and all had undergone multiple attempts to clear Mf, including the repeated administration of high-dose IVM and topical MOX. Some of the dogs had also received a 30-day course of doxycycline (10mg/kg PO BID), either prior to or following melarsomine injections. The practitioner contacted LSU in order to report these suspect ML resistant cases, as well as to inquiry about other possible Mf treatment options, in hopes of successfully relocating these dogs, as well as avoiding the possible spread of ML resistant DI strains to regions outside the SE USA.

Plan:

LSU agreed to assist with these cases, and based on previous literature and clinical experience, the following plan was implemented:

- A thorough case history and blood would be sent to LSU from all suspect ML resistant cases at that time, for DI antigen testing (DiroCheck®), both prior to and following the
heat treatment of plasma samples [52], as well as quantitative Mf testing (via modified Knott’s assay). New cases would also be enrolled, as they were identified.

- After Ag and Mf status was determined, enrolled animals would receive levamisole HCl 5mg/kg PO SID x 10 days (LevaMed® Soluble Drench Power at concentration 12.4mg/ml)
- Ag and Mf status of each dog would be monitored, with administration of additional levamisole treatments as needed

**Outcome:**

Over 6 months, 17 dogs were enrolled in the study; circulating Mf were eliminated in 12 of the cases (based on Knott’s), while Mf were still identified in 5. However, all 5 of those animals had very low Mf counts (<50Mf/mL), and none had Mf detectable on blood smears. Detailed case histories from 3 representative animals are outlined in Figure 6.

**Conclusions:**

- The use of LEV in suspect ML resistant DI infections was successful in eliminating circulating Mf in approximately 70% of cases
- Of those that still had detectable Mf; Mf numbers were highly reduced, especially when compared to reductions observed following microfilaricidal doses of ML
- The practitioner continues to use LEV as part of her ML resistant management strategy in *D. immitis* infections; however she reports that LEV treatments can be challenging, as the drug is extremely bitter and can be difficult to administer, especially to large dogs.
- Since presenting this case series, other clinics outside of Louisiana have reported using LEV in suspected ML resistant heartworm infections, with more success administering the drug when compounded.
Figure 6. Detailed Case Histories From 3 Representative Animals - Timeline of drug treatment with moxidectin (MOX), ivermectin (IVO), doxycycline (Doxy), levamisole, and DI antigen (Ag- or Ag+) and/or microfilariae status
Appendix. Copyright Information
List of References

This list of references cited is divided into two sections: 1) References cited in Chapter 3 based on dissertation research results originally published in 2014, in the refereed journal ‘Parasites and Vectors’[35]; and 2) Select references (up to 2019) citing the 2014 P&V article, demonstrating the impact of validating the existence of macrocyclic lactone drug resistance in *Dirofilaria immitis* in accordance with Koch’s Postulates.

Section I


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Section II


37. McTier, Six, Pullins, Chapin, Kryda, Mahabir, Woods, Maeder.. Preventive efficacy of oral moxidectin at various doses and dosage regimens against macrocyclic lactone-resistant heartworm (Dirofilaria immitis) strains in dogs Parasit Vectors. 2019; 12: 444.

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42. Hodgkinson, Kaplan, Kenyon, Morgan, Park, Paterson, Babayan, Beesley, Britton, Chaudhry, Doyle, Ezenwa, Fenton, Howell, Laing, Mable, Matthews, McIntyre, Milne, Morrison, Prentice, Sargison, Williams, Wolstenholme, Devaney. Refugia and


Vita

Cassan Pulaski grew up in Mandeville, Louisiana, just outside New Orleans. She earned a B.S. in Biology and a B.A. in Psychology from the Sally McDonnell Barksdale Honors College at the University of Mississippi in 2008. She then completed her MPH and DVM degrees at Louisiana State University in 2012 and 2013, respectively. Following graduation from veterinary school, she began a PhD program at LSU in the department of Pathobiological Sciences, and in January of 2015, she began a NCVP residency program in clinical veterinary parasitology. At the time of final submission, she is a Clinical Assistant Professor and the Diagnostic Parasitology Section Head at the University of Georgia College of Veterinary Medicine and Athens Veterinary Diagnostic Laboratory.