BIOLOGY, EPIDEMIOLOGY, AND GENETICS OF MULTIPLE DRUG RESISTANCE IN THE CANINE HOOKWORM (*ANCYLOSTOMA CANINUM)* by

PABLO D. JIMENEZ CASTRO

(Under the Direction of Ray M. Kaplan)

ABSTRACT

There have been increasing reports of *Ancylostoma caninum* infections that do not clear after multiple treatments in different dog breeds in the United States over the past few years. We have determined that the cause of the lack of clearance of these infections in most cases was due to multiple drug resistant (MDR) isolates of *A. caninum*. The emergence of this resistance in hookworms infecting dogs in the US warranted investigating several epidemiological questions. To start, an extensive literature review was performed that described the natural history of *A. caninum* including the biology of the parasite, history of the disease, and taxonomy and life cycle of the nematode, among other topics. The review also provided a history of anthelmintic resistance in general, as well as focusing specifically on parasites of dogs. In the first research section, we attempted to diagnose and describe for the first time, cases of MDR in *A. caninum* to all the major drug classes used for the treatment of hookworms of dogs in the US. The second study attempted to evaluate the efficacy of a product registered in Europe as well as three other anthelmintic products commonly used for the treatment of canine hookworms registered in the US, against a MDR isolate of *A. caninum*, Worthy 4.1F3P. The third and fourth study evaluated the range of resistant phenotypes in isolates infecting greyhounds in the US and used the deep amplicon sequencing assay to investigate the molecular epidemiology of these *A. caninum* infecting

greyhounds as well as in the canine pet population across the US to be able to provide insights with regards to the origin(s) and spread of this resistance.

INDEX WORDS: Hookworm, *Ancylostoma caninum*, canine health, anthelmintic resistance, zoonosis

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DEDICATION

This dissertation is dedicated to my mother and father for always supporting me on my dreams and pushing me to be better in life. But specially this is dedicated to my wife, Karen, the partner I chose to go through life. You have encouraged and supported me throughout this rollercoaster of an endeavor, and I will always be grateful for that.

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CHAPTER 1

A REVIEW OF THE BIOLOGY AND ANTHELMINTIC RESISTANCE IN THE CANINE HOOKWORM, *ANCYLOSTOMA CANINUM*

INTRODUCTION

Hookworms are one of the most common soil-transmitted helminths and can infect a wide variety of mammals. Taxonomically, these belong to the superfamily Ancylostomatoidea, and family Ancylostomatidae. Two subfamilies comprise these group, the Ancylostomatinae, which are parasites of carnivorous hosts, and Bunostominae which are parasites of herbivorous hosts. Omnivorous hosts can have parasites from any of these two subfamilies. The subfamily Ancylostomatinae comprises the genera *Ancylostoma, Uncinaria, Globocephalus,* and *Placoconus*. Whereas the Bunostominae comprise the genera *Bunostomum, Necator, Bathmostomum,* and *Grammocephalus* (Bowman, 2014). Currently there are 15 species that comprise the *Ancylostoma* genus, these are *Ancylostoma caninum* (Ercolani, 1859), *Ancylostoma duodenale* (Dubini, 1843), *Ancylostoma braziliense* (Gomes de Faria, 1910) and *Ancylostoma ceylanicum* (Looss, 1911), *Ancylostoma tubaeforme* (Zeder, 1800), *Ancylostoma malayanum* (Alessandrini, 1905), *Ancylostoma pluridentatum* (Alessandrini, 1905), *Ancylostoma paraduodenale* (Biocca, 1951), *Ancylostoma kusimaense* (Nagayosi, 1955), *Ancylostoma buckleyi* (Le Roux &

Biocca, 1957), *Ancylostoma taxideae* (Kalkan & Hansen, 1966), *Ancylostoma genettae* (Macchioni, 1995), *Ancylostoma protelesis* (Macchioni, 1995), *Ancylostoma somaliense* (Macchioni, 1995), and *Ancylostoma ailuropadae* (Yang, Hoberg & Xie, 2017) (Xie et al., 2017).

The etymology of *Ancylostoma* comes from: *Ancylo* = curved + stoma = mouth and hookworms are called as such because in the majority of cases the anterior region is bent dorsally, thus having the shape of a "hook". For nearly 100 years, the common hookworm of both dogs and cats, first described as *Sclerostoma caninum* and *Strongylus tubaeformis*, respectively, were referred to as *Ancylostoma caninum* (Burrows, 1962). Only in 1954, Biocca published his results from detailed studies working with *Ancylostoma* of dogs and cats in Italy, provided the original descriptions of Zeder in 1800 for *Ancylostoma tubaeforme (Strongylus tubaeformis)* and Ercolani in 1859 for *Ancylostoma caninum (Sclerostoma caninum)*, redescribed *A. tubaeforme,* and showed that in fact these were two different species, as the latter had a shorter oesophagus, smaller lateral lobes of the copulatory bursa, different distribution of lateral rays and far longer spicules (Biocca, 1954).

The canine hookworm, *A. caninum* (Nematoda: Strongylida: Ancylosmastoidea) (Figure 1), is the most prevalent and important intestinal nematode parasite of dogs in the United States. A survey published in 1996 evaluating 6,458 fecal samples from animal shelters across the US yielded a prevalence for *A. caninum* of 19.19% (Blagburn et al., 1996). Followed by a prevalence of only 2.5% from 1,199,293 fecal samples of pet dogs evaluated in 2006, with the prevalence depending on age, level of care, and geographic location of the dog (Little et al., 2009). A recent study evaluating archived data of over 39

million dog fecal samples from 2012-2018, showed a stable prevalence of hookworm infections from 2012-2014 ranging between 1.5% - 2.3%, but then starting in 2015 the prevalence began to steadily rise each year, with an overall increase from 2015-2018 of 47% (Drake and Carey, 2019). This decrease in prevalence of more than 75% from 1996 to 2012-2014 even though were examining samples from different populations of dogs, could be largely because in this time frame the awareness of anthelmintics for companion animals was very different than at the beginning of the 1990's, and a more frequent treatment to cover gastrointestinal nematodes was pushed by both veterinarians and parasitologists. However, in a recent large scale study evaluating the prevalence of intestinal parasites in dog parks throughout the USA, 7.1% of the samples were positive for hookworms and of those, 98.2% were positive for *A. caninum* (Stafford et al., 2020). Interestingly, this prevalence is more than twice as high as that reported for 2018 by (Drake and Carey, 2019), and is more than 70% higher than the mean prevalence for 2017-2019 reported by (Sweet et al., 2021). There could have been some degree of underdiagnosing thus underestimating these prevalence rates, due to technical factors such as not all of the eggs being detected at the fecal floatation because a centrifugal step was not used, or biological factors such as different maturity of infections, as some could have been too early to detect, or too old and the females could be dying off due to senescence. However, taken together these data suggest that hookworm prevalence is rapidly increasing, and that dogs that visit dog parks are at a higher risk of infection. Also, this shows that anthelmintics labelled for hookworms are showing not to be efficacious, as these pets are most likely to be on a monthly preventive for the canine heartworm, Dirofilaria immitis, and all of these products have a drug with a label efficacy against hookworms.

Several families of mammal wildlife species are affected by parasites of the genus *Ancylostoma*; however, to date only individuals of the families Canidae (*Canis latrans, Vulpes vulpes, Canis lupus, Canis lupus dingo, Canis aureus, Urocyon cinereoargenteus* and *Canis rufus*), Felidae (*Lynx rufus and Lynx canadensis*), and Ursidae (*Ursus americanus*) have been reported to harbour patent infections of *A. caninum* (Seguel and Gottdenker, 2017). Recently, patent infections by *A. caninum* have been reported in the black-eared opossums, *Didelphis aurita* (Bezerra-Santos et al., 2020) from Brazil, which is relevant due to the synanthropic nature of these animals, as these are well adapted to urban environments, and their opportunistic dietary behavior facilitates their role as reservoirs (Cunningham et al., 2017; Teodoro et al., 2019).

Hookworms are blood-feeding nematodes that use a cutting apparatus to attach to the intestinal mucosa and submucosa, and contract their muscular esophagus to create negative pressure, which sucks a plug of tissue into their buccal capsules (Hotez et al., 2004). Bleeding is facilitated by both mechanical damage and chemical action by hydrolytic enzymes that cause rupture of capillaries and arterioles. Additionally, hookworms release an assortment of anticlotting agents to ensure blood flow (Stassens et al., 1996), which have been found to be more effective at prolonging the time of conversion from prothrombin to thrombin when compared to both recombinant hirudin, a natural occurring peptide found in the saliva of leeches, and tick anticoagulant peptide (Cappello et al., 1995). The adult worms of *A. caninum* are voracious bloodsuckers, with sucking movements of the oesophagus reported as high as 120-150 per minute, and blood intake of 0.84 mL/adult worm with feeding taking place even during the copula and after resection behind the oesophagus (Wells, 1931b, a). Pathological consequences of infection include

iron-deficiency anemia, hypoalbuminemia, and enteritis, characterized by diarrhea that may contain fresh (haematochezia) or digested blood (melena) (Kalkofen, 1987; Epe, 2009; Taylor et al., 2016).

Hookworm biology

Hookworms have evolved to be very successful parasites. An example of this are the recent findings showing that infective larvae of *Nippostrongylus brasiliensis*, a hookworm of rodents, can evade the host´s immune system through the secretion of a DNase II enzyme, and degrading the DNA backbones of the neutrophil extracellular traps (Bouchery et al., 2020). However, the main reason likely is the multiple routes by which these parasites can infect their hosts. *A. caninum* has a direct life cycle (Figure 2). The first stage larvae hatch after 24-48 hours after the eggs are passed in the feces, and after 3-4 days, moult to the second stage larvae. By 5-7 days, the larvae moult to the third stage but retains the previous cuticle (Bowman, 2014). Third stage larvae can infect new-born puppies by the transmammary route (Stone and Girardeau, 1968), percutaneously (Looss, 1905), orally (Looss, 1897; Foster and Cross, 1934), or via ingestion of paratenic or transport hosts, such as rodents (Matsusaki, 1951) and insects, such as cockroaches (Harada, 1954; Little, 1961). Transmammary infection results from reactivation of arrested tissue-stage larvae from peripheral organs and muscle in pregnant bitches, which then travel to the mammary glands, where are passed in the colostrum and milk to new-born puppies peaking at 6 days, for up to 20 days, and for up to three subsequent litters (Enigk and Stoye, 1967). In puppies infected via skin penetration there is a blood-lung migration pathway, where larvae enter the bloodstream, travel to the lungs, penetrate lung capillaries to invade the alveoli, migrate up the bronchial tree and trachea, reach the pharynx to be

coughed up and finally reach the small intestine, the parasite's predilection site (Anderson, 2000; Bowman, 2014). The pre-patent period of *A. caninum* in naïve puppies, young and adult dogs is 15-18, 15-20, and 15-26 days with an average of 15.3, 17.2 and 18.4 days, respectively (Herrick, 1928).

This pathway and developmental cycle through the tissues are substantially modified in dogs older than three months, especially in females. Rather than the lungs, most of the larvae penetrate peripheral organs (Sarles, 1929b; Miller, 1965) such as muscle (unpublished observations, M.D. Little), lung, liver, stomach (Scott, 1928), and gut wall (Schad, 1979), entering into an arrested state and capable of surviving for several years (Schad and Page, 1982). Consequently, in these cases, few worms establish directly in the lumen of the small intestine (Herrick, 1928; Michel, 1974). An interesting biological feature, commonly referred to as 'larval leak', and which is not associated with pregnancy has been described in this parasite. In these cases, the arrested somatic larvae continuously leak out and migrate to the intestine where they mature to adults. These dogs chronically shed hookworm eggs, usually in low numbers, and treatment provides only a temporary respite of egg shedding, as new 'leaking' larvae repopulate the gut and begin a new round of egg shedding within a few weeks of treatment (Schad and Page, 1982). The actual mechanism responsible for this phenomenon is thought to be an immunological deficit, however, a specific cause has not been elucidated (Loukas and Prociv, 2001).

Females of *A. caninum* are very fecund, producing on average, up to 10,000 eggs per day (Herrick, 1928). Previous studies have shown that female *A. caninum* dwelling in the small intestine at an intensity of 200 worms can produce twice as many eggs per worm than those present in an infection of 300 worms, demonstrating a density-dependent

phenomenon (Krupp, 1961). Earlier research also showed an inverse relationship between egg production and the number and age of worms but did not find a correlation between intensity of infection and size of worms (Sarles, 1929a; McCoy, 1931). Another study investigating pyrantel resistance in *A. caninum* demonstrated a density dependent fecundity of the female worms, whereby female worms increased their individual egg output due to reductions in the number of worms in the lumen of the small intestine (Kopp et al., 2007). This phenomenon has also been reported in other species of hookworms that are phylogenetically closely related to *A. caninum*, such as *Necator americanus* (Sweet, 1925; Hill, 1926) and *A. duodenale* (Earle and Doering, 1932), as well as in mixed infections of these two parasites (Anderson and Schad, 1985).

Anthelmintic drugs

There are three major anthelmintic drug classes currently available for treatment of hookworms in canines in the USA. The benzimidazoles (e.g., febantel and fenbendazole), are a class of anthelmintics whose mechanism of action is to bind tubulin, the eukaryotic cytoskeletal subunit of the microtubule, thus preventing the self-association of subunits onto the growing microtubules by the cell. Tubulin is a dimeric protein comprised of α and β monomers. Microtubules exist in a dynamic equilibrium with tubulin with the ratio of dimeric tubulin to polymeric microtubules being controlled by a range of endogenous regulatory proteins and co-factors (Lacey, 1988, 1990; McKellar and Benchaoui, 1996). The first report of this mechanism was in *Ascaris suum* as the normal microtubule matrix of intestinal cells was disintegrated after treatment with mebendazole (Borgers et al., 1975).

The avermectins/milbemycins (e.g., ivermectin, moxidectin, and milbemycin oxime), are macrocyclic lactones that are used to control nematodes of human and

veterinary importance (Campbell and Benz, 1984; Shoop et al., 1995). The mode of action is to selectively paralyze the parasite by increasing muscle permeability to Cl^- ions in glutamate-gated chloride channels (GluCl) located in motor neurons (Cully et al., 1994). This mechanism paralyzes the worm, interfering with pharyngeal pumping, motility, and reproduction (Yates et al., 2003).

Tetrahydropyrimidines (e.g., pyrantel), are acetylcholine receptor agonists which bind selectively to synaptic and extrasynaptic receptors on nematode muscle cells and open Ca^{2+} ion channels. This forces contraction of the cell and induces a spastic paralysis in the worm (Martin, 1997). Intracellular recordings made with micropipettes from *A. suum* body muscles showed that pyrantel produces depolarization, increased spike activity and muscle contraction (Aubry et al., 1970).

In registration studies, febantel, moxidectin and milbemycin oxime all demonstrated efficacy of >99% (F.D.A, 1994, 2006, 2012), fenbendazole demonstrated efficacy of >98% (F.D.A, 1983) and pyrantel demonstrated a somewhat variable efficacy, with a mean across studies of approximately 94%, where more than half of those studies yielded >99% (F.D.A, 1993)

Anthelmintic resistance

Strongylid nematodes have a number of genetic features, which favour the development of anthelmintic resistance, such as rapid rates of nucleotide sequence evolution and exceedingly large effective population sizes, leading to remarkably high levels of genetic diversity (Nadler, 1987; Blouin et al., 1995; Gilleard and Redman, 2016). Using mtDNA restriction site data in *Ostertagia ostertagi*, results showed that 98% of the

total genetic diversity in this species is partitioned within populations, showing very high gene flow among these populations and more importantly, the average number of nucleotide substitutions per site among individuals from the same population was five to 10 times higher than typical estimates reported for species in other taxa (Blouin et al., 1992). Furthermore, in a phylogenetic reconstruction of the metazoans using nuclear 18S rRNA sequences, the longest branches were nematodes (*Haemonchus, Strongyloides,* and *Caenorhabditis)* (Philippe et al., 1994). This suggests that nematode DNA does indeed evolve faster than DNA from other taxa. In a study looking into the sequence diversity in the nicotinamide adenine dinucleotide dehydrogenase subunit 4 (nad4) gene in five species of trichostrongyloid nematodes from four or five different locations in the United States (i.e., *Haemonchus contortus, Teladorsagia circumcincta, O. ostertagi, H. placei,* and *Mazamostrongylus odocoilei*), the within-population diversity was 0.026 substitutions per site of the nad4 sequence, which was much higher than that observed for other taxa (Lynch and Crease, 1990; Blouin et al., 1995).

Anthelmintic resistance is a heritable trait (Prichard et al., 1980), and is defined as occurring 'when a greater frequency of individuals in a parasite population, usually affected by a dose or concentration of compound, are no longer affected, or a greater concentration of drug is required to reach a certain level of efficacy' (Wolstenholme et al., 2004). The combination of a high density of animals in one place, and high transmission rates produces large effective populations of worms which increases the probability that mutations conferring resistance will occur (Gilleard, 2006; Redman et al., 2015). Combined with increased selection pressure with anthelmintics treatments, and the lack of refugia (i.e., the proportion of a worm population that is not exposed to drugs and which

goes on to reproduce to parent the next generation of parasites, and help to maintain susceptible genes in the population) will lead to a rapid increase in their frequency (Martin et al., 1981; van Wyk, 2001). This combination of factors is known to place heavy selection pressure for drug resistance in nematodes (Wolstenholme et al., 2004). The first reports of anthelmintic resistance began to appear very soon after the introduction of the first broad spectrum anthelmintic, thiabendazole (Brown, 1961), in *H. contortus* (Conway, 1964; Drudge et al., 1964; Smeal et al., 1968), with high levels of multiple-drug resistance (MDR) in nematodes of sheep and goats, worldwide (Kaplan and Vidyashankar, 2012). Recent studies looking into the cost of anthelmintic resistance in gastrointestinal nematodes in dairy cattle, dairy sheep, dairy goats, beef cattle, and meat sheep, estimated that this was around € 38 million annually (Charlier et al., 2020).

The widespread development of resistance in nematode parasites of livestock was presumed for many years to be caused by anthelmintic selection acting on mutations that occurred before the start of drug use or in the standing variation (Roos, 1990; Silvestre and Humbert, 2002), so a beneficial mutation arised once and increased in frequency called a "hard" selective sweep due to selection pressure with a subsequent loss of marker polymorphism, known as the "hitchhiking" effect (Maynard Smith and Haigh, 1974). It is important to note that a hard selective sweep can also result from the rapid emergence of a mutation in a single genetic background after the selection was introduced, indicating a *de novo* mutation (Hermisson and Pennings, 2005). However, this hypothesis has been challenged as mutations already present in the standing variation or *de novo* mutations that take place after the onset of selection, are important as well (Silvestre et al., 2009; Skuce et al., 2010; Redman et al., 2015), based in that for a rapid adaptive change in eukaryotes

to occur, an availability of new mutations had to be present as multiple adaptive alleles sweeping through a population leading to a more complex genetic signature of selection (Messer and Petrov, 2013). The frequency in which anthelmintic resistance arises and the spread are important considerations for the prevention and management (Silvestre and Humbert, 2002; Skuce et al., 2010; Redman et al., 2015). Evidence of such "soft" selective sweeps has already been reported in trichostrongylid species of ruminants in European (Redman et al., 2015), North and South American countries (Brasil et al., 2012; Barrere et al., 2012; Barrere et al., 2013), as well as Asian countries (Chaudhry et al., 2015).

The origin of a resistant allele can be inferred by using haplotype networks. Mutations of recent origin will be found at the tips of branches on the haplotype network, whereas longer-standing resistant genotypes will have accumulated further mutations, forming more extensive subtrees. Geographical information can be included as well as resistance spread through migration will be found in the same haplotypic backgrounds as in the source population, whereas resistance emerging in situ will be found in haplotypic backgrounds common in the local sensitive population (Karasov et al., 2010). It is currently not known how anthelmintic resistance would arise in *A. caninum* and spread through canine populations.

Anthelmintic resistance in companion animals

The issue of whether resistance is likely to become a problem in parasites of companion animals has received relatively little attention, and when addressed, it has been viewed as an issue relating to the increased use of prophylactic helminth treatments in pets (Thompson, 2001). Reports of anthelmintic resistance in parasites of small animals different from hookworms are very scarce. This include *Toxocara canis* (Dryden and

Ridley, 1999), *Dipylidium caninum* (Jesudoss Chelladurai et al., 2018), and *Dirofilaria immitis* (Pulaski et al., 2014; Wolstenholme et al., 2015). This is attributable to the lack of surveillance (i.e., measurement of treatment efficacy), indirect life cycles or the presence of paratenic hosts which increase the amount of refugia, but mainly this is due to the different epidemiological factors that are present in the livestock situation but are not present in household pets. Such as large numbers of animals in one place, high transmission rates, and increased frequency of anthelmintic treatment.

However, the epidemiology of nematode transmission on greyhound farms much more closely resembles the epidemiological conditions present on livestock farms, than to the epidemiological conditions present in a pet home environment. Consequently, it would not be surprising if anthelmintic resistance also were to become a common problem on greyhound farms. Anthelmintic resistance in *A. caninum* was first reported in 1987 against pyrantel in a greyhound puppy that was imported to New Zealand from Australia (Jackson et al., 1987). Subsequently, several additional cases were diagnosed in Australia (Hopkins et al., 1988; Hopkins and Gyr, 1991; Kopp et al., 2007; Kopp et al., 2008a, b). However, there were no further cases of anthelmintic resistance reported in *A. caninum* between 2008 to 2019, when a report provided evidence of a case of resistance to benzimidazoles and macrocyclic lactones in an isolate of *A. caninum* obtained from a greyhound dog originating from Florida, USA in 2016 (Kitchen et al., 2019).

Current management of hookworm infections in dogs

Typically, when a dog presents to a veterinarian with a fecal examination (Zajac et al., 2002) or coproantigen-detection ELISA test (Elsemore et al., 2017) positive for hookworms, the dog is treated with one or more drugs from the benzimidazole, macrocyclic

lactone or tetrahydropyrimidine classes. If the dog then tests positive again in a future exam, the infection is attributed to reinfection or reactivation of encysted/arrested larvae (larval leak). Consequently, the same treatment regimen is often repeated, or the veterinarian may choose to use a drug from a different drug class. As a result, anthelmintic resistance is not diagnosed, and most often is not even considered as a likely cause of the recurrent hookworm infections. Therefore, as resistance evolves and leads to more recurrent hookworm infections, veterinarians typically treat more often, and rotate and/or combine drugs. But they do not perform fecal egg count reduction test (FECRT) to measure the efficacy of the various drugs administered. Thus, as long as one drug remains efficacious, the problem will appear to be managed, and recurrent infections will continue to be attributed to reinfection or reactivation of encysted/arrested larvae. However, once MDR to all drugs evolves, it is no longer possible to manage the infections, and the problem of anthelmintic resistance becomes more obvious.

In vitro assays to diagnose anthelmintic resistance

The first *in vitro* assay reported for this purpose was the egg hatch assay (EHA). This is a bioassay used for detecting resistance to benzimidazole anthelmintics (Le Jambre, 1976). Based on the ovicidal properties of the benzimidazole drug class (Hunt and Taylor, 1989), the aim is to incubate undeveloped eggs in serial concentrations of the anthelmintic, usually thiabendazole as this compound is the most soluble within the class. The percentage of eggs that hatch (or conversely die) at each concentration is determined, corrected for natural mortality from control wells, and a dose-response line plotted against drug concentration (Taylor et al., 2002). This assay has been used successfully to detect resistance against benzimidazoles in multiple nematode parasites of livestock (Varady et

al., 1996; von Samson-Himmelstjerna et al., 2009; Rialch et al., 2013). Additionally, the EHA was assessed in *A. caninum* (Diawara et al., 2013), and used to evaluate drug susceptibility/resistance to benzimidazoles in the human hookworm, *N. americanus* (Kotze et al., 2005; Albonico et al., 2005; Diawara et al., 2013).

The larval development assay (LDA) (Coles et al., 1988) is a commonly used *in vitro* bioassay used for detecting resistance to multiple different classes of anthelmintics in gastrointestinal (GI) nematode parasites of sheep and goats (Kaplan et al., 2007; Howell et al., 2008; Raza et al., 2016) and cattle (Kotze et al., 2014). The LDA is based on the ability of anthelmintics to prevent free-living pre-parasitic nematode stages from developing to the infective third larval stage (L3) (Coles et al., 1988; Gill et al., 1995).

The adult development assay for culturing L3s to the adult stages has been reported for *H. contortus* (Stringfellow, 1986), and has been used to diagnose resistance to benzimidazoles, macrocyclic lactones, and closantel. However, it was only successful at discriminating between susceptible and resistant thiabendazole isolates (Stringfellow, 1988; Small and Coles, 1993).

The larval paralysis/motility test (LMA) was first reported in nematodes in work performed in L3s of *Teladorsagia circumcincta* after incubation for 24 hrs in serial dilutions of either levamisole or morantel. The percentage of paralysed larvae was then visually determined at each concentration and a dose-response line plotted (Martin and Le Jambre, 1979). A modification of this assay using eserine as an acetylcholinesterase inhibitor, was shown to be successful at discriminating thiabendazole susceptible and resistant isolates of *H. contortus*, *Trichostrongylus colubriformis,* and *T. circumcincta* (Sutherland and Lee, 1990). The first non-subjective motility assay was first reported when motility was

measured by an automated micromotility meter was used to measure the motor function of larval (e.g., *Nippostrongylus brasiliensis* and *Ascaris suum)* and adult nematodes (e.g., *Caenorhabditis elegans* and *Brugia pahangi*) after incubation with anthelmintics. Originally this used a microprocessor technology to measure light refraction at the meniscal interface. So, as movement of larvae in solution altered the angle of light refraction entering the photodiode, the light deviation is measured and information passed to a computer to give a motility index (Bennett and Pax, 1986). This test was successful in discriminating between benzimidazole susceptible and resistant isolates of *H. contortus*, but was not able to diagnose resistance to levamisole (Coles et al., 1989). A subjective motility assay based on the assessment of the L3 movement by the researcher was successful in discriminating ivermectin susceptible and resistant isolates of *H. contortus.* The larvae were incubated in serial concentrations of ivermectin on agar in the dark at 25C, and stimulated to move by exposure to light (Gill et al., 1991). A similar technique has been reported to evaluate anthelmintics effects on *Necator americanus, Ancylostoma ceylanicum, Strongyloides ratti*, and *S. stercolaris* (Kotze et al., 2004). However, when this technique was used to discriminate between susceptible and pyrantel resistant isolates of *A. caninum*, a biphasic response was noted (i.e., tachyphylaxy), with recovery reported at higher concentrations (Kopp et al., 2008a). Interestingly, using newer platform technologies for image analysis to measure motility (Storey et al., 2014), no consistent significant differences were found in the dose-response between resistant and susceptible *Cooperia* spp. and *H. contortus* third-stage larvae (George et al., 2018). Similar results were reported when performed in L4 stages of *Cooperia* spp. (Paras and Kaplan, 2020). In terms of other nematode parasites of dogs, this technology has been applied to microfilariae

(Maclean et al., 2017), and L3s and L4s (Jimenez Castro, unpublished results) of *Dirofilaria immitis*, but reported no significant differences between susceptible and resistant isolates as well.

The migration inhibition assay (MIA) was first reported based on the activity of benzimidazoles in *Oesophagostomum dentatum* (Petersen et al., 1997) and was able to discriminate between susceptible and pyrantel-resistant isolates (Petersen et al., 2000) using the adult stages of this parasite. This method is based on the ability of parasites to migrate through a mesh following incubation with different concentrations of the anthelmintic. However, modifications to use the larval stages of trichostrongylids of ruminants (LMIA) (Kotze et al., 2006) and migration through agar and not mesh have been reported successful at diagnosing resistance to the macrocyclic lactones in *Haemonchus contortus*, but not in *T. colubriformis* nor *T. circumcincta* (Kotze et al., 2006). These have been evaluated for cattle parasites, including *Cooperia* spp. and *O. ostertagi* but have not consistently demonstrated differences between resistant and susceptible populations (Demeler et al., 2012; Areskog et al., 2014). Additionally, this test has been applied to parasites of dogs such as *D. immitis* (Evans et al., 2013), and *A. caninum* (Kopp et al., 2008a), but with similar results for either parasite in terms of not being able to discriminate between resistant and susceptible isolates (Kopp et al., 2008a; Evans et al., 2017).

The larval arrested morphology assay (LAMA) (Kopp et al., 2008a) was developed to detect resistance to pyrantel in *A. caninum*. This was based on the shape that the L3s assume in the quiescent state at different concentrations of pyrantel following incubation of 48 hrs at 25°C. Thus, the effect of pyrantel is quantified by comparing the percentage of 'normally quiescent' or larvae that did not have any kinking or only mild deviations in that

well to the average of the control well percentages for that plate. This assay yielded a resistance ratio of 17.2 (Kopp et al., 2008a).

The larval feeding inhibition assay (LFIA) (Kopp et al., 2008a) was developed from a larval feeding assay (Hawdon and Schad, 1990) for *A. caninum* to detect resistance to pyrantel. Briefly, following activation of the plates by addition of a feeding activator solution, L3s are added to each well along with different concentrations of pyrantel and incubated for at least 32 hrs at 37°C. Fluorescein isothiocyanate-conjugated bovine serum albumin is added to the wells and left to incubate for an additional 3 hrs. Larvae are then transferred to new plates and counted on a fluorescence inverted microscope and only larvae with fluorescein visualized along at least 50% of their gastrointestinal tract were scored as feeding. Numbers of feeding larvae in drug-treated wells were expressed as a percentage of the mean for control wells. This assay was able to yield resistance ratios between 6 and 9.3 (Kopp et al., 2008a).

In terms of biochemical assays, the tubulin binding assay (Lacey and Snowdon, 1988) for the detection of benzimidazole resistant nematodes using the binding of tritiated benzimidazole carbamates to tubulin extracts of L3s was successful in detecting resistant isolates of *H. contortus, T. colubriformis*, and *T. circumcincta*. This test was based on the reduced affinity of tubulin for the benzimidazole, so a higher amount of drug and unbound tubulin will be present in resistant isolates. However. because it needs relatively high numbers of L3s, it would not be appropriate for routine field assays. Colorimetric assays based on tests developed to detect insecticide resistance in aphids, comparing the degree of color change due to non-specific esterases and acetylcholinesterases, were able to discriminate between benzimidazole resistant and susceptible isolates of *H. contortus*, *T.*

colubriformis, and *T. circumcincta*. Samples were compared either by visual examination or with the use of a densitometer. Significantly greater esterase or acetylcholinesterase activity was found in the benzimidazole resistant strains. (Sutherland et al., 1989)

In summary, the only *in vitro* tests that have been used in *A. caninum* to date are the EHA, LFIA, LMIA, LMA, and the LAMA.

Molecular assays for anthelmintic resistance

The only mechanism of resistance currently known for anthelmintics is for the benzimidazole drugs. These drugs block the polymerization of parasite microtubules, and they do this by binding to the nematode β-tubulin protein monomers (Cleveland and Sullivan, 1985; Lacey, 1988, 1990). Mutations in a β-tubulin gene that could give rise to benzimidazoles resistance were fueled by the findings in fungi of reduced fungicide binding (Davidse and Flach, 1977) and altered electrophoretic properties (Sheir-Neiss et al., 1978) of that specific protein in resistant mutants. This was followed by gene cloning in *Neurospora crassa* (Orbach et al., 1986*)* and sequencing in *Saccharomyces cerevisiae* (Thomas et al., 1985*)*, and *Aspergillus nidulans* (Jung and Oakley, 1990; Jung et al., 1992) of β-tubulin from resistant mutants, identifying the individual mutations responsible. This was then followed by mutagenesis work in the model nematode *C. elegans*, where all 28 independently isolated resistant mutations mapped to one single locus, *ben-1* (Driscoll et al., 1989). Subsequently, evidence for selection on the β-tubulin locus was first shown for *H. contortus* (Roos, 1990; Lubega et al., 1994) and *T. colubriformis* (Grant and Mascord, 1996). This was followed by sequence analysis that found that the only consistently different polymorphism between susceptible and resistant populations of *H. contortus* was a tyrosine substitution at the codon 200 (Kwa et al., 1993a; Kwa et al., 1993b). The next

substitutions that were found to be associated with the resistance phenotype were at codon 167 in *H. contortus* (Prichard, 2001), *T. circumcincta* (Silvestre and Cabaret, 2002) and in cyathostomins (Drogemuller et al., 2004), and subsequently at codon 198 in *H. contortus* (Ghisi et al., 2007). Briefly, single nucleotide polymorphisms (SNPs) in the isotype-1 of the β- tubulin gene family located at codons F167Y (TTC/Phe → TAC/Tyr), E198A (GAG/Glu → GCG/Ala) and F200Y (TTC/Phe → TAC/Tyr) have been found to be associated with benzimidazole resistance. Several methods have been developed to detect and measure these mutations, such as allele-specific PCR (Silvestre and Cabaret, 2002), PCR-RFLP (Ghisi et al., 2007), real-time PCR (Alvarez-Sanchez et al., 2005), pyrosequencing (Demeler et al., 2013; Chaudhry et al., 2014; Knapp-Lawitzke et al., 2015; Ramunke et al., 2016), and PCR microsatellite amplification (Redman et al., 2015), but these all have limitations that affect their usefulness. However, a recently developed deep-amplicon sequencing assay for measuring benzimidazole-associated resistance mutations in nematode communities of cattle, sheep, bison, and horses provides a powerful new tool that enables unparalleled sensitivity of detection and allows screening for the emergence of resistance mutations (Avramenko et al., 2019)

Additionally, high throughput sequencing techniques enabled the detection of additional mutations at codon 198, such as E198V, E198L, E198K, E198I, and E198Stop (Redman et al., 2015; Avramenko et al., 2019; Avramenko et al., 2020; Mohammedsalih et al., 2020) and at codons 167 and 200 with a change from phenylalanine to leucine (Avramenko et al., 2020). Structural modelling studies suggest that the SNPs at codons 167 and 200 reduce the binding affinity of benzimidazoles by increasing the polarity and hydrophilicity in the binding pocket, while the polymorphisms at codon 198 are predicted

to disrupt the formation of a key Hydrogen-bond interaction and reduces the length of the aliphatic chain, which causes the formation of an oversized hydrophobic cavity in the binding site (Aguayo-Ortiz et al., 2013). SNPs at these codons have been reported in multiple species of Strongylid nematode parasites of livestock such as *H. contortus* (Kwa et al., 1994; Prichard, 2001), *T. circumcincta* (Elard et al., 1996), *Nematodirus battus* (Melville et al., 2020), *Trichostrongylus axei* (Avramenko et al., 2019), *T. colubriformis* (Grant and Mascord, 1996), *Cooperia oncophora* (Njue and Prichard, 2003), *C. punctata, Ostertagia ostertagi, H. placei* (Avramenko et al., 2019), *C. pectinata, Orloffia bisonis* (Avramenko et al., 2020), and cyathostomins (von Samson-Himmelstjerna et al., 2001; Drogemuller et al., 2004; Blackhall et al., 2011). Stop codons, a mutation predicted to result in a null allele, thus removing the major drug target, have been reported at codons 144, 153, 157, 158, 166, 172, 181, 183, 194, 208 in all the trichostrongylid species, and in *C. oncophora* at codon 198, with the highest frequency being at position 183 (Avramenko et al., 2020). These SNPs have also been reported in other parasite orders such as Trichocephalida with *Trichuris trichiura* (Diawara et al., 2009; Diawara et al., 2013) and Spirurida with *Wucheria bancrofti* (Schwab et al., 2005), both for 200 codon (TTC/Phe → TAC/Tyr).

Specifically for hookworms, there have been reports of such SNPs in the 200 codon (TTC/Phe → TAC/Tyr) (Diawara et al., 2013; Zuccherato et al., 2018) and the 198 codon (GAG/Glu → GCG/Ala) (Rashwan et al., 2016), both for *Necator americanus*. It is noteworthy that others have looked for benzimidazole-resistance associated SNPs in *A. caninum* without success (Furtado and Rabelo, 2015). However, studies performed in Brazil did report finding a SNP at codon 198 in *A. braziliense* (Furtado et al., 2018) and at

codon 200 in *A. caninum* (Furtado et al., 2014) at very low frequencies, 1.2 and 0.8%, respectively using PCR-RFLP. However, these findings were not confirmed by sequencing. Finally, for the 167 codon (TTC/Phe → TAC/Tyr) a recent study using CRISPR/Cas 9, the authors were successful in replicating this SNP in the homologous *ben-1* gene of *C. elegans* in *A. caninum* (Kitchen et al., 2019).

Interestingly, SNPs at these codons have also been reported in other taxonomical kingdoms, such as Fungi (Sheir-Neiss et al., 1978; Orbach et al., 1986; Foster et al., 1987; Fujimura et al., 1992; Jung et al., 1992; Zou et al., 2006; Banno et al., 2008; Msiska and Morton, 2009; Aguayo-Ortiz et al., 2013; Liu et al., 2014; Zhang et al., 2016) and Protozoa (Edlind et al., 1994; Franzen and Salzberger, 2008).

However, there has been increasing evidence of non-β-tubulin loci that are involved in resistance to benzimidazole drugs. Evidence of this has already been reported in *Caenorhabditis* spp., where a quantitative trait loci that did not overlap with β-tubulin genes was identified in two genetically divergent isolates (Zamanian et al., 2018). Also, using genome wide association mappings in *C. elegans*, novel genomic regions independent of *ben-1* and other β-tubulin loci were correlated with resistance to albendazole (Hahnel et al., 2018). Additionally, disparity in responses in *C. elegans* to fenbendazole and albendazole showed evidence that the former could have additional targets beyond β-tubulin, such as genes that encode β-tubulin interacting proteins (Dilks et al., 2020).

Public health concerns

Beyond the concerns for canine health, multiple-drug resistance in canine hookworms could present serious public health concerns since *A. caninum* is zoonotic.

Humans infected percutaneously may develop cutaneous larva migrans (CLM) (Leeming and Oxon, 1966). Cases of eosinophilic enteritis (Prociv and Croese, 1996; Landmann and Prociv, 2003), folliculitis (Miller et al., 1991; Opie et al., 2003), localized myositis (Little et al., 1983), erythema multiforme (Vaughan and English, 1998), ophthalmological manifestations (Garcia et al., 2008), as well as patent infections have also been described (Ngcamphalala et al., 2019; Furtado et al., 2020).

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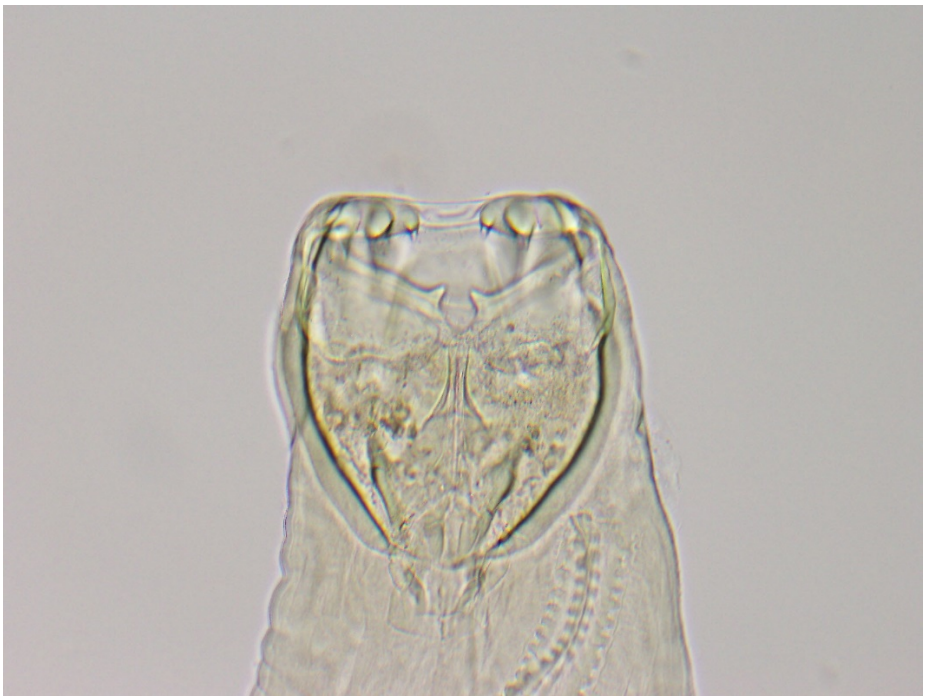

Fig. 1: Cephalic or anterior extremity of a female adult *Ancylostoma caninum*, showing the large buccal capsule and characteristic three pairs of teeth.

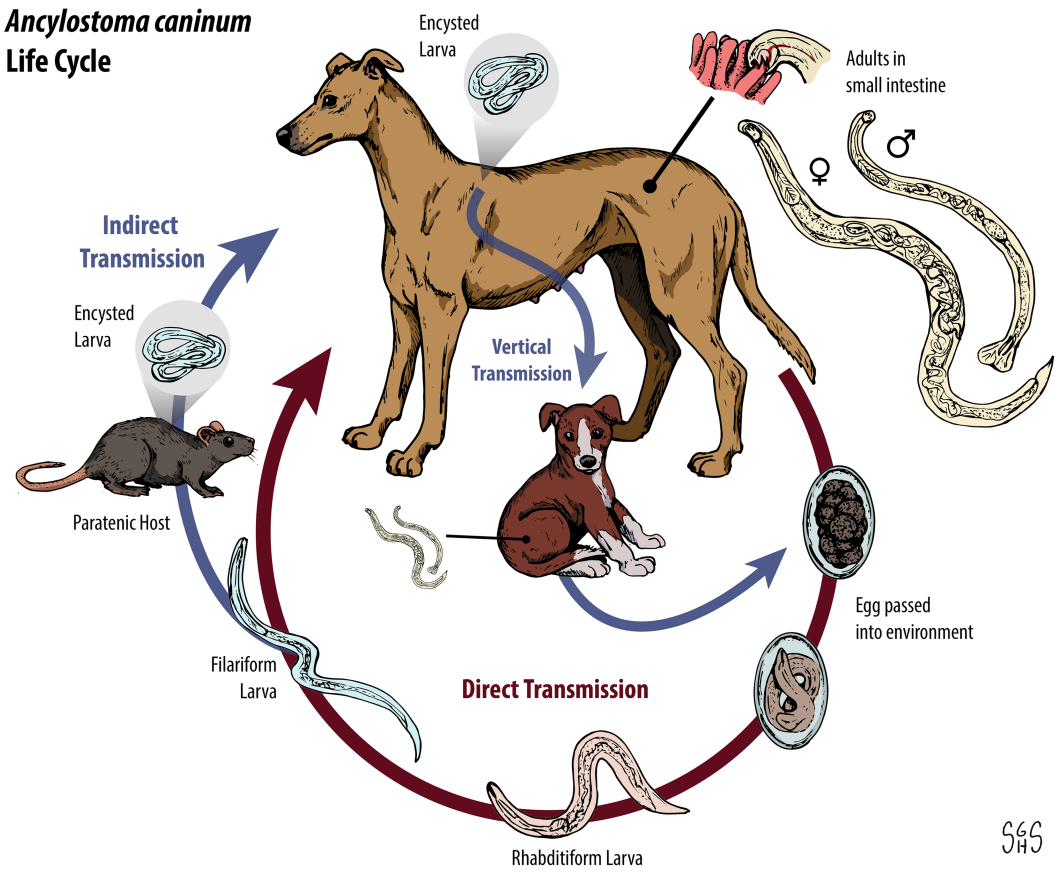

Fig. 2: Life cycle of *Ancylostoma caninum.* Artwork by Dr Sarah Sapp. U.S. Centers for Disease Control and Prevention (CDC).

CHAPTER 2

MULTIPLE DRUG RESISTANCE IN THE CANINE HOOKWORM, *ANCYLOSTOMA CANINUM*: AN EMERGING THREAT? [1]

[1] Jimenez Castro, P.D., Howell, S.B., Schaefer, J.J., Avramenko, R.W., Gilleard, J.S., Kaplan, R.M., 2019. Multiple drug resistance in the canine hookworm *Ancylostoma caninum*: an emerging threat? *Parasites & Vectors* 12, 576. doi: 10.1186/s13071-019-3828-6. Reprinted here with permission of publisher.

ABSTRACT

Background: The canine hookworm, *Ancylostoma caninum* is the most prevalent and important intestinal nematode parasite of dogs in the United States. Hookworms are typically well controlled by treatment with all commonly used anthelmintics that are approved for this use in dogs. However, in the past few years, cases of recurrent/persistent canine hookworm infections appear to have dramatically increased, suggesting that anthelmintic resistance (AR) may have evolved in this parasite. These cases are highly overrepresented by greyhounds, but multiple other breeds are also represented. The aim of this study was to characterize several of these suspected resistant isolates using *in vitro*, genetic, and clinical testing to determine if these cases represent true anthelmintic resistance in *A. caninum*.

Methods: Fecal samples containing hookworm eggs from three cases of persistent hookworm infections; one from a greyhound, one from a miniature schnauzer, and one from a hound-mix, were received by our laboratory. These were then used to establish infections in laboratory dogs, and to perform egg hatch assays (EHA) and larval development assays (LDA) for detecting resistance to benzimidazoles and macrocyclic lactones, respectively. Additional EHA and LDA were performed on eggs recovered from the laboratory-induced infections. Fecal egg count reduction tests were performed to detect resistance to pyrantel. Deep amplicon sequencing assays were developed to measure the frequency of non-synonymous single nucleotide polymorphisms (SNP) at codons 167, 198 and 200 of the *A. caninum* isotype-1 β-tubulin gene**.**

Results: Resistance ratios for the three *A. caninum* isolates tested ranged from 6.0 to >100 and 5.5 to 69.8 for the EHA and LDA, respectively. Following treatment with pyrantel, reduction in faecal egg counts were negative or 0%. Deep amplicon sequencing of the isotype-1 β-tubulin gene identified a high frequency of resistance-associated SNPs at codon 167 in all three resistant isolates and in two additional clinical cases.

Conclusions: These data conclusively demonstrate multiple anthelmintic resistance in multiple independent isolates of *A. caninum*, strongly suggesting that this is an emerging problem in the United States. Furthermore, evidence suggest that these resistant hookworms originate from racing greyhound farms and kennels, though additional research is needed to confirm this.

INTRODUCTION

The canine hookworm, *Ancylostoma caninum* is the most prevalent and important intestinal nematode parasite of dogs in the United States (Little et al., 2009). A recent study evaluating over 39 million fecal samples from 2012-2018, showed evidence of a steady yearly increase in prevalence from 2015 onwards, with an overall increase of 47% (Drake and Carey, 2019). Anthelmintic drugs approved for the treatment of *A. caninum* in the United States include, febantel, moxidectin, milbemycin oxime, fenbendazole and pyrantel. In registration studies, febantel, moxidectin and milbemycin oxime all demonstrated efficacy of >99% (F.D.A, 1994, 2006, 2012), fenbendazole demonstrated efficacy of >98% (F.D.A, 1983) and pyrantel demonstrated a somewhat variable efficacy, with a mean across studies of approximately 94%, where more than half of those studies yielded >99% (F.D.A, 1993)**.** Pathological consequences of hookworm infection include iron-deficiency anemia, hypoalbuminemia, and an enteritis, characterized by diarrhea that

may contain fresh or digested blood (melena) (Kalkofen, 1987; Epe, 2009; Taylor et al., 2016).

Hookworms are very successful parasites, and one of the main reason is the multiple routes by which they can infect their hosts. *Ancylostoma caninum* is transmitted by the transmammary route to new-born puppies (Stone and Girardeau, 1968), percutaneously (Granzer and Haas, 1991), orally (Epe, 2009), or via ingestion of paratenic hosts, such as rodents (Matsusaki, 1951) and insects (Little, 1961). Transmammary infection results from reactivation of arrested tissue-stage larvae in pregnant bitches, which then travel to the mammary glands, where they are passed in the colostrum and milk to new-born puppies for up to 18 days (Enigk and Stoye, 1967).

In puppies infected via skin penetration there is a blood-lung migration pathway (Anderson, 2000; Bowman, 2014). However, in older dogs , this pathway and developmental cycle is substantially modified; rather than the lungs, most of the larvae penetrate peripheral organs (somatic tissues) such as muscle (unpublished observations, M.D. Little) or gut wall (Schad, 1979), where they enter into an arrested state and are capable of surviving for several years (Schad and Page, 1982).

An interesting biological feature of *A. caninum* infection is the phenomenon known as “larval leak”, where arrested somatic larvae continuously migrate to the small intestine where they develop to the adult stage (Schad and Page, 1982; Epe, 2009). These cases are not associated with pregnancy and dogs with “larval leak” will chronically shed hookworm eggs, often in low numbers, with treatment only providing a temporary break of egg shedding, due to new reactivated hypobiotic larvae repopulating the gut and beginning a new round of egg shedding within a few weeks of treatment (Bowman, 2014). The actual

mechanism responsible for this phenomenon is thought to be an immunological deficit, however, a specific cause has not been elucidated (Loukas and Prociv, 2001).

Because this larval reactivation is a well-described phenomenon, dogs presenting with recurrent hookworm infections are presumed to be suffering from this problem. Even though, no data is available to document the historical number of cases of recurrent hookworm infection in dogs, parasitologists at several veterinary colleges in the US who we have communicated with, including our laboratory, have received increasing numbers of communications in the last 2-3 years. These cases are heavily overrepresented by greyhounds but include many other breeds as well. The emergence of anthelmintic resistance in *A. caninum* would give a plausible explanation for these recent observations.

Parasitic strongylid nematodes have a number of genetic features, which favour the development of anthelmintic resistance, such as rapid rates of nucleotide sequence evolution and exceedingly large effective population sizes, leading to remarkably high levels of genetic diversity (Blouin et al., 1995; Gilleard and Redman, 2016). Anthelmintic resistance is a heritable trait (Prichard et al., 1980), and is defined as occurring 'when a greater frequency of individuals in a parasite population, usually affected by a dose or concentration of compound, are no longer affected, or a greater concentration of drug is required to reach a certain level of efficacy' (Wolstenholme et al., 2004). *A. caninum* is the most common nematode parasite of greyhounds on breeding farms (Ridley et al., 1994); this high prevalence is likely a consequence of the unrestricted access to exercise runs made out of sand and dirt, which produces an ideal environment for the development and survival of the infective larvae (Bowman, 2014). To address the problem of nematode infections, the dogs on these breeding farms are subject to a very intense deworming protocol; puppies

are often treated weekly with an anthelmintic until three months of age, then tri-weekly until sixth months of age, and then monthly for the rest of their breeding or racing lives (Ridley et al., 1994). This would present a very high drug selection pressure on the hookworm population on these farms and racing kennels.

In livestock, the intensive use and near complete reliance on anthelmintic drugs for control of nematode infections has led to high levels of anthelmintic resistance and multi-drug resistant (MDR) populations of nematodes on a global scale (Kaplan, 2004). In contrast, anthelmintic resistance in *A. caninum* has developed much more slowly, with few cases reported, and until this year, only to pyrantel. The first report of pyrantel resistance was from New Zealand in a greyhound puppy that was imported from Australia (Jackson et al., 1987), with several more cases subsequently diagnosed in Australia (Hopkins et al., 1988; Hopkins and Gyr, 1991; Kopp et al., 2007; Kopp et al., 2008a, b). The issue of whether resistance is likely to become a problem in parasites of dogs has received relatively little attention, and when addressed, it has been viewed as an issue relating to the increased use of prophylactic helminth treatments in pets (Thompson, 2001). However, the epidemiology of nematode transmission on greyhound farms much more closely resembles the epidemiological conditions present on livestock farms, than to the epidemiological conditions present in a pet home environment. Consequently, it would not be surprising if anthelmintic resistance also were to become a common problem on greyhound farms. Interestingly, coincident with our investigations, a recent publication reported resistance to benzimidazoles and macrocyclic lactones in an isolate of *A. caninum* obtained from a greyhound dog (Kitchen et al., 2019). The dog in that case presented to a veterinary clinic

with a hookworm infection that was subsequently refractory to multiple treatments with fenbendazole.

Beyond the concerns for canine health, multiple-drug resistance in canine hookworms could present serious public health concerns, since *A. caninum* is zoonotic. Humans infected percutaneously may develop cutaneous larva migrans (CLM) (Leeming and Oxon, 1966). Cases of eosinophilic enteritis (Prociv and Croese, 1996), as well as patent infections have also been described (Ngcamphalala et al., 2019; Furtado et al., 2020).

Given the increasing frequency of reports by veterinarians that our laboratory has been receiving of recurrent hookworm infections that are poorly responsive to anthelmintics, it seemed likely that anthelmintic resistance had evolved in *A. caninum*. The aim of this study was to characterize several of these suspected resistant isolates using *in vitro*, genetic, and clinical testing

MATERIAL AND METHODS

Parasite isolates

Three fecal samples containing hookworm eggs were received from veterinarians who were treating cases of recurrent hookworm infections in canine patients. These three "suspected-resistant" isolates of *A. caninum* were designated Worthy, Lacy and Tara. Two additional fecal samples from *A. caninum* isolates isolated from dogs with no history of anthelmintics treatments also received. One designated ETCR, was previously cycled in the laboratory and confirmed as susceptible, and a second was acquired from a local dog shelter, which was confirmed as susceptible during the study. For the experimental

infections, eggs recovered from fecal samples were placed onto NGM plates (Sulston and Hodgkin, 1988) and cultured for seven days to obtain third-stage infective larvae, which were used subsequently to orally infect purpose-bred research dogs (University of Georgia AUP # A2017 10-016-Y1-A0).

In order to distinguish different passages and treatment events of the hookworm isolates, we established a naming convention as follows: name of the isolate followed by a number that corresponds to the number of passages the isolate has undergone. The letters F, P and M after the dot correspond to any treatments applied with either fenbendazole, pyrantel or milbemycin oxime, respectively. The number preceding the letter indicates the passage in which this treatment took place. For example, Worthy 4.1F2P3M would correspond to the fourth passage of the Worthy isolate and treatment with fenbendazole in the first passage, treatment with pyrantel in the second passage and treatment with milbemycin oxime in the third passage. Available diagnostic and treatment histories of the dogs from which we obtained the hookworm isolates are as follows:

Worthy: Three-year-old greyhound, adopted December 10, 2016 from Florida and currently residing in Tennessee. Prior to adoption, the dog was treated with pyrantel and administered heartworm prophylaxis (not specified).

January 11, 2017: New pet exam at University of Tennessee College of Veterinary Medicine Community Practice Clinic, fecal positive for hookworms. Administered fenbendazole (50 mg/kg) daily for 10 days and started monthly Heartgard® Plus (Merck, Kenilworth, NJ) (ivermectin/pyrantel).

January 31, 2017: Fecal positive for hookworms. Administered fenbendazole (50 mg/kg) daily for 10 days

February 21, 2017: Fecal negative

April 20, 2017: Fecal positive for hookworms, reporting many eggs seen. Administered fenbendazole (50 mg/kg) daily for 10 days.

July 26, 2017: Administered fenbendazole (50 mg/kg) daily for 10 days and switched from Heartgard® Plus (Merck, Kenilworth, NJ) (ivermectin/pyrantel) to monthly Advantage Multi® (Bayer, Leverkusen, Germany) (imidacloprid/moxidectin).

August 7, 2017: Administered fenbendazole (50 mg/kg) daily for 10 days

August 21, 2017: Fecal positive for hookworms. Administered Advantage Multi® (Bayer, Leverkusen, Germany) (imidacloprid/moxidectin).

September 21, 2017: Fecal positive for hookworms. Administered Advantage Multi® (Bayer, Leverkusen, Germany) (imidacloprid/moxidectin).

October 16, 2017: Fecal positive for hookworms. Sample sent to the University of Georgia. Fecal egg count (FEC) of 160 eggs per gram (EPG).

December 20, 2017: Research purpose-bred beagle was infected with 201 third-stage larvae.

Tara: Adult miniature schnauzer breeding bitch from St. Augustine, Florida

Spring 2017: Fecal examination was positive for hookworm eggs. Adult dogs started on Drontal® Plus (Bayer, Leverkusen, Germany) (praziquantel/pyrantel pamoate/febantel) once per month, with puppies receiving treatment at two, four, six and eight weeks of age, and then once per month afterwards. In addition, all dogs received Heartgard® Plus (Merck, Kenilworth, NJ) (ivermectin/pyrantel) monthly. Therefore, all dogs were being treated twice monthly with pyrantel and once monthly with febantel.

November 2017: Fecal examination positive for hookworms and sample sent to UGA. FEC of 100 EPG.

December 20, 2017: Research purpose-bred beagle was infected with 250 third-stage larvae.

Lacy: Adult hound mix from Griffin, Georgia.

Mid October-Mid November 2017: Treated twice, three weeks apart with a compounded combination of pyrantel, praziquantel and mebendazole.

December 11, 2017: Dog was treated with a compounded combination of praziquantel, pyrantel, and oxantel.

December 13 and December 15, 2017: Treated with pyrantel

December 16, 2017: Adult hookworm specimens were found whilst taking rectal temperature and hookworm eggs were present in feces. Treated with fenbendazole for three days (December 16-18, 2017).

December 18, 2017: Fecal sample submitted to UGA containing live adult worms and eggs present in the feces. No FEC was performed

January 25, 2018: Research purpose-bred beagle was infected with 250 third-stage larvae.

ETCR (Susceptible lab-isolate): From a naturally-infected adult dog residing in Cumberland County, Tennessee, in June 2016 with a history of no anthelmintic treatments ever being given. This isolate had subsequent passages in research purpose-bred beagles and a sample was received at UGA on October 17, 2017, with further propagation in a research purpose-bred beagle.

Barrow (Susceptible lab-isolate): A pooled fecal sample from an unknown number of naturally-infected adult shelter dogs residing in Barrow County, Georgia with no history

of anthelmintic treatments. Sample was received at UGA on March 13, 2018. Research purpose-bred beagle was infected with 250 third stage larvae on April 17, 2018.

***In vitro* assays**

Fresh feces from laboratory beagles infected with the Worthy, Tara, Lacy, ETCR and Barrow isolates were collected and made into a slurry with water, followed by filtration through 425 µm and 180 µm sieves, and then again through 85 µm and 30 µm nylon filters. The fecal material containing the eggs was then rinsed from the 30 µm filter with distilled water, and reduced to a volume of 10-15 mL. This was then layered on top of saturated sucrose and centrifuged at 1372 x *g* for seven mins at 4°C. Following centrifugation, eggs were recovered, rinsed with distilled water through a 20 µm sieve, transferred to a tube, and then the volume was adjusted to yield 50-60 eggs per 20 µL using distilled water.

Egg hatch assay (EHA): Fresh feces containing undeveloped eggs were used, as partial egg development may affect the dose response (Coles and Simpkin, 1977). Assays were performed using both agar and liquid-based methods with no significant difference detected between methods. Agar-based assays were performed using 96-well plates using a previously described agar-matrix technique (Diawara et al., 2013) with minor modification. Liquid-based assays were also performed using a 96-well plate format (Kotze et al., 2009) with minor modifications. A stock solution of 80 mM of thiabendazole (Sigma-Aldrich, St. Louis, MO) was prepared using 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO), and then was serially diluted using distilled water to produce 10 final concentrations ranging from 36 to 0.001125 µM in 1% DMSO. The first two wells of each row were negative controls containing only 0.5% DMSO for the agar plates and 1% DMSO for the liquid based plates, and wells 3-12 contained increasing concentrations of

thiabendazole. Agar-based assay plates were prepared by adding 70 µl of 2% Agar (Bacto Agar, VWR, Becton Dickinson Sparks, MD) and 70 µL of thiabendazole solution to each well. Liquid-based plates were prepared by just adding 100 µL of thiabendazole solution to each well with no agar. Agar plates were sealed with Parafilm (Bemis NA, Neenah, WI), and stored in the refrigerator at 4°C for a maximum of one week. Prior to performing the assays, plates were removed from the refrigerator and permitted to reach room temperature. Approximately 50-60 eggs in a volume of 10 µL were then added to each well. Plates were incubated for 48 hrs at 25°C, and assays were terminated by adding 20 µL of 10% Lugols iodine to all wells. Numbers of eggs and larvae in each well were counted, and hatching was corrected for the average hatching rate in the control wells. The initial assays using ETCR, ETCR 1.0, Barrow, Tara, Lacy, Worthy, Worthy 1.1F and Worthy 2.1F were performed singly with each thiabendazole concentration tested in triplicate. In order to improve the accuracy of our measurement of IC_{50}, and permit us to more accurately calculate 95% confidence intervals, we repeated the assays using three biological replicates of Barrow 1.0 and Worthy 4.1F3P, with three technical replicates per concentration in each assay.

Larval development assay (LDA): Larval development assays were performed initially using DrenchRite® LDA (Horizon Technology, Australia) assay plates (Howell et al., 2008). The DrenchRite® LDA evaluates resistance to benzimidazoles, macrocyclic lactones and levamisole using the drugs, thiabendazole, ivermectin aglycone and levamisole, respectively. Subsequently, LDA plates were prepared using only ivermectin aglycone. The three-drug plates had concentrations of ivermectin aglycone ranging from 0.97 – 10,000 nM and the ivermectin aglycone-only plates had concentrations ranging from

1.9 – 1000 nM. After isolating the eggs as described for the EHA, 90 μL/mL of amphotericin B (250 μg/mL, supplied by Horizon Technology) were added, and 20 μL containing approximately 50 - 70 eggs were dispensed into each well. Assay plates were sealed with Parafilm and incubated at 25°C. After 24 hr, 20 μL of nutritive media, composed of 0.87% Earle´s balanced salts, (Sigma-Aldrich, St. Louis, MO), 1% yeast extract (BD Difco, VWR, Becton Dickinson Sparks, MD), 0.76% NaCl (Sigma-Aldrich, St. Louis, MO), with an addition of 1% *E. coli* OP50, were added to each well. The plates were resealed and incubated for six additional days, after which the assays were terminated by adding 20 μL of 50% Lugols iodine to all wells. The contents of each well were transferred to a clean 96-flat well plate, and all eggs and larvae in each well were counted using an inverted microscope as previously described (Tandon and Kaplan, 2004). Development to L3 was corrected for all drug wells based on the average development in the control wells. The LDA does not evaluate pyrantel, which is the other anthelmintic approved for the treatment of hookworms of dogs in the United States. However, levamisole, which is used in the DrenchRite® plate, has a similar mechanism of action to pyrantel (Martin, 1997). The initials assays performed with ETCR 1.0, Lacy and Worthy 1.0 were performed singly with each ivermectin concentration tested in duplicate. In order to improve the accuracy of our measurement of IC_{50}, and allow us to more accurately calculate 95% confidence intervals, we repeated the assays using three biological replicates of lab isolates Barrow 1.0 and Worthy 4.1F3P, with two technical replicates per concentration in each assay.

***In vivo* measurements**

One laboratory dog infected with the initial Tara isolate (first passage) and two dogs infected with larvae from the second passage of the Worthy isolate (Worthy 2.1F), were treated orally with pyrantel (10 mg/kg, Strongid ®, Parsippany-Troy Hills, NJ). Reductions in fecal egg counts (FEC) were measured at day 10 for the Tara isolate and at day 13 for the Worthy isolate. The average of the FEC from the two dogs infected with the Worthy isolate was used for the reduction calculation. All FEC were performed in triplicate using the Mini-FLOTAC (University of Naples Federico II, Naples, Italy) procedure with a detection threshold of 5 EPG (Maurelli et al., 2014; Lima et al., 2015), adding two grams of feces to 18 ml of sodium nitrate (Feca-Med® , Vedco, Inc. St. Joseph; MO, USA specific gravity = 1.25 to 1.30). Fecal egg count reduction was calculated using the following formula: ((Pre-treatment FEC – Post-treatment FEC) / (Pre-treatment FEC)) x 100. For the pre-treatment FEC, we used the two-day mean of the day prior to treatment and the day of treatment or the average of the two days before treatment if FEC were not performed on the day of treatment.

***Ancylostoma caninum* isotype-1 β-tubulin deep amplicon sequencing**

DNA was extracted from pools of eggs, third-stage larvae or adults using a previously described lysis protocol (Avramenko et al., 2015). Deep amplicon sequencing assays were developed to determine the frequency of non-synonymous single nucleotide polymorphisms (SNP) at codons 167, 198 and 200 of the *A. caninum* isotype-1 β-tubulin gene. The approach and methods were as previously described for ruminant trichostrongylid nematodes except for the primer design (Avramenko et al., 2019). The presence of a large intron between exons 4 and 5 (1217 bp in reference sequence DQ459314.1 (GenBank accession)) meant that a single amplicon encompassing the three

codons of interest would be too long for reliable Illumina sequencing. Consequently, primers were designed to amplify two separate regions of the *A. caninum* isotype-1 β-tubulin gene; a 293 bp fragment between exons 3 and 4 encompassing codon 167 and a 340 bp fragment between exons 5 and 6 encompassing codons 198 and 200 (Table 1).

Using these primers, adapted primers suitable for Illumina next-generation sequencing were designed as previously described (Avramenko et al., 2019). The following PCR conditions were used to generate both fragments appropriate for sequencing: 5 µL of 5× NEB Q5 Reaction Buffer (New England Biolabs Ltd, USA), 0.5 µL of 10 mM dNTPs, 1.25 µL of 10 µM Forward primer mixture, 1.25 µL of 10 µM Reverse primer mixture, 0.25 µL of NEB Q5 polymerase, 13.75 µL of molecular grade water, and 3 µL of DNA lysate. The thermocycling parameters were 98 °C for 30 s, followed by 45 cycles of 98 °C for 10 s, 65 °C for 15 s, and 72 °C for 25 s, followed by 72°C for 2 min. Samples were purified and barcoded primers added following the protocols outlined in Avramenko et al., 2019 (Avramenko et al., 2019). Library preparation was as previously described and library sequencing performed using the Illumina MiSeq platform with the 2x250 v2 Reagent Kit (Illumina Inc., San Diego, CA, USA) (Avramenko et al., 2015). The average read depth was ~14,000 for each sample fragment, ranging between 10,000 and 19,000 reads. Sequence analysis was performed following the bioinformatic pipeline outlined in Avramenko et al., 2019 (Avramenko et al., 2019). Generated sequences were compared against a susceptible genotype *A. caninum* isotype-1 β-tubulin reference sequence (GenBank: DQ459314.1). Only observed variants resulting in non-synonymous changes at codons 167, 198 and 200 that are known to be associated with benzimidazole resistance in other Strongylid nematodes are reported. The isolates examined were ETCR, Barrow,

Worthy, Worthy 1.1F, Worthy 2.1F, Tara, Tara 1.1F and Lacy. Additionally, two clinical samples with a history of recurrent infections despite repeated anthelmintic treatments were included; Fame Taker (retired racing greyhound residing in Georgia) and Dolores (lab-mix, Worthy´s housemate companion).

***Ancylostoma caninum* ITS-2 rDNA deep amplicon sequencing**

In order to confirm the hookworm species represented in the various samples, we used an ITS-2 rDNA deep amplicon sequencing assay (Avramenko et al., 2015). This method is capable of discriminating between different nematode species based upon the sequence identity of the ITS-2 region of the rDNA. The samples were prepared and sequenced as described in Avramenko et al. 2015 (Avramenko et al., 2015), and analysed with the bioinformatic pipeline described in Avramenko et al. 2017 (Avramenko et al., 2017). Several *A. caninum* and *A. braziliense* ITS-2 sequences were added to the analysis database for the purposes of this analysis (GenBank accession: DQ438050, DQ438051, DQ438052, DQ438053, DQ438054, DQ438060, DQ438061, DQ438062, DQ438065, DQ438066, DQ438067, AB751614, AB751615, AB751616, DQ438072, DQ438073, DQ438074, DQ438075, DQ438076, DQ438077, DQ438078, DQ438079).

Data analyses

All dose-response analyses were performed after log transformation of the drug concentrations and constraining the bottom value to zero. Data were then fitted to a four-parameter non-linear regression algorithm with variable slope (GraphPad Prism® version 8.0, GraphPad Software, San Diego, CA, USA). The IC_{50} values, which represent the concentration of drug required to inhibit hatching (EHA) or development to the third larval

stage (LDA) by 50% of the maximal response, and corresponding resistance ratios (IC_{50} resistant isolate / IC_{50} susceptible isolate) were calculated.

RESULTS

Adult worms recovered from a hookworm case confirmed as being multiple-drug resistant in this study were identified using morphological criteria as being *A. caninum* (not shown). Additionally, all samples analysed were assessed with an ITS-2 deep amplicon sequencing assay as described in the methods section, confirming that they were *A. caninum* based upon sequence identity of the generated amplicons. This assay uses a taxonomy-based approach to assess the identity of generated amplicons, based upon a provided reference database. Compared to an example *A. caninum* reference sequence (GenBank Accession: AB751614), generated ITS-2 amplicons had between 96.9 and 100% sequence identity compared to the reference. Additionally, compared to an example *A. braziliense* reference sequence (GenBank Accession: DQ438050), generated ITS-2 amplicons had 80.6-82.7% sequence identity, further supporting classification as *A. caninum* rather than *A. braziliense*.

In vitro assays

The EHA yielded high R^2 values for the dose response and provided excellent discrimination between the susceptible and resistant isolates. In the initial testing using samples from the original source dogs, the resistance ratios for Lacy, Tara and Worthy, as

compared to the ETCR susceptible isolate were 10.9, 11.8 and 14.5, respectively, indicating that these isolates had a high level of resistance to benzimidazole anthelmintics (Fig. 1, Table 2).

Interestingly, a second EHA performed on the first passage of the Worthy isolate 13 days following treatment with fenbendazole demonstrated a large shift in dose response as compared to the original test. The IC_{50} for Worthy increased more than 10-fold, from 3.35 μM to greater than 36 μM, yielding a resistance ratio of greater than 100. An accurate IC_{50} could not be calculated since 36 μM was the highest concentration tested. Subsequent testing using the laboratory isolates Barrow 1.0 and Worthy 4.1F3P, also yielded high R^2 values, but the slope of the dose response for Worthy 4.1F3P had changed as compared to previous assays, and this impacted the calculated value for IC_{50}. Though the IC_{50} for the susceptible Barrow 1.0 isolate (0.17 μM) was similar to that of the susceptible ETCR isolate, the IC_{50} for Worthy 4.1F3P decreased to 1.01 μM; this yielded a resistance ratio of only 6. In comparison, the resistance ratio for the IC_{95} was 41.25; this difference from the resistance ratio for the IC_{50} is largely due to the difference in the slope of the dose response (Fig. 1, Table 2).

The LDA failed to provide good discrimination between the benzimidazole-susceptible and -resistant isolates, yielding resistance ratios of less than 2.0 (Table 2). Using levamisole, the LDA yielded dose response curves with low R^2; this prevented both the calculation of accurate IC_{50} values and any useful discrimination between pyrantel-susceptible and -resistant isolates (data not shown). In contrast, ivermectin aglycone, yielded strong discrimination for detecting resistance to macrocyclic lactones, with resistance ratios of 5.5 and 63.2 for Lacy and Worthy 1.0, respectively (Fig. 2, Table 3).

Assays performed using multiple biological replicates of Barrow 1.0 and Worthy 4.1F3P yielded high R^2 values for the dose response and a resistance ratio of 69.8, which was quite similar to the resistance ratio for the macrocyclic lactones in the earlier assays (Fig. 2, Table 3).

***In vivo* measurements**

Reductions in FEC were measured on the Tara and Worthy isolates following treatment with pyrantel. For both isolates there was essentially no reduction in FEC following treatment with pyrantel; FEC in Tara actually increased (negative reduction) and FEC in Worthy remained unchanged (3% reduction) (Fig. 3 and 4, respectively).

Benzimidazole resistance-associated SNP frequencies determined by deep amplicon sequencing

Two PCR amplicons, encompassing codons 167 and 198/200 of the isotype 1 β- tubulin gene respectively, were sequenced at depth to investigate the presence, and determine the frequency of SNP associated with benzimidazole resistance in ruminant trichostrongylid species (Table 4).

SNP associated with benzimidazole resistance were only seen at position 167. All three phenotypically resistant isolates had a high frequency of the benzimidazole resistance associated F167Y (TTC>TAC) SNP in the samples tested, ranging from 13% to almost 100% (Table 4). In the samples from the susceptible isolates, the allele frequencies were 0%, 1% and 9% (Table 4). In the Tara isolate, following a single treatment with fenbendazole the SNP frequency increased from 13% to 51%. For the Lacy isolate, the adults that were expelled after treatment with fenbendazole had allele frequencies of around 50% indicating these worms were heterozygous for the SNP, whereas the eggs

recovered from the same feces as the adults had SNP frequencies close to 100%. For the clinical cases Fame taker and Dolores, the SNP frequency was around 90%.

DISCUSSION

In this work, we conclusively demonstrate for the first time the presence of multiple-resistance to benzimidazoles, macrocyclic lactones and pyrantel in *A. caninum*. Coincident with our studies, a separate recent study reported resistance to benzimidazoles and macrocyclic lactones in *A. caninum* recovered from a greyhound dog (Kitchen et al., 2019). The origins of these resistant hookworms remain to be determined, however, evidence suggests that they originated from racing greyhound farms. *Ancylostoma caninum* is the most prevalent parasitic nematode in racing greyhounds (Jacobs and Prole, 1976; Ash et al., 2019), and this is attributed to the near constant exposure of these dogs to infective third stage larvae in the sand/dirt exercise run/pens (Ridley et al., 1994). Racing greyhounds are also treated extremely frequently with multiple different anthelmintics throughout their lives (Ridley et al., 1994). The intervals between these treatments often are less than the pre-patent period for hookworms. This high intensity of treatment will minimize the amount of refugia (parasite life stages that are not exposed to anthelmintic treatment). Consequently, any worms surviving treatment will have a large reproductive advantage and will rapidly increase in frequency (Martin et al., 1981). This combination of factors is known to place heavy selection pressure for drug resistance (Wolstenholme et al., 2004), and is very similar to the epidemiological factors that have led to high levels of multiple-drug resistance in nematodes of sheep and goats, worldwide. The EHA is an *in*

vitro bioassay used for detecting resistance to benzimidazole anthelmintics (Le Jambre, 1976). Based on the ovicidal properties of the benzimidazole drug class (Hunt and Taylor, 1989), this assay has been used successfully to detect resistance against benzimidazoles in multiple nematode parasites of livestock (Varady et al., 1996; von Samson-Himmelstjerna et al., 2009a; Rialch et al., 2013). Additionally, the EHA was assessed in *A. caninum* (Diawara et al., 2013), and used to evaluate drug susceptibility/resistance to benzimidazoles in the human hookworm, *Necator americanus* (Kotze et al., 2005; Albonico et al., 2005; Diawara et al., 2013). The IC_{50} values we measured for the two susceptible isolates we tested were very similar to that previously reported for *A. caninum* (Diawara et al., 2013), but in the resistant isolates, there was a clear shift to the right in the dose-response with resistance ratios greater than 6.0 in all isolates tested. Interestingly, when the EHA was repeated on parasite eggs collected from the resistant Worthy 1.0 isolate soon after treatment with fenbendazole, the right shift in the dose response increased dramatically, producing a resistance ratio of greater than 100. Given that the high β-tubulin SNP frequencies measured for Worthy, had no significant change in the SNP frequencies in the before and after treatment samples, this dramatic increase in IC_{50} and resistance ratio suggests that the treatment triggered the induction of another resistance mechanism(s). The fact that the allele frequency did not change and the increase in the levels of resistance was only temporary suggests that is not due to heterogeneity, but instead a change in the parasite population, otherwise, it would have been a permanent change. However, this high level of induced resistance was only temporary, as testing of the same isolate on the next passage produced IC_{50} values similar to the original Worthy isolate prior to fenbendazole treatment. However, these observations demand further study. Overall, these data demonstrate clearly

that the EHA is able to effectively discriminate between benzimidazole-susceptible and -resistant isolates, and that the isolates tested have high levels of benzimidazole resistance.

The LDA is a commonly used *in vitro* bioassay used for detecting resistance to multiple different classes of anthelmintics in gastrointestinal (GI) nematode parasites of sheep and goats (Kaplan et al., 2007; Howell et al., 2008; Raza et al., 2016). The LDA is based on the ability of anthelmintics to prevent free-living pre-parasitic nematode stages from developing to the infective third larval stage (L3) (Gill et al., 1995). Testing the LDA using multiple isolates of *A. caninum*, both multiple-drug resistant and susceptible, we found the LDA to provide excellent discrimination between our susceptible and resistant isolates for the macrocyclic lactones, but did not provide useful levels of discrimination for benzimidazoles, or for pyrantel. The poor discrimination for resistance to benzimidazoles was similar to that recently reported for *A. caninum* (Kitchen et al., 2019). Thus, unlike for GI nematodes of sheep where the LDA provides good discrimination for multiple drug classes, when used with *A. caninum*, the LDA appears only useful for measuring resistance to macrocyclic lactone drugs. This finding builds on previous works demonstrating that *in vitro* bioassays used for detection of anthelmintic resistance in parasitic nematodes are highly species-specific and drug class-specific in their ability to provide useful levels of discrimination between susceptible and resistant isolates (Varady et al., 1996; Craven et al., 1999; Tandon and Kaplan, 2004).

Interestingly, we found a wide range in the level of resistance in the two resistant isolates we tested, and those differences seem to correlate with the clinical case histories of the source dogs prior to our receipt of the samples. The IC_{50} for the first passage of the Worthy isolate yielded a resistance ratio of 63.2, which is more than 11 times greater than

the resistance ratio of 5.5 that we measured for Lacy. As noted in the clinical case histories, there was no history of recent use of macrocyclic lactones in Lacy, whereas Worthy had received three consecutive monthly treatments with moxidectin (Advantage Multi® (Bayer, Leverkusen, Germany)) just prior to our receipt of the sample. Furthermore, at the time the LDA data were collected, the Worthy isolate had not received treatment with a macrocyclic lactone drug after being established in the lab. This difference in clinical history likely is relevant for several reasons. First, to the best of our knowledge, greyhound farms and kennels have been administering ivermectin for parasite control for decades, but did not begin using moxidectin until very recently. Thus, it is unlikely that any of the dogs infected with the resistant isolates evaluated in this study were treated with moxidectin prior to adoption. Second, moxidectin is considerably more potent than ivermectin against many nematodes (Prichard et al., 2012). In *H. contortus*, ivermectin resistant worms that are naïve to moxidectin are typically killed at very high efficacy following administration of moxidectin (Craig et al., 1992; Oosthuizen and Erasmus, 1993); however, once moxidectin is used regularly in an ivermectin-resistant population, resistance to moxidectin can develop rapidly (Kaplan et al., 2007). A study investigating the emergence of moxidectin resistance in *H. contortus* found that a farm naïve to moxidectin but with ivermectin resistance had an LDA resistance ratio of 5.3, whereas farms with resistance to moxidectin had resistance ratios of 32 – 128, which is 6 – 24 fold higher (Kaplan et al., 2007). These similarities in the *A. caninum* and *H. contortus* data suggest that the resistant hookworms originating with the greyhounds and now spreading into the pet population have a clinically relevant level of resistance to macrocyclic lactones even without further selection, such as those infecting Lacy. However, as evidenced by the data from Worthy,

additional selection with moxidectin can rapidly lead to very high levels of field-derived resistance.

The other recent report of resistance in *A. caninum* (Kitchen et al., 2019) also used the LDA to measure resistance to macrocyclic lactones; however, the data of the two studies are dramatically different. The IC_{50} and corresponding resistance ratio we measured in *A. caninum* for both macrocyclic-resistant and -susceptible isolates were fairly comparable to those previously reported for *H. contortus* (Kaplan et al., 2007). However, Kitchen et al., (2019) reported values that are vastly different, both in terms of IC_{50} level and in magnitude of resistance ratio. The IC_{50} they reported for their resistant isolate was lower than what we measured in our susceptible isolate, and the IC_{50} reported for their susceptible isolate was at pM levels, almost 5,000-fold lower than what we measured. This yielded resistance ratio of greater than 1,000; a level that is greater than what has been reported, even in the most resistant *Haemonchus* isolates. Given the available clinical histories, the resistant isolate they studied was likely similar to the Lacy isolate, with little to no previous exposure to moxidectin. We measured a 5.5 resistance ratio for the Lacy isolate, thus their analyses demonstrated a resistance ratio more than 200 times greater than what we measured. Additionally, we consistently generated sigmoidal dose response curves with high R^2, and readily achieved 100% inhibition of development for our susceptible isolate. In contrast, the data shown in Kitchen et al., (2019) indicates that inhibition greater than 80% was not achieved, and shapes of dose response curves were not sigmoidal. The cause of these differences is not readily apparent, but likely are due to differences in the methods used in the two studies.

An additional interesting observation was that following treatment with fenbendazole, the egg counts in dogs infected with both the Tara and Worthy isolates initially decreased by greater than 99%, but then steadily increased after treatment to rather high levels (Additional file 1). Additionally, the mild clinical signs of enteritis that one of the dogs was displaying prior to treatment did not improve post-treatment. Given the EHA and β-tubulin SNP frequency data demonstrating extremely high levels of resistance in the surviving worms, the egg count and clinical response data suggest that the treatment was poorly effective in killing the worms, but induced a temporary inhibition of egg production. A similar temporary deleterious effect on worm fecundity has been reported previously for benzimidazoles in *H. contortus* in sheep (Scott et al., 1991), but is not recognized as an usual effect in nematodes of livestock following treatment with benzimidazoles. In contrast, this phenomenon has been reported on multiple occasions following treatment with ivermectin and moxidectin (McKellar et al., 1988; Sutherland et al., 1999; Condi et al., 2009). Regarding the reductions in FEC measured for pyrantel, for both isolates, it was clear that there was no effect of treatment (Figure 3 and 4).

Currently, the mechanisms of resistance to macrocyclic lactones and pyrantel in nematodes are unknown. Consequently, there are no molecular diagnostics available to detect resistance to these drug classes. However, the mechanism of resistance to benzimidazole drugs is well-described. Benzimidazoles work by blocking the polymerization of parasite microtubules, and they do this by binding to the nematode β-tubulin protein monomers (Lacey, 1988, 1990). SNPs in the isotype-1 β- tubulin gene located at codons 167(TTC/Phe → TAC/Tyr), 198GAG/Glu → GCG/Ala) and 200(TTC/Phe → TAC/Tyr) are associated with benzimidazole resistance in multiple

species of Strongylid nematode parasites such as *Haemonchus contortus* (Kwa et al., 1994), *Teladorsagia circumcincta* (Elard et al., 1996) and cyathostomins (von Samson-Himmelstjerna et al., 2001)**.** Several PCR and pyrosequencing assays have been developed to detect and measure these mutations, (Álvarez-Sánchez et al., 2005; von Samson-Himmelstjerna et al., 2009b; Demeler et al., 2013; Chaudhry et al., 2014; Knapp-Lawitzke et al., 2015; Redman et al., 2015; Ramünke et al., 2016) but these all have limitations that affect their usefulness.

However, a recently developed deep-amplicon sequencing assay for measuring benzimidazole-associated resistance mutations in nematode communities of cattle, sheep, bison and horses provides a powerful new tool that enables unparalleled sensitivity of detection and permits screening for the emergence of resistance mutations (Avramenko et al., 2019). We modified and used this deep amplicon-sequencing assay in *A. caninum* and here we report the first use of this approach in a hookworm. Of the SNP associated with benzimidazole resistance in trichostrongylid nematodes, only F167Y (TTC>TAC) was detected. This same SNP has been commonly found in other nematode Strongylid nematode parasites such as equine cyathostomins (Hodgkinson et al., 2008), *Haemonchus contortus* (Prichard, 2001)**,** *Haemonchus placei* (Brasil et al., 2012), and *Teladorsagia circumcincta* (Silvestre and Cabaret, 2002), and has only been rarely reported in *Ascaris lumbricoides* and *Trichuris trichuira* (Diawara et al., 2013)**.** Recently, this same SNP was also reported in a resistant isolate of *A. caninum* that was originally isolated from a racing greyhound from Florida. Furthermore, using CRISPR/Cas 9, the authors of that work were successful in replicating this SNP in the homologous *ben-1* gene of *C. elegans,* and saw a

doubling of the resistance ratio in the EHA, which was similar to the resistance ratio measured in their *A. caninum* resistant isolate using the LDA (Kitchen et al., 2019).

Using deep amplicon sequencing, we found low allele frequencies for the benzimidazole resistance-associated SNPs in the susceptible isolates; in Barrow, the frequency was 1.2%, and the two analyses for ETCR yielded highly variable results of 0% and 8.8%. The reason for this discrepancy is not known and further analyses are in progress. In contrast, high SNP frequencies were recorded for all resistant isolates. The original isolate of Worthy had a SNP frequency of 92.2%, which is consistent with the high selection pressure produced by the five rounds of intensive (10-day) fenbendazole treatment the dog received in the year prior to us collecting the sample. The lowest frequency measured in a resistant isolate was 12.7% in Tara, however, following a single treatment with fenbendazole, the SNP frequency increased to 50.9%. It is unclear why Tara had a relatively low SNP frequency relative to the other resistant isolates, given that Tara had a history of multiple treatments with febantel prior to our receipt of the sample. Further analyses are in progress with all of our archived samples to address these issues. Interestingly, three single adult worms recovered from the feces of Lacy that we sequenced (out of many that were expelled alive after treatment with fenbendazole) had F167Y (TTC>TAC) SNP frequencies of approximately 50% indicating that these worms were heterozygous at codon 167. This was an interesting finding, as it suggests that heterozygous worms were able to survive the treatment, but could not maintain their position in the GI tract. In comparison, eggs recovered from the same feces demonstrated a SNP frequency of almost 100%, suggesting that the worms that survived the treatment and maintained their position in the intestine were virtually all homozygous for resistance.

It is noteworthy that others have looked for benzimidazole-resistance associated SNP in *A. caninum* without success (Furtado and Rabelo, 2015)**.** However, studies performed in Brazil did report finding a SNP at codon 198 in *A. braziliense* (Furtado et al., 2018) and at codon 200 in *A. caninum* (Furtado et al., 2014) at very low frequencies, 1.2 and 0.8%, respectively using PCR-RFLP. However, these findings were not confirmed by sequencing.

Here we report compelling evidence using *in vitro*, *in vivo* and genetic analyses that convincingly demonstrate that recent cases of hookworm in dogs that appear refractory to treatment, are due to *A. caninum* that are MDR. Though larval leak is likely involved in most of these cases, our data indicate strongly that MDR is the primary cause. This is an important and concerning development, as the emergence and spread of MDR *A. caninum* to all three major anthelmintic classes, would pose a serious threat to canine health, as there are no other effective drug classes currently approved for the treatment of hookworms in dogs in the United States. Though a recent study reported success in treating several cases of recurrent hookworm infections in greyhounds recently retired from racetracks using a combination therapy of moxidectin, pyrantel pamoate and febantel at monthly intervals (Hess et al., 2019), we have recently diagnosed multiple cases at a greyhound adoption kennel where this same regimen appears to be ineffective (data not shown). The disparity in these findings is consistent with the rapid evolution of moxidectin resistance when moxidectin is used against ivermectin resistant worms (Kaplan et al., 2007).

Conclusions

MDR in *A. caninum* is an emerging problem in dogs. Evidence suggests that the problem originated in the greyhound racing industry and has since begun to spread through the pet

population. Nevertheless, we still lack definitive evidence to infer that these resistant hookworms are spreading into the pet dog population. Clearly, further epidemiological and molecular epidemiological investigations are needed in order to gain knowledge on the origin, prevalence, and distribution of MDR *A. caninum*. Furthermore, new treatments approved for use in dogs are greatly needed. These results also provide proof of concept that anthelmintic resistance can arise in hookworm species. *Ancylostoma caninum* is extremely close phylogenetically to the human hookworm species *Ancylostoma duodenale*, *Ancylostoma ceylanicum* and *Necator americanus* (Blaxter, 2000). Consequently, these findings should provide some concern to the global health community, as the scale-up of mass drug administration for soil-transmitted helminths (STH) is now placing similar selection pressures for benzimidazole resistance in human hookworms, and reduced efficacies are widely reported (Albonico et al., 1994; De Clercq et al., 1997; Albonico et al., 2003; Albonico et al., 2004; Flohr et al., 2007; Speich et al., 2016). The deep amplicon sequencing assay used in this work, also can be used to perform global-level surveillance for the detection of benzimidazole resistance in human hookworms, and with minor modifications, in roundworms (*Ascaris lumbricoides*) and whipworms (*Trichuris trichiura*).

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Table 1. *Ancylostoma caninum* isotype-1 β-tubulin primers

Primer	Sequence 5'-3'	Length (bp)	Forward /Reverse	Codons
ACB1_167_F	GGYGCAGGAAACAACTG	17	Forward	167
ACB1_167_R	CTTTGGTGAGGGGACAACA	19	Reverse	167
ACB1_200_F	GTRGTGGAGCCATACAATGC	20	Forward	198, 200
ACB1_200_R	GGCATGAAGAAGTGAAGACGT	21	Reverse	198, 200

Table 2. IC_{50} data for benzimidazoles in *Ancylostoma caninum* isolates. ETCR was the susceptible isolate used for calculating resistance ratios in the initial assays, and Barrow 1.0 was used for the EHA, and ETCR 1.0 for the LDA in subsequent assays. Inital assays were perfomed singly using ETCR, ETCR 1.0, Barrow, Tara, Lacy, Worthy, Worthy 1.1F and Worthy 2.1F, and subsequent assays were performed in triplicate using Barrow 1.0 and Worthy 4.1F3P, in order to improve the precision of the estimate and reduce the width of the 95% confidence intervals (CI). The values for Barrow 1.0 and Worthy 4.1F3P represent the mean IC_{50} of three biological replicate assays, with each thiabendazole concentration measured in triplicate. IC_{95} values were also calculated for the EHA. Resistance ratios (RR) were calculated as $IC_{50/95}$ resistant isolate / $IC_{50/95}$ susceptible isolate; RR are not provided for the susceptible isolates or where assays were not performed, as this value has no relevance in those instances. EHA: Egg hatch assay. LDA: Larval development assay.

Isolate	EHA (uM) IC_{50} (95% CI)	EHA IC_{50} RR	LDA (uM) IC_{50}	LDA IC_{50} RR	EHA (uM) IC_{95} (95% CI)	EHA IC_{95} RR

ETCR	**0.23**		**-**		**0.49**	
ETCR 1.0	**0.25**		**0.07**		**1.13**	
Barrow	**0.24**		**-**		**3.46**	
Tara	**2.73**	**11.8**	**0.12**	**1.7**	**7.60**	**15.5**
Lacy	**2.51**	**10.9**	**0.13**	**1.8**	**31.14**	**63.6**
Worthy	**3.35**	**14.5**	**-**		**10.07**	**20.6**
Worthy 1.1F	**> 36**	**> 100**	**-**		**> 36**	**> 70**
Worthy 2.1F	**2.65**	**11.5**	**-**		**> 36**	**> 70**
Barrow 1.0	**0.17 (0.16-0.19)**	**-**	**-**		**0.36 (0.28-0.46)**	
Worthy 4.1F3P	**1.02 (0.92–1.12)**	**6.0**	**-**		**14.85 (9.96–23.22)**	**41.3**

- Assays not performed

Table 3. DrenchRite LDA dose response data for macrocyclic lactones in *Ancylostoma caninum* isolates. Inital assays were perfomed singly using ETCR 1.0, Lacy and Worthy 1.0, and subsequent assays were performed in triplicate using Barrow 1.0 and Worthy 4.1F3P, in order to improve the precision of the estimate and reduce the width of the 95% confidence intervals (CI). In all assays each ivermectin aglycone concentration was measured in duplicate. Resistance ratios (RR) were calculated as IC_{50} resistant isolate / IC_{50} susceptible isolate; RR are not provided for the susceptible isolates, as this value has no relevance. LDA: Larval development assay.

Isolate	LDA IC_{50} (nM) (95% CI)	R^2	LDA RR
ETCR 1.0	**16.62**	**0.93**	
Lacy	**91.53**	**0.53**	**5.5**
Worthy 1.0	**1052**	**0.45**	**63.2**

Barrow 1.0	**12.31 (10.42-14.70)**	**0.98**	
Worthy 4.1F3P	**859 (411.3-3426)**	**0.92**	**69.8**

Table 4. Single nucleotide polymorphism frequencies for *A. caninum* isolates at the three different codons associated with resistance to benzimidazoles.

Isolate/Patient	BZ phenotype (S/R)	Sample Sequenced	F167Y Freq (%)	E198A Freq (%)	F200Y Freq (%)
ETCR	**S**	**250 Eggs**	**0**	**0**	**0**
ETCR	**S**	**300 L3**	**8.8**	**0**	**0**
Barrow	**S**	**250 L3**	**1.2**	**0**	**0**
Worthy	**R**	**L3**	**92.2**	**0**	**0**
Worthy 1.1F	**R**	**300L3**	**87.6**	**0**	**0**
Worthy 2.1F	**R**	**100 L3**	**94.5**	**0**	**0**
Tara	**R**	**375 Eggs**	**12.7**	**0**	**0**
Tara 1.1F	**R**	**250 L3**	**50.9**	**0**	**0**
Lacy	**R**	**Single adult**	**47.4**	**0**	**0**
Lacy	**R**	**Single adult**	**52.9**	**0**	**0**
Lacy	**R**	**Single adult**	**46.0**	**0**	**0**
Lacy	**R**	**Eggs**	**99.7**	**0**	**0**
Fame Taker	**R**	**350 L3**	**90.7**	**0**	**0**
Dolores (Worthy house companion)	**R**	**300 L3**	**88.9**	**0**	**0**

BZ: Benzimidazoles
Freq: Frequency
L3: Third stage larvae
S/R: Susceptible/Resistant

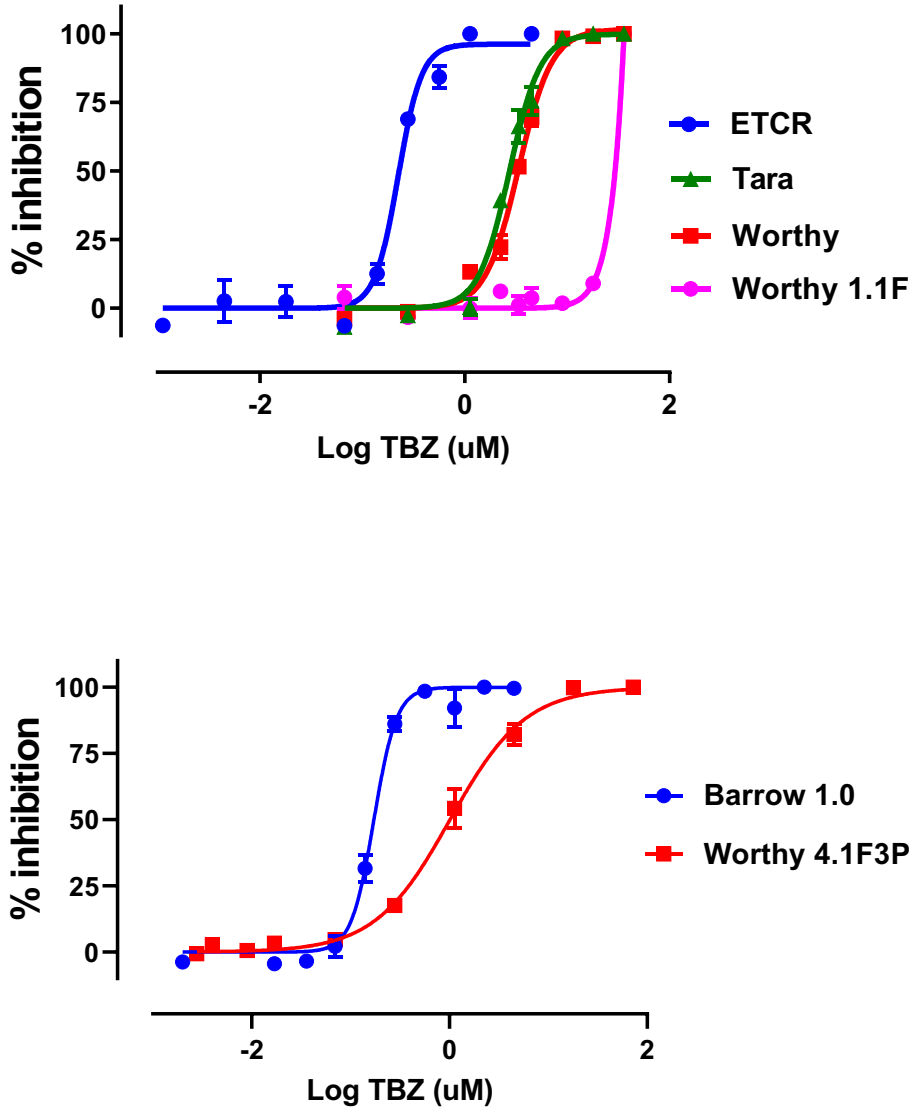

Fig. 1. Dose-response curves for the Egg Hatch Assay. Initial assays were performed singly using ETCR, Tara, Worthy and Worthy 1.1F. Subsequent assays were performed in triplicate using the Barrow 1.0 and Worthy 4.1F3P isolates with three replicates per

concentration. Curves were generated using the variable slope nonlinear regression model analysis contained in GraphPad 8.

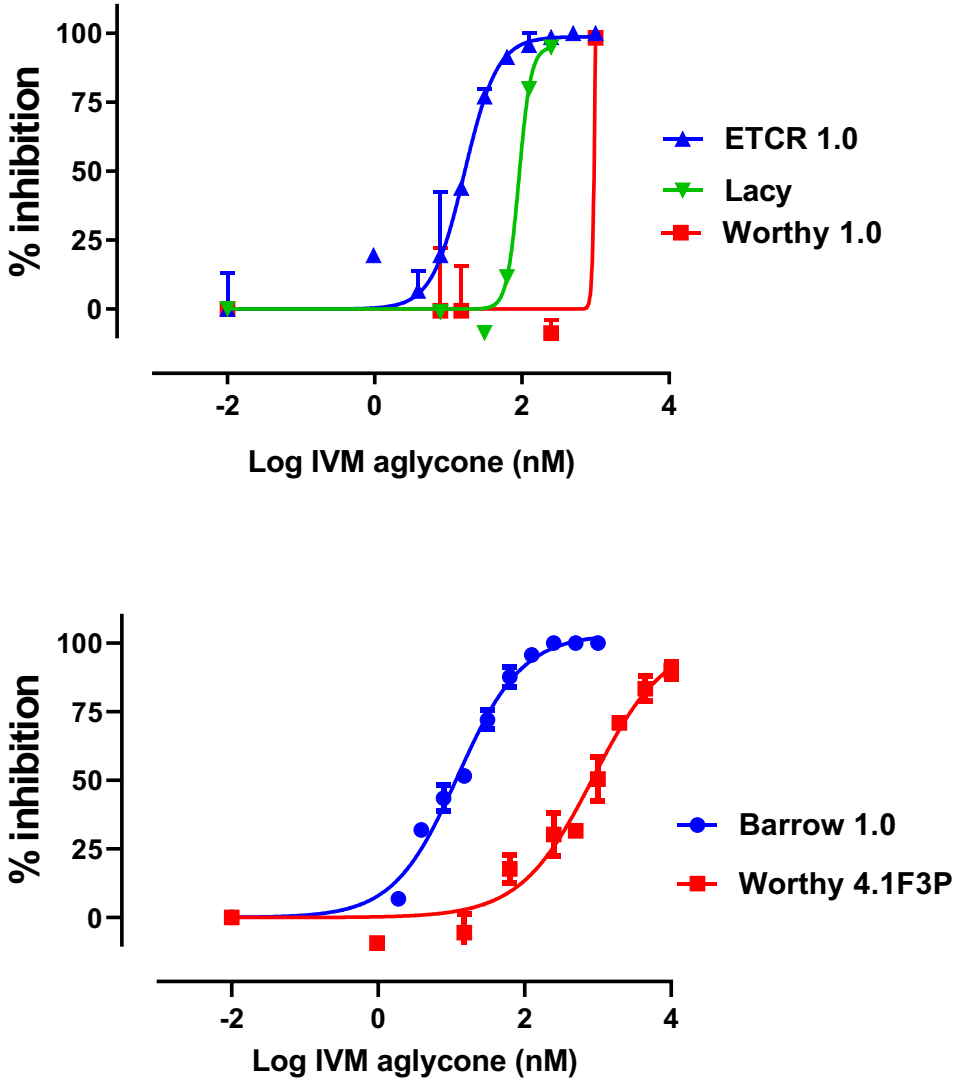

Fig. 2. Dose-response curves for the Larval Development Assay. Initial assays were performed singly using ETCR 1.0 Lacy and Worthy 1.0. Subsequent assays were performed in triplicate using Barrow 1.0 and Worthy 4.1F3P isolates with two replicates

per concentration. Curves were generated using the variable slope nonlinear regression model analysis contained in GraphPad 8.

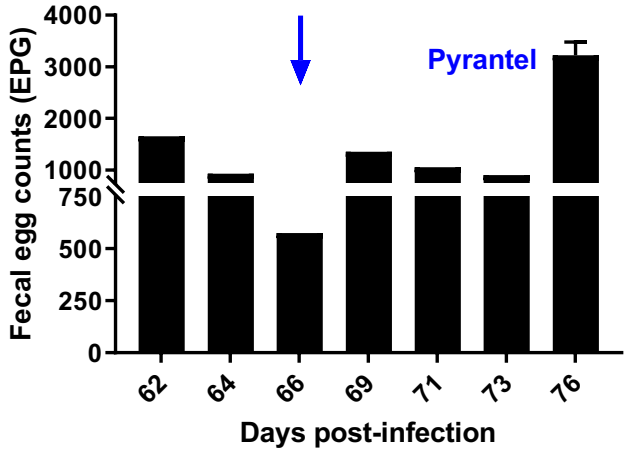

Fig. 3. Fecal egg counts (FEC) over the course of infection of a dog infected with the Tara isolate. Treatment with pyrantel was administered on day 66 (23 Feb 2018) and post- treatment FEC was performed on day 10 post-treatment.

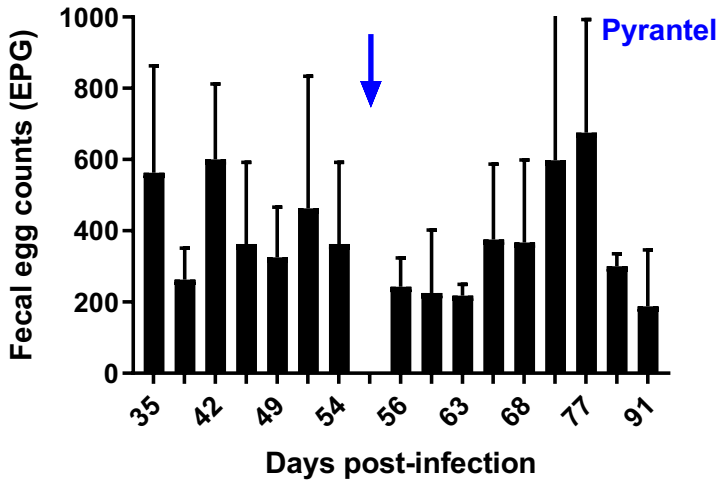

Fig. 4. Average of fecal egg counts (FEC) over the course of infection of two dogs infected with larvae from the second passage of the Worthy isolate, with a treatment event with fenbendazole on the first passage (Worthy 2.1F). Treatment with pyrantel was administered on day 55 (25 Oct 2018) and post- treatment FEC was performed on day 13 post-treatment.

CHAPTER 3

EFFICACY EVALUATION OF ANTHELMINTIC PRODUCTS AGAINST AN INFECTION WITH THE CANINE HOOKWORM (*ANCYLOSTOMA CANINUM*) ISOLATE WORTHY 4.1F3P IN DOGS [2]

[2] Jimenez Castro PD, Mansour A, Charles S, Hostetler J, Settje T, Kulke D, Kaplan RM. 2020. Efficacy evaluation of anthelmintic products against an infection with the canine hookworm (*Ancylostoma caninum*) isolate Worthy 4.1F3P in dogs *International Journal*

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ABSTRACT

Ancylostoma caninum is the most prevalent intestinal nematode of dogs, and has a zoonotic potential. Multiple-drug resistance (MDR) has been confirmed in a number of *A. caninum* isolates, including isolate Worthy 4.1F3P, against all anthelmintic drug classes approved for hookworm treatment in dogs in the United States (US). The cyclooctadepsipeptide emodepside is not registered to use in dogs in the US, but in a number of other countries/regions. The objective of this study was to evaluate the efficacy of emodepside + praziquantel, as well as three commercial products that are commonly used in the US for treatment of hookworms, against a suspected (subsequently confirmed) MDR *A. caninum* isolate Worthy 4.1F3P. 40 dogs infected on study day (SD) 0 with 300 third-stage larvae, were randomly allocated to one of five treatment groups with eight dogs each: pyrantel pamoate (Nemex®-2), fenbendazole (Panacur® C), milbemycin oxime (Interceptor®), emodepside + praziquantel tablets and non-treated control. Fecal egg counts (FEC) were performed on SDs 19, 20, 22, 27, 31 and 34. All treatments were administered as per label requirements on SD 24 to dogs in Groups 1 through 4. Two additional treatments were administered on SDs 25 and 26 to dogs in Group 2 as per label requirements. Dogs were necropsied on SD 34 and the digestive tract was removed/processed for worm recovery and enumeration. The geometric mean (GM) worm counts for the control group was 97.4, and for the pyrantel pamoate, fenbendazole, milbemycin oxime, and emodepside + praziquantel groups were 74.8, 72.0, 88.9, and 0.4, respectively. These yielded efficacies of 23.2%, 26.1%, and 8.8%, and 99.6%, respectively.

These data support previous findings of the MDR status of Worthy 4.1F3P as treatments with pyrantel pamoate, fenbendazole and milbemycin oxime lacked efficacy. In sharp contrast, Worthy 4.1F3P was highly susceptible to treatment with emodepside + praziquantel.

INTRODUCTION

The canine hookworm, *Ancylostoma caninum* is the most prevalent and important intestinal nematode parasite of dogs in the United States (US) with the prevalence depending on age, level of care and geographic location of the dog (Little et al., 2009). A recent study evaluating over 39 million fecal samples from 2012-2018, showed evidence of a steady yearly increase in prevalence from 2015 onwards, with an overall increase of 47% (Drake and Carey, 2019). Anthelmintic drugs currently approved for treatment of *A. caninum* in the United States include, febantel and fenbendazole of the benzimidazole class, moxidectin and milbemycin oxime of the macrocyclic lactone class (sub-class milbemycin), and pyrantel of the tetrahydropyrimidine class. In registration studies, febantel, moxidectin and milbemycin oxime all demonstrated efficacies >99% (F.D.A, 1994, 1998, 2006), fenbendazole demonstrated an efficacy >98% (F.D.A, 1983) and pyrantel demonstrated a somewhat variable efficacy, with a mean across studies of approximately 94% with more than half of those studies with efficacies greater than 99% (F.D.A, 1993).

Hookworms are blood-feeding nematodes that use a cutting apparatus to attach to the intestinal mucosa and submucosa, and contract their muscular esophagus to create negative pressure, which sucks a plug of tissue into their buccal capsules (Hotez et al., 2004). Bleeding is facilitated by both mechanical damage and chemical action by

hydrolytic enzymes that cause rupture of capillaries and arterioles. Additionally, hookworms release an assortment of anticlotting agents to ensure blood flow (Stassens et al., 1996). Pathological consequences of infection include iron-deficiency anemia, hypoalbuminemia, and an enteritis characterized by diarrhea, which may contain fresh (hematochezia) or digested blood (melena) (Kalkofen, 1987).

In the past few years, there is empirical evidence that veterinarians are diagnosing increasing numbers of cases of persistent hookworm infections that appear refractory to standard anthelmintic therapy available in the United States. Recently retired racing greyhounds are highly over-represented among the cases of persistent/recurrent hookworm infection being reported to our laboratory (University of Georgia). To further investigate these observations, three isolates of *A. caninum* from clinical cases with persistent infections were established in laboratory beagles and evaluated using *in vitro* (egg hatch assay, larval development assay), molecular (deep amplicon sequencing), and *in vivo* testing (fecal egg count reduction). Data from these studies confirmed that all three isolates were resistant to the benzimidazole, avermectin/milbemycin and tetrahydropyrimidine classes of anthelmintics (Jimenez Castro et al., 2019). One isolate that was characterized in the above- mentioned studies by Jimenez Castro et al. (2019) was Worthy 4.1F3P. Though still unproven, clinical evidence strongly suggests that the MDR status of these *A. caninum* isolates evolved on greyhound breeding farms and kennels (Jimenez Castro et al., 2019). Spread of these isolates could pose a serious threat to dogs in the US.

These recent findings are particularly interesting given the history of anthelmintic resistance in *A. caninum,* where only very few cases are reported. The first report of

anthelmintic resistance in *A. caninum* was to pyrantel in a greyhound puppy that was imported from Australia (Jackson et al., 1987). Several additional cases of resistance to pyrantel in dogs were subsequently diagnosed in Australia (Hopkins et al., 1988; Hopkins and Gyr, 1991; Kopp et al., 2007; Kopp et al., 2008a, b). However, subsequent to 2008 there were no further cases of anthelmintic resistance reported in *A. caninum* until 2019, when a report provided evidence of a case of resistance to benzimidazoles and macrocyclic lactones in an isolate of *A. caninum* obtained from a greyhound dog originating from Florida, USA (Kitchen et al., 2019). This was followed shortly thereafter by the report mentioned above, which demonstrated MDR against all three major drug classes most commonly used for the treatment of this parasite in dogs (Jimenez Castro et al., 2019).

Additionally, this could also pose a public health concern, as *A. caninum* is a zoonotic parasite that can cause cutaneous larva migrans (Leeming and Oxon, 1966; Bowman et al., 2010; Del Giudice et al., 2019), eosinophilic enteritis (Prociv and Croese, 1996), as well as patent infections in humans (Ngcamphalala et al., 2019; Furtado et al., 2020). Long-term treatment protocols composed of triple drug combinations of febantel, pyrantel and moxidectin, combined with strict environmental hygiene, were recently reported as being effective against persistent hookworm infections in greyhound dogs (Hess et al., 2019). However, diagnostic surveillance performed by our laboratory (University of Georgia) over the past year on actively racing and recently retired greyhounds originating from different locations has shown that this same triple anthelmintic combination most often fails to treat and control infections.

Emodepside is a drug in the cyclooctadepsipeptide class, a semisynthetic derivative of PF1022A (Sasaki et al., 1992). PF1022A itself is a fermentation product of the fungus

Rosellinia spp. PF1022, which is found on the leaves of the plant *Camellia japonica* (Terada, 1992; Harder et al., 2003; Kulke, 2014). Historically the presynaptic latrophilin-like receptor (LAT-1), called depsiphilin in *A. caninum* (Krüger et al., 2009), and the ionotropic $GABA_A$ receptors (Chen et al., 1996; Miltsch et al., 2012) have been considered as putative molecular targets of emodepside in nematodes. However, various studies confirmed the calcium-activated and voltage-gated potassium nematode channels, SLO-1, as the most relevant and direct molecular drug target (Guest et al., 2007; Welz et al., 2011; Kulke et al., 2014; Crisford et al., 2015).

Emodepside has proven efficacy against a large and diverse number of nematode parasites infecting multiple different hosts, including *A. caninum* (Akyol et al., 1993; Zahner and Schares, 1993; Kachi et al., 1998; von Samson-Himmelstjerna et al., 2000; Zahner et al., 2001; Reinemeyer et al., 2005; Schmahl et al., 2007; Schroeder et al., 2009; Schimmel et al., 2009). Furthermore, due to its unique mechanism of action, emodepside has proven effective against nematode isolates with resistance to drugs from the other major classes of anthelmintics (von Samson-Himmelstjerna et al., 2005). Emodepside is sold for dogs as two separate products in several markets outside the USA; Profender® tablets for Dogs, containing emodepside and praziquantel and Procox® oral suspension containing emodepside and toltrazuril. A field study performed in several European countries in dogs ranging from one month to 11 years old using Profender® Tablets for Dogs demonstrated an efficacy of 99.9% against *A. caninum* and *U. stenocephala,* (Altreuther et al., 2011).

The objective of this study was to evaluate the efficacy of emodepside + praziquantel, as well as three other anthelmintic products commonly used for the treatment

of canine hookworms, against *A. caninum* isolate Worthy 4.1F3P. This isolate was later confirmed as an MDR isolate of *A. caninum* (Jimenez Castro et al., 2019).

MATERIALS AND METHODS

2.1 Study Design

The study was performed as a randomized, blinded, controlled efficacy study. The study protocol was approved by the study site Institutional Animal Care and Use Committee. All dogs were individually identified by ear tattoo, individually housed throughout the study, fed a balanced commercial dry dog food once daily, and provided with water *ad libitum.* Physical examinations were performed during the acclimation period to ensure dogs were healthy and eligible for enrolment into the study. The inclusion criteria included three negative fecal egg counts (FECs) performed on SDs -7, - 6, and -3 and good health. All personnel that were involved in data collection or assessment were blinded to the treatment assignment of the animals throughout the entire study period.

2.2 *Ancylostoma caninum* isolate

The *A. caninum* isolate used in this study was designated as Worthy 4.1F3P, the fourth laboratory passage of an isolate that was originally isolated from a retired racing greyhound in October, 2017 (Jimenez Castro et al., 2019). During the course of laboratory passage, this isolate underwent treatment selection with fenbendazole and pyrantel on the first and third passages, respectively.

2.3 Parasitological methods

Two laboratory beagles were infected with the *A. caninum* isolate Worthy 3.1F3P to provide the infective larvae used in this study. Feces containing hookworm eggs were

then cultured by mixing with activated charcoal (Black Diamond Media, Tinley Park, IL), and incubated at 76-80° F and 56-92% relative humidity for at least five days. Third-stage larvae were then harvested using the Baermann technique (Baermann, 1917), placed into gelatin capsules and administered to the dogs. Fecal egg counts were performed on SD -7, -6, -3, 19, 20, 22, 27, 31 and 34, with all FECs performed using the McMaster procedure with a lower limit of detection of 25 eggs per gram (EPG) (Gordon and Whitlock, 1939).

2.4 Experimental inoculations

Forty-two purpose-bred Beagles sourced from a USDA licensed vendor (mix of male/female; 2.5 months of age at infection; 2.6 – 4.6 kg) were acclimated for 7 days. On SD 0, 42 dogs were inoculated orally both in the morning and in the afternoon, with 150 third-stage infective larvae (a total of 300 L3) of the Worthy 4.1F3P *A. caninum* isolate. Just prior to inoculation, L3 were placed into gelatin capsules to insure uniformity of inoculation. At the time of inoculation, capsules were placed in the back of the throat, and the dog was then administered 20-30 mls of deionized water to ensure that the capsule was swallowed. All dogs were checked for vomiting at 1 h ± 15 min post-inoculation and were examined at least once daily for any abnormal clinical signs or adverse events. On SD 22, all dogs were weighed followed by a physical examination, then 40 of the 42 dogs confirmed as hookworm-infection positive by having FEC higher than 25 EPG on SDs 19, 20 and 22, were completely randomized to one of five treatment groups, each composed of eight dogs. The two dogs not included in the study went back to the colony maintained at the study facility.

2.5 Drug administrations

All dogs were weighed using a certified scale two days before their scheduled treatment on SD 24. All treatments were administered orally as per the label requirements. Treatment groups were as follows: Group 1: pyrantel pamoate (Nemex®-2: Zoetis, Kalamazoo, MI) at a minimum of 5 mg/kg bodyweight (BW), Group 2: fenbendazole (Panacur® C: Merck Animal Health, Madison, NJ) at a minimum of 50 mg/kg BW for three consecutive days), Group 3: milbemycin oxime (Interceptor®: Elanco, Greenfield, IN) at a minimum of 0.5 mg/kg BW, Group 4: emodepside + praziquantel tablets at a minimum of 1 mg + 5 mg/kg BW, and Group 5: non-treated control.

2.6 Necropsy/worm counts

All dogs were humanely euthanized on SD 34, and the entire gastrointestinal tract, from the stomach to the rectum, was removed and processed in accordance with the relevant laboratory standard operating and parasitological procedures from TRS Labs, Inc. Briefly, the tract was opened longitudinally and the mucosa was scraped twice with 12" tissue forceps with gross serrated jaws, rinsed with tap water and left to soak for 2-3 hours in plastic containers. The entire content of the container was then passed through a standard testing sieve #60 with a pore size of 250 μm. All recovered worms were placed into a labelled container containing warm normal saline, left overnight at room temperature and counted and sexed on SD 35, and then placed in 70% ETOH for storage.

2.7 Efficacy calculation/statistical analysis

The adult hookworm counts at necropsy were used to evaluate the efficacy of the treatment groups against *A. caninum*. Percent efficacy was calculated using the formula:

$$\% \text{ Efficacy} = \left(\frac{\text{Geometric mean control - Geometric mean treated}}{\text{Geometric mean control}} \right) \text{x } 100$$

Effectiveness would be claimed against the parasite (i.e., calculated for both sexes combined on the basis of the addition of small and large intestinal counts) if the following criteria were met:

1. At least six adequately infected non-treated dogs. From the parasitological perspective a number ≥5 worms per dog was considered adequate.
2. Calculated percent efficacy of at least 90% for each treatment group.
3. Significant difference between the treatment group and the non-treated control group using a 5% level of significance and appropriate statistical analyses.

A non-parametric statistical method (Wilcoxon's Rank Sum test) was used to test for group differences in worm counts using a 5% significance level. Only pair-wise comparisons using the non-treated group were analyzed and reported. All analyses were performed using programs in SAS® version 9.4.

RESULTS

No treatment related adverse events were recorded. The geometric mean (GM) worm count for the control group was 97.4, whereas GM worm counts for the pyrantel pamoate, fenbendazole, milbemycin oxime, and emodepside + praziquantel treatment groups were 74.8, 72.0, 88.9, and 0.4, respectively. These yielded efficacies of 23.2%, 26.1%, and 8.8%, and 99.6% respectively. The control group had significantly higher worm counts compared to the fenbendazole, pyrantel pamoate and emodepside + praziquantel treatment groups. The emodepside + praziquantel treated group had a higher efficacy when compared with each of the other three treatment groups. (Table 1)

FEC were recorded for each dog on SD 19, 20, 22, 27, 31 and 34, and are shown in Table 2, with averages for each day shown in figure 1. For the pyrantel and milbemycin groups, the post-treatment FECs increased in a similar fashion as observed in the non-treated control group. Interestingly, the pattern of FEC reduction was very different for fenbendazole compared to the other drugs. Dogs treated with fenbendazole initially showed a high level of FEC reduction on SD 27, but this effect was temporary. In contrast, only one dog in the emodepside + praziquantel group was positive for hookworm eggs on SD 27, and on SD 31 and 34 all dogs were negative for hookworm eggs.

DISCUSSION

The present study supports previous findings of the MDR status of the *A. caninum* Worthy 4.1F3P as treatments with pyrantel pamoate, fenbendazole and milbemycin oxime lacked efficacy. Additionally, we demonstrate that emodepside + praziquantel tablets were highly effective against this isolate, yielding an efficacy of 99.6%. Emodepside has already demonstrated high efficacy against MDR nematode isolates of *Haemonchus contortus* and *Cooperia oncophora* in ruminants (von Samson-Himmelstjerna et al., 2005).

The benzimidazoles are one of the most important broad-spectrum classes of anthelmintics available to control parasitic nematodes of both animals and humans (Stepek et al., 2006). An interesting observation in this study was that following treatment with fenbendazole (Panacur® C), there was a large reduction in egg counts one day after completion of the three-day treatment regimen (SD 27), but FEC gradually increased on SDs 31 and 34 (Table 2). We also reported similar findings in the two isolates tested in our other recent work (Jimenez Castro et al., 2019). These data demonstrate that treatment with

fenbendazole caused a temporary suppression in egg shedding. Interestingly, this phenomenon appears to be relatively unique to *A. caninum*. Benzimidazole anthelmintics have been used for many decades in a multitude of hosts against numerous parasite species, and high prevalences of resistance to benzimidazoles are reported worldwide in many species of gastrointestinal nematode parasites (Kaplan, 2004; Kaplan and Vidyashankar, 2012). Yet, to our knowledge, this egg suppression phenomenon has only been reported once previously, in *H. contortus* in sheep (Scott et al., 1991). In contrast, egg output suppression has been reported on several occasions following treatment with ivermectin and moxidectin in several different parasite species (McKellar et al., 1988; Sutherland et al., 1999; Condi et al., 2009; Macrelli et al., 2019).

The milbemycin oxime (Interceptor®) treated group had the lowest efficacy of all the treatments (8.8%). This clearly demonstrates that milbemycin oxime lacks efficacy against this MDR isolate of *A. caninum*, and based on previously published data (Jimenez Castro et al., 2019), it is highly likely that this isolate is also less susceptible to all other macrocyclic lactone anthelmintics. Macrocyclic lactones, particularly ivermectin, have a long history of being used intensively by the greyhound industry for parasite control (Ridley et al., 1994). Though, there are no recent citable publications confirming this ongoing intensive use, multiple communications with people familiar with the greyhound industry, including one of the authors of the paper cited above, have confirmed the ongoing nature of this practice. In contrast, to our knowledge, moxidectin, a substantially more potent member of this drug class (Prichard et al., 2012), has only recently started to be used on greyhound farms and kennels. The dog from which the Worthy isolate was originally isolated was a recently adopted retired racing greyhound that had multiple failed treatments

with moxidectin prior to us collecting the parasites from this dog. However, after being established in the laboratory this isolate received no further treatment selection with macrocyclic lactones (Jimenez Castro et al., 2019).

A further interesting observation was the large increase in egg production per female worm in the milbemycin group compared to the control and pyrantel-treated groups. We have made similar observations on multiple occasions in both small ruminants and cattle infected with macrocyclic-resistant isolates of *Haemonchus* following treatment with ivermectin (unpublished observations). Currently, we do not have an explanation for this increased egg output, but it seems to be a common occurrence and warrants further investigation.

Previous work with both drug-susceptible and pyrantel-resistant *A. caninum* demonstrated a density dependent fecundity of the female worms, whereby female worms increased their individual egg output due to reductions in the number of worms in the lumen of the small intestine (Kopp et al., 2007). This phenomenon was not seen in this study.

Based on work in our laboratory, both published and unpublished, as well as frequent communications with veterinarians dealing with cases of persistent hookworm infections, Worthy 4.1F3P appears to be representative of the worms currently circulating in greyhounds. The lack of efficacy demonstrated by the most commonly used products in the US for the treatment of hookworms in dogs therefore portends a very serious situation, and threatens not just canine health, but also human health due to its zoonotic potential. Further research investigating the molecular epidemiology is warranted in order to gain a deeper understanding of the origin(s) of this MDR to commercial products used in the US, as well as its prevalence and geographic distribution.

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Table 1. Numbers of worms recovered and percent efficacy for each treatment group. All dogs were infected with 300 *A. caninum* L3 on day 0, were treated on day 24, and were euthanized and worms recovered on day 34. Statistical comparisons were performed using the Wilcoxon's Rank Sum test.

Treatment Group[1]	No. of dogs	Numbers of worms per dog (range)	Geometric mean[2] number of worms per dog	% Efficacy[3]
Pyrantel pamoate	8	63 - 105	74.8 [a]	23.2
Fenbendazole	8	57 – 94	72.0 [a]	26.1
Milbemycin oxime	8	55 – 115	88.9	8.8
Emodepside + praziquantel	8	0 – 1	0.4 [a]	99.6
Non-treated control	8	71 - 132	97.4	NA

[1] Nemex®-2 (pyrantel pamoate), Panacur® C (fenbendazole), Interceptor® (milbemycin oxime)
[2] Worm counts were logarithmically transformed (ln [count + 1]), averaged and then back-transformed to approximate the geometric means
[3] Efficacy was calculated using the formula: [(geometric mean worm count control group – geometric mean worm count treated group) / (geometric mean worm count control group)] x 100
[a] Statistically significant compared to the non-treated control group ($p < 0.05$)
NA: Not applicable

Table 2 | Fecal egg counts (FEC) in eggs per gram for each dog on each study day (SD) that FEC were performed.

	Post-infection			Post-treatment		
Treatment	**SD 19**	**SD 20**	**SD 22**	**SD 27**	**SD 31**	**SD 34**
Pyrantel pamoate	2375	2450	3900	3850	1875	5575
	2775	2250	2025	2075	1825	4850
	2875	3450	2550	2050	2675	1475
	1275	2325	2200	1950	1475	2275
	1200	3875	2450	2800	3025	7450
	2725	2125	1750	4425	4950	5150
	625	1875	2500	4600	1525	1050

	725	825	2875	3425	1775	1175
Fenbendazole	1150	1425	1975	0	425	375
	4250	2425	2550	25	500	2550
	1450	1350	1125	225	700	2100
	2375	3075	2350	25	700	875
	1700	1750	1450	0	650	1325
	3050	2350	3275	0	475	2350
	1025	2425	3075	0	50	425
	2500	2100	3000	0	225	1025
Milbemycin oxime	3625	3500	1875	4775	2425	17875
	1225	2925	2300	3575	2025	1375
	2400	2200	1550	2100	1725	2075
	2025	3200	3800	2575	1575	2375
	1175	1525	2350	1850	1425	3875
	1625	2075	2175	2200	2475	6075
	1475	1250	1375	1950	2975	13400
	1650	2300	2325	1500	1325	2875
Emodepside + praziquantel	1475	2275	3950	0	0	0
	1575	1750	3250	0	0	0
	925	875	2000	0	0	0
	3050	3475	3125	0	0	0
	1725	2525	2025	0	0	0
	1300	1825	3200	0	0	0
	1800	2650	3025	0	0	0
	1250	1650	2175	3475	0	0
Non-treated control	500	1225	2075	1625	3175	4850
	925	3300	2775	4250	3450	6225
	1650	4225	2125	2325	2375	3050
	950	2950	2000	275	1250	1475
	925	2575	5550	6825	2350	11550
	2150	3725	2650	2425	2325	2775
	2375	1650	1525	3650	2000	2100
	1025	1925	1450	2000	2350	1300

[1] Nemex®-2 (pyrantel pamoate), Panacur® C (fenbendazole), Interceptor® (milbemycin oxime)

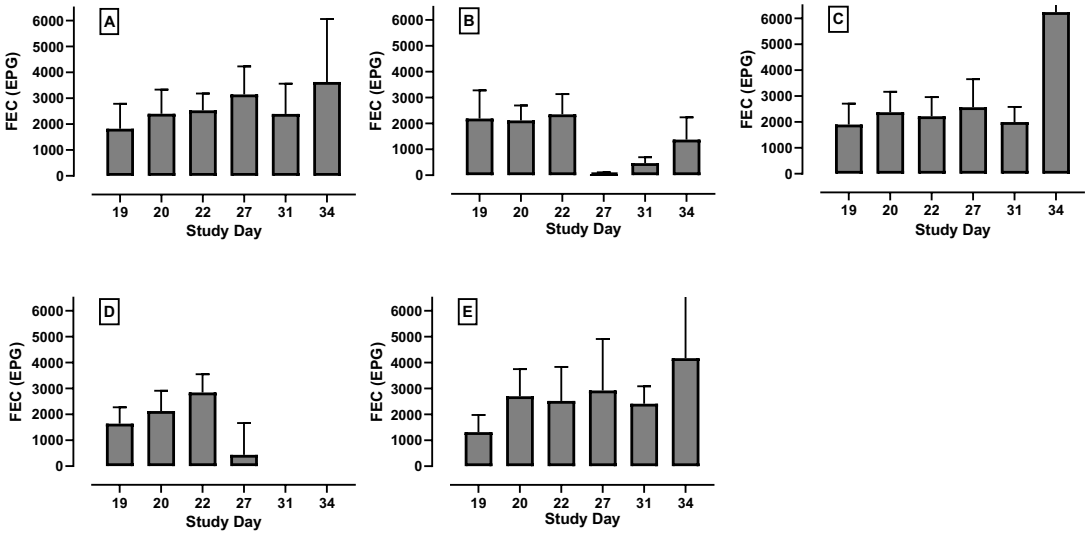

Figure 1. | Arithmetic mean fecal egg counts (FEC) and standard deviation for groups of eight dogs infected with the Worthy 4.1F3P isolate of *Ancylostoma caninum* on study days (SD) 19, 20, 22, 27, 31 and 34. Treatments with pyrantel pamoate (A), fenbendazole (B), milbemycin oxime (C), emodepside + praziquantel (D), and non-treated control (E) were administered on SD 24 per approved label instructions.

CHAPTER 4

MULTIPLE DRUG RESISTANCE IN HOOKWORMS INFECTING GREYHOUND DOGS IN THE USA[3]

[3] Jimenez Castro PD, Venkatesan A, Redman E, Chen R, Malatesta A, Huff H, Zuluaga Salazar DA, Avramenko R, Gilleard JS, Kaplan RM. Multiple drug resistance in hookworms infecting greyhound dogs in the USA. *Submitted to International Journal of Parasitology: Drugs and Drug Resistance, April 30th, 2021.*

ABSTRACT

Ancylostoma caninum is the most prevalent nematode parasite of dogs. We confirmed multiple-drug resistance (MDR) in several A. caninum isolates to all anthelmintic drug classes approved for the treatment of hookworms in dogs in the USA. Cases of MDR hookworms appear to be highly overrepresented in greyhounds. The aims of this study were to evaluate the drug-resistant phenotypes and genotypes of the A. caninum infecting greyhounds. Fecal samples from greyhounds of the USA were acquired from two greyhound adoption kennels, one active greyhound racing kennel, and three veterinary practices. Fecal egg counts (FECs) were performed on fecal samples from 219 greyhounds, and despite treatment with anthelmintics, the mean FEC was 822.4 eggs per gram (EPG). Resistance to benzimidazoles and macrocyclic lactones were measured using the egg hatch assay (EHA) and the larval development assay (LDA), respectively. We performed 23 EHA and 22 LDA on either individual or pooled feces, representing 54 animals. Mean and median IC50 and IC95 values for the EHA were 5.3 μM, 3.6 μM, and 24.5 μM, 23.4 μM, respectively. For the LDA, the median IC50 value was >1000 nM. These values ranged 62-81 times higher than our susceptible laboratory isolates. Only post-treatment samples representing 219 greyhounds were collected. For samples collected <10 days post-treatment with albendazole, moxidectin, or a combination of febantel-pyrantel-moxidectin, the mean FEC were 349, 333, and 835 EPG, respectively. We obtained DNA from hookworm eggs isolated from 70 fecal samples, comprised of 60 individual dogs and 10 pools. Deep sequencing of the isotype 1 β-tubulin gene only revealed the presence of the F167Y (TTC>TAC) resistance polymorphism in 99% of these samples. These clinical, in

vitro, and genetic data provide strong evidence that greyhound dogs in the USA are infected with MDR A. caninum at very high levels in prevalence and infection intensity.

Keywords: *Ancylostoma caninum*, hookworms, multiple-drug resistance (MDR), deep-amplicon, greyhounds

1. Introduction

The canine hookworm, *Ancylostoma caninum* is the most prevalent and important intestinal nematode parasite of dogs in the USA, with the prevalence depending on age, level of care and geographic location of the dog (Little et al., 2009). A recent study evaluating over 39 million fecal samples from 2012-2018, found that the prevalence of hookworms remained very stable from 2012-2014 at around 2%, but then from 2015 onwards, there was a steady yearly increase, with an overall increase of 47% by 2018 (Drake and Carey, 2019). Moreover, in a study assessing intestinal parasites from 3006 dog fecal samples collected in 288 off-leash dog parks across the USA in 2019, the prevalence of *A. caninum* was 7.1% (Stafford et al., 2020). Interestingly, this prevalence is more than twice as high as that reported for 2018 by (Drake and Carey, 2019), and is more than 70% higher than the mean prevalence for 2017-2019 reported by (Sweet et al., 2021). Taken together these data suggest that hookworm prevalence is rapidly increasing, and that dogs that visit dog parks are at a higher risk of infection.

Anthelmintic drugs currently approved for the treatment of *A. caninum* in the United States include, febantel and fenbendazole, moxidectin and milbemycin oxime, and pyrantel, of the benzimidazole, avermectin/milbemycin, and tetrahydropyrimidine classes,

respectively. In registration studies, febantel, moxidectin and milbemycin oxime all demonstrated efficacies of >99% (F.D.A, 1994, 1998, 2006), fenbendazole demonstrated efficacy of >98% (F.D.A, 1983) and pyrantel demonstrated a slightly variable efficacy, with a mean across studies of approximately 94%, where more than half of those studies yielded >99% (F.D.A, 1993).

Hookworms are blood-feeding nematodes that use a cutting apparatus to attach to the intestinal mucosa and submucosa, and contract their muscular esophagus to create negative pressure, which sucks a plug of tissue into their buccal capsules (Hotez et al., 2004). Bleeding is facilitated by both mechanical damage and chemical action by hydrolytic enzymes that cause rupture of capillaries and arterioles (Stassens et al., 1996). Pathological consequences of infection in dogs include iron-deficiency anaemia, hypoalbuminemia, and an enteritis characterized by diarrhoea, that may contain fresh (haematochezia) or digested blood (melena) (Kalkofen, 1987; Epe, 2009; Taylor et al., 2016).

In the past few years, there is empirical evidence that veterinarians are diagnosing increasing numbers of cases of persistent hookworm infections, primarily in greyhound dogs, that appear refractory to typical anthelmintic therapy. Recent work in our laboratory confirmed that many, if not most, of these persistent hookworm cases are likely due to multiple-drug resistance (MDR) in *A. caninum*, with retired racing greyhounds highly over-represented among the cases reported to our laboratory (Jimenez Castro et al., 2019). Our laboratory established one of these *A. caninum* isolates (Worthy), which we obtained from a recently adopted retired racing greyhound dog. In a controlled efficacy study, we confirmed high levels of resistance to all classes of drugs approved for treatment of

hookworm in dogs; fenbendazole, pyrantel pamoate and milbemycin oxime yielded efficacies of 26%, 23% and 9%, respectively (Jimenez Castro et al., 2020). *A. caninum* is also zoonotic in humans, and MDR *A. caninum* will not be susceptible to usual anthelmintic treatments administered by physicians.

At its peak in 1991, greyhound racing was rated the sixth most popular sporting activity in the USA, was legal in 19 states, and generated around 100,000 jobs with wager revenues of $3.5 billion USD (Theil, 2021). At that time there were 38,000 individual pups and 5,700 registered racers, but by 2020 those numbers had dramatically decreased to 4,300 and 850, respectively (Gartland, 2021). Kansas (KS), the state with the most greyhound breeding farms, had 274 greyhound breeding farms in the 1990's, but by 2015 this number had decreased by more than half to 130 (Hall, 2016), and continues to fall. These farms tend to have large dog populations; more than 60% and 80% of all racing greyhounds come from farms with >250 dogs and >100 dogs, respectively (Hall, 2016).

In November, 2018, voters in Florida (FL) passed Amendment 13, a constitutional amendment which banned wagering on live dog races, including greyhound racing in the state as of January 1st, 2021 (State, 2018). In 2018, 65% of the greyhound tracks in the USA were in FL. However, with the closure of these tracks and others in several other states in the past few years, to our knowledge there currently are only 7 tracks in 5 states remaining. This change likely represents the beginning of the end for greyhound racing in the USA. In parallel, a large network of greyhound adoption groups has been active for many years, having over 160 organizations across the USA and Canada with the majority being in FL followed by New York (NY) and Ohio (OH). This results in tens of thousands of retired racing greyhounds being adopted as pets every year (Lord et al., 2007), and with

the demise of the greyhound racing industry, the number of adoptions are rapidly increasing. Thus, it is important for the health of both racing greyhounds and pet dogs to determine the extent of the MDR hookworm problem in racing greyhounds.

The aims of this study were to investigate the prevalence of infection, the range of *in vitro* and *in vivo* drug susceptible/resistant phenotypes, and the frequency of benzimidazole-resistant beta-tubulin genotypes in greyhound dogs infected with *A. caninum*.

2. Materials and Methods

2.1 Sample collection

From February 2019 to February 2020, fecal samples were acquired from two greyhound adoption kennels located in Birmingham, Alabama (AL), and Dallas, Texas (TX), one active greyhound racing kennel in Sanford, FL, and three veterinary practices located in Acworth, Georgia (GA), Columbia, South Carolina (SC), and St. Petersburg, FL USA that work with greyhound adoption organizations. Most samples were collected from individual dogs, but from the Sanford, FL site, only anonymous samples from the ground were available. The dogs residing in these kennels originated from 16 different locations in 8 different states. These included five breeding farms located in Kansas (KS), Colorado (CO), Arkansas (AR), Texas (TX), or Oklahoma (OK), and 11 racing tracks located in Alabama (AL), FL, AR, or West Virginia (WV).

2.2 *In vitro* assays

To evaluate drug response phenotypes, the egg hatch assay (EHA) and larval development assay (LDA) were used for benzimidazoles (BZs), and macrocyclic lactones (MLs), respectively as previously described (Jimenez Castro et al., 2019). The concentration

ranges for ivermectin aglycone (1.9 – 1000 nM) in the LDA, and thiabendazole (0.075 – 40 μM) in the EHA were selected based on our previous work to allow the discrimination of susceptible versus resistant isolates of *A. caninum* (Jimenez Castro et al., 2019). EHA plates were sealed with parafilm and stored in the refrigerator at 4°C for a maximum of one week. LDA plates (Microbial Screening Technologies, Armidale, New South Wales, Australia) were vacuum sealed upon arrival and stored in the refrigerator at 4°C for a maximum of 10 months. Previous experience in our laboratory has demonstrated that LDA plates containing ML stored in this manner provided consistent results for well over a year. Prior to performing the assays plates were removed from the refrigerator and permitted to reach room temperature. Eggs were isolated using 50 mL tubes containing activated charcoal granules (0.5 – 1 cm) and specialized lids, which essentially were a filter that could attach firmly to 15 mL centrifuge tubes. Five to ten grams of feces and approximately 15 mL of water were added to the 50 mL tube containing approximately 5 grams of activated charcoal and vigorously shaken by hand to break up the feces. The lid of the 50 mL tube was removed and replaced with the specialized lid, and a 15 mL tube was attached to the other end. The apparatus was then shaken again which allowed the filtered fecal suspension to fill the 15 mL tube, which was then centrifuged at 240 x *g* for 10 mins. The supernatant was discarded, 10 ml of sodium nitrate (Feca-Med®, Vedco, Inc. St. Joseph; MO, USA specific gravity = 1.2) were added and the tube was vortexed to disperse the 1-2 mls of fecal material left as sediment. The tube was then centrifuged again at 240 x *g* for 10 mins. Following centrifugation, the supernatant containing the eggs was passed through a 20 μm stainless steel sieve, rinsed with distilled water, transferred to a new tube, and then the volume was adjusted to yield 50-60 eggs per 20 μl using distilled water. If insufficient

eggs were recovered to perform both the EHA and the LDA, then only the EHA was performed.

2.3 *In vivo* measurements

Every dog sampled in this study was treated with anthelmintics either every two weeks or once a month; therefore, all of the samples were collected relatively recently post-treatment. Samples were refrigerated immediately after collection and shipped to the Kaplan lab at the University of Georgia in a container with ice packs. In order to account for the differences in timeframe since the previous anthelmintic treatment, dogs were assigned to one of three categories based on the following biological factors: (A) = <10 days, as this can be too soon to measure an accurate fecal egg count reduction (FECR) and can lead to false positives due to temporary inhibition of egg production (Jimenez Castro et al., 2019; Jimenez Castro et al., 2020), (B) = 10 – 21 days, as this would be an optimal timeframe for measuring the FECR, and (C) = > 21 days, as there is the possibility that eggs could be shed from reactivated encysted/arrested larvae that migrated to the small intestine and completed development to sexually mature adults following the anthelmintic treatment (due to "larval leak") (Jimenez Castro and Kaplan, 2020). Fecal egg counts (FEC) were performed using the Mini-FLOTAC (University of Naples Federico II, Naples, Italy) procedure with a detection threshold of 5 EPG (Maurelli et al., 2014; Lima et al., 2015), adding two grams of feces to 18 ml of sodium nitrate (Feca-Med®, Vedco, Inc. St. Joseph; MO, USA specific gravity = 1.25 to 1.30). Positive samples were defined as having a FEC of ≥ 5 EPG. All anthelmintic treatments were administered by either kennel or veterinary practice personnel. Where products approved for use in dogs were used,

treatments were administered according to label instructions; these included febantel-pyrantel pamoate, Drontal® Plus (Elanco, Greenfield, IN), moxidectin, Advantage® Multi (Elanco, Greenfield, IN) and pyrantel pamoate, Nemex-2® (Zoetis, Kalamazoo, MI). In some instances, products labelled for large animals were used; these included moxidectin, Quest® Plus (Zoetis, Kalamazoo, MI) and albendazole, Valbazen® (Zoetis, Kalamazoo, MI). These products were administered orally at approximately 3.3 mg/kg and 19 mg/kg, respectively. In some cases, compounded drugs were used, such as pyrantel pamoate, praziquantel, and mebendazole which are included in the PPM Triwormer (Roadrunner pharmacy, Phoenix, AZ).

2.4 *Ancylostoma caninum* isotype-1 beta-tubulin deep amplicon sequencing

2.4.1 DNA preparation

After setting up the *in vitro* assays, the remaining eggs were transferred to 2 ml cryotubes (Sigma-Aldrich, St. Louis, MO), suspended in a final concentration of 70% ETOH and stored at -80°C until further use. DNA lysates were prepared from individual or pooled egg samples. Briefly, 3 freeze-thaw cycles were carried out at -80°C and at 55°C respectively, followed by adding 180 µL of DirectPCR (Cell) Lysis Buffer (Catalog No. 301-C, Viagen Biotech, St. Louis, MO) and 20 µL of Proteinase K (Catalog No. 19133, QIAGEN, Hilden, Germany). Samples were then incubated for at least 12h at 65°C, then 1h at 95°C, and were then cooled to 4°C. DNA was purified from the crude DNA lysates using the QIAGEN QIAmp DNA mini kit (Cat# 51306), following the manufacturer's recommended protocol, and stored at -80°C.

2.4.2 Deep- amplicon sequencing assay analysis

Deep amplicon sequencing assays developed to evaluate the frequency of non-synonymous single nucleotide polymorphisms (SNP) at codons 167, 198 and 200 of the *A. caninum* isotype-1 β-tubulin gene were applied to 70 samples ranging from 200 - 20,000 eggs (mean of 1670, standard error of mean 296.9) from the two adoption kennels, one active racing kennel, and from one of the veterinary practices. Using adapted primers suitable for Illumina deep-sequencing, two separate regions of the *A. caninum* isotype-1 β-tubulin gene, comprising 293 bp and 340 bp which encompass codon 167, and codons 198 and 200, respectively, were PCR amplified (Jimenez Castro et al., 2019). The following PCR conditions were used: 5µL KAPA HiFi Hotstart fidelity buffer (5X) (KAPA Biosystems, USA), 1.25µL forward primer (10µM), 1.25µL reverse primer (10µM), 0.75µL dNTPs (10µM), 0.5µL KAPA HiFi polymerase (0.5U), 0.1µL bovine serum albumin (Thermo Fisher Scientific), 14.15µL H2O, and 2µL of DNA lysate. The thermocycling parameters were 95 ℃ for 3 min, followed by 45 cycles of 98 ℃ for 20 s, 65 ℃ for 15 s, and 72 ℃ for 30 s, followed by 72 ℃ for 2 min. Sample purification and addition of barcoded primers followed the protocols defined in (Avramenko et al., 2019). Library preparation was as previously described and library sequencing performed using the Illumina MiSeq platform with the 2 × 300v3 Reagent Kit (Illumina Inc., San Diego, CA, USA) (Avramenko et al., 2015). For the fragment encompassing codon 167, two independent PCR reactions were performed on 66 samples and the libraries were sequenced in two independent sequencing runs using the Illumina MiSeq platform with the 2 x 300 v3 Reagent Kit.

2.4.3 Sequence analysis

Cutadapt v3.2 (Martin, 2011) was used to remove the *A. caninum* forward and reverse primer sequences. Following adapter trimming, all the forward and reverse reads were processed using the DADA2 bioinformatic pipeline to obtain Amplicon Sequence Variants (ASVs) (Callahan et al., 2016). During the quality filtering step of the DADA2 pipeline, the default setting with the following additional settings were used: (i) forward and reverse reads were trimmed to a length of 280 bp and 190 bp, respectively and (ii) reads shorter than 50 bp or with an expected error of > 1 or > 2 in the forward and reverse reads respectively were removed. The DADA2 algorithm was then applied to the filtered and trimmed reads to identify the ASVs. Following this, the overlapping forward and reverse reads were merged, allowing a maximum mismatch of 4 bp in the overlap region. The ASVs generated using the DADA2 pipeline were then aligned to the *A. caninum* isotype-1 β-tubulin reference sequence (Genbank Accession: DQ459314.1) using a global (Needleman-Wunsch) pairwise alignment algorithm without end gap penalties. Following alignment, the ASVs were discarded if they were <180 bp or >350 bp long, or if they had a percentage identity <70% to the reference sequence, or if the ASVs had fewer than 200 reads in a sample, or if they were not present in two or more samples. This additional filtering ensures the removal of spurious sequences. In summary, of 2,081,485 total paired-end reads inputted into the DADA 2 analysis pipeline, 822,023 merged paired-end reads were outputted for ASV generation and analysis. Following mapping of ASVs to the *A. caninum* isotype-1 β-tubulin reference sequence, and removal of ASVs that had less than a total of 200 mapped reads or were present in only one sample, 783,793 reads remained for variant calling. The codons 167, 198 and 200 were then analyzed for the presence of any variants resulting in non-synonymous changes. The MUSCLE alignment tool was used

to align the filtered ASVs from both the fragments with isotype-1 and 2 β-tubulins from other nematodes present in Clade V of the nematode phylogeny. Using the Geneious tree builder, a neighbour-joining tree, utilizing the Jukes Cantor tree building method, was constructed from the trimmed alignment, and having *H. contortus* isotype-3 β-tubulin as the outgroup (Genbank Accession: HE604101) and 2000 bootstrap replicates.

2.5 Data analyses

Dogs treated solely with albendazole and moxidectin were represented in each of the post-treatment timeframe categories, therefore for each drug individually, statistical analyses were performed to determine if the FEC of the dogs differed between those categories. For this a Kruswal-Wallis test was performed for the overall comparison with a Benjamini-Krieger-Yekutielli procedure for individual pairwise comparisons. For the EHA and LDA, dose-response analyses were performed after log transformation of the drug concentrations and constraining the bottom value to zero. The top parameter for samples that did not reach 100% inhibition was constrained to 100 to avoid introducing artificial bias to the model. Data were then fitted to a four-parameter non-linear regression algorithm with variable slope. The IC_{50} or IC_{95} values, which represent the concentration of drug required to inhibit hatching (EHA) or development to the third larval stage (LDA) by 50% or 95% of the maximal response were calculated.

For comparing the infection prevalence between states, a Chi-square test was performed for the overall comparison, followed by a Fisher's exact test with a Bonferroni procedure for individual pairwise comparisons between states. All analyses were designed to maintain the overall type I error rate at 5%. To evaluate the level of agreement between the two independent PCR amplifications and separate sequencing runs of the fragment

encompassing codon 167, a Bland-Altman analysis was performed. To quantify the relationship between the EHA and the deep-amplicon sequencing assay to measure levels of BZ resistance, a Spearman correlation analysis was performed comparing both IC_{50} and IC_{95} values with the F167Y SNP frequencies. All statistical analyses were performed in GraphPad Prism® version 9.0.2, GraphPad Software, San Diego, CA, USA.

3. Results

3.1 Fecal egg count data

171 of the 219 fecal samples from racing or recently retired greyhounds were positive for hookworm eggs, yielding an overall prevalence of 79%, with a mean FEC of 822.4 EPG (Table 1). The prevalence of infection for the Birmingham, AL ($p = 0.0063$) and Sanford, FL ($p < 0.0001$) kennels were significantly higher than for the Dallas, TX kennel. Percent reductions in FEC following treatments could not be calculated since pre-treatment FEC were not available. However, in the 197 (89.9%) samples collected 2 – 21 days post-treatment, the mean FEC was 721 EPG, indicating a major lack of efficacy across the different treatments. For the group of samples collected following moxidectin treatment, samples collected > 21 days post-treatment had a statistically significant higher mean FEC when compared to the other two categories (Table 2). For the group of samples collected following albendazole treatment, the 10-21 and >21 days categories had a statistically significant higher mean FEC when compared to the <10 days. Interestingly, a single group of samples from nine dogs recently acquired from a breeding farm in KS had <5 EPG following treatment. For all other sites, mean FEC were 330 EPG or greater.

3.2 *In vitro* assays

EHA and LDA were performed on 35 samples, yielding dose-response data on 23 and 22 samples, respectively, which represented samples collected from 54 greyhounds (Fig. 1).

Mean hatching rates of eggs in the assays ranged from 82 – 98%. For the EHA, mean and median IC_{50} and IC_{95} values were 5.3 µM, 3.6 µM, and 24.5 µM, 23.4 µM, respectively (Table 3). For the LDA, IC_{50} and IC_{95} values were calculated in a subset of assays, however for the majority of assays, IC_{50} values could not be calculated since this value was greater than the highest concentration tested. Likewise, IC_{95} values could not be calculated for the majority of assays performed. Consequently, the mean and median values for both parameters have no real usefulness and are not reported. IC_{50} and IC_{95} values were calculated for 22 samples with a range of 28.1 nM to > 1000 nM. 16 samples (73%) had IC_{50} > 1000 nM.

3.3 Relative frequencies of the isotype-1 β-tubulin benzimidazole resistance associated polymorphisms

The three codons in the isotype-1 β-tubulin gene known to have BZ resistance-associated polymorphisms (167, 198 and 200) in Strongylid nematodes were examined using deep-amplicon sequencing. The average read depth for the fragment containing codon 167 was ~10,400, ranging between 3,180 to 24,732 reads across the samples. For the fragment containing codons 198 and 200, the average read depth was ~22,700, ranging between 7,890 to 37,060 reads. Only the F167Y (TTC>TAC) resistance polymorphism was detected and this was present in 99% (69/70) of the samples that were sequenced, and this polymorphism was found at high frequencies in most positive samples (Fig. 2). In 48 out of the 70 samples (69%), the frequency of the resistant allele was ≥75%. In 29% of the

samples, and in at least one sample from each greyhound kennel, the allele frequency was ≥90%. All greyhound kennels had samples with at least a 60% frequency of the F167Y SNP. Only 7% of the samples had <25% of the resistant allele, and only one sample had 100% frequency of the susceptible allele. These frequencies had a high level of agreement (bias = -0.02 and 95% limits of agreement of -0.14 to 0.10) between the two independent sequencing runs for the fragment containing codon 167 (Supplementary Fig 1). No SNPs were detected at codons 198 nor 200 (Supplementary Fig. 2). The ASVs for the amplicons spanning codons 167 and codons 198 and 200 were aligned to other nematode β-tubulins using MUSCLE alignment tool. This alignment was trimmed, and a neighbour-joining tree was constructed using Geneious tree builder with *H. contortus* isotype-3 β-tubulin (Genbank accession: HE604101). All the ASVs formed a monophyletic cluster with *Ancylostoma* isotype-1 β-tubulin (supplementary Fig. 3 and 4).

3.4 Comparison of F167Y (TTC>TAC) frequency and egg hatch assay phenotype

Both EHA (phenotypic) and β-tubulin allele (genotypic) data were only available for 15 samples. There was not a statistically significant correlation between the IC_{50} and resistant SNP F167Y allele frequency (p = 0.08), (r = 0.48), however, there was a significant correlation with the IC_{95} (p = 0.04), (r = 0.54) (Fig. 3).

4. Discussion and conclusions

The present study provides strong and conclusive evidence that racing greyhounds in the USA are infected with MDR *A. caninum* at a very high prevalence, and with wide geographic distribution. Very high IC_{50} and IC_{95} values were measured for both the benzimidazoles and macrocyclic lactones when compared to the susceptible isolate from

our previous work (Jimenez Castro et al., 2019), indicating that almost every sample was resistant to both drugs. The F167Y (TTC>TAC) benzimidazole resistance polymorphism was detected, in 99% of the samples, and at high frequencies in more than 2/3 of the samples. All three greyhound kennels had samples with at least a 60% frequency of the F167Y SNP, and every sample from the Sanford, FL site had at least a 70% frequency of the resistant SNP. These data are consistent with levels we reported for our MDR (Worthy) lab isolate, which yielded F167Y SNP frequencies of 87.6 - 94.5% over several different passages (Jimenez Castro et al., 2019). Furthermore, in a controlled efficacy study using Worthy 4.1F3P, we measured an efficacy of 26% for fenbendazole, confirming that this high F167Y SNP frequency was associated with a very low *in vivo* efficacy (Jimenez Castro et al., 2020).

Although additional genetic analyses are required for confirmation, the available clinical and genetic evidence strongly suggests that these MDR *A. caninum* evolved on greyhound breeding farms and kennels. Thus, it is germane to this issue to examine the clinical and epidemiological factors that may be responsible for the development of these MDR worms, and to hypothesize why/how this problem became so prevalent and widespread before it was recognized. *Ancylostoma caninum* is the most prevalent parasitic nematode in racing greyhounds (Jacobs and Prole, 1976; Ash et al., 2019), and this is attributed to the near constant exposure of these dogs to infective third stage larvae in the sand/dirt exercise run/pens, which are ideal for hookworm transmission (Ridley et al., 1994). The combination of large numbers of animals and high transmission rates produces large effective populations of worms and increases the probabilities that resistance mutations will occur (Gilleard, 2006; Redman et al., 2015). Racing greyhounds are also

treated extremely frequently with multiple different anthelmintics (e.g., fenbendazole, ivermectin, pyrantel) throughout their lives (Ridley et al., 1994). The intervals between these treatments often are less than the pre-patent period for hookworms, which will minimize the amount of refugia. Thus, genetically-resistant worms surviving treatment will have a large reproductive advantage, and the lack of refugia will lead to a rapid increase in their frequency (Martin et al., 1981; van Wyk, 2001). This combination of factors is known to place heavy selection pressure for drug resistance in nematodes (Wolstenholme et al., 2004), and is very similar to the epidemiological factors that have led to high levels of MDR in gastrointestinal nematodes of sheep and goats, worldwide (Kaplan and Vidyashankar, 2012).

Given the fact that resistance is likely to have developed independently to each drug class, and the fact that MDR *A. caninum* to all three major anthelmintic classes seem to be virtually ubiquitous in racing greyhounds in the USA, one must ask the question; "why has there not been any published reports of resistance to any single drug class previously in the USA greyhound population"? To answer this question, we must examine how veterinarians typically manage hookworm infections in dogs. Typically, when a dog presents to a veterinarian with a fecal positive for hookworms, the dog is treated with one or more drugs from the benzimidazole, macrocyclic lactone or tetrahydropyrimidine classes. If the dog then tests positive again in a future exam, the infection is attributed to reinfection or reactivation of encysted/arrested larvae (larval leak). Consequently, the same treatment regimen is often repeated, or the veterinarian may choose to use a drug from a different drug class. One thing that was never done by small animal clinicians in the past, is measuring the efficacy of the treatment in a fecal egg count reduction test (FECRT)

(Jimenez Castro and Kaplan, 2020), by performing both pre- and post-treatment FEC. As a result, anthelmintic resistance (AR) is not diagnosed, and most often is not even considered as a likely cause of the recurrent hookworm infections. Therefore, as resistance evolves and leads to more recurrent hookworm infections, veterinarians typically treat more often, and rotate and/or combine drugs. But they do not perform FECRT to measure the efficacy of the various drugs administered. Thus, as long as one drug remains effective, or several drugs each retain some modest level of efficacy, the problem will appear to be managed by the drug(s), and recurrent infections will continue to be attributed to reinfection or reactivation of encysted/arrested larvae. However, once high levels of MDR to all drugs evolves, it becomes difficult to manage the infections, and the likelihood that anthelmintic resistance will be recognized increases. Our data demonstrate evidence of very high IC_{50} and IC_{95} values for benzimidazoles and the macrocyclic lactones. With regards to pyrantel, no *in vitro* or molecular assays currently exist for measuring resistance. However, in every suspected MDR case we have treated with pyrantel pamoate there is virtually no efficacy based on FEC reduction, (Jimenez Castro et al., 2019), and in a controlled efficacy study using Worthy 4.1F3P, pyrantel pamoate yielded an efficacy of only 23% (Jimenez Castro et al., 2020).

Regarding benzimidazole resistance, the Sanford, FL kennel applied the greatest benzimidazole selection pressure of any of the kennels, treating all dogs twice a month with albendazole, and all dogs tested had F167Y (TTC>TAC) frequencies of at least 70%. When comparing the phenotypic and genotypic data for benzimidazole, the IC_{95} yielded a significant correlation ($p = 0.04$) but the IC_{50} did not ($p = 0.08$). The lack of significance for the IC_{50} may be due to low power, as a consequence of only having 15 samples with

both types of data. However, this finding is consistent with our previous work where we found that the IC_{95} was more appropriate for discriminating susceptible vs resistant isolates using the EHA (Jimenez Castro et al., 2019). Interestingly, there were two samples, Dl 13 and Dl 14 that had IC_{95} values 13 and 18 times higher than the susceptible isolate from our previous work (Jimenez Castro et al., 2019), but had a resistant F167Y SNP allele frequency of only 5%, and 0%, respectively (Supplementary Table 1). This lack of correlation between phenotype and genotype raises three possible explanations: (1) the EHA has a high interassay variability, (2) there are mutations, other than at codons 167, 198 and 200, that are involved with resistance to benzimidazole drugs, or (3) there are loci other than β-tubulin that are involved with resistance to benzimidazole drugs. However, in on our previous work we tested multiple isolates and biological replicates and had rather low interassay variability (Jimenez Castro et al., 2019). Additionally, we previously measured a >100-fold increase in the EHA IC_{50} in the Worthy isolate following treatment with fenbendazole, but the SNP allele frequency remained relatively unchanged (Jimenez Castro et al., 2019). Together, these findings lend support to the hypothesis that there are non-β-tubulin mutations that are involved in resistance to benzimidazole drugs in *A. caninum*. Evidence of this has already been reported in *Caenorhabditis* spp. where a quantitative trait loci that did not overlap with β-tubulin genes was identified in two genetically divergent isolates (Zamanian et al., 2018). Also, using genome wide association mappings in *C. elegans*, novel genomic regions independent of *ben-1* and other β-tubulin loci were correlated with resistance to albendazole (Hahnel et al., 2018). Additionally, disparity in responses in *C. elegans* to fenbendazole and albendazole provided evidence that the former could have additional targets beyond β-tubulin, such as genes that encode

β-tubulin interacting proteins (Dilks et al., 2020). Thus, our observations demand further study.

Our previous work demonstrated that the LDA provided excellent discrimination between susceptible and resistant *A. caninum* isolates for the MLs (Jimenez Castro et al., 2019). In the current study the median IC_{50} value was >83 times higher than the susceptible isolate value from our previous work. This is both extremely high and an underestimation, since the IC_{50} of many samples could not be accurately measured given that they exceeded the highest concentration tested. Furthermore, of all the samples that had LDA assays with IC_{50} values >1000 nM, all but one had moxidectin as the last treatment administered, and that one group was administered moxidectin in the previous treatment. Macrocyclic lactones, particularly, ivermectin, have been used intensively by the greyhound industry for parasite control for the past several decades (Ridley et al., 1994). However, to our knowledge, moxidectin, which is a substantially more potent member of this drug class (Prichard et al., 2012), has only recently started to be used on greyhound farms and kennels. In *H. contortus*, ivermectin resistant worms that are naïve to moxidectin are typically killed at very high efficacy following administration of moxidectin (Craig et al., 1992; Oosthuizen and Erasmus, 1993); however, once moxidectin is used regularly in an ivermectin-resistant population, resistance to moxidectin can develop rapidly (Kaplan et al., 2007). Evidence suggests this same pattern is occurring in hookworms as well.

When examining the FEC data for moxidectin and albendazole by timeframe post-treatment, we found significant differences for both drugs, but in a different pattern. For the group of samples collected following albendazole treatment, the 10-21 (p = 0.0003) and >21 days (p = 0.0004) categories had a statistically significant higher mean FEC when

compared to the <10 days category. This finding is consistent with our previous observations in the Worthy isolate, where we documented a temporary suppression on worm fecundity following treatment with fenbendazole (Jimenez Castro et al., 2019; Jimenez Castro et al., 2020). In contrast, for the dogs treated with moxidectin, there was no difference between the <10 and 10-21 day timeframes, but the >21day period had significantly higher FEC ($p = 0.0002$) and ($p < 0.0001$), respectively. This is also consistent with our previous observations where no reduction in fecundity was seen in moxidectin-resistant *A. caninum* following treatment with ML drugs. The higher EPG in the >21day period is likely due to reinfection and/or reactivation of arrested larvae.

The almost ubiquitous presence of MDR worms in recently retired greyhounds, combined with the demise of the greyhound racing industry and increasing numbers of greyhound adoptions, poses a serious risk to the health of pet dogs. From 2009-2019, the number of dog parks increased by 74% in the USA (TPL, 2019), and a recent survey showed that the prevalence of *A. caninum* in dogs visiting these parks (Stafford et al., 2020) was more than 70% higher as compared to the prevalence in all pet dogs recorded over the same general timeframe (Sweet et al., 2021). These relative prevalence data should not be surprising; a fecal pile deposited by a 30 kg dog with an *A. caninum* FEC of ~1000 EPG will contain approximately 500,000 eggs. If not picked up, tens to hundreds of thousands of infective larvae are likely to contaminate the surrounding soil from this one defecation. Consequently, there is a high probability of transmission to other dogs visiting the dog park, and once infected, anthelmintic treatment of these dogs with the three most commonly used drug classes will have little efficacy. The end result will be a continual cycle of infection and transmission that is not interrupted by monthly treatments with

heartworm preventive products, or with anthelmintics administered specifically to treat the hookworm infections. When this is considered in light of the fact that resistance in *A. caninum* was not reported in greyhounds until the worms were already MDR, we believe it is likely that anthelmintic-resistant *A. caninum* are already quite common in pet dogs.

Finally, given these new alarming data, it is urgent that studies be performed to determine the prevalence and geographic distribution of drug-resistant *A. caninum* in the general pet dog population. Additionally, studies investigating the haplotype diversity of the susceptible and resistant alleles in *A. caninum* isolates from both greyhounds and the general pet dog population from different geographic regions are likely to provide deeper insights into the molecular epidemiology and the origin(s) of these MDR worms.

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Conflict of interest statement

The authors do not report any conflict of interests.

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Table 1. Mean fecal egg count (FEC) data and percent prevalence of hookworm infections from 219 greyhound dog samples obtained from two greyhound adoption kennels, one greyhound racing kennel, and veterinary practices. Data from the three veterinary practices were combined for reporting purposes. Positive samples were defined as having a FEC of ≥ 5 eggs per gram (EPG).

Adoption kennel	FEC (EPG)	Prevalence (%) (95%CI) *	Total # of samples
Birmingham, AL	527.8	76.3 (65.6,84.2) [a]	80
Sanford, FL	1288.6	87.5 (79.1,93.5) [a]	80
Dallas, TX	683	48.1 (30.1,66.5) [b]	27
Veterinary practices[1]	529.3	90.6 (77.5,97.6)	32
Overall means	822.4	79 (73.3,84.0)	219

[1] Three veterinary practices located in Acworth, GA, Columbia, SC, and St. Petersburg, FL
*Shared superscripts denote non-significant differences

Table 2. Median fecal egg counts (FEC) in eggs per gram (EPG) with the range for greyhound dogs. Samples were obtained following treatments with several different drugs at multiple times points posttreatment. State of origin of the dogs is provided, and where more than one city was represented and city was known, a subscript number indicates the number of cities represented. Timeframe since the previous anthelmintic treatment were categorized as: (A): <10 days, (B): 10 – 21 days, and (C): > 21 days.

Treatment	No. of greyhounds	Categories post-treatment	FEC (range) (EPG)	State of origin[5]
Single drugs				
Moxidectin[1]	20	A	90 (1675) [a]	AL, FL_1, FL_2, FL_3, AR, TX, WV, KS
Moxidectin[1]	41	B	100 (2560) [a]	AL, AR, FL_1, FL_2, FL_4, WV, AR, CO, TX
Moxidectin[1]	53	C	560 (6000) [b]	AL, AR, FL_1, FL_3, FL_4, FL_5, FL_6, WV
Pyrantel pamoate[2]	3	C	370 (820)	FL, AL
Albendazole[3]	20	A	12.5 (2700) [c]	AR, FL
Albendazole[3]	21	B	2075 (6785) [d]	AR, FL
Albendazole[3]	10	C	1225 (4575) [d]	FL
Combinations				
Febantel-pyrantel-moxidectin[4]	20	A	200 (4725)	AL, AR, AR_1, FL_1, FL_3, FL_4, FL_7
Moxidectin[1] + pyrantel[2]	9	C	0	KS

[1] Quest® Plus
[2] Nemex®-2
[3] Valbazen®
[4] Drontal® Plus and Advantage® Multi
[*]Only nematocidal drugs from the commercial products are mentioned

[5] FL_1 = Palm Beach, FL_2 = Daytona Beach, FL_3 = Jacksonville, FL_4 = Naples-Ft. Myers, FL_5 = Sanford, FL_6 = Clermont, FL_6 = Pensacola, AR_1 = West Memphis

Table 3. Geographic origin of the dogs, fecal egg counts (FEC) in eggs per gram (EPG), egg hatch assay (EHA) IC_{50} and IC_{95} values (uM), larval development assay (LDA) IC_{50} values and IC_{95} values (nM), last anthelmintic treatment administered, and days from treatment to sample collection. All dose-response analyses were performed after log transformation of the drug concentrations and constraining the bottom value to zero. Data were then fitted to a four-parameter non-linear regression algorithm with variable slope. Data for the susceptible isolate, Barrow 1.0, was taken from Jimenez Castro et al., 2019.

Sample ID code[1]	FEC (EPG)	EHA IC_{50}	EHA IC_{95} (BZ)	LDA IC_{50}	LDA IC_{95}	Last treatment administered	Days since last	City and State o sample origin[7]
Barrow	N/A	0.17	0.36	12.3	241.8	N/A	N/A	N/A
Bh 2	4725	1.2	16.8	255.5	>1000	Febantel-pyrantel-moxidectin[2]	4	Birmingham, AI
Bh 7	1895	2.4	21.5	43.3	>1000	Febantel-pyrantel-moxidectin[2]	4	Palmbeach, FL
Bh 19	1825	2	24	28.1	>1000	Febantel-pyrantel-moxidectin[2]	4	Palmbeach, FL
Bh 20	2635	1.14	>40	NA	NA	Febantel-pyrantel-moxidectin[2]	4	Birmingham, AI
Bh 25	1440	3.3	15.3	75.8	315.6	Moxidectin[3]	16	Birmingham, AI
Bh 28	1410	2.9	22.7	438.5	>1000	Moxidectin[3]	16	Birmingham, AI
Bh 32	520	5.8	26.1	>1000	>1000	Moxidectin[3]	16	Birmingham, AI
Bh 40	690	4	>40	>1000	>1000	Moxidectin[3]	16	Daytona beach, F
Bh 41	615	8.5	16.3	>1000	>1000	Moxidectin[3]	2	Birmingham, AI
Bh 43	1675	10.3	>40	>1000	>1000	Moxidectin[3]	2	Birmingham, AI
Bh 51	205	14.6	26.9	>1000	>1000	Moxidectin[3]	18	Birmingham, AI
Bh 53	2560	NA	NA	>1000	>1000	Moxidectin[3]	11	Birmingham, AI
Sf 10-20*	472.7	NA	NA	375.4	>1000	Moxidectin[3]	31	Sanford, FL

Sf 61-70*	1353	10.2	>40	>1000	>1000	Moxidectin[3]	30	Sanford, FL
Sf 71-80*	1819	NA	NA	>1000	>1000	Albendazole[4]	30	Sanford, FL
Dl 12	4450	4.8	33.8	>1000	>1000	Unknown	25	KS
Dl 13	600	2.1	6.7	NA	NA	Unknown	25	KS
Dl 14	620	1.5	4.8	NA	NA	Unknown	25	KS
Cl 9	1110	4.8	33.8	>1000	>1000	Pyrantel-mebendazole-moxidectin[5]	4	Sanford, FL
Cl 12	490	NA	NA	>1000	>1000	Pyrantel-mebendazole-moxidectin[5]	9	Sanford, FL
Cl 14	1180	2.4	18.9	>1000	>1000	Moxidectin[3]	5	Birmingham, AL
Cl 16	1665	1.9	6.8	NA	NA	Moxidectin[3]	24	Fort Myers, FL
Sp 1	905	19.6	>40	>1000	>1000	Moxidectin[3]	23	FL
Sp 4	1125	6.1	16.9	NT	NT	Pyrantel[6]	23	FL
Sp 5	2870	6.9	31.5	>1000	>1000	Unknown	23	FL
Sp 6	560	7.3	>40	>1000	>1000	Moxidectin[3]	23	FL
Ac 1	390	2.5	19.4	>1000	>1000	Unknown	Unknown	FL

[1] Samples were received from these locations, greyhound adoption kennels: Bh = (Birmingham, AL), and Dl = (Dallas, TX); greyhound racing kennel: Sf = (Sanford, FL), and veterinary practices: Cl = (Columbia, SC), Sp= (St. Petersburg, FL), and Ac = (Acworth, GA)
[2] Drontal® Plus and Advantage® Multi
[3] Quest® Plus
[4] Valbazen® (albendazole)
[5] PPM Triwormer and Advantage® Multi
[6] Nemex®-2
[7] For some samples the city of origin was not available. City/state refers only to the origin of the sample tested but does not necessarily reflect the origin of the dogs prior to arriving at the respective kennels or veterinary practices.
* Pooled samples comprising ten dogs
NA: Assay data not available due to assay failure
NT: Assay data not available due to insufficient amount of eggs
N/A: Not applicable

Table 4. Single nucleotide polymorphism frequencies for *Ancylostoma caninum* isolates at the three different codons associated with resistance to benzimidazoles from 70 greyhound dog samples comprised of 60 individuals and 10 pools ranging from 5-10 with an average of 8.6 representing a total of 165 greyhounds, obtained from two greyhound adoption kennels, one greyhound racing kennel, and one veterinary practice.

Sample code*	Sample sequenced (# of eggs)	F167Y Freq (%)	SNPs at codon 198 Freq (%)	F200Y Freq (%)
Bh1	1000	72.8	0	0
Bh10	400	85.0	0	0
Bh13	400	76.2	0	0
Bh14	400	100.0	0	0
Bh17	1000	100.0	0	0
Bh19	4000	89.6	0	0
Bh2	2000	92.1	0	0
Bh20	2000	72.4	0	0
Bh25	1800	5.8	0	0
Bh26	1400	90.9	0	0
Bh28	1800	42.1	0	0
Bh29	1200	73.5	0	0
Bh31	1200	16.0	0	0

Bh32	1200	91.8	0	0
Bh33	1200	81.2	0	0
Bh39	1200	75.2	0	0
Bh4	1000	96.2	0	0
Bh40	1000	81.1	0	0
Bh41	1600	78.2	0	0
Bh43	1000	95.2	0	0
Bh44	900	71.0	0	0
Bh45	300	43.0	0	0
Bh47	300	48.0	0	0
Bh48	900	66.4	0	0
Bh49	300	100.0	0	0
Bh51	1000	100.0	0	0
Bh53	1000	88.4	0	0
Bh54	1200	91.8	0	0
Bh55	225	100.0	0	0
Bh61	1600	67.0	0	0
Bh62	1000	86.6	0	0
Bh63	1000	100.0	0	0
Bh65	2300	59.7	0	0
Bh66	1200	83.1	0	0
Bh67	6200	77.2	0	0

Bh68	7000	90.7	0	0
Bh7	1000	86.0	0	0
Bh70	1000	91.9	0	0
Bh71	1700	65.1	0	0
Bh73	800	90.1	0	0
Bh74	1000	82.0	0	0
Bh76	2800	77.8	0	0
Bh77	1200	88.6	0	0
Bh79	1200	73.6	0	0
Bh8	1000	86.9	0	0
Bh80	1100	65.2	0	0
Bh9	2000	70.4	0	0
Cl3	800	80.4	0	0
Cl6	2400	98.2	0	0
Dl11	1000	73.9	0	0
Dl12	2000	87.6	0	0
Dl13	900	5.3	0	0
Dl14	1200	0.0	0	0
Dl17	1000	92.9	0	0
Dl18	1000	23.2	0	0
Dl4	20000	70.6	0	0
Dl7	750	51.8	0	0

Dl8	1000	76.2	0	0
Dl9	1600	100.0	0	0
Sf 10-20	1200	87.6	0	0
Sf 21-30F	1500	85.7	0	0
Sf 21-30Fp	2000	87.5	0	0
Sf 21-30Mp	1050	80.1	0	0
Sf 31-40F	1400	85.0	0	0
Sf 31-40M	1400	81.3	0	0
Sf 41-45	200	97.8	0	0
Sf 51-55M	1200	83.6	0	0
Sf 56-60F	1200	71.9	0	0
Sf 61-70	3000	88.8	0	0
Sf 71-80	1000	83.4	0	0

*Samples were received from these locations, greyhound adoption kennels: Bh = (Birmingham, AL), and Dl = (Dallas, TX); greyhound racing kennel: Sf = (Sanford, FL), and veterinary practice: Cl = (Columbia, SC)

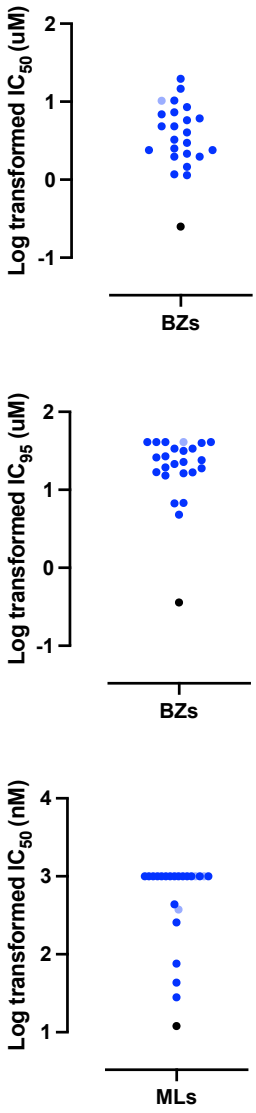

Fig. 1. Scatter dot plots of greyhound samples showing the log transformed Egg Hatch Assay (EHA) IC50 (a) and IC95 (b) values, and the Larval Development Assay (LDA) IC50 (c) values for the benzimidazoles (BZs) and macrocyclic lactones (MLs), respectively. Each dark blue and light blue dot represent an assay performed on an individual or a pooled sample, respectively. The black dot represents the value of our susceptible laboratory isolate for reference. Dose-responses were analysed using the variable slope nonlinear regression model analysis contained in GraphPad 9.0.2.

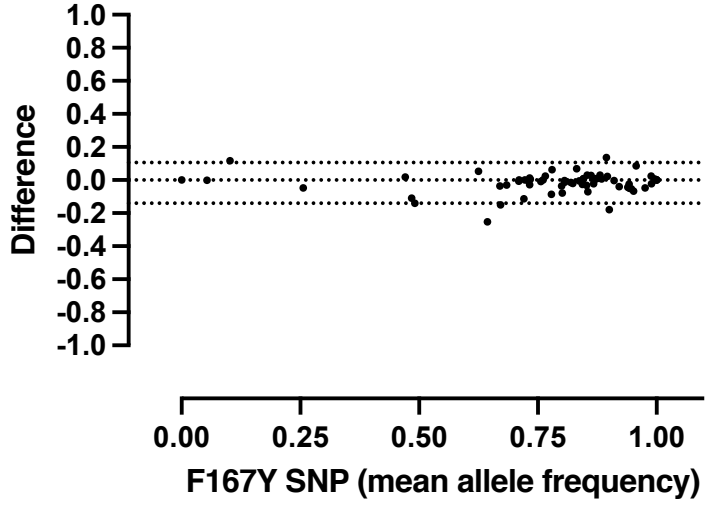

Fig. 2. Bland-Altman plot showing the level of agreement between the resistant allele frequencies reported in two independent PCR amplifications and separate sequencing runs (i.e., Run1 and Run2) of the fragment containing codon 167 on a subset of 66 samples.

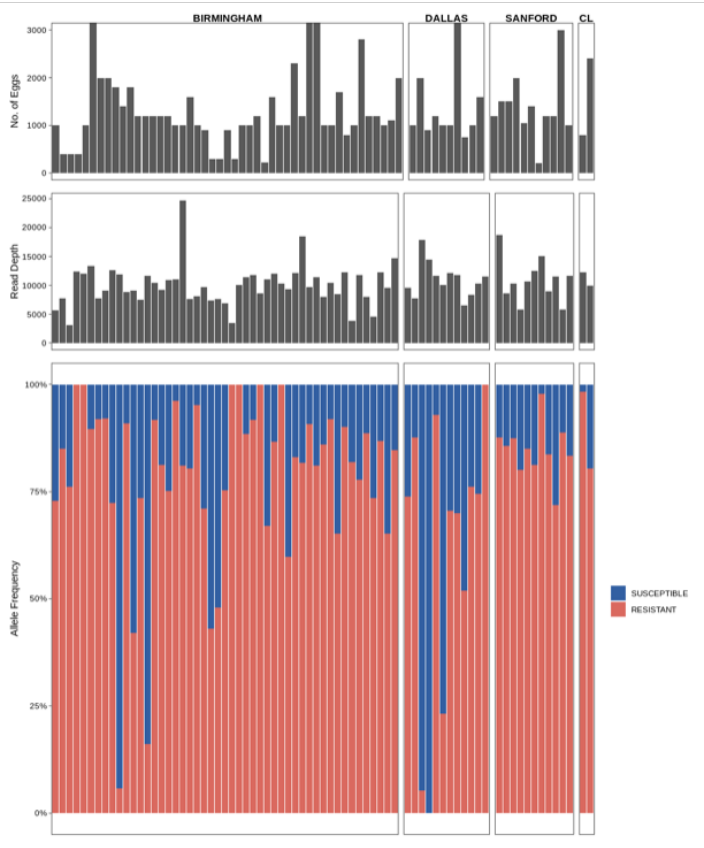

Fig. 3. The relative proportions of isotype-1 ß-tubulin alleles encoding resistance conferring polymorphisms at F167Y vs wild type susceptible as measured by deep-amplicon sequencing in 70 samples from greyhounds that originated from 16 different locations in 8 different states. Only four samples had more than 3000 eggs, so we set the max at 3000 eggs because those outliers with large numbers would distort the overall chart.

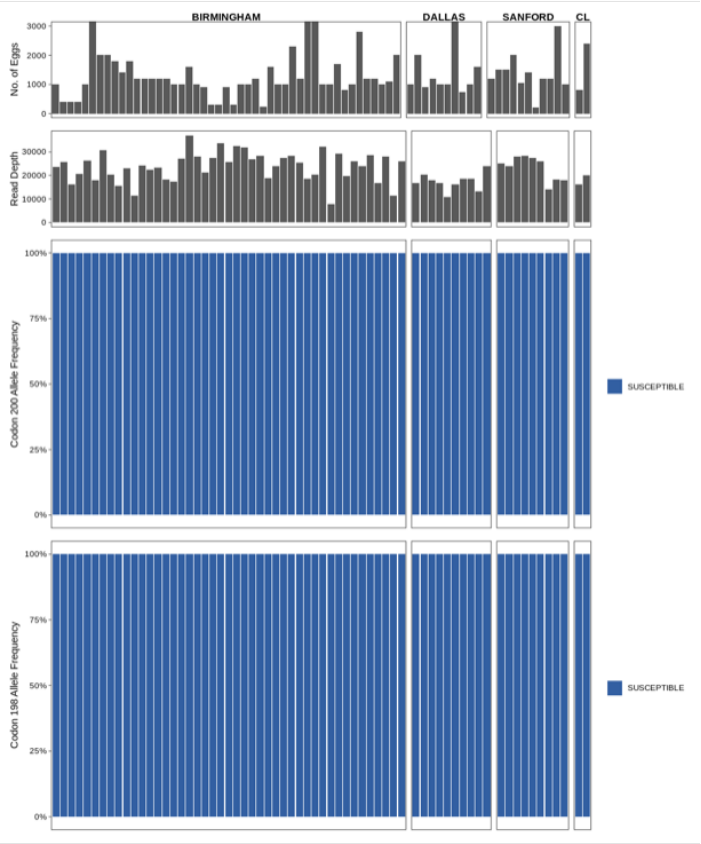

Fig. 4. The relative proportions of isotype-1 ß-tubulin alleles in the fragment containing codons 198 and 200 as measured by deep-amplicon sequencing in 70 samples from greyhounds that originated from 16 different locations in 8 different states. Only four samples had more than 3000 eggs, so the maximum was set at 3000 eggs so those outliers with large numbers would not distort the overall chart.

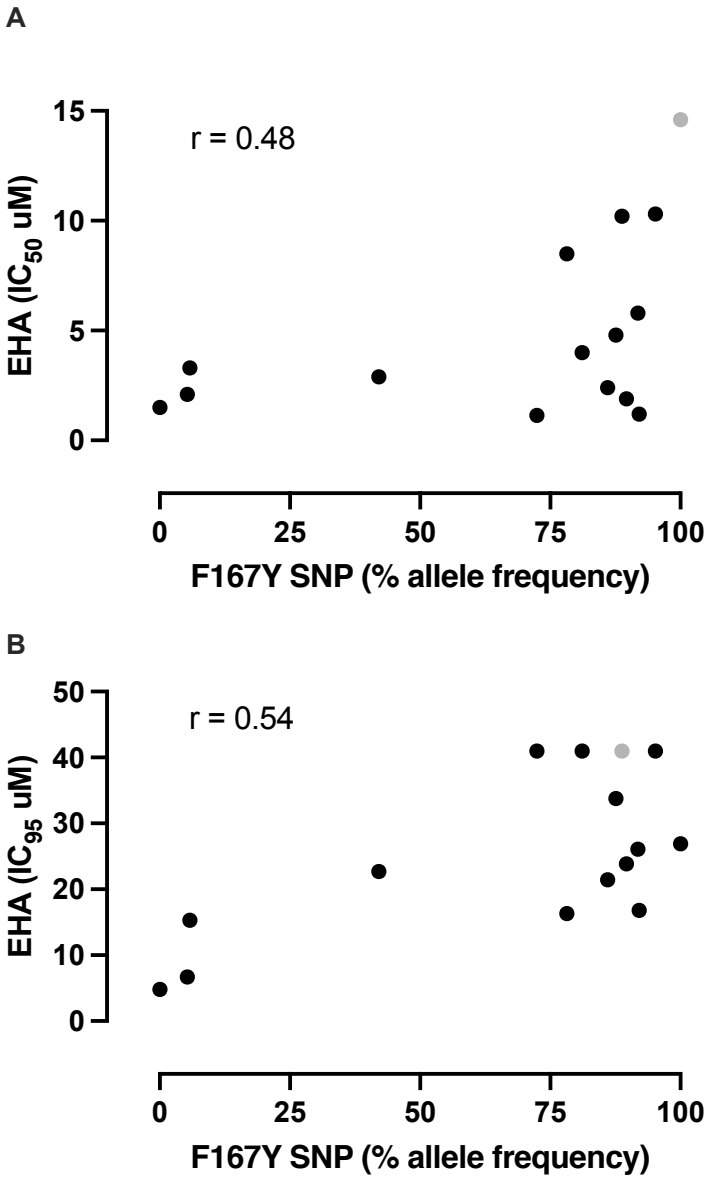

Fig 5. Scatterplots of EHA (A) IC50 values (r = 0.48) or (B) IC95 values (r = 0.54) vs. F167Y SNP frequency based on deep-amplicon sequencing. Only 15 samples had results from both assays. The gray dot represents a pooled sample. The highest concentration tested in the EHA was 40 μM, therefore the four values with IC95 of 40 μM likely would have been greater if higher concentrations were tested.

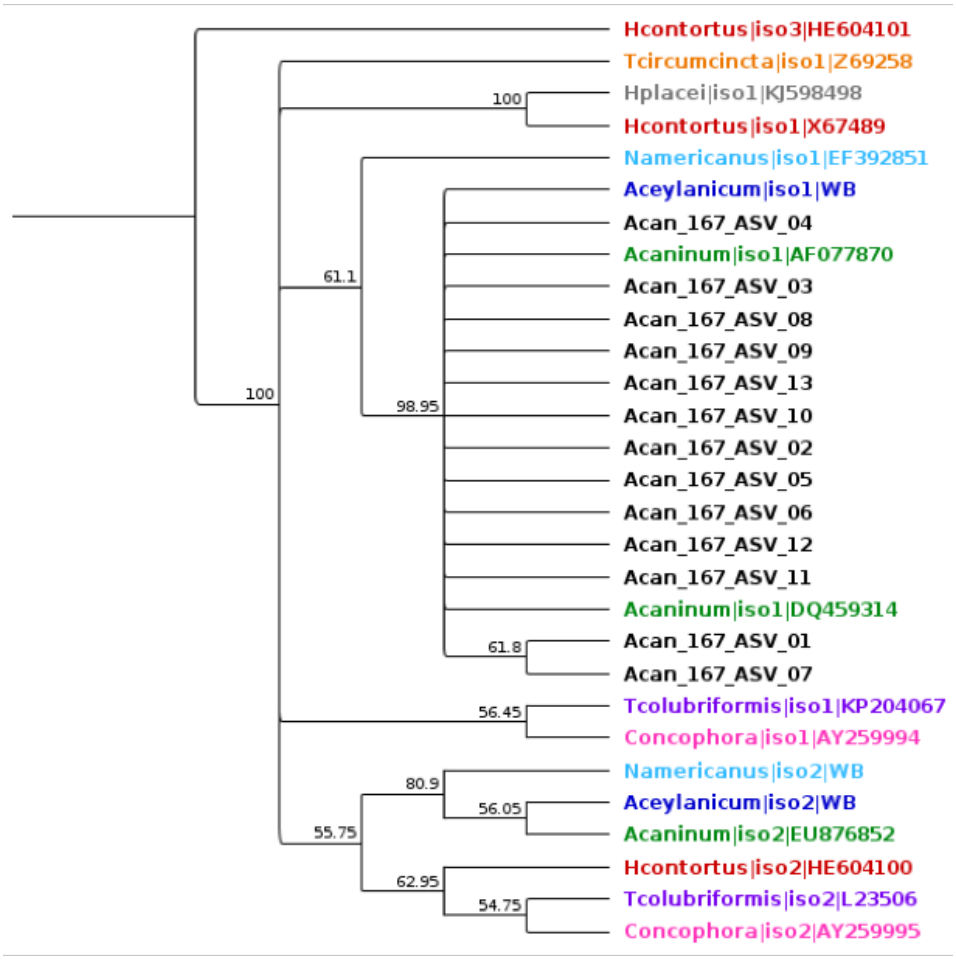

Fig 6. Neighbor-joining tree, utilizing the Jukes Cantor tree building method, with isotype-1 and 2 β-tubulins from other nematodes present in Clade V of the nematode phylogeny, and having *H. contortus* isotype-3 β-tubulin as the outgroup for the fragment containing codon 167.

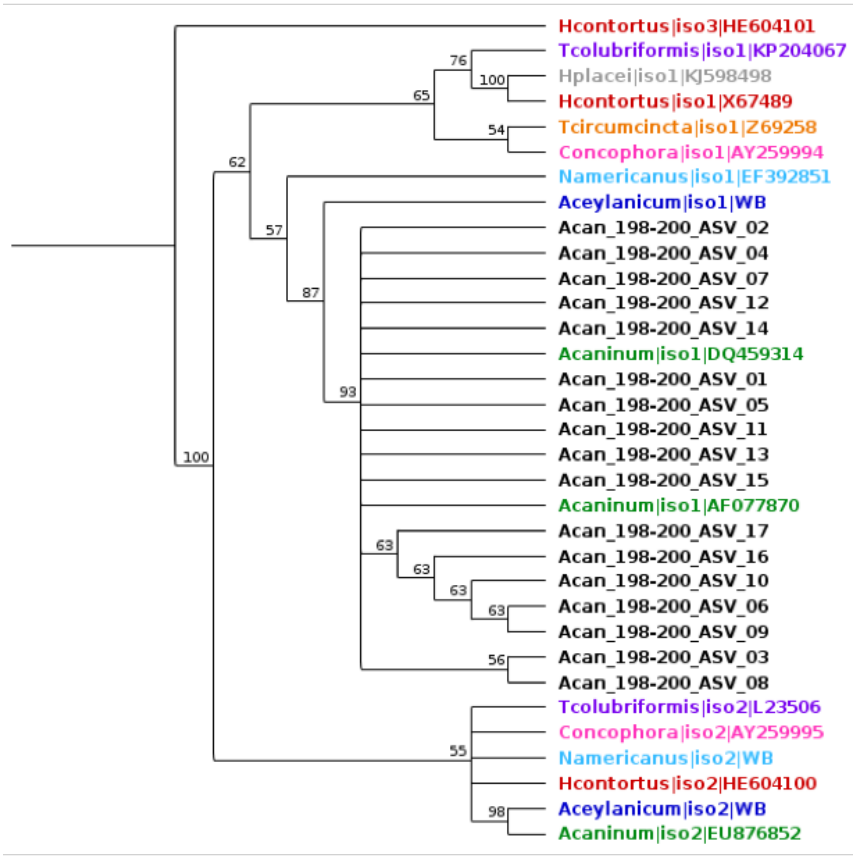

Fig 7. Neighbour-joining tree, utilizing the Jukes Cantor tree building method, with isotype-1 and 2 β-tubulins from other nematodes present in Clade V of the nematode phylogeny, and having H. contortus isotype-3 β-tubulin as the outgroup for the fragment containing codons 198 and 200.

CHAPTER 5

Investigating the molecular epidemiology of multiple drug resistance in the canine hookworm, *Ancylostoma caninum*

INTRODUCTION

The canine hookworm, *Ancylostoma caninum* is the most prevalent and important intestinal nematode parasite of dogs in the United States, with the prevalence depending on age, level of care and geographic location of the dog (Little et al., 2009).

Anthelmintic resistance is an ever-increasing threat and understanding the emergence patterns is an important goal in order to develop effective programs to delay the onset and spread of resistance. To date, the only mechanism of molecular resistance we know is for the benzimidazole class. Three non-synonymous single nucleotide polymorphisms (SNPs), F167Y (TTC>TAC), E198A (GAA>GCA) and F200Y (TTC>TAC), in the isotype-1 beta-tubulin gene are associated with benzimidazole resistance in several sheep trichostrongylid gastrointestinal nematodes (Kwa et al., 1994; Kwa et al., 1995; Prichard, 2001; Ghisi et al., 2007; Redman et al., 2015; Avramenko et al., 2019), and recently the F167Y SNP has been identified in multi-drug resistant (MDR) isolates of *A. caninum* (Jimenez Castro et al., 2019). These amino acid codons (167, 198 and 200) have been shown to be important residues in the benzimidazole binding pocket and their effect on drug sensitivity has been functionally demonstrated (Aguayo-Ortiz et al., 2013).

An interesting feature of DNA sequences is that these provide haplotypes, or the genes on a chromosome that were inherited together from a single parent which provides the genetic background in which a SNP is found. This can be used to reconstruct phylogenies of families within and between populations. As a consequence, the spread of particular genes, their origin by recombination, and evidence of their introduction into the population by migration can be inferred because a historical reconstruction of actual evolutionary events within populations (Lewontin, 1985). Three processes could account for the presence of resistance-conferring alleles in a parasitic nematode population. Firstly, migrations and thus gene flow can allow the dispersion of the resistance alleles from one population to another. This has been observed for insecticide resistance genes in mosquitoes (Raymond et al., 1991) and also for resistance alleles in *Haemonchus contortus* (Chaudhry et al., 2015). Secondly, resistance alleles could have been present in the population for years/decades. For example, the selection of pre-existing resistance alleles in *H. contortus, Teladorsagia circumcincta*, and *Trichostrongylus colubriformis* (Silvestre and Humbert, 2002). Thirdly, new resistance alleles can arise by spontaneous mutations. The study of the diversity of benzimidazole-resistance alleles in several populations should make it possible to determine the origin of these benzimidazole-resistance alleles. On the one hand, a small number of resistance alleles can be expected if these alleles have been dispersed from a population to another by gene flow or if pre-existing alleles were present before the isolation of populations. On the other hand, a far greater diversity of the resistance alleles can be expected if these alleles result from spontaneous mutations arising after the isolation of the populations. To the best of our knowledge, no data are available on the origin of the benzimidazole-resistance alleles in the *A. caninum* populations.

The widespread development of resistance in nematode parasites of livestock was presumed for many years to be caused by anthelmintic selection acting on mutations that occurred before the start of drug use (Roos, 1990; Silvestre and Humbert, 2002), so a beneficial mutation arised once and increased in frequency due to selection pressure with a subsequent loss of marker polymorphism, known as the "hitchhiking" effect (Maynard Smith and Haigh, 1974). However, this hypothesis has been challenged as recurrent mutations that take place after the onset of selection are important as well, known as *de novo* mutations (Silvestre et al., 2009; Skuce et al., 2010; Redman et al., 2015), based in that for a rapid adaptive change in eukaryotes to occur, an availability of new mutations had to be present as multiple adaptive alleles sweeping through a population leading to a more complex genetic signature of selection (Messer and Petrov, 2013). The frequency in which anthelmintic resistance arises and the spread are important considerations for the prevention and management (Silvestre and Humbert, 2002; Skuce et al., 2010; Redman et al., 2015). Evidence of such soft selective sweeps has already been reported in trichostrongylid species of ruminants in European (Redman et al., 2015), North and South American (Brasil et al., 2012; Barrere et al., 2012; Barrere et al., 2013), as well as Asian countries (Chaudhry et al., 2015).

The first report of pyrantel resistance was from New Zealand in a greyhound puppy that was imported from Australia (Jackson et al., 1987), with several more cases subsequently diagnosed in Australia (Hopkins et al., 1988; Hopkins and Gyr, 1991; Kopp et al., 2007; Kopp et al., 2008a, b). The issue of whether resistance is likely to become a problem in parasites of dogs has received relatively little attention, and when addressed, it has been viewed as an issue relating to the increased use of prophylactic helminth

treatments in pets (Thompson, 2001). However, the epidemiology of nematode transmission on greyhound farms much more closely resembles the epidemiological conditions present on livestock farms, than to the epidemiological conditions present in a pet home environment (Jimenez Castro et al., 2021). Consequently, it would not be surprising if anthelmintic resistance also were to become a common problem on greyhound farms. Our recent work shows that the F167Y (TTC>TAC) benzimidazole resistance polymorphism was detected, in 99% of samples collected from greyhounds that originated from 16 different locations in 8 different states, and at high frequencies in more than 2/3 of the samples (Jimenez Castro et al., 2021).

To date we do not know the extent of the resistance problem in *A. caninum* and therefore how high the level of concern should be, the manner of genetic inheritance of the resistant phenotype, and whether resistance emerged once and then spread as a hard selective sweep, or if there were multiple independent occurrences of resistance that then spread as a soft selective sweep. Both patterns may occur in the emergence and spread of drug resistance in parasites, but which predominates is dependent on a variety of factors (Nair et al., 2007; Redman et al., 2015).

Beyond the concerns for canine health, MDR in canine hookworms could present serious public health concerns since *A. caninum* is zoonotic and MDR *A. caninum* will not respond to usual anthelmintic treatments administered by physicians. Humans infected percutaneously may develop cutaneous larva migrans (CLM) (Leeming and Oxon, 1966). Cases of eosinophilic enteritis (Prociv and Croese, 1996), as well as patent infections have also been described (Ngcamphalala et al., 2019; Furtado et al., 2020).

The main purpose of this study was to investigate the prevalence and geographic distribution of drug-resistant hookworms in pet dogs as well as the haplotype diversity of the resistant alleles and implications of animal movement in their spread.

Materials and Methods

Sample collection

From February 2020 - December 2020, 1269 positive hookworm samples were collected from four different IDEXX diagnostic laboratories located in Memphis, TN (South), North Grafton, MA (North), Elmhurst, IL (Midwest), Irvine, CA (West). Samples were refrigerated immediately after collection and stored until shipped to the laboratory at the University of Georgia in a container with ice packs. For this study, only positive samples were examined. These were classified based on the semi-quantitative classification used by IDEXX: “rare”, 1-2 ova; “few”, 3-10 ova; “moderate”, 11-30 ova; “many”, >30 ova per slide.

Geographical regions

Samples were assigned to four diverse geographic areas as previously described (Blagburn et al., 1996; Little et al., 2009). The states comprising each region were: Northeast (CT, DE, MA, MD, ME, NH, NJ, NY, PA, RI, VT), Midwest (IA, IL, IN, KS, MI, MN, MO, ND, NE, OH, SD, WI), South (AL, AR, FL, GA, KY, LA, MS, NC, OK, SC, TN, TX, VA, WV), and West (AK, AZ, CA, CO, HI, ID, MT, NM, NV, OR, UT, WA, WY).

Breed and age categorization

Dogs were categorized according to the American Kennel Club (AKC, 2021) into small, medium and large breeds, and age grouped in alignment with recent American Animal

Hospital Association guidelines, with consistent years applied to each category: puppy, < 1 year-old; young adult, 1 to 3 years-old; mature adult, 4 to 6 years-old; and senior, ≥ 7 years-old (Creevy et al., 2019).

Ancylostoma caninum isotype-1 beta-tubulin deep amplicon sequencing

DNA preparation

A total of 207 samples comprised of 142 individual and 65 pools, representing a total of 499 dogs, were included for the initial analysis. Samples from all four geographical regions of the USA, all dog breed categories, as well as all age groups were included in this initial analysis (Fig. 1). Hookworm eggs from samples classified as "many", "moderate", and "few" were isolated individually. Whereas "rare" samples were isolated as pools within regions and if too many per state. These pools were grouped in "few", "moderate", and "large" depending on the number of dogs that comprised the pool. Samples classified as "few" that had less than 1 gr of feces, were included in pools. Using wooden tongue depressors and water the feces were mixed to form a fecal slurry. This was then filtered through Cheesecloth grade 40 bleached (GRAINGER, Lake Forest, IL) and transferred to disposable cups. The fecal solution was then transferred to a 15 mL tube and centrifuged at 240 x *g* for 10 mins. The supernatant was discarded and 10 mL of sodium nitrate (Feca-Med®, Vedco, Inc. St. Joseph; MO, USA specific gravity = 1.25 to 1.30) were added followed by vortexing to break up the pellet. This was then centrifuged again at 240 x *g* for 10 mins. Following centrifugation, eggs were recovered, rinsed with distilled water through a 20 µm sieve, transferred to a tube, and then three 20 ul aliquots were taken to estimate

the number of eggs. These were then transferred to 2 mL cryotubes (Sigma-Aldrich, St. Louis, MO), suspended in a final concentration of 70% ethanol and stored at -80°C until further use. DNA lysates were prepared from individual or pooled egg samples as previously described with minor modifications (Jimenez Castro et al., 2021). Briefly, 3 freeze-thaw cycles were carried out at -80°C and at 55°C respectively, followed by adding 180 µL of DirectPCR (Cell) Lysis Buffer (Catalog No. 301-C, Viagen Biotech, St. Louis, MO) and 20 µL of Proteinase K (Catalog No. 19133, QIAGEN, Hilden, Germany). Samples were then incubated for at least 12h at 65°C, then 1h at 95°C, and were then cooled to 4°C. DNA was purified from the crude DNA lysates using the QIAGEN QIAmp DNA mini kit (Cat# 51306), following the manufacturer's recommended protocol, and stored at -80°C. Final elutions of DNA were made in 50 µl of Tris-EDTA buffer solution (Sigma-Aldrich, St. Louis, MO).

Deep- amplicon sequencing assay analysis

Deep amplicon sequencing assays evaluating the frequency of non-synonymous SNPs at codons 167, 198 and 200 of the *A. caninum* isotype-1 beta-tubulin gene were performed on 207 samples from four geographical regions of the USA. These were comprised of 142 individually isolated samples ranging from 300 - 14,125 eggs with an average of 1865 eggs, and 65 pools ranging from 300 - 10,350 eggs with an average of 1808 eggs. Using adapted primers suitable for Illumina next-generation sequencing, two separate regions of 293 bp and 340 bp which encompass codon 167, and codons 198 and 200, respectively, were PCR amplified (Jimenez Castro et al., 2019). PCR conditions were as previously described (Jimenez Castro et al., 2019). Sample purification and addition of barcoded primers followed the protocols defined in (Avramenko et al., 2019). Library preparation

was as previously described and library sequencing performed using the Illumina MiSeq platform with the 2 × 250 v2 Reagent Kit (Illumina Inc., San Diego, CA, USA) (Avramenko et al., 2015). The bioinformatic pipeline for sequence filtering and variant identification was performed as previously described (Jimenez Castro et al., 2021). 70 DNA samples comprised of 60 individually isolated samples and 10 pools from greyhounds of three different adoption kennels and one veterinary practice from (Jimenez Castro et al., 2021) were included for the haplotype distribution analysis.

Data analyses

For comparing the prevalence between geographical regions, a Chi-square test was performed for the overall comparison, followed by a Fisher's exact test with a Bonferroni procedure for individual pairwise comparisons between geographical regions. For comparing the F167Y SNP frequency a Kruskal-Wallis test was performed for the overall comparison, followed by a correction with a the Benjamini-Krieger-Yekutieli procedure for individual pairwise comparisons between geographical regions. All analyses were designed to maintain the overall type I error rate at 5%.

Results

The three sites in the isotype-1 beta-tubulin gene known to have BZ resistance-associated polymorphisms (F167Y, E198A and F200Y) in strongylid nematodes were examined using deep-amplicon sequencing. The average read depth for the fragment containing 167 was ~26,000. Whereas for 198 and 200 was ~31,000. Only the F167Y (TTC>TAC) resistance polymorphism was detected in 50% (71/142) and 77% (50/65) of the individual and pooled

samples that were sequenced, respectively (Fig. 2 and 3). Overall, 58% (120/207) of the samples tested had the resistant F167Y allele. The overall prevalence for the individual and pooled samples was 49.3%, and 28.3%. In 24 of the 71 individual samples (34%), and 7 of the 50 (14%) pooled samples that had the resistant allele, the frequency was ≥75%. In 21% (15/71) and 4% (2/50) of individual and pooled samples, respectively, the allele frequency was ≥90%. If we break this down by region, for the Northeast, Midwest, South, and West, 41% (18/44) and 89% (24/27) (Fig. 4), 45% (17/38) and 67% (12/18) (Fig. 5), 55% (23/42) and 65% (11/17) (Fig. 6), and 67% (12/18) and 100% (3/3) (Fig. 7) of the individual and pooled samples that were sequenced, respectively had the F167Y (TTC>TAC) resistance polymorphism.

No significant differences were detected either in terms of prevalence nor frequency of the F167Y resistance polymorphism between regions when comparing amongst individuals (p = 0.2, and p = 0.09) (Table 1). Regarding the pooled samples, no significant differences were detected between the sample size groups (p = 0.11) (Table 2), nor between the mean allele frequencies (Table 3). Only 139 individual samples had breed information in order to divide the breeds by size; no significant differences were detected either in terms of prevalence (p = 0.41) nor frequency (p = 0.42) of the F167Y resistance polymorphism (Table 4 and 5). Only 107 individual samples had age information; no significant differences were detected either in terms of prevalence (p = 0.06) nor frequency (p = 0.72) of the F167Y resistance polymorphism (Table 5 and 6).

Regarding the haplotype distribution analysis, initial analysis of the ASVs revealed very interesting findings. In the greyhound samples from (Jimenez Castro et al., 2021), 11 different haplotypes were identified in the fragment containing codon 167 of which two

and nine had the resistant and susceptible allele, respectively (Fig. 8), with only a single predominant resistant haplotype (ie., HT_001) across all samples with the resistant allele (Fig. 9). For codon 198 and 200, 15 individual haplotypes were identified but none with the resistant E198A or F200Y polymorphism (Fig. 10). For the 207 samples collected from the IDEXX laboratories from four different geographical regions, 227 different haplotypes were identified overall in the fragment containing codon 167, of which 3 and 224 had the resistant and susceptible allele, respectively. If we break this by region, for the Northeast, Midwest, South, and West there were 2 and 177 (Fig. 11), 1 and 149 (Fig. 12), 3 and 141 (Fig. 13), and 1 and 66 (Fig. 14) haplotypes with the resistant and susceptible allele, respectively. The frequencies of the resistant haplotypes were 99.8% for HT_001, 0.01% for HT_269, and 0.001% for HT_385 across the US. For codon 198 and 200, 115 individual haplotypes were identified but none with the resistant E198A or F200Y polymorphism (Fig. 15).

Fig 16. shows the frequency of the resistant haplotypes for each region. Only one resistant haplotype was present across regions with more than 99% frequency in all the samples that had a resistant allele.

Discussion and conclusions

The present study provides strong and conclusive evidence that MDR *A. caninum* are highly prevalent in the pet dog population of the USA. More than half of all the hookworm positive samples we tested had the F167Y (TTC>TAC) benzimidazole resistance polymorphism, and of those, 25% had ≥75% resistance allele frequencies. Taking these data together with the data from the greyhound samples we collected previously (Jimenez

Castro et al., 2021), we have strong genetic evidence that almost all greyhounds are infected with drug-resistant hookworms, and that resistance evolved as a single hard selective sweep in greyhounds and then crossed over into the pet dog population.

Understanding the nature of adaptive changes occurring in response to anthelmintic selection in parasite populations is key to understanding the origin and spread of resistance mutations (Redman et al., 2015). Rapid adaptation in response to selection results in so-called "selective sweeps" at the loci under selection. There are essentially two different types of selective sweep (Pennings and Hermisson, 2006; Martínez-Valladares et al., 2012; Messer and Petrov, 2013). The classic "hard selective" sweep is characterised by a single resistance haplotype in the populations(s) resulting from a single mutation arising and sweeping through the population(s) eventually reaching fixation (Messer and Petrov, 2013). A classic example is the spread of insecticide resistance in *Drosophila melanogaster* (Daborn et al., 2002). In that case, the same resistance allele is present globally in dichlorodiphenyltrichloroethane (DDT)-resistant *D. melanogaster* populations. In contrast, soft selective sweeps are characterised by the presence of multiple resistance haplotypes in the population(s) and can arise in two ways. First, from recurrent *de novo* mutations appearing on different susceptible haplotype backgrounds after the onset of the selection. Second, from polymorphisms already present in the standing genetic variation before the onset of selection (Messer and Petrov, 2013). The effective population sizes of strongylid nematodes can be very large, thus it should not be surprising for independent resistance mutations to arise and become selected in these organisms (Redman et al., 2015). The population genetic parameter = $N_e\mu$ describes the rate at which new mutations arise in a population, where N_e = effective population size and μ = the mutation rate per generation

(Nielsen, 2000). If greater than 0.1, then the probability of soft selective sweeps to occur is suggested to be higher (Pennings and Hermisson, 2006; Messer and Petrov, 2013). Thus, a population size of 10^7 would be enough for recurrent mutations to repeatedly arise and produce soft selective sweeps if the mutation rate per generation is similar to that of *C. elegans* (Denver et al., 2000). However, the evidence from our current study clearly shows that for the benzimidazole resistance in *A. caninum*, there was a hard selective sweep that increased in frequency and spread through the pet population. Only one resistant haplotype (HT_001) was present in all the greyhound samples from our previous work (Jimenez Castro et al., 2021) at frequencies greater than 99%, which is the same haplotype that was present at a frequency >99% in all the samples with the F167Y (TTC>TAC) resistant polymorphism from the pet dog population across different geographic regions of the USA. Moreover, *A. caninum* infections with this same haplotype showed very high resistance against macrocyclic lactones and pyrantel (Jimenez Castro et al., 2019; Jimenez Castro et al., 2020; Jimenez Castro et al., 2021). However, this does not translate necessarily into pet dogs having triple drug resistant *A. caninum* isolates, as this will depend on the household having adopted a greyhound, or if the dog goes to a dog park that greyhounds frequent. More widespread geographic surveys looking into the other drug classes hopefully will become available as progress is made towards resistance markers in clade V nematodes.

Here we report compelling evidence that shows the origin of the MDR *A. caninum* isolates in the greyhound industry, and how common these MDR isolates are across different regions of the USA. Further analyses looking into the haplotype networks as well as the genetic diversity are likely to provide deeper insights into the relatedness of the resistant and susceptible alleles and the origin (s) of these MDR worms.

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Table 1. Percent prevalence of the F167Y (TTC>TAC) resistance polymorphism from 142 hookworm positive samples obtained from four regions of the USA. No significant differences were detected

Geographic region	Prevalence (%) (95%CI)	Total # of samples
Northeast	40.9 (27.2, 55.6)	44
Midwest	44.7 (29.6, 60.5)	38
South	54.7 (39.7, 69.1)	42
West	66.6 (43.7, 85.1)	18
Overall prevalence	49.3 (41.1, 57.5)	142

Table 2. Percent positives of the F167Y (TTC>TAC) resistance polymorphism from 65 pools of hookworm positive samples obtained from four regions of the USA, representing a total of 357 dogs. Pools were composed of 2 – 13 samples with an average of 5.5. Pools were grouped in "few", "moderate", and "large" depending on the number of dogs that comprised the pool. No significant differences were detected.

Pool groups	Mean	Range	% positives	Total # of samples
Few	2	2	61.1	18
Moderate	4.1	3-6	77.2	22
Large	9.2	7-13	91.7	25
Overall means	5.1	NA	76.7	65

NA: not applicable

Table 3. Mean percent allele frequencies with the standard error of the mean (SEM) of 120 samples obtained from four regions of the USA positive for the F167Y (TTC>TAC) resistance polymorphism. Samples were comprised of 70 individuals and 50 pools, representing a total of 299 dogs. Pools were composed of 2 – 13 samples with an average of 6.

Geographic region	Individuals positive	Mean allele frequency (%) (SEM)*	Pools positive	Mean allele frequency (%) (SEM)*	Total # of samples
Northeast	18	48.8 (10.2) [a]	24	23.4 (4.4) [a]	42
Midwest	17	47.5 (10.1) [a]	12	28.1 (7.3) [a b]	29
South	23	32.5 (5.9) [a]	11	47.2 (10.1) [b]	34
West	12	68.8 (7.9) [a]	3	66.3 (18.2) [b]	15
Overall means	17.5	49.4	12.5	41.3	120

*Shared superscripts denote non-significant difference

Table 4. Percent prevalence of the F167Y (TTC>TAC) resistance polymorphism from 139 individually isolated hookworm positive samples with breed information obtained from four regions of the USA. Breeds were divided into small, medium, and large according to the American Kennel Club classification (AKC, 2021). No significant differences were detected.

Breed category	Prevalence (%) (95%CI)	Total # of samples
Small	40 (24.9, 56.5)	35
Medium	53.7 (41.8, 65.3)	67
Large	48.6 (33.0, 64.4)	37
Overall prevalence	48.9 (40.7, 57.2)	139

Table 5. Percent allele frequency with the standard error of the mean (SEM) from 68 samples with breed information obtained from four regions of the USA positive for the F167Y (TTC>TAC) resistance polymorphism. Breeds were divided into small, medium, and large according to the American Kennel Club classification (AKC, 2021). No significant differences were detected.

Breed category	Mean allele frequency (%) (SEM)	Total # of samples
Small	40.3(10.4)	14
Medium	41.6(6.1)	36
Large	58.7(8.8)	18
Overall mean	46.9	68

Table 6. Percent prevalence of the of the F167Y (TTC>TAC) resistance polymorphism from 107 individually isolated hookworm positive samples with age information obtained from four regions of the USA. Groups were divided into puppy, < 1 year-old; young adult, 1 to 3 years-old; mature adult, 4 to 6 years-old; and senior, ≥ 7 years-old. No significant differences were detected.

Age category	Prevalence (%) (95%CI)	Total # of samples
Puppy	60.7 (42.3, 77.3)	28
Young adult	50 (34.1, 65.9)	36
Mature adult	34.8 (17.6, 55.1)	23
Senior	30 (13.2, 51.6)	20
Overall prevalence	46.7 (37.4, 56.2)	107

Table 7. Percent allele frequency with the standard error of the mean (SEM) from 50 samples with breed information obtained from four regions of the USA positive for the F167Y (TTC>TAC) resistance polymorphism. Groups were divided into puppy, < 1 year-old; young adult, 1 to 3 years-old; mature adult, 4 to 6 years-old; and senior, ≥ 7 years-old. No significant differences were detected.

Age category	Mean allele frequency (%) (SEM)	Total # of samples
Puppy	38.5(8.8)	18
Young adult	32.7(7.9)	18
Mature adult	58.3(13.4)	8
Senior	66.8(17.1)	6
Overall mean	49.1	50

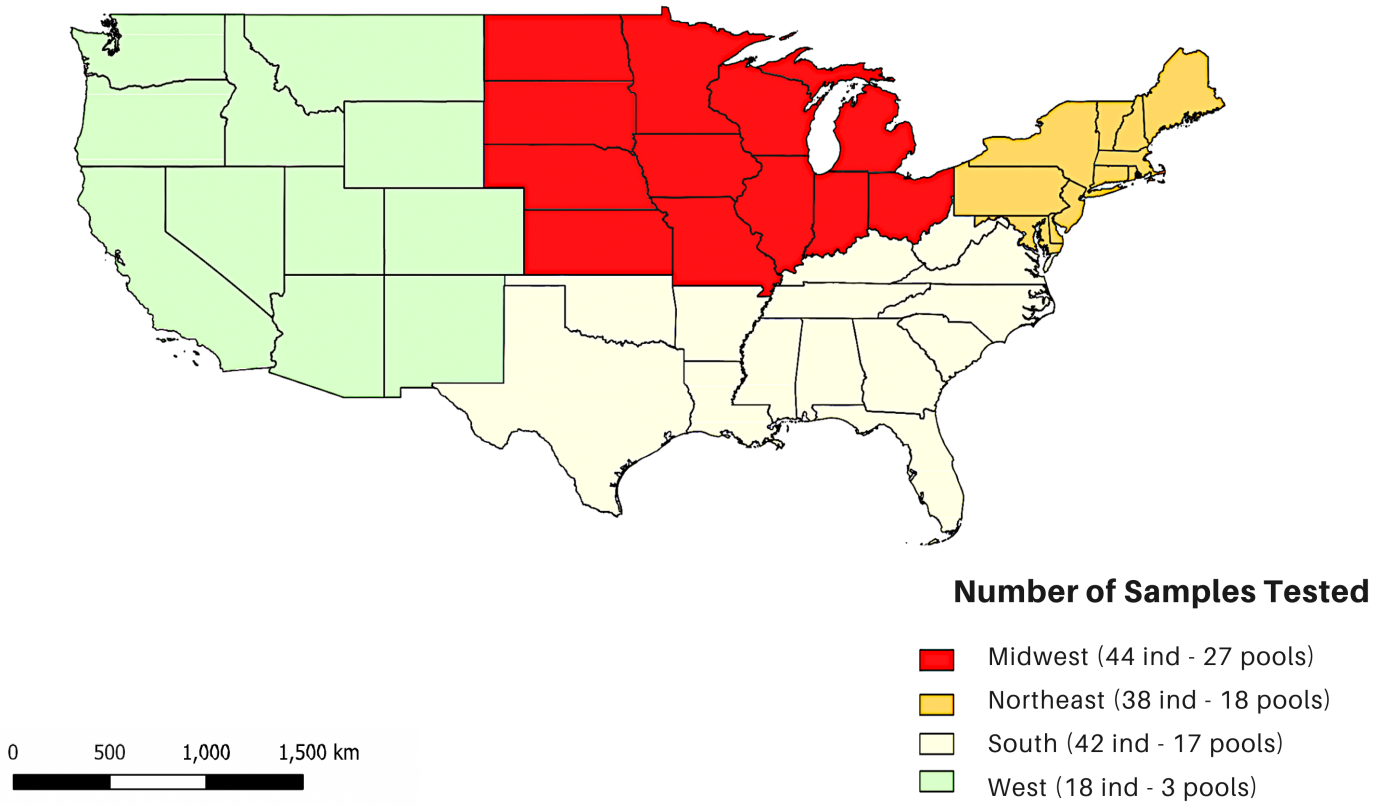

Fig. 1. The four geographical regions of the USA and the numbers of individuals and pool samples tested in the initial analysis.

Fig. 2. The relative proportions of the isotype-1 ß-tubulin alleles encoding resistance conferring polymorphisms at F167Y vs wild type susceptible as measured by deep-amplicon sequencing in 207 samples, comprised of 142 individual and 65 pooled samples from dogs across four different regions of the USA. Samples that did not amplify for this fragment are indicated by a white bar

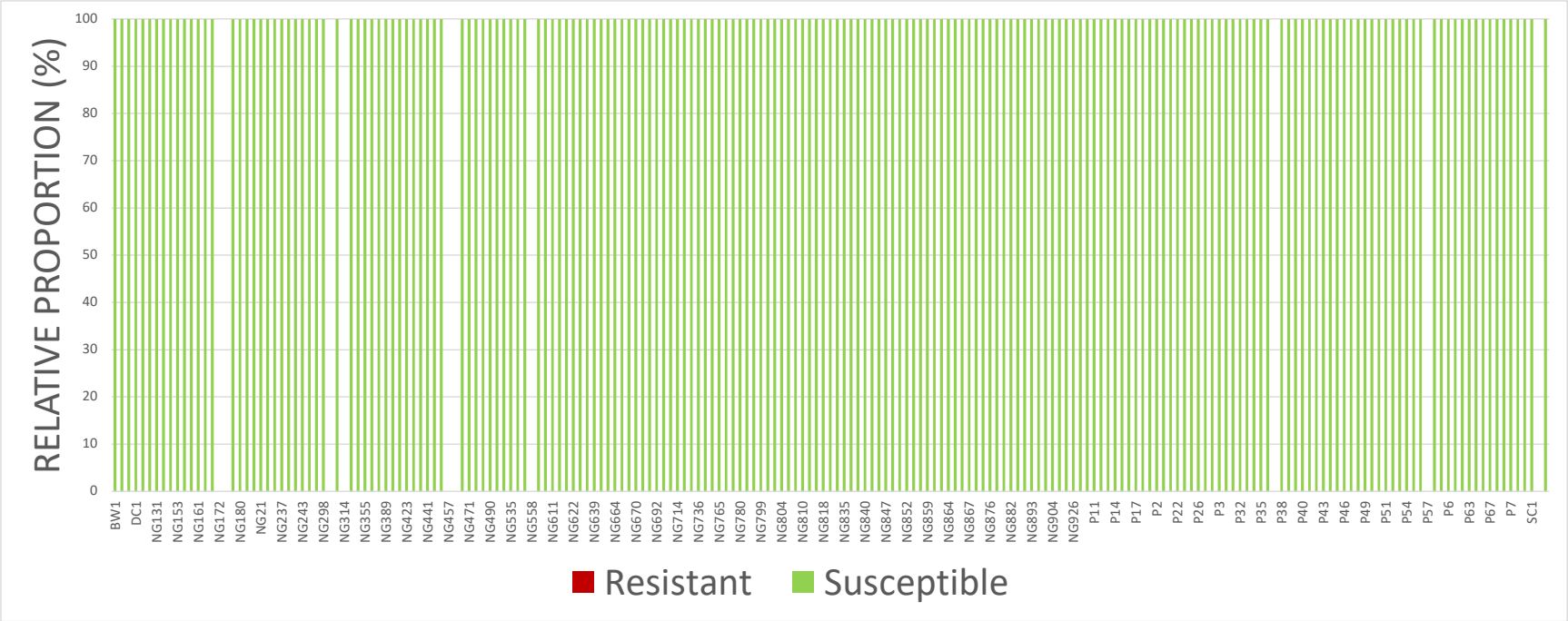

Fig. 3. The relative proportions of the isotype-1 ß-tubulin alleles in the fragment containing codons 198 and 200 in 207 samples, comprised of 142 individual and 65 pooled samples from dogs across four different regions of the USA. Samples that did not amplify for this fragment are indicated by a white bar

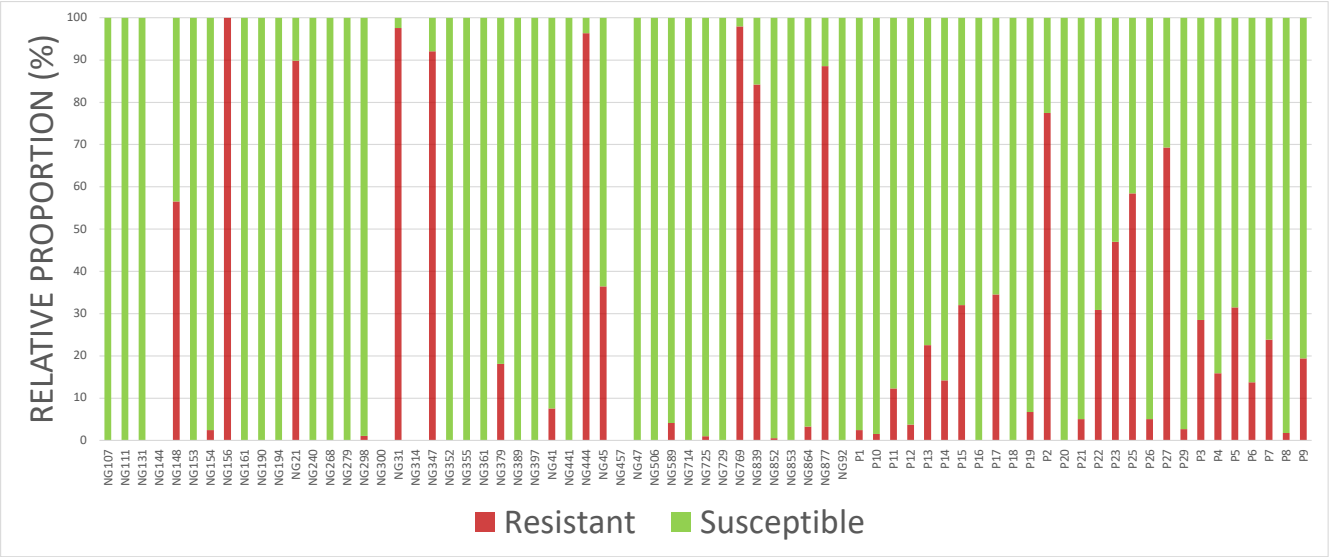

Fig. 4. The relative proportions of the isotype-1 ß-tubulin alleles encoding resistance conferring polymorphisms at F167Y vs wild type susceptible as measured by deep-amplicon sequencing in 71 samples, comprised of 44 individual and 27 pooled samples from dogs of the Northeast region. Samples that did not amplify for this fragment are indicated by a white bar

Fig. 5. The relative proportions of the isotype-1 ß-tubulin alleles encoding resistance conferring polymorphisms at F167Y vs wild type susceptible as measured by deep-amplicon sequencing in 56 samples, comprised of 38 individual and 18 pooled samples from dogs of the Midwest region. Samples that did not amplify for this fragment are indicated by a white bar

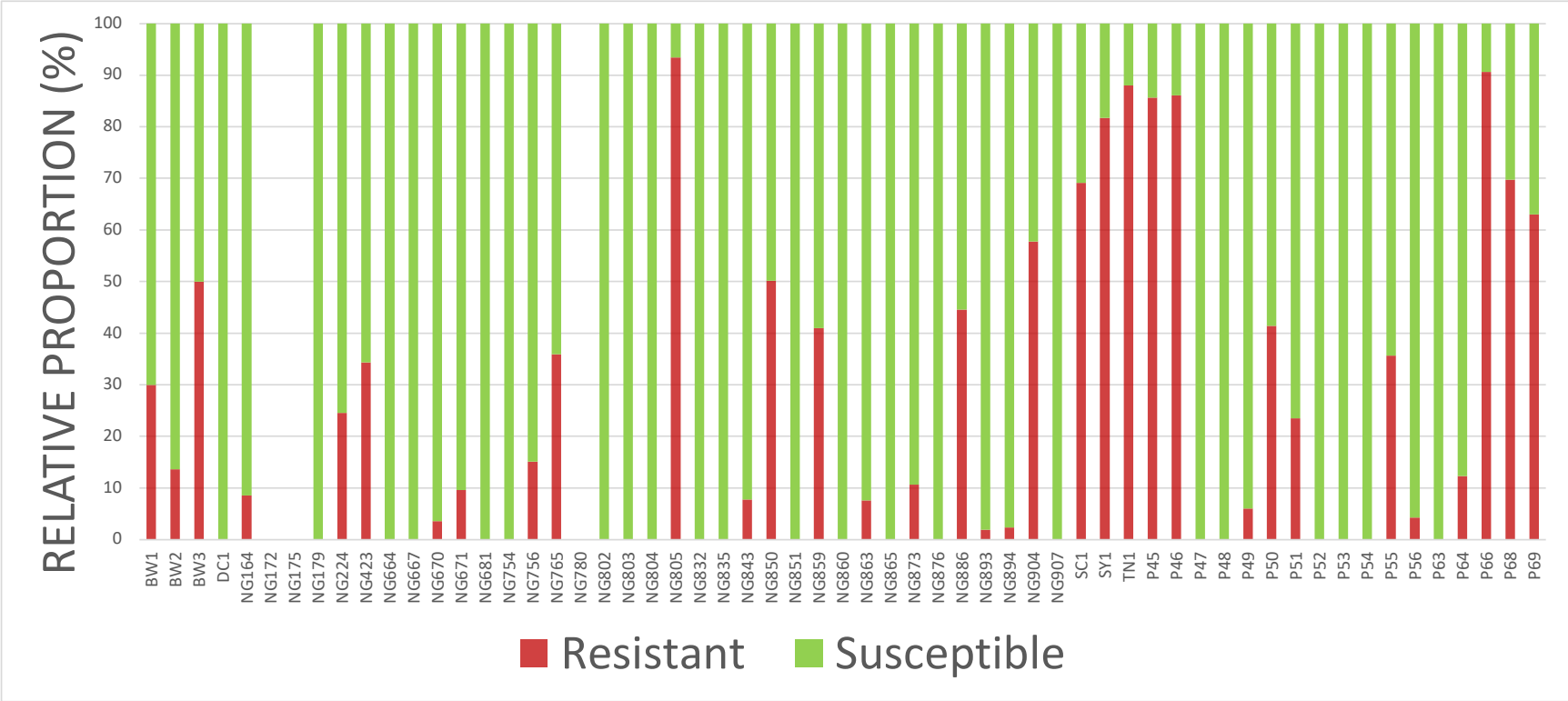

Fig. 6. The relative proportions of the isotype-1 ß-tubulin alleles encoding resistance conferring polymorphisms at F167Y vs wild type susceptible as measured by deep-amplicon sequencing in 59 samples, comprised of 42 individual and 17 pooled samples from dogs of the South region. Samples that did not amplify for this fragment are indicated by a white bar

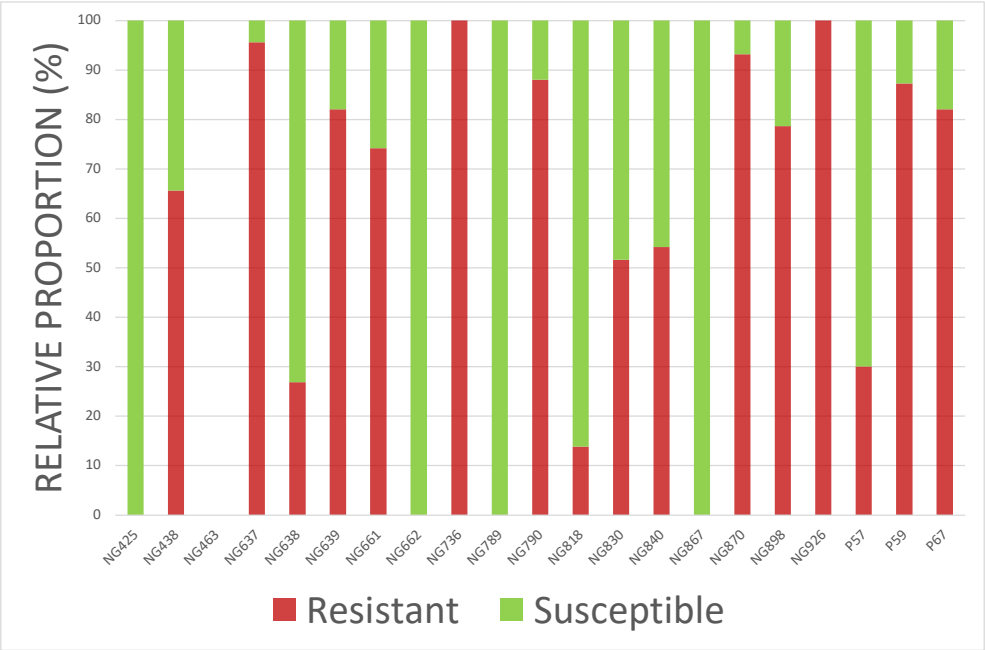

Fig. 7. The relative proportions of the isotype-1 ß-tubulin alleles encoding resistance conferring polymorphisms at F167Y vs wild type susceptible as measured by deep-amplicon sequencing in 21 samples, comprised of 18 individual and 3 pooled samples from dogs of the West region. Samples that did not amplify for this fragment are indicated by a white bar

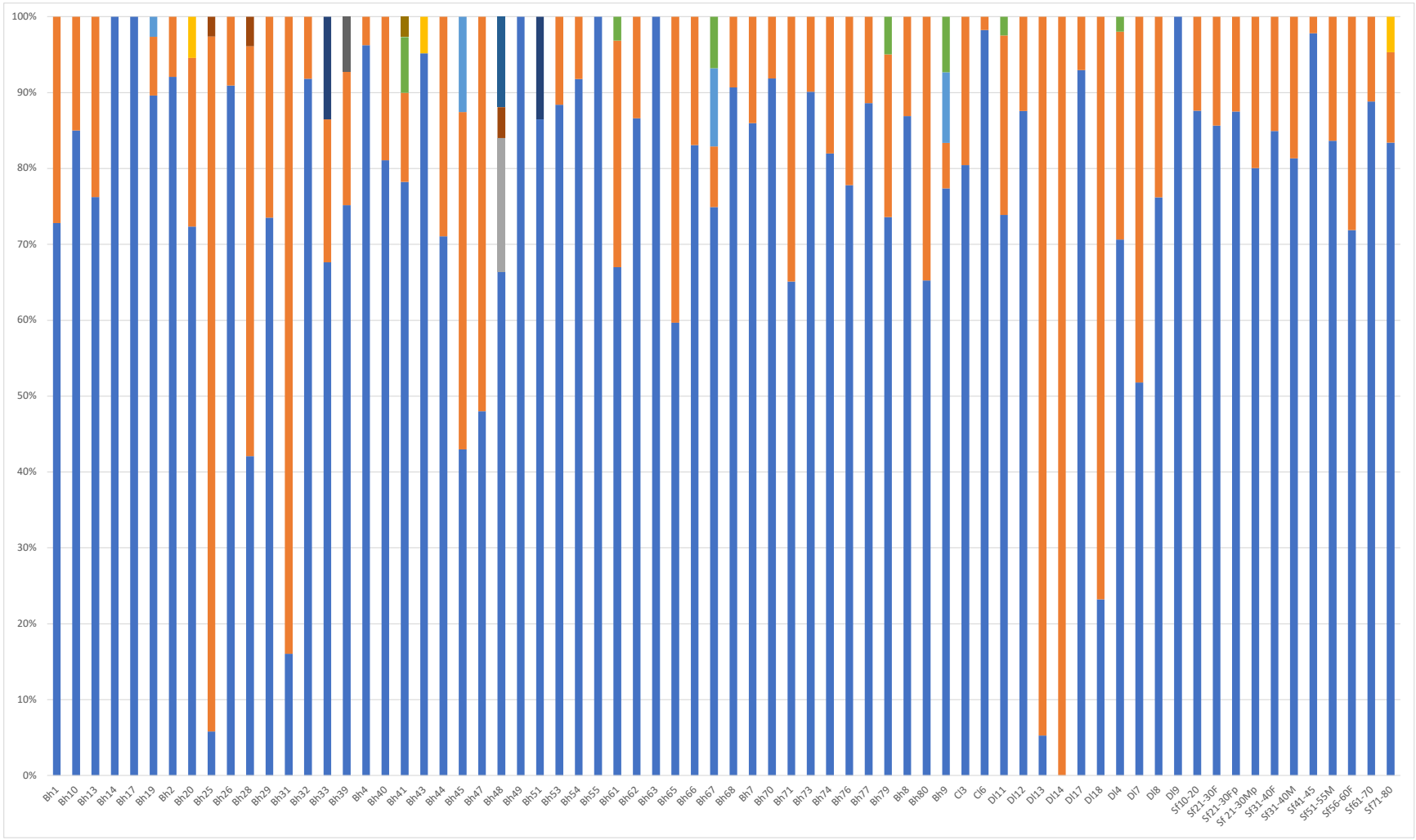

Fig. 8. Distribution of haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codon 167 in *A. caninum* populations infecting two greyhound adoption kennels and one greyhound active racing kennel, which dogs originated from 16 different locations in the USA. One color corresponds to each different haplotype.

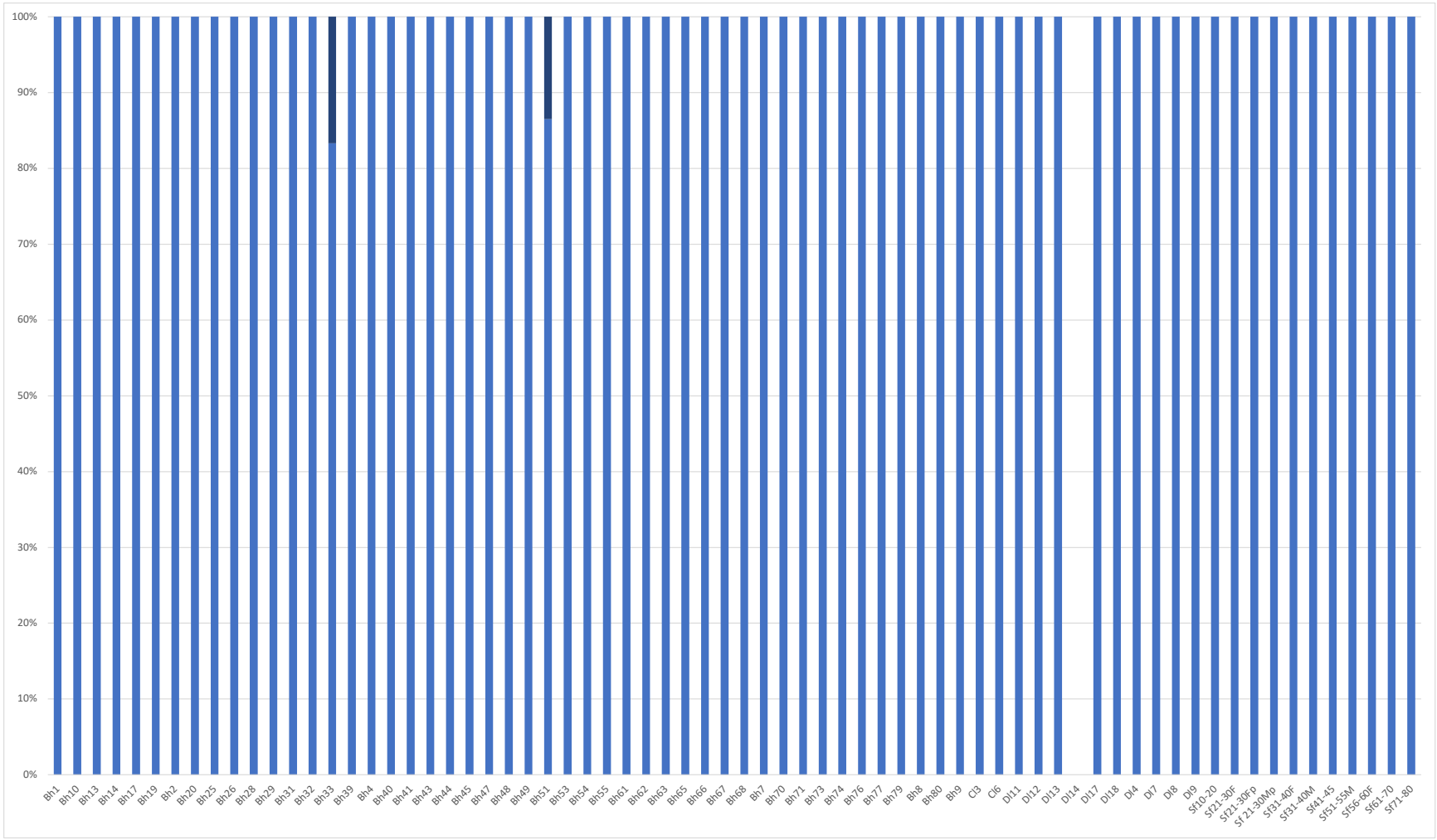

Fig. 9. Distribution of resistant haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codon 167 in *A. caninum* populations infecting two greyhound adoption kennels and one greyhound active racing kennel, which dogs originated from 16 different locations in the USA. Sample DL14 was the only sample with 100% of the susceptible allele.

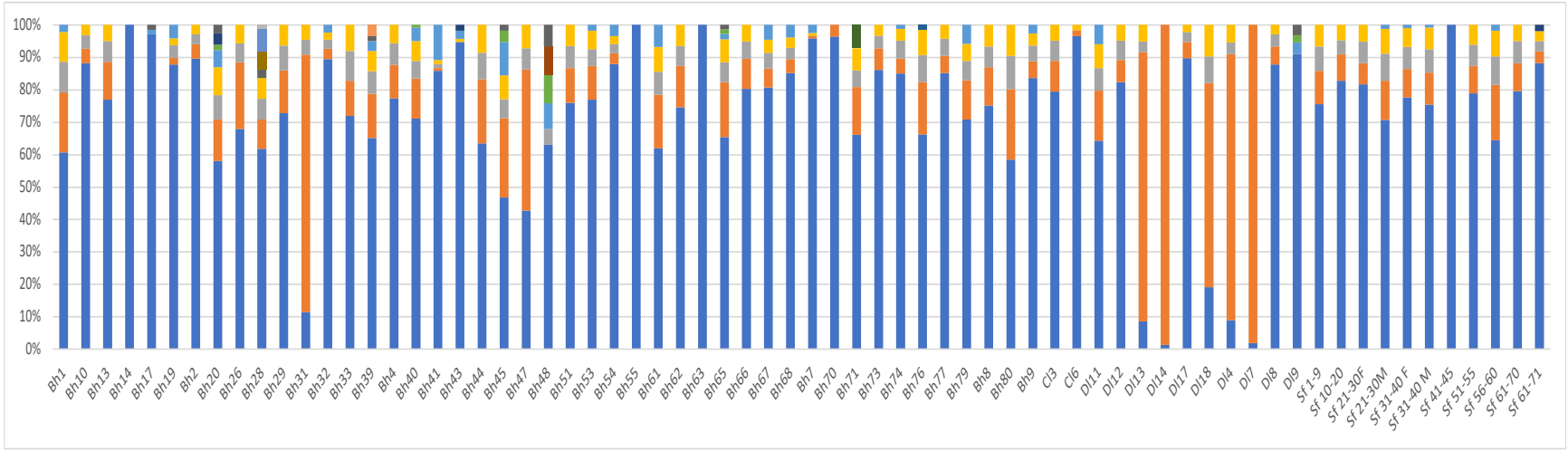

Fig. 10. Distribution of haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codons 198 and 200 in *A. caninum* populations infecting two greyhound adoption kennels and one greyhound active racing kennel, which dogs originated from 16 different locations in the USA.

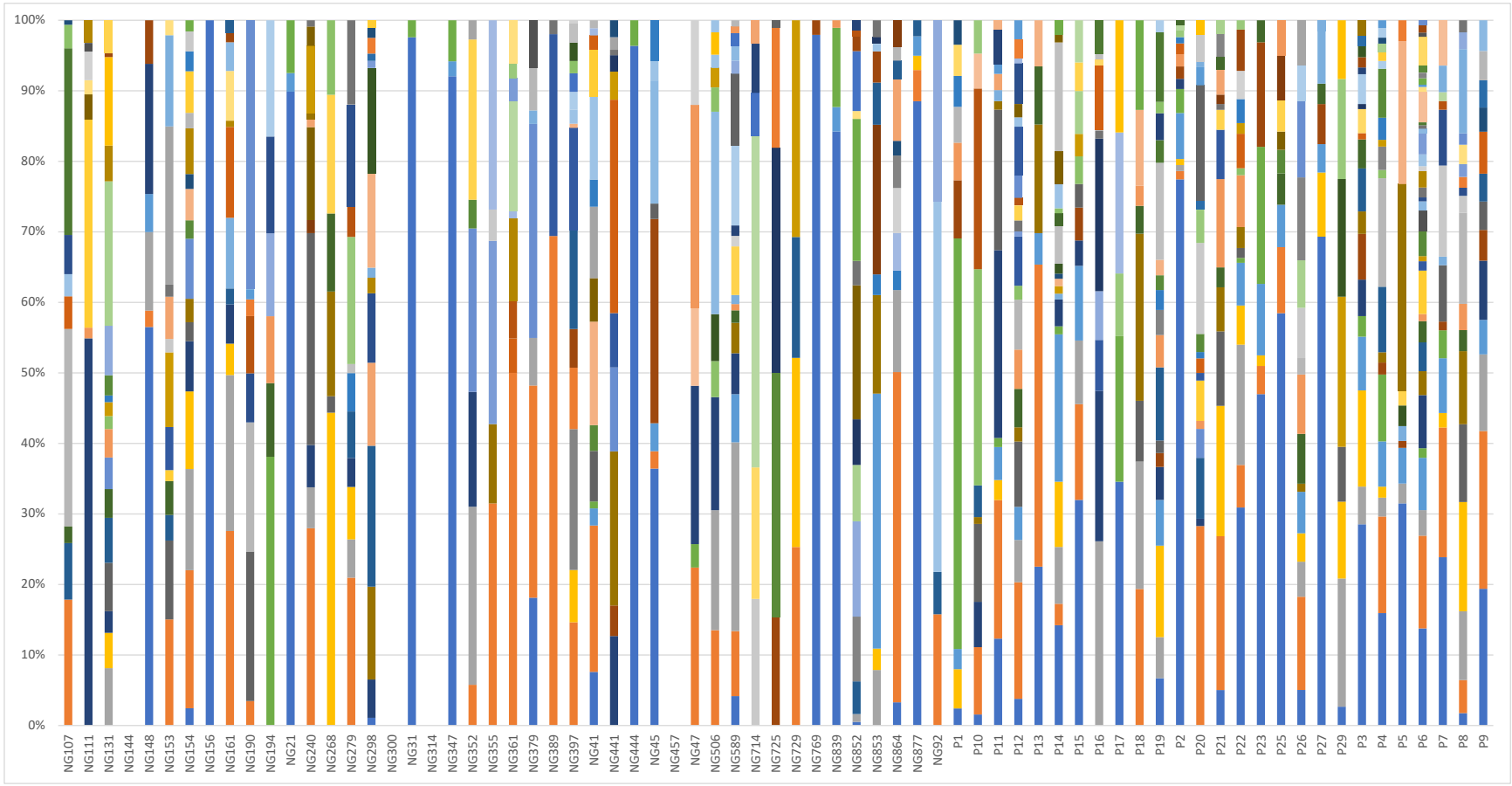

Fig. 11. Distribution of haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codon 167 in *A. caninum* populations from 71 samples, comprised of 44 individual and 27 pooled samples from dogs of the Northeast region. Samples that did not amplify for this fragment are indicated by a white bar

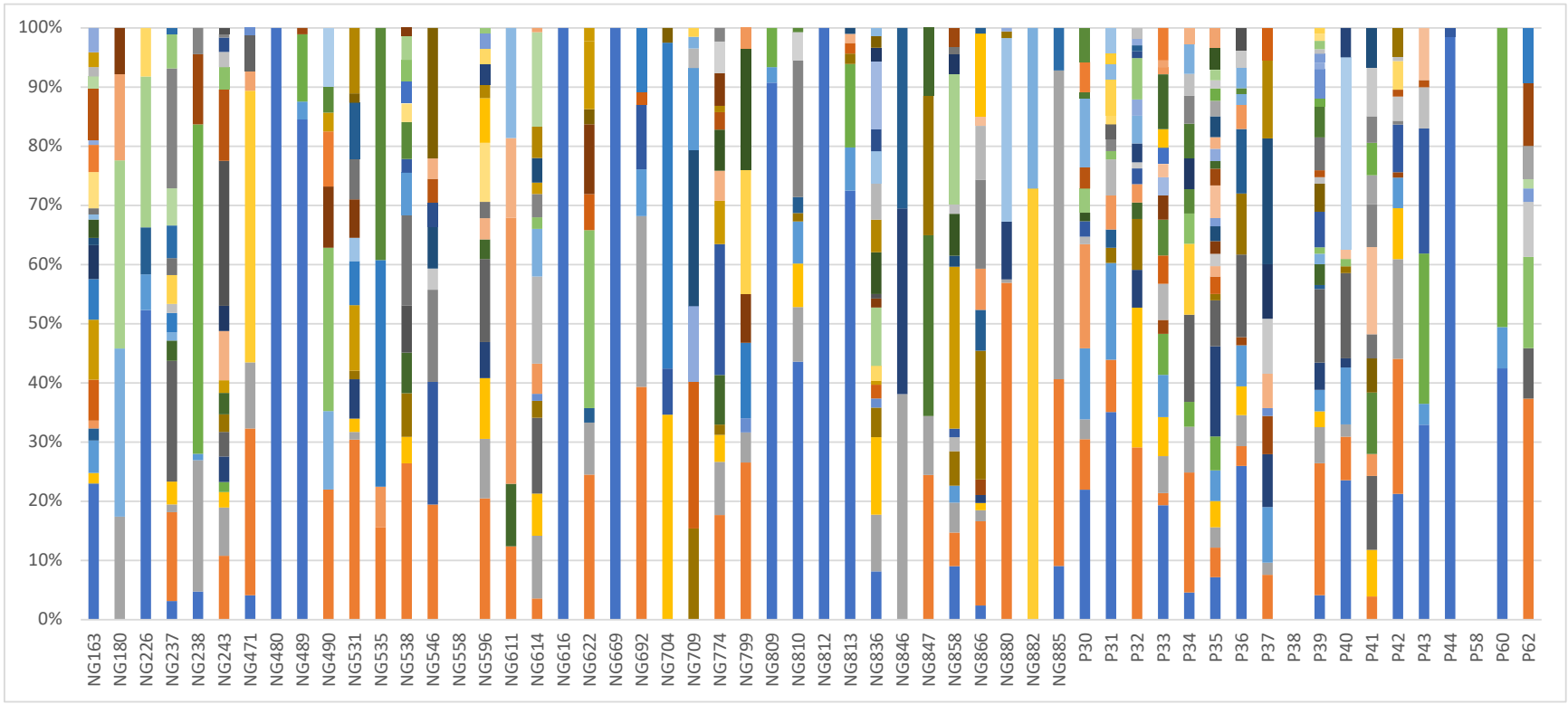

Fig. 12. Distribution of haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codon 167 in *A. caninum* populations from 56 samples, comprised of 38 individual and 18 pooled samples from dogs of the Midwest region. Samples that did not amplify for this fragment are indicated by a white bar

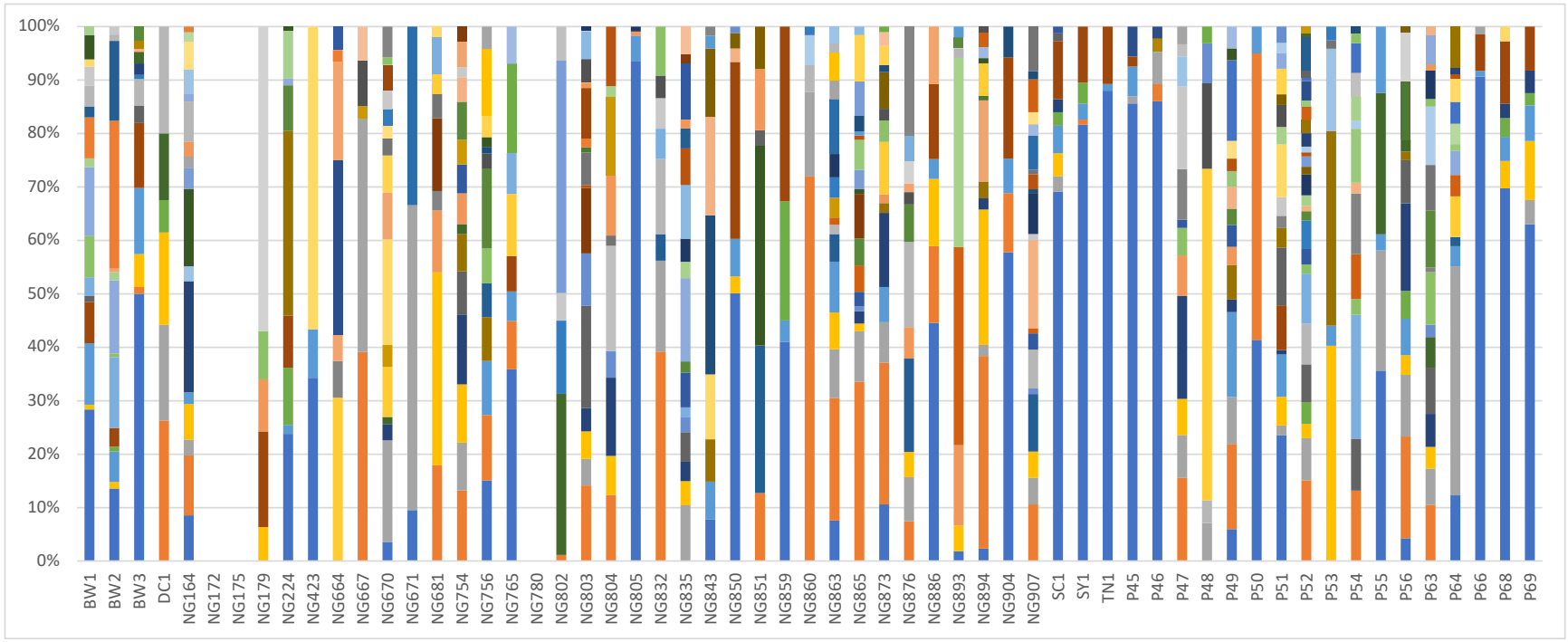

Fig. 13. Distribution of haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codon 167 in *A. caninum* populations from 59 samples, comprised of 42 individual and 17 pooled samples from dogs of the South region. Samples that did not amplify for this fragment are indicated by a white bar

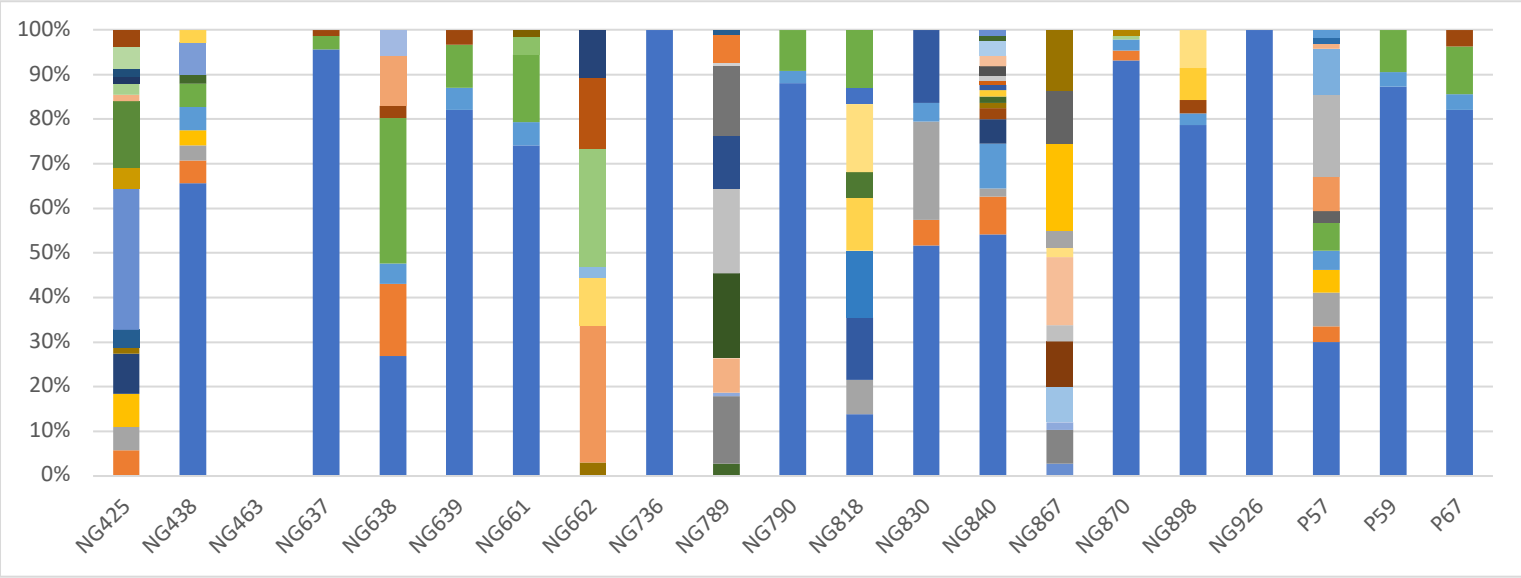

Fig. 14. Distribution of haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codon 167 in *A. caninum* populations from 21 samples, comprised of 18 individual and 3 pooled samples from dogs of the West region. Samples that did not amplify for this fragment are indicated by a white bar

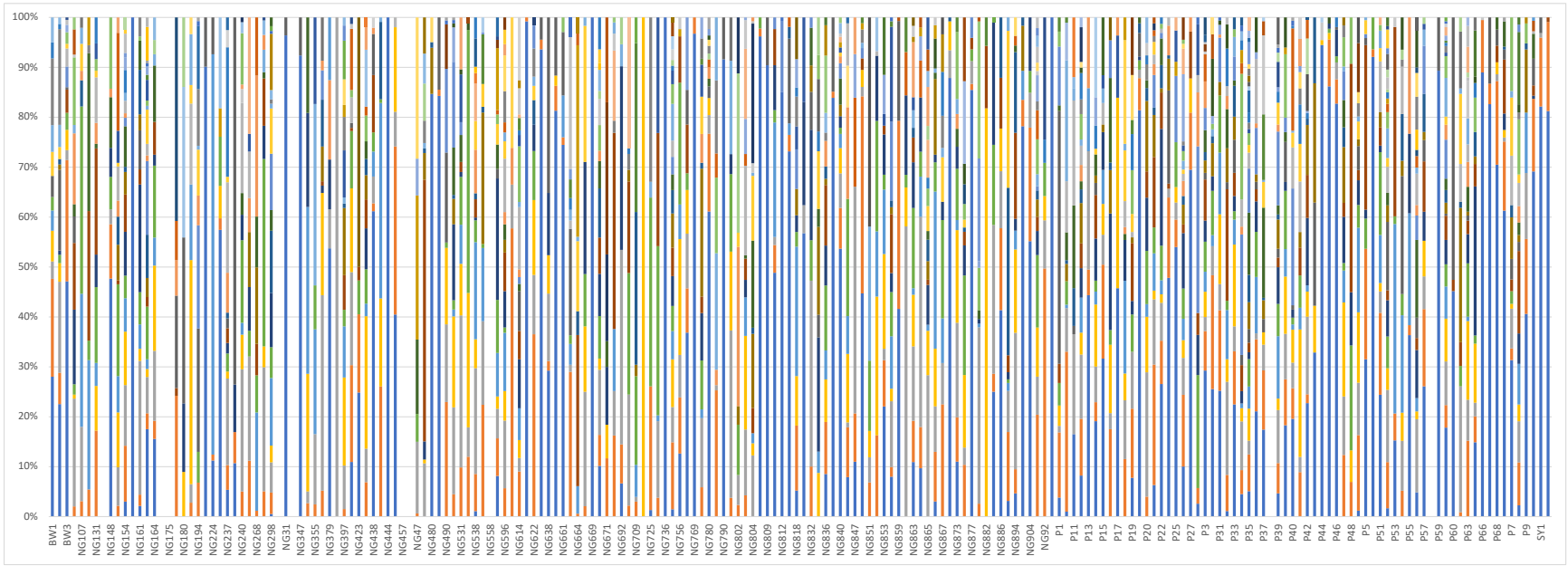

Fig. 15. Distribution of haplotypes identified in the fragment of the isotype-1 ß-tubulin gene containing codons 198 and 200 in *A. caninum* populations in 207 samples, comprised of 142 individual and 65 pooled samples from dogs across four different regions of the USA. Samples that did not amplify for this fragment are indicated by a white bar

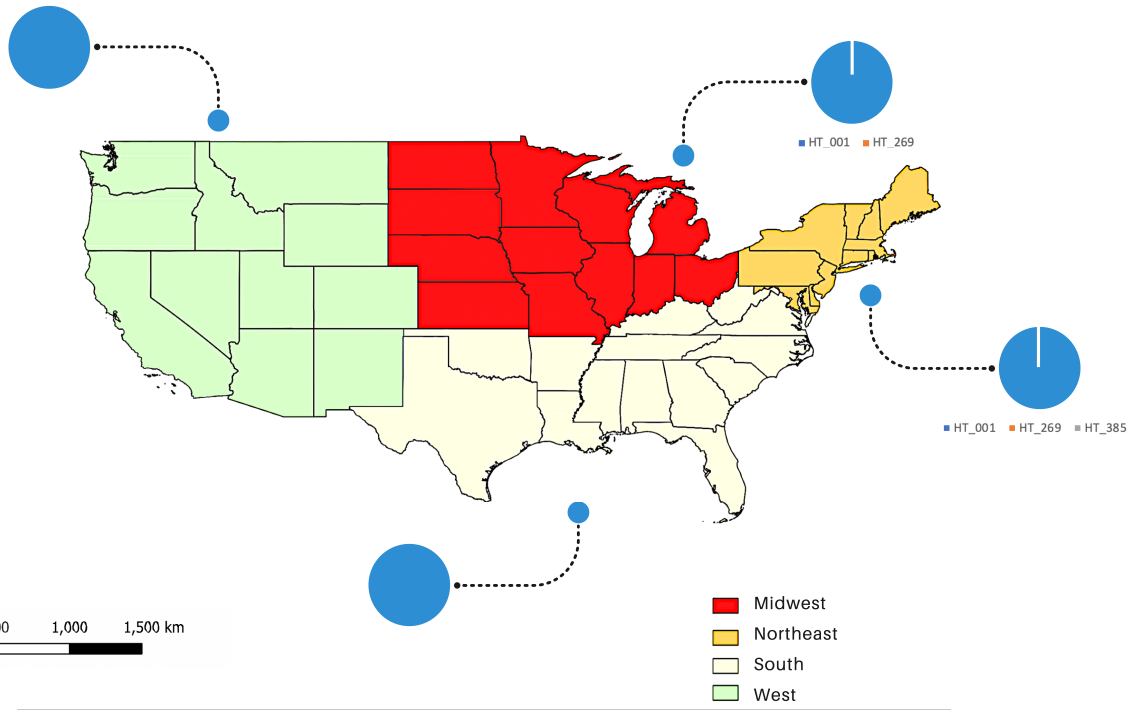

Fig. 16. Relative frequency of individual resistant F167Y haplotypes in *A. caninum* populations in 207 samples, comprised of 142 individual and 65 pooled samples from dogs across four different regions of the USA. A total number of three resistant haplotypes were identified

CHAPTER 6

SUMMARY AND CONCLUSIONS

Ancylostoma caninum is the most prevalent nematode parasite in dogs across the USA. A recent study found that the prevalence of hookworms remained very stable from 2012-2014, but then from 2015 onwards, there was a steady yearly increase, with an overall increase of almost half by 2018. Moreover, in a study assessing intestinal parasites from fecal samples collected in dog parks across the USA in 2019, the prevalence of *A. caninum* was 7.1%. Interestingly, this prevalence is more than twice as high as that reported for 2018 and is more than 2/3 higher than the mean prevalence for 2017-2019. Taken together these data suggest that hookworm prevalence is rapidly increasing, and that dogs that visit dog parks are at a higher risk of infection.

We have convincingly reported MDR in *A. caninum* to all major drug classes commonly used to treat hookworms of dogs in the USA. Additionally, we confirmed the MDR status of the *A. caninum* isolate, Worthy 4.1F3P, against pyrantel pamoate, fenbendazole and milbemycin oxime and susceptibility against emodepside.

We also provided strong and conclusive evidence that racing greyhounds in the USA are infected with MDR *A. caninum* at a very high prevalence, and with wide geographic distribution. Furthermore, very high IC_{50} and IC_{95} values were measured for both the benzimidazoles and macrocyclic lactones when compared to the susceptible isolate from our previous work, indicating that almost every sample was resistant to both

drugs. Additionally, the F167Y (TTC>TAC) benzimidazole resistance polymorphism was detected, in 99% of the samples, and at high frequencies in more than 2/3 of the samples.

Furthermore, more than half of all the hookworm positive samples we tested collected from four geographical regions carry resistant alleles, and about 25% of them had frequencies ≥75%. No differences were detected in breed size or age group in prevalence nor SNP frequency, so no risk factors associated with being infected with resistant worms, nor the % of those resistant alleles were identified.

Viewing together, these data from the greyhound samples as well as the samples collected from the diverse geographic regions of the USA, there is strong genetic evidence that almost all greyhounds are infected with drug-resistant hookworms, that resistance evolved as a single hard selective sweep in greyhounds and then crossed over into the pet dog population, and that more than half of all pet dogs with hookworm infections have worms with resistant alleles. It is important to perform further analyses looking into the haplotype networks as well as the genetic diversity as these will likely provide deeper insights into the relatedness of the resistant and susceptible alleles and the geographic origin(s) of these MDR worms.

This dissertation describes a handful of studies that attempt not only to answer several fundamental questions about anthelmintic resistance in the canine hookworm, *A. caninum*, but also provide data that could be useful for veterinarians and parasitologists to diagnose and manage infections with these MDR isolates. This dissertation is only the beginning of the story of anthelmintic resistance in *Ancylostoma caninum* and should provide the groundwork for additional studies looking into the development of new diagnostic tests, mechanism of resistance for the macrocyclic lactones and

tetrahydropyrimidines drug classes, and new drug therapies to control these MDR infections.