# Suspect macrocyclic lactone resistance cases with the canine heartworm (*Dirofilaria immitis*): mismatch between microfilarial suppression test and SNP results in Australia

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**Abstract**

Canine heartworm disease is a potentially deadly cardiopulmonary disease caused by the mosquito-borne filarial nematode *Dirofilaria immitis*. In Australia, the administration of macrocyclic lactone (ML) drugs has successfully reduced the prevalence of *D. immitis* infection. However, the recent re-emergence of *D. immitis* in dogs in Queensland, Australia and the identification of ML-resistant isolates in the USA poses an important question of whether ML-resistance has emerged in this parasite in Australia. The aim of this study was to utilise phenotypic and genotypic assays to determine the presence of ML-resistant *D. immitis* in Australia. To do this, we surveyed 45 (n=45) dogs from Queensland and New South Wales across 3 years (2019-2022) for the presence of *D. immitis* infection using an antigen test, quantitative Modified Knott's test, and qPCR targeting both *D. immitis* and the *D. immitis* symbiont *Wolbachia*. A phenotypic assay utilising a microfilarial suppression test for 23/45 dogs was coupled with genetic testing of filtered microfilariae for SNPs previously associated with ML-resistance in isolates from the USA. Sixteen (16/45) dogs tested positive for *D. immitis* infection despite reportedly receiving 'rigorous' heartworm prevention for 12 months prior to the study. The phenotypic and genotypic assays in this study did not unequivocally demonstrate the presence of ML-resistant *D. immitis* in Australia. Although the failure of 16 dogs to reduce microfilaremia by >90% after ML treatment was considered phenotypic evidence of suspect ML-resistance, no genotypic evidence was discovered using the genetic SNP analysis. It is possible that the presence of ML-resistance in this study was masked by the high initial dose of moxidectin (microspheres, 500 µg/kg s.c.) administered to eighteen (18/23) dogs at the time of diagnosis, which had the potential to clear any ML-resistant *D. immitis* microfilariae prior to post-ML treatment sampling.

## 1. Introduction

Canine heartworm disease is a potentially deadly cardiopulmonary disease caused by the mosquito-borne filarial nematode *Dirofilaria immitis*, which is distributed across many parts of the world (Simón et al., 2012; Genchi and Kramer, 2020; Lau et al., 2021; Noack et al., 2021). Although the prevalence of *D. immitis* in Australia is low, the parasite is endemic to the country and has recently re-emerged in dogs in Queensland (Nguyen et al., 2016; Orr et al., 2020; Panetta et al., 2021). For the past 30 years in Australia and the USA, the main strategy for canine heartworm prevention has been the administration of macrocyclic lactone (ML) anthelmintics (Orr et al., 2020; Diakou and Prichard, 2021; Lau et al., 2021; Noack et al., 2021; Prichard, 2021). These ML preventatives include monthly oral or topical formulations of ivermectin, milbemycin oxime, moxidectin and selamectin, or extended-release injections of moxidectin microspheres (Nguyen et al., 2016; McTier et al., 2019a). When used according to product instructions, these preventatives are approved to be 100% effective against less than 30-day old *D. immitis* (Hampshire, 2005). Over the past 20 years, lack of efficacy (LOE) reports for all heartworm product categories have occurred in the Lower Mississippi River Valley region of the USA (Hampshire, 2005). Most of these LOE reports were associated with compliance issues and extenuating circumstances, which prevented dogs from receiving sufficient product every 30 days (Atkins et al., 2014). A small number of LOE cases were not linked to noncompliance, indicating the potential development of ML-resistance in canine heartworms (Atkins et al., 2014). Laboratory studies have now identified several ML-resistant *D. immitis* isolates circulating in the Lower Mississippi Valley region of the USA (Blagburn et al., 2011; Pulaski et al., 2014; Bourguinat et al., 2015; Blagburn et al., 2016; Maclean et al., 2017; McTier et al., 2017b; Diakou and Prichard, 2021). These studies involved experimentally infecting ML-naïve dogs with infective L3 obtained from field LOE cases (Pulaski et al., 2014). In addition, <100%

efficacy has been experimentally demonstrated for all currently marketed MLs (Blagburn et al., 2016; McTier et al., 2019b).

MLs are essentially the only class of drugs currently available for canine heartworm prevention in Australia, in addition to the daily diethylcarbamazine citrate (Noack et al., 2021). Hence, the emergence of ML-resistance in *D. immitis* in the USA presents a significant concern for the veterinary industry by limiting the usefulness of ML products and jeopardising canine health. Prolonging the efficacy of MLs for canine heartworm prevention is warranted. With the absence of rapid biological tests that would demonstrate LOE of the target population (<30-day old *D. immitis*), discovery of heritable DNA molecular markers is needed for monitoring ML-resistance in adult *D. immitis* populations or their microfilaria within the dog bloodstream. Previous studies from the USA have compared the genotypes of different groups of *D. immitis* and identified single nucleotide polymorphisms (SNPs) potentially linked to ML-resistance (Bourguinat et al., 2011; Bourguinat et al., 2015). A whole genome approach was used in Bourguinat et al. (2015) to identify 186 SNP loci associated with the inability of dogs to remove circulating microfilariae after ML administration. From these 186 SNPs, a subset of 42 SNPs was initially validated as being highly associated with the ML-resistance phenotype in *D. immitis* (Bourguinat et al., 2015). The 42 SNPs were further evaluated in 10 additional ML-susceptible and ML-resistant isolates to generate 2, 3, 5 and 10-SNP predictive models for ML-resistance (Bourguinat et al., 2017). The 10 SNP approach that most effectively separated ML-susceptible and ML-resistant isolates was validated in an extensive study from south-east USA with field-collected *D. immitis* microfilariae, demonstrating high levels of sensitivity and specificity (Ballesteros et al., 2018). The SNP-based genotype predicted the phenotype which was defined by the inability of MLs to clear microfilariae using a suppression test and thus implied the presence of ML-resistant *D. immitis* infection (Ballesteros et al., 2018).

In contrast to the USA, it is currently unknown whether ML-resistance has emerged in *D. immitis* in Australia. A recent study revealed a large proportion of Australian dogs that were microfilaria-positive despite 'rigorous' administration of MLs in far-north Queensland (Nguyen et al., 2016). However, evidence of ML purchase history or ML administration was not provided by clients (Nguyen et al., 2016). A study that adopted the predictive SNP models developed in the USA to determine ML-resistance status did not find evidence of SNPs associated with ML-resistance in adult *D. immitis* from Sydney, Australia (Lau et al., 2021). As *D. immitis* prevention remains critical and prevalent across tropical Australia, further inquiry into ML-resistance in Australian *D. immitis* populations is necessary (Orr et al., 2020; Panetta et al., 2021). New screening tools and surveillance are required that apply the microfilarial suppression test originally proposed by Geary et al. (2011) and subsequently utilised by Ballesteros et al. (2018).

The aim of this study was to utilise phenotypic and genotypic assays to determine the presence of ML-resistant *D. immitis* in Australia. To do so, we surveyed dogs across 3 years (2019-2022) for the presence of *D. immitis* using an antigen test, quantitative Modified Knott's test, and qPCR targeting both *D. immitis* as well as the *D. immitis* symbiont *Wolbachia*. We utilised phenotypic assays using a sequential Modified Knott's test for the microfilarial suppression test, coupled with filtration of microfilariae for genetic testing of SNPs previously associated with ML-resistance in isolates from the USA.

## 2. Material & Methods

### 2.1. Blood collection from dogs in Queensland and New South Wales

Blood samples were collected opportunistically from dogs in Queensland and New South Wales, Australia between September 2019 and January 2022. The dogs selected for this study

were privately-owned animals from participating veterinary practices and had confirmed or suspected *D. immitis* infections. Blood samples (≤4 mL) were collected by registered veterinarians and shipped in EDTA tubes to the Sydney School of Veterinary Science, University of Sydney, Australia for diagnostic *D. immitis* testing. The following details were obtained for each dog: sex, age, breed, desexing status, locality, heartworm prevention history for the previous 12 months, and whether the animal had lived or visited other states or territories. Dog owners were required to complete a consent form prior to blood collection.

### 2.2. Microfilarial suppression testing

To determine the ML-resistance phenotypes of *D. immitis* from infected dogs, we adopted a microfilarial suppression test. This test utilises a Modified Knott's test to enumerate the number of *D. immitis* microfilariae in dog blood before and ideally 30 days after ML treatment. This allows us to observe reductions in microfilaremia levels. The type of ML product administered was at the veterinarian's discretion, according to their best practice in heartworm disease management. We calculated the percentage (%) reduction of microfilariae between the pre-treatment and post-treatment samples. A microfilariae reduction of <75% is suggestive of ML-resistant *D. immitis* (Geary et al., 2011). In cases in which dogs showed <90% reductions in microfilariae, additional blood samples were collected where possible to observe whether microfilariae levels eventually declined.

To perform the Modified Knott's test, 1 mL of whole blood was mixed with 10 mL of 2% formalin in a 15 mL tube (Knott, 1939; Mylonakis et al., 2004). The mixture was centrifuged at maximum speed for 5 min using a benchtop centrifuge (POCD Scientific, Australia). The pellet was mixed with 1% methylene blue stain and enumerated microscopically. The slide was examined under a microscope (BX40, Olympus, Australia) using 10× objective and the number of microfilariae observed in the entire slide was counted.

### 2.3. Packed cell volume and total plasma protein

The packed cell volume (PCV) and total plasma protein (TPP) of each blood sample were measured to provide additional information on the general health status of the animal. To do this, blood was drawn into a micro-haematocrit capillary tube until ¾ full. One end of the tube was sealed with 3 mm of clay before being centrifuged at 13,000 rpm for 3 min using a micro-haematocrit centrifuge (Clements, Australia). After centrifugation, any presence of haemolysis, jaundice, or lipaemia in the plasma layer was noted. The PCV was measured using a micro-haematocrit reader (Hawksley, England). A diamond pen was used to create an indent above the buffy coat in the capillary tube. The tube was broken at the indent site to release the plasma. A few drops of plasma were placed onto a refractometer (Leica Inc., USA) and the TPP was measured.

### 2.4. Detection of *D. immitis* antigen using DiroCHEK® ELISA

Prior to performing DiroCHEK® ELISA (Zoetis, Australia) testing, the stored plasma samples were thawed at room temperature and vortexed for 3 sec. Both 'neat' and 'heated' aliquots of each plasma sample were tested. To heat the plasma samples, a 100 μL aliquot of plasma was diluted with 100 μL of phosphate buffered saline (PBS; pH = 7.4) in a plain 1.5 mL tube. For samples with insufficient plasma volume, only 50 μL of plasma was diluted with 50 μL of PBS. The diluted plasma samples were heated at 103°C for 10 min using a dry heat block (Major Science). Heated samples were immediately centrifuged at maximum speed for 5 min using a benchtop centrifuge (Eppendorf, Australia) set at 4°C. The supernatant was transferred into a separate 1.5 mL tube for use in DiroCHEK® ELISA testing. To load the DiroCHEK® ELISA plate, 50 μL of heated and unheated plasma for each sample were transferred into separate wells. Positive and negative controls provided by the manufacturer were included, and samples with leftover heated plasma were included as

repeats. The DiroCHEK® ELISA test was then performed according to manufacturer's instructions. The optical density (OD) of each sample in the DiroCHEK® ELISA plate was measured at 620 nm in a Halo LED 96 microplate reader (Dynamica). To distinguish between negative and positive samples, the following equation was applied as a threshold:

$$Threshold = 3\ x\ SD\ (Mean[OD\ of\ negative\ controls])$$

### 2.5. Microfilariae extraction

To isolate microfilariae from dog blood, a filtration procedure was adopted and modified (Bourguinat et al., 2015). Prior to filtration, 1 mL of whole blood was diluted with 35 mL of Red Blood Cell lysis buffer (8 g/L sodium carbonate / 5 mL/L Triton X-100) in a 50 mL tube. For one sample (HW52), only 100 µl of blood was diluted. The diluted blood was then pushed through a syringe containing a polycarbonate membrane filter (pore size 20 µm, filter diameter 25 mm; Sterlitech® Corporation, WA). This allowed the microfilariae to be collected on the filter membrane whilst other blood components passed through the syringe. The syringe was then flushed with 50 mL of phosphate buffered saline (PBS). The filter membrane was removed from the syringe, placed into a 1.5 mL tube, and stored at -20°C until further use.

### 2.6. DNA isolation

DNA was isolated from 100 µL of whole blood using the blood protocol from the Monarch® Genomic DNA Purification Kit (New England Biolabs, Australia). In addition, DNA was isolated from microfilariae on filter membranes using the same kit. DNA isolation from filter membranes followed the animal tissue sample protocol and used Proteinase K. Each DNA isolation batch contained an extraction blank. The eluted DNA was stored at -20°C until further use in the qPCR and next generation sequencing (NGS) assays.

### 2.7. Quantitative PCR targeting dog, *D. immitis* and *Wolbachia* sp. of *D. immitis*

A qPCR targeting the partial canine glyceraldehyde3-phosphate dehydrogenase (*GAPDH*) region was performed to verify the presence of dog DNA using primers with a probe, as previously described (Orr et al., 2020; Panetta et al., 2021) (Supplementary Table S1). A diagnostic TaqMan probe qPCR targeting the *cox1* gene was performed to detect *D. immitis* DNA using primers with a probe, as previously described (Panetta et al., 2021) (Supplementary Table S1). A qPCR targeting the *ftsZ* gene of *D. immitis*-associated *Wolbachia* was performed using primers and probe described in Laidoudi et al. (2020) (Supplementary Table S1).

The dog, *D. immitis* and *Wolbachia* qPCR assays were carried out in 10 µL final volumes of Luna® Universal Probe qPCR Master Mix (New England Biolabs, Australia) containing 1 µL of template DNA isolated from whole dog blood and primers and probe at a final concentration of 400 nM and 100 nM, respectively. To confirm the presence of *D. immitis*, qPCRs targeting dog and *D. immitis* DNA were repeated using DNA from extracted microfilariae. For these reactions, the volumes of primer, probe, PCR-grade water, and template DNA were doubled to reach final volumes of 20 µL. All qPCRs were carried out on a CFX96 Real-Time PCR system with the corresponding Bio-Rad CFX Maestro Software (BioRad, Australia). Cycling conditions for all three assays consisted of an initial denaturation at 95°C for 1 min followed by 40 two-step cycles at 95°C for 15 sec and 60°C for 30 sec. Each qPCR run included DNA extraction blanks and a no template control (NTC) of PCR-grade water. The arbitrary qPCR baseline threshold was set to 100 RFU and $C_t$-values were reported for all samples. A qPCR targeting the *GAPDH* region of the dog genome confirmed the presence of dog DNA in all samples. All No Template Control (NTC) reactions remained negative.

**2.8. Statistical analysis of phenotypic data**

A paired t-test was performed to compare the OD between neat and heated plasma samples in the DiroCHEK® ELISA. Additional paired t-tests were performed to observe phenotypic differences between pre-ML treatment and post-ML treatment samples. To evaluate linear relationships between phenotypic datasets, Pearson's correlation coefficients were generated. Prior to performing correlation analyses with the qPCR data, 'N/A' results were replaced with a $C_t$-value of '40'. All statistical analyses of phenotypic data were performed using GraphPad Prism (version 9.3.1).

### 2.9. Selection of SNP markers

Studies from the USA identified 10 SNPs that best differentiated ML-susceptible *D. immitis* from ML-resistant isolates (Bourguinat et al., 2017). Seven of these SNPs were selected for analysis in this study, with 5 of these SNPs belonging to the 5-SNP predictive model generated by Ballesteros et al. (2018). Additional information about the 7 SNPs selected for this study including their position in the genome, various names used in other studies, primer sequences, fragment sequences, and specific polymorphisms at each locus are available in Supplementary Table S2.

### 2.10. Pyrosequencing targeting 5 SNPs associated with ML-resistance in USA

A total of 43 DNA samples from extracted microfilariae were selected for analysis using pyrosequencing technology. Out of the 7 SNPs selected for this study, the following 5 SNPs were targeted: L42411, L21554, L45689, L9400, and L20587 using newly designed primers (Supplementary Table S3). For each DNA sample, 15 µL was submitted to Macrogen Inc., Seoul, Korea for sample preparation, assay design, PCR amplification, pyrosequencing and data analysis on the PyroMark Q48 Autoprep and the associated PyroMark Q48 Advanced Software (Qiagen).

## 2.11. Illumina PCR amplicon Next Generation Sequencing targeting 7 SNPs associated with ML-resistance in USA

Illumina overhang adapters were added to all 7 primer sets which targeted regions encompassing the SNPs of interest (L42411, L21554, L45689, L9400, L20587, L15709_A, and L30575) according to Bourguinat et al. (2015). Their reference sequences were mapped to *D. immitis* genome nDi.2.2 scaffolds (Godel et al., 2012). The first stage PCRs for Illumina Next Generation Sequencing (NGS) were performed in-house according to the '16S Metagenomic Sequencing Library Preparation' instructions for the Illumina MiSeq system (Illumina, Australia). Conventional PCR assays were carried out with Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Australia). For some samples, dilutions of 1:10 or 1:100 were used due to low $C_t$-values obtained from the *D. immitis*-specific qPCR. Conventional PCRs were performed on a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Australia). The cycling conditions included an initial denaturation step at 98°C for 30 sec followed by 27 – 35 cycles of 98°C for 5 sec, 55°C for 10 sec and 72°C for 20 sec with a final extension at 72°C for 2 min. The number of cycles varied so that each sample reached the exponential phase of amplification, as previously described (Power et al., 2021). To increase efficiency of the first stage PCRs, conventional PCR assays were replaced with qPCR assays with SensiFAST™ SYBR® No-ROX Kit (Bioline, Australia). The cycling conditions included an initial denaturation at 95°C for 3 min followed by 28 - 40 cycles of 95°C for 5 sec, 55°C for 10 sec and 72°C for 20 sec with a final melt curve. The inclusion of a melt curve allowed us to confirm the presence of the desired PCR product. DNA of an adult *D. immitis* collected from a dog in Sydney (P6/20D), Australia was used as a positive control (Lau et al., 2021). A no template control (NTC) of PCR-grade water was included for each SNP assay. Presence of expected PCR products was confirmed by visualisation on agarose gel for conventional PCR and qPCR products with ambiguous melt profiles. To determine

whether comparable results were obtained using the two PCR approaches, three samples (HW19, HW20, HW32) were run as both conventional PCR and qPCR reactions for 5 loci (L42411, 21554, 45689, 9400, 20587), along with additional samples for L42411 (HW33) and L20587 (HW63). To investigate the impact of DNA concentration on the NGS results, four samples in the L45689 assay (HW20, HW41, HW45, HW66) and four samples in the L9400 assay (HW20, HW31, HW66, P6/20D) were repeated using alternative dilutions or cycle numbers. As one sample (HW52) was analysed using filtered microfilariae from 100 µL of blood, repeats for three loci (L20587, L15709A, L30575) were included using filtered microfilariae from 1 mL of blood. There was no difference that wound compromised SNP calls between the approaches irrespective of the dilution, blood volume, cycle number or PCR approach used.

After the first-stage PCR, products were submitted to the Ramaciotti Centre for Genomics, University of New South Wales, Australia for indexing (384), library preparation and next generation sequencing (NGS) using Illumina MiSeq v2 250PE sequencing run.

### 2.12. SNP analysis of NGS samples

Data as FastQ files were analysed in R 4.0.5 within RStudio 2022.07.1 Build 554 (RStudio Team, 2022). For each SNP locus, primers were identified and removed from all sequencing reads using Cutadapt 4.0 (Martin, 2011) followed by DADA2, an open-source R package (https://github.com/benjjneb/dada2). DADA2 pipeline (1.18.0; Callahan et al. (2016)) was used to infer the *D. immitis* SNP amplicon sequence variants (ASVs) present in the filtered microfilariae DNA samples. The quality profiles of the cut sequencing reads were visualised and inspected using the “plotQualityProfile” tool. The cut forward and reverse reads (FastQ) were filtered, denoised and trimmed to 180 and 150 nucleotides, respectively, using the “filterAndTrim” tool. Overall, >92%, >75%, >97%, >71%, >96%, >91%, and >40% of input

reads were retained and 0.03%, 0.01%, 0.58%, 0.01%, 0.01%, 0.00%, and 0.00% of merged paired reads were removed as chimeras for L42411, L21554, L45689, L9400, L20587, L15709_A, and L30575, respectively. For data cleaning purposes, the proportion (%) of each ASV in each sample was calculated. In an effort to remove misassigned reads caused by sample bleeding or barcode hopping, a threshold of <2.5% was applied (Mitra et al., 2015). This involved removing the reads of ASVs whose proportions represented <2.5% in a sample and replacing them with '0'. Alternative thresholds of <1% and <0.5% were tested but not selected for further downstream analysis. A reference table containing frequencies of sequence fragments of all 7 SNP loci was created, with options for each possible SNP polymorphism. This reference table was imported into DADA2 and the "AssignSpecies" tool was used to assign the remaining ASVs to a reference sequence if they were a perfect match. ASVs were imported into CLC Main Workbench (version 22.0) (CLC bio, Qiagen, Australia) and "BLAST at NCBI" was performed. Based on the BLAST results, ASVs that were not identified as *D. immitis* were excluded and ASV proportions for each sample were re-calculated. The remaining *D. immitis* ASVs were aligned to their respective SNP reference sequences and visually inspected using CLC Main Workbench.

### 2.13. Animal Ethics

Research performed at the University of Sydney was approved by the Animal Research Ethics Committee, which follows the NSW Animal Research Act 1985 and the Australian code for the care and use of animals for scientific purposes 8$^{th}$ edition (2013).

### 2.14. Data accessibility

Raw FastQ sequences were deposited at (details). Additional data for this study is available at LabArchives (https://dx.doi.org/10.25833/ygs4-ha97).

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## 3. Results

### 3.1. Blood collection pre- and post-ML treatment

A total of 45 dogs (Dog 1 to Dog 45) and their 78 blood samples (HW01 to HW63, HW65 to HW79) were collected and followed up over 2.5 years (2019-2022) as suspect to be infected with *D. immitis* based on the veterinarians' judgement (Supplementary Table S4). At the beginning of the study, infection with *D. immitis* was confirmed in 44/45 dogs using at least one diagnostic test. Dogs testing positive for *D. immitis* were domiciled at eleven locations across eastern Australia, with thirty-eight dogs from Queensland and six dogs from New South Wales (Figure 1). All dog bloods were processed using exhaustive diagnostic laboratory workflow (Figure 2). Twenty-three (n=23) dogs were sampled at the time of diagnosis (pre-ML treatment) and at least once post diagnosis; 14 dogs once after treatment (post-ML), eight dogs twice after treatment, and one dog (Dog 40) three times after treatment (Figure 2, 3). In addition, twenty-two dogs were only sampled pre-ML treatment and following samples were not possible to obtain. A mean PCV of 0.52 (min 0.36 to max 0.66) and a mean TPP of 88.4 g/mL (min 60 to max 120 g/mL) were obtained from 69 blood samples.

### 3.2. Delays in >90% *Dirofilaria immitis* microfilariae count reduction post-ML treatment

A total of 37/44 (84.1%) *D. immitis*-positive dogs were microfilaraemic at the beginning of the study, with a mean initial microfilaria count of 7,689 microfilariae/mL. Microfilarial suppression testing was performed to demonstrate the suspect ML-resistance phenotypes of *D. immitis* (Figure 2). The percentage (%) reduction of microfilariae counts after ML administration was obtained for 22 dogs that had sequential blood samples. The mean initial microfilaria count for 21 dogs was 6,617 mf/mL (min. 38 to max. 38,250 microfilariae/mL)

plus one had 1 microfilaria/mL (Dog 1) (Figure 3, Table 1). In addition, one dog (Dog 19) had 0 microfilariae/mL, but tested positive on PCR and serology so a post-ML sample was also obtained and processed.

Using eight dogs sampled pre-ML which were then administered moxidectin (ProHeart SR-12, s.c.) and sampled again at least twice post-ML, we show a 43% reduction of microfilaria by day 7-49 (pre-ML: mean 6,115 microfilariae/mL; post-ML: mean 3,462 microfilariae/mL). This was reduced to 98% (post-ML: mean 133 microfilariae/mL) by 60-136 days (Figure 3A). Five of these dogs were subjectively considered to be on 'rigorous' heartworm prevention by their owners (Figure 3A). All remaining dogs but three were administered moxidectin (ProHeart SR-12, s.c.) at the time of diagnosis (Figure 3B,C,D). Three dogs were administered ivermectin (HeartGard Plus, p.o.). Dog 21 on ivermectin (HeartGard Plus) completely reduced their microfilariae by day 28 from a relatively low initial microfilaremia of 175 microfilariae/ml (Figure 3C). The remaining two dogs (Dog 2, 13) on ivermectin experienced (HeartGard Plus) <90% reductions in microfilaremia post-ML, with Dog 2 experiencing an increase in microfilariae by 181% (Figure 3C).

Sixteen dogs (73%, 16/22) had ≤ 90% reduction in microfilariae count, including three dogs (Dog 25, Dog 2, Dog 7) which had an increase in microfilariae (Figure 3B,C). At the first blood collection post-ML treatment, six dogs (27%, 6/22) had > 90% reduction in microfilariae count. A paired t-test revealed that there was no statistically significant reduction in microfilariae levels in the first blood collection after ML treatment (Figure 4A). We then used only samples with initial microfilaria counts of >1,000 and <10,000 microfilariae/mL, and a statistically significant reduction in microfilariae levels was observed (Figure 4B).

Of the 16 dogs that had ≤90% microfilariae reduction in the first post-ML blood collection, nine dogs underwent a second blood collection after treatment to determine whether circulating microfilariae were eventually cleared from their systems (Figure 3A,B). In this second blood collection, 8/9 dogs had > 90% reduction in microfilariae count and 1/9 dogs (Dog 25) had ≤90% reduction in microfilariae count. Dog 25 had >20,000 microfilariae/mL on both occasions, however an additional microfilaria count one week later from Dog 25 revealed a ≤90% reduction (Figure 3B). For this second blood collection, a statistically significant reduction in microfilariae levels was observed from the raw data (Figure 4C). When three outliers (Dog 24, Dog 25, Dog 35) were excluded from this analysis, a statistically significant reduction in microfilariae was maintained (Figure 4D).

### 3.3. Presence of heartworm antigen using DiroCHEK® ELISA

Out of all dogs with confirmed *D. immitis* infection at the start of this study, 41/44 (93.2%) dogs initially tested positive for *D. immitis* antigen using DiroCHEK® ELISA with neat plasma samples and 43/44 (97.7%) initially tested positive using DiroCHEK® ELISA with heated plasma samples. Out of all blood samples collected in this study, 88.2% (67/76) of neat plasma samples and 98.7% (75/76) of heated plasma samples tested positive for *D. immitis* antigen using DiroCHEK® ELISA, respectively (Figure 5A, Table 1). A paired t-test was performed on the raw DiroCHEK® ELISA data, revealing a significant difference in $OD_{620}$ between neat and heated plasma samples (Figure 5B). Moderate positive correlations were found between raw microfilariae counts and the $OD_{620}$ of neat and heated dog plasma in DiroCHEK® ELISA (Figure 6A). These moderate positive correlations were maintained after excluding samples with microfilariae counts greater than 20,000 microfilariae/mL (Figure 6B).

### 3.4. Presence of *Dirofilaria immitis* and *Wolbachia* sp. of *D. immitis* using qPCR

DNA samples obtained from dog blood were subjected to qPCR targeting the *cox1* gene of *D. immitis* and the *ftsZ* gene of *D. immitis*-associated *Wolbachia*. *D. immitis* and *Wolbachia* DNA were initially detected in 39/44 (88.6%) and 34/44 (77.3%) dogs with confirmed *D. immitis* infection, respectively. At Day 0 (pre-ML), *D. immitis* and *Wolbachia* were detected in 100% (23/23, $C_t$ mean: 24.63, $C_t$ range: 19.48-37.68) and 82.61% (19/23, $C_t$ mean: 28.21, $C_t$ range: 25.07-31.75) of dogs, respectively. At ~Day 30 (post-ML), *D. immitis* was detected in 82.61% (19/23, $C_t$ mean: 24.37, $C_t$ range: 21.80-32.13) of dogs and *Wolbachia* was detected in 65.22% (15/23, $C_t$ mean: 33.10, $C_t$ range: 28.78-35.48) of dogs. By Day 30+, 77.8% (7/9, $C_t$ mean: 29.00, $C_t$ range: 25.89-36.45) of dogs were positive for *D. immitis* and 33.33% (3/9, $C_t$ mean: 30.27, $C_t$ range: 21.01-35.78) of dogs were positive for *Wolbachia*. The *D. immitis*-specific qPCR on extracted microfilariae DNA found one additional *D. immitis* DNA positive dog (Dog 31: 0 microfilaria/mL) that tested negative for *D. immitis* DNA on a sample from whole blood. In addition, two dogs (Dog 1: 1 microfilaria/mL, Dog 20: 0 microfilaria/mL) were *D. immitis* DNA positive only on whole blood but *D. immitis* DNA-negative using extracted microfilariae (Supplementary Table S4).

A strong negative correlation was demonstrated between the *D. immitis* qPCR $C_t$-values of whole blood DNA and microfilariae counts that were <20,000 mf/mL (Figure 6C). Similarly, a strong negative correlation was observed between the *Wolbachia* qPCR $C_t$-values of whole blood DNA and microfilariae counts (Figure 6D). Including five samples with microfilariae counts greater than 20,000 mf/mL, a moderate negative correlation was shown between the *D. immitis* qPCR $C_t$-values of whole blood DNA and microfilariae counts (Figure 6E). For all *Wolbachia* qPCR of whole blood DNA, a moderate negative correlation was observed with microfilaria counts (Figure 6F). A strong positive correlation was found between the *D. immitis* and *Wolbachia* qPCR $C_t$-values of whole blood DNA samples (Figure 6G). Twenty-

six samples with $C_t$-values of '40' were removed as outliers, resulting in a moderate positive correlation (Figure 6H).

Paired t-tests of the raw *D. immitis* and *Wolbachia* qPCR data revealed statistically significant increases in $C_t$-value from pre-ML to first post-ML sampling (Figure 7A,B). Three samples from the *D. immitis* qPCR with pre-ML $C_t$-values > 30 were excluded, and a significant increase in $C_t$-value remained (*D. immitis* paired t-test, t[19]=2.159, p=0.0439). In the *Wolbachia* qPCR, four samples with Day 0 $C_t$-values >35 were excluded, and a significant increase in $C_t$-value was still observed (*Wolbachia* qPCR paired t-test, t[18]=9.290, p <0.0001). Similarly, paired t-tests of the raw *D. immitis* and *Wolbachia* qPCR data showed statistically significant increases in $C_t$-values from pre-ML to second post-ML sampling (Figure 7C,D).

### 3.5. Illumina PCR amplicon NGS identification of ML-susceptible *D. immitis* in Australian dogs

Illumina NGS amplicon metabarcoding from 64 extracted microfilariae DNA samples from 37 dogs and one DNA sample from an adult *D. immitis* (P6/20D, positive control) was performed to determine the nucleotide proportions at 7 SNPs of interest (65 DNA samples×7 loci = 455 PCRs) (Figure 8). Successful amplification of the target region was achieved for 96.5% (439/455) of PCR samples. Three DNA samples had loci which failed the first-stage PCR amplification and were not submitted for Illumina NGS amplicon sequencing (HW34: 1/7 SNPs submitted, 0 microfilariae/mL; HW50: 5/7 SNPs submitted, 1 microfilariae /mL; HW51: 1/7 SNPs submitted, 0 microfilariae/mL). Only 5/7 SNPs were submitted for P6/20D. FastQ files were obtained, and SNP calling was successful for 98.2% (431/439) of submitted PCR samples. Four DNA samples had loci which failed to produce reads for SNP calling (HW29: 6/7 SNPs successful; HW34: 0/1 SNPs successful; HW50: 0/5 SNPs successful;

HW51: 0/1 SNPs successful). For the purpose of reproducibility, an additional 28 PCR samples were submitted as 'repeats'. These repeat samples demonstrated identical outcomes in terms of SNP calling. Overall, a total of 8,742,348 merged non-chimeric paired reads were obtained to acquire amplicon sequence variants (ASVs) which were matched to the reference ML 'susceptible' and ML 'resistant' allele for their respective SNP (Figure 8).

#### 3.5.1. *L42411 (Di.2.2.scaf00001:466197)*

A total of 1,368,512 merged non-chimeric paired reads were obtained for L42411 with an average of 20,426 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. All 62 samples had 100% of their reads assigned to a single ASV (L42411_ASV1) which was a perfect match to the ML 'susceptible' reference sequence.

#### 3.5.2. *L21554 (nDi.2.2.scaf00046:222254)*

A total of 1,167,570 merged, non-chimeric paired reads for 362 ASVs were obtained for L21554 with an average of 17,690 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. Of these 62 samples, 14 had 100% of their reads assigned to a single ASV (L21554_ASV1) which was a perfect match to the 'susceptible' reference sequence. The remaining 48 samples contained L21554_ASV1 along with low levels of other ASVs (L21554_ASV2, L21554_ASV3) which possessed a SNP at another position in the sequence.

#### 3.5.3. *L45689 (nDi.2.2.scaf00185:10639)*

A total of 1,664,418 merged, non-chimeric paired reads for 101 ASVs were obtained for L45689 with an average of 23,777 paired reads per sample. Overall, 61/65 DNA samples had 100% of the susceptible nucleotide at the suspect position. Of these 61 samples, 15 had 100% of their reads assigned to a single ASV (L45689_ASV1) which was a perfect match to the

‘susceptible’ reference sequence. The remaining 46 samples contained multiple ASVs (L45689_ASV1, L45689_ASV2, L45689_ASV3, L45689_ASV4, L45689_ASV8, L45689_ASV10, L45689_ASV11) which possessed 1-2 SNPs at other positions in the sequence. One sample (Dog 25 – HW39) contained low levels (3.8%) of L45689_ASV6 which possessed a nucleotide of unknown ML association at the suspect position (Figure 9).

#### 3.5.4. *L9400 (nDi.2.2.scaf00005:662854)*

A total of 908,148 merged, non-chimeric paired reads for 543 ASVs were obtained for L9400 with an average of 12,613 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. Of these 62 samples, 57 had 100% of their reads assigned to a single ASV (L9400_ASV1) which was a perfect match to the ‘susceptible’ reference sequence. The remaining 5 samples contained L9400_ASV1 along with other ASVs (L9400_ASV2, L9400_ASV30) which possessed a SNP at another position in the sequence.

#### 3.5.5. *L20587 (nDi.2.2.scaf00185:62174)*

A total of 1,600,533 merged, non-chimeric paired reads for 90 ASVs were obtained for L20587 with an average of 23,537 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. Of these 62 samples, 57 had 100% of their reads assigned to a single ASV (L20587_ASV1) which was a perfect match to the ‘susceptible’ reference sequence. The remaining five samples contained L20587_ASV1 along with other ASVs (L20587_ASV2, L20587_ASV4, L20587_ASV5, L20587_ASV6, L20587_ASV8, L20587_ASV11) which possessed a SNP at another position in the sequence.

#### 3.5.6. *L15709_A (nDi.2.2.scaf00046:76278)*

A total of 1,526,468 merged, non-chimeric paired reads for 78 ASVs were obtained for L15709_A with an average of 24,620 paired reads per sample. Overall, 61/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. All 61 samples had 100% of their reads assigned to a single ASV. Thirty samples had all reads assigned to L15709A_ASV1 which was a perfect match to the 'susceptible' reference sequence. The other 31 samples had all reads assigned to L15709A_ASV2 which possessed a deletion elsewhere in the sequence.

#### 3.5.7. *L30575 (nDi.2.2.scaf00046:22857)*

A total of 506,699 merged, non-chimeric paired reads for 542 ASVs were obtained for L30575 with an average of 8,173 paired reads per sample. Overall, 60/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. All 60 samples had 100% of their reads assigned to a single ASV (L30575_ASV1) which was a perfect match to the 'susceptible' reference sequence.

### 3.6. Pyrosequencing identification of ML-'susceptible' *D. immitis* in Australian dogs

Pyrosequencing of extracted microfilariae DNA was performed to analyse 5 SNPs. Automated calls for the nucleotide at the known SNP using pyrosequencing for L42411, L21554, L45689, L9400, and L20587 revealed that samples had >81%, >98%, >83%, >98%, and >87% of reads containing the 'susceptible' SNP nucleotide, respectively (Supplementary Table S4). For L21554 and L9400, 21/43 and 38/43 samples had 100% of reads containing the "susceptible" SNP nucleotide, respectively. Samples contained low levels (0.00-12.24%) of reads with an alternative nucleotide at the SNP position. Manual inspection of the pyrogram did not reveal presence of an alternative nucleotide that was previously recognised to be associated with the ML-resistance in the USA.

## 4. Discussion

The use of MLs over the past 30 years has successfully reduced the prevalence of canine heartworm infection (Orr et al., 2020; Lau et al., 2021). However, the re-emergence of *D. immitis* infection in Queensland, Australia and the confirmation of ML-resistant isolates in the USA poses an important question of whether ML-resistance exists in Australia (Blagburn et al., 2011; Pulaski et al., 2014; Bourguinat et al., 2015; Blagburn et al., 2016; Nguyen et al., 2016; Maclean et al., 2017; McTier et al., 2017b; Orr et al., 2020; Panetta et al., 2021). By utilising phenotypic assays and genotypic tests adopted from the USA, this study did not unequivocally demonstrate the presence of ML-resistant *D. immitis* in Australia. Although the canine heartworm microfilarial suppression test revealed some phenotypic evidence of suspect ML-resistance, no genotypic evidence was discovered. The approach in this study was similar to that of Ballesteros et al. (2018), where 27 dogs underwent microfilarial suppression testing and SNP analysis with moxidectin (Elanco, Advantage Multi for dogs, spot on). By doing so, they validated the association between ML microfilaricidal response phenotype and SNP loci genotype (Ballesteros et al., 2018). The current study obtained a comparable sample size to Ballesteros et al. (2018), and 5 of the 7 SNPs analysed in this study were adopted from their 5-SNP predictive model which had 94.1% sensitivity and 100% specificity. We initially used a pyrosequencing approach to detect the susceptible and resistant SNPs, but the SNP approach using Illumina amplicon metabarcoding coupled with DADA2 analysis provided reproducible results directly comparable to previous studies such as Ballesteros et al. (2018).

To treat canine heartworm disease, infected dogs are administered doxycycline and ML at the time of diagnosis (Day 0) to target the *Wolbachia* endosymbiont of *D. immitis*, prevent further infection and clear circulating microfilariae (Nelson et al., 2018). To target the adult stages of *D. immitis*, clinicians in Australia typically follow the three-dose protocol

recommended by the American Heartworm Society (Nelson et al., 2018). This three-dose protocol involves an injection of an adulticide (melarsomine) on Day 60, a second injection on Day 90, and a third injection 24 hours later (Nelson et al., 2018). Clinicians see their patients on Day 0, Day 60 and Day 90/91 that present opportunities not just for clinical examination, but for blood collection and subsequent *D. immitis* diagnostics. Such sampling is particularly important if we are inquiring about the clearance of microfilaria as a proxy to ML response. The current study suggests that the microfilarial suppression test is an ambiguous indicator for the detection of ML-resistance in Australian *D. immitis*. The microfilarial suppression test was initially suggested to be used with spot-on moxidectin in the USA based on the label claim for microfilaria and 99% clearance within 14 days in an experimental trial (Geary et al., 2011; Bowman et al., 2015). We adopted the suppression test by extending the interval to ~30 days for two reasons. Firstly, to suit the veterinary practice when owners brought their dog back for health check-up, and secondly to utilise the published microfilaricidal effect of yearly injectable moxidectin (Zoetis, ProHeart SR-12) used in Australia. The choice of ML-preventative, however, was at the veterinarian's discretion. Using this microfilarial suppression test, the failure of 16 dogs (ML at diagnosis; moxidectin microspheres: 14 dogs, ivermectin: 2 dogs) to reduce microfilaremia by >90% was considered phenotypic evidence of suspect ML-resistance. This finding was not corroborated by genetic SNP analysis or phenotypic testing beyond 30 days, as dogs that were re-tested eventually reached a >90% reduction in microfilaremia by Day 55 to Day 136. Thus, if the microfilarial suppression test is to be performed in Australia, a longer time period (>30 days) is required between pre-ML and post-ML testing to observe adequate reductions in microfilariae levels. Delays in reaching >90% microfilarial reduction following ML treatment have been previously observed. In McTier et al. (2017a), single doses of ProHeart 6 (Zoetis, moxidectin microspheres, 170 µg/kg) and oral moxidectin (250 µg/kg) on Day 0

resulted in ≤90% reductions in mean microfilariae counts for the resistant isolate ZoeMO-2012 at Day 28 (ProHeart 6: 86.5%, oral moxidectin: 83.6%), whereas ProHeart 12 (Zoetis, moxidectin microspheres, equivalent to ProHeart SR-12 registered in Australia, 500 µg/kg) led to a 91.2% reduction in microfilariae. At Day 84, all three MLs reached >90% reductions in microfilariae (ProHeart 6: 96.8%, ProHeart 12: 96.9%, oral moxidectin: 92.6%) (McTier et al., 2017a). The study tested only one *D. immitis* isolate under experimental conditions (McTier et al., 2017a). In contrast, the current study sampled privately-owned dogs which most likely carried a population of *D. immitis*, rather than a single isolate. It would be advisable to consider both the 60-day interval or even at the day 90/91, for a longer-term microfilarial suppression test to determine the microfilaricidal effects of the administered MLs in the *D. immitis* infected dogs.

The microfilarial suppression test ambiguity in the present study can be attributed to the varying effectiveness of MLs to clear circulating microfilaria (Prichard, 2021). In Australia, no product has a label claim for microfilaria, unlike in the USA where Advantage Multi for dogs (10% imidacloprid + 2.5% moxidectin topical solution) has such a claim and was therefore used by Ballesteros et al. (2018) in their study (Bowman et al., 2015). A microfilaricidal effect was noted early on for ivermectin in dogs at a dose of 50 µg/kg, but due to toxicity in dolichocephalic breeds, this high dose is not on any label (Blair and Campbell, 1979). The current preventative dose against <30-day old *D. immitis* with ivermectin is only 6 µg/kg, which is considered insufficient to clear microfilaria efficiently. Milbemycin oxime at 500 µg/kg is considered microfilaricidal, but no registered product currently has such a claim (Prichard, 2021). Moxidectin in the spot on formulation (Elanco, Advantage Multi for dogs/Advocate for dogs at a minimum dose of 2,500 µg/kg) was demonstrated to be effective against microfilaria, which has been claimed as such in the

Advantage Multi for dogs (Elanco) marketed in the USA (McCall et al., 2014; Bowman et al., 2015).

The traditional microfilarial suppression test utilises a Modified Knott's test to enumerate the number of circulating *D. immitis* microfilariae pre- and post-ML treatment. This enables clinicians to determine whether the ML is successfully clearing microfilaremia in the infected dog. However, performing a Knott's test is not practical for clinicians as it can be time-consuming, labour-intensive, and prone to human error. Alternative tools that are more rapid and accurate are required to measure changes in post-treatment microfilaremia levels. The blood samples in this study were screened for *D. immitis* using DiroCHEK® and qPCR targeting *D. immitis* and its associated *Wolbachia* endosymbiont. Based on our data, performing DiroCHEK® on neat or heated plasma was not suitable for the assessment of ML-treatment efficacy due to its lower correlation with the modified Knott's test. The *D. immitis* and *Wolbachia* qPCRs produced higher correlations with the modified Knott's test and were able to demonstrate the success of ML-treatment for reducing microfilariae levels. We therefore suggest clinicians to use these faster molecular tools as a substitute for the Knott's test when monitoring the success of MLs in canine heartworm cases. The *D. immitis*-specific qPCR detected a larger proportion of infected dogs in this study compared to the *Wolbachia*-specific qPCR and may therefore be a better tool for measuring microfilariae levels. Future studies will benefit from a triplex qPCR targeting *D. immitis*, its associated *Wolbachia*, and dog DNA to reduce time and cost. The same *Wolbachia* qPCR assay was recently used to monitor the *Wolbachia* levels in dogs from Italy following doxycycline and Advocate (moxidectin) treatment (Laidoudi et al., 2020; Louzada-Flores et al., 2022). By doing so, they found that all dogs tested negative for *Wolbachia* after one month. Contrastingly, the current study saw only 8/28 dogs test negative for *Wolbachia* approximately 30 days after ML-

treatment, with 4 of these dogs already being negative on Day 0. When infected dogs were re-sampled on Day 30+, only 6/9 tested negative for *Wolbachia*.

The SNPs analysed in Ballesteros et al. (2018) were able to distinguish between ML-susceptible and ML-resistant *D. immitis* in the USA. However, in the current study and Lau et al. (2021), no evidence of ML-resistance in Australian *D. immitis* was found using these same ML resistance-associated SNPs. In Lau et al. (2021), the genome data of five adult *D. immitis* exhibited marked genetic differences compared to those previous phenotypically characterised *D. immitis* isolates from the USA. It is therefore likely that the SNPs developed in the USA are not the best markers for association with ML-resistance in Australia (Ballesteros et al., 2018; Lau et al., 2021). Locally defined SNPs from whole genome sequence data are required to associate and specifically predict the ML-resistance status of Australian *D. immitis*. This will enable the early detection of ML resistance in Australia, allowing clinicians, researchers and the animal pharmaceutical industry to develop effective control measures to limit and manage the spread of resistant parasites.

Successful prevention of *D. immitis* heartworm can only be achieved with rigor in prevention due to inability to successfully remove older than 30-day *D. immitis* with current ML-based preventatives (Prichard, 2021). For veterinarians, however, the inquiry into rigor with ML-prevention will always be difficult with products administered by owners as they can unintentionally skip dosing if dogs regurgitate the dose (Atkins et al., 2014). Unintentionally missed ML-dose may lead to infection – apparent lack of efficacy (LOE) reports - despite owners' assumed rigor with prevention. In our study, despite reportedly receiving 'rigorous' heartworm prevention with either moxidectin, milbemycin oxime or ivermectin for 12 months prior to the veterinarian's diagnosis, 16 dogs tested positive for canine heartworm infection. Similar findings were encountered in Nguyen et al. (2016) where microfilaraemic

dogs in Queensland previously received 'rigorous' prevention. In this study, owner compliance could not be confirmed and was solely based on the owner's reports.

## 5. Conclusion

In summary, this study could not demonstrate ML resistance using the available tools in Australia. An alternative scenario, however, exists if the aforementioned SNPs are considered unreliable markers. Focusing on the dogs that were administered moxidectin (microspheres, 500 µg/kg s.c.) at the time of diagnosis of *D. immitis* infection, the high initial dose was potentially sufficient to clear even ML-resistant *D. immitis* microfilariae. Higher doses such as 24 µg/kg of moxidectin or higher have been experimentally shown to improve *D. immitis* prevention with known ML-resistant *D. immitis* and are already formulated and approved for use in dogs (McTier et al., 2019a; Savadelis et al., 2022). Moxidectin (microspheres) has been shown to significantly improve prevention in a field trial in the Lower Mississippi River Valley region, which is known for its high prevalence of ML-resistance (McTier et al., 2019b). If the alternative scenario is considered, then ML resistance has been masked by the use of moxidectin (microspheres) as a potentially effective approach to counter such infections.

To confirm the presence of drug resistance in a parasite population, we must demonstrate that resistance is genetically inherited (Kohler, 2001). Since 2005, lack of efficacy complaints for heartworm preventatives led to speculation and controversy on the potential emergence of ML-resistant *D. immitis* in the USA (Hampshire, 2005; Atkins et al., 2014). It was not until 2014 that the first ML-resistant strain (LSU 10) was isolated in the USA and resistance to MLs was unequivocally confirmed (Pulaski et al., 2014). To do this, a laboratory dog was experimentally infected with L3s obtained from a field case, and then treated with ivermectin

(Pulaski et al., 2014). Infective larvae from this dog were then successfully passaged into a second laboratory dog, which confirmed the genetic heritability of drug resistance (Pulaski et al., 2014). In Australia, we currently lack these types of specialised facilities where laboratory dogs can be experimentally infected with canine heartworm. Due to this limitation, it remains unknown whether ML-resistance is present in Australia, much like the 2005-2014 time period in the USA. Until we can demonstrate the heritability of ML-resistance in Australian *D. immitis* isolates via experimental infection studies, we can only speculate and test for resistance using the available tools as a proxy.

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## Supplementary Tables

**Supplementary Table S1:** Primers used throughout the study

**Supplementary Table S2:** SNPs in this study adopted from previous 5-SNPs models from the USA

**Supplementary Table S3:** Pyrosequencing assays for *Dirofilaria immitis* SNP associated with ML-resistance

**Supplementary Table S4:** Summary of Australian dogs infected with *Dirofilaria immitis* monitored over 2019-2022

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## Legends to Figures

**Fig. 1.** Location of dogs infected with *Dirofilaria immitis* included in this study from Eastern Australia. These dogs (n=44) were sampled between 2019-2022 for presence of *D. immitis* antigen, *D. immitis* DNA and associated *Wolbachia* DNA. The red circles indicate a location that is associated with the town name and the number indicates the number of *D. immitis*-positive dogs using any test. On the right, two close-ups of Townsville, Queensland and Greater Sydney, New South Wales are shown. Image from Google Earth; data from SIO, NOAA, U.S Navy, NGA, GEBCO (Image Landsat/Copernicus, CNES/Airbus, Maxar Technologies).

**Fig. 2.** Diagnostic methods for the diagnosis of *Dirofilaria immitis* infection and response to macrocyclic lactone administration in dogs. Blood samples were collected at the time of diagnosis, followed by the administration of doxycycline antibiotic and macrocyclic lactone, and then the administration of adulticide injections. Each blood sample was assayed using a Modified Knott's test to quantify microfilariae, microfilariae were extracted from blood via filtration for DNA isolation, plasma samples were assayed for the presence of *D. immitis* antigen and DNA isolated directly from blood was assayed quantitatively for the presence of *D. immitis* DNA and endosymbiotic *Wolbachia* DNA. DNA isolated from filtered microfilariae were tested for the presence of *D. immitis* DNA and endosymbiotic *Wolbachia* DNA in addition to single nucleotide polymorphisms (SNPs) associated with macrocyclic lactone resistance from the USA. SNPs were assayed using Illumina amplicon metabarcoding next generation sequencing (NGS) and pyrosequencing.

**Fig. 3.** Microfilarial suppression testing on 21 dogs positive for *Dirofilaria immitis*. (A) Dogs (n=8) that exhibited <90% (red square %) microfilarial reduction at the first post-ML sampling, but consecutive second post-ML sampling demonstrated >90% microfilarial reduction (green square %). (B) Dog 25 at the first post-ML sampling had an increased number of microfilariae, but one week later the microfilariae were reduced by <90% (red squares); however, if comparing the 1$^{st}$ and 2$^{nd}$ post-ML samples, the reduction was >90%. (C) Dogs (n=9) that were sampled only once post-ML with variable reduction of microfilariae. (D) Dogs (n=3) that were sampled once, yet this sampling was delayed to 70 or more days post-ML. QLD – Queensland, Australia, NSW – New South Wales, Australia, yellow star – dogs with at least two consecutive post-ML blood samples tested, ML – macrocyclic lactone.

**Fig. 4.** Paired t-test analyses on microfilariae counts pre- and post-ML treatment. (A) Including all dogs (n= 23), there was no statistically significant reduction in microfilariae

(mf) levels in the first post-ML sampling (t[22]=0.6594, p=0.5165). (B) Including only dogs (n=13) with pre-ML microfilariae counts >1,000 and <10,000 mf/mL, a statistically significant reduction in microfilariae levels was observed in the first post-ML sampling (t[12]=4.108, p=0.0015). (C) Including all dogs (n=9), a statistically significant reduction in microfilariae levels was observed in the second post-ML sampling (t[8]=t=3.991, p=0.0040). (D) Including only dogs (n=6) with pre-ML microfilariae counts >1,000 and <10,000 mf/mL, a statistically significant reduction in microfilariae levels was maintained (t[5]=4.790, p=0.0049). Data analysis and graphs were produced using GraphPad Prism (version 9.3.1).

**Fig. 5.** *Dirofilaria immitis* antigen testing (DiroCHEK® ELISA, Zoetis, Australia) on 76 blood samples collected from 44 dogs in Australia. (A) Neat plasma samples from 41/44 dogs and heated plasma samples from 43/44 dogs initially tested positive for *D. immitis* antigen. Out of all blood samples tested, 67/76 neat samples and 75/76 heated samples were positive for *D. immitis* antigen. A threshold of 3×SD (Mean[optical density of negative controls]) was applied to each run to distinguish between *D. immitis*-negative and *D. immitis*-positive samples. Thresholds of 0.0784 (HW1-HW10), 0.0596 (HW11-HW17, HW19), 0.0786 (HW20-HW49, HW51-HW57), and 0.0532 (HW58-HW63, HW65-HW75, HW77-HW79) were applied. (B) Paired t-test on DiroCHEK® ELISA data demonstrated a significant difference in optical density between neat and heated plasma samples (Paired t-test, t[74]= 4.220, p <0.0001). Data analysis and graphs were produced using GraphPad Prism (version 9.3.1).

**Fig. 6.** Correlations between microfilariae count (mf/mL), DiroCHEK® ELISA (Zoetis, Australia) of neat and heated plasma, and qPCR of whole blood DNA targeting *D. immitis* and associated *Wolbachia*. (A) Moderate positive correlations between microfilariae counts and $OD_{620}$ of neat (n=76) and heated (n=76) plasma. (B) Moderate positive correlations between microfilariae counts and $OD_{620}$ of neat (n=71) and heated (n=71) plasma after the

exclusion of five samples with microfilariae counts >20,000 Mf/mL. (C) Strong negative correlation between *D. immitis* qPCR $C_t$-values of whole blood DNA and microfilariae counts (n=72) after the exclusion of five samples with microfilariae counts <20,000 mf/mL. (D) Strong negative correlation between *Wolbachia* qPCR $C_t$-values of whole blood DNA and microfilariae counts (n=72) after the exclusion of five samples with microfilariae counts <20,000 mf/mL. (E) Moderate negative correlation between *D. immitis* qPCR $C_t$-values of whole blood DNA and microfilariae counts (n=77). (F) Moderate negative correlation between *Wolbachia* qPCR of whole blood DNA and microfilaria counts (n=77). (G) Strong positive correlation between *D. immitis* and *Wolbachia* qPCR $C_t$-values of whole blood DNA (n=78). (H) Moderate positive correlation between *D. immitis* and *Wolbachia* qPCR $C_t$-values of whole blood DNA (n=52) after the exclusion of 26 samples with $C_t$-values of '40'. Data analysis and graphs were produced using GraphPad Prism (version 9.3.1).

**Fig. 7.** Paired t-test analyses on *D. immitis* and *Wolbachia* qPCR $C_t$-values pre- and post-ML treatment. (A) *D. immitis* qPCR demonstrated statistically significant increase in $C_t$-value between pre-ML and first post-ML sampling (n=23). (B) *Wolbachia* qPCR demonstrated statistically significant increase in $C_t$-value between pre-ML and first post-ML sampling (n=23). (C) *D. immitis* qPCR demonstrated statistically significant increase in $C_t$-value between pre-ML and second post-ML sampling (n=9). (D) *Wolbachia* qPCR demonstrated statistically significant increase in $C_t$-value between pre-ML and second post-ML sampling (n=9). Data analysis and graphs were produced using GraphPad Prism (version 9.3.1).

**Fig. 8.** Australian dogs infected with *Dirofilaria immitis* and response to ML treatment. Dog identification number is indicated at the top. Upper-most bar chart represents log transformed microfilariae (mf) counts pre-ML and post-ML (black to grey; Modified Knott's test). Yellow stars – dogs with at least two consecutive post-ML blood samples tested (2+ post-ML), blue stars – dogs with one post-ML blood sample tested (1 post-ML). Percent (%) suppression of

mf at the first and second post-ML time point is plotted (purple bars, n/a – not applicable due to absence of sequential sample). Percentage (%) reduction at 1st post-ML timepoint is written along the year these samples were collected. Nucleotide proportions at SNP regions (identified according to nDi2.2. genome assembly contigs) are shown for all samples analysed. The blue, red, and orange represent the nucleotides, with the reference nucleotides associated with ML-resistance indicated for each SNP on the right. Dog 25 has microfilaria count and L45689 highlighted by dotted line rectangles.

**Fig 9.** Nucleotide percentages at a SNP region (L45689) previously associated with ML-resistance in the USA for Dog 25. Blood samples were collected at 0-, 30- and 36-days post-ML treatment and DNA was isolated from filtered *Dirofilaria immitis* microfilariae. The blue, red, and orange boxes in the key represent the nucleotides and their associated ML-resistance status.

**Table 1. Microfilarial suppression test, antigen and qPCR diagnostics on *Dirofilaria immitis* infected dogs from Australia**

| Dog | Location (Australia) | *D. immitis* prevention | | Microfilarial suppression test | | | DiroCHEK® ELISA | | qPCR $C_t$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Compliance (past 12 months) | ML at diagnosis | pre-ML/post-ML | | % mf change | pre-ML/post-ML | | pre-ML/post-ML | |
| | | | | ID | mf/ml | | Neat | Heat | *D. immitis* | *Wolbachia* |
| >90% microfilariae (mf) reduction | | | | | | | | | | |
| 1 | Cairns, QLD | Sporadic | HeartGard Plus | HW1/HW2 | 1/0 | -100 | +/- | +/+ | 37.7/- | -/- |
| 15 | Deeragun, QLD | No | ProHeart SR-12 | HW25/HW36 | 380/0 | -100 | +/- | +/+ | 26.0/- | -/- |
| 21 | Tugun, QLD | Yes | HeartGard Plus | HW31/HW34 | 175/0 | -100 | -/+ | +/+ | 32.2/32.1 | -/- |
| 38 | Deeragun, QLD | n/a | ProHeart SR-12 | HW63/HW75 | 1,500/0 | -100 | +/+ | +/+ | 23.4/- | 29.5/- |
| 16 | Deeragun, QLD | n/a | ProHeart SR-12 | HW26/HW33 | 7,625/600 | -92 | +/+ | +/+ | 23.1/25.3 | 28.1/34.7 |
| 5 | Deeragun, QLD | Sporadic | ProHeart SR-12 | HW7/HW13 | 38,250/3,512 | -91 | +/+ | +/+ | 20.0/22.4 | 25.1/32.3 |
| <90% microfilariae (mf) reduction | | | | | | | | | | |
| 33 | Deeragun, QLD | Yes | ProHeart SR-12 | HW55/HW59 | 1,570/470 | -70 | +/+ | +/+ | 24.6/25.2 | 30.3/- |
| 13 | Cairns, QLD | No | HeartGard Plus | HW20/HW22 | 7,450/2,475 | -67 | +/+ | +/+ | 20.5/21.8 | 25.2/32.8 |
| 6 | Deeragun, QLD | Yes | ProHeart SR-12 | HW8/HW15 | 7,560/2,518 | -67 | +/+ | +/+ | 21.3/22.3 | 26.7/32.0 |
| 34 | Deeragun, QLD | Yes | ProHeart SR-12 | HW56/HW58 | 9,400/3,180 | -66. | +/+ | +/+ | 21.2/22.9 | 27.3/35.5 |
| 28 | Westbrook, QLD | No | ProHeart SR-12 | HW44/HW49 | 3,180/1,100 | -65 | +/+ | +/+ | 25.1/24.9 | 28.8/33.7 |
| 27 | Westbrook, QLD | No | ProHeart SR-12 | HW43/HW48 | 3,420/1,350 | -61 | +/+ | +/+ | 25.8/23.9 | 29.8/33.8 |
| 37 | Deeragun, QLD | Sporadic | ProHeart SR-12 | HW61/HW65 | 2,300/960 | -58 | +/+ | +/+ | 22.6/25.4 | 29.9/- |
| 35 | Deeragun, QLD | Yes | ProHeart SR-12 | HW57/HW62 | 11,990/7,760 | -35 | +/+ | +/+ | 19.5/22.7 | 27.3/32.6 |
| 40 | Nana Glen, NSW | No | ProHeart SR-12 | HW68/HW71 | 3,410/2,380 | -30 | +/- | +/+ | 22.7/27.0 | 28.2/- |
| 24 | Deeragun, QLD | Yes | ProHeart SR-12 | HW38/HW40 | 420/300 | -29 | +/+ | +/+ | 26.6/26.0 | 31.7/33.5 |
| 4 | Deeragun, QLD | Sporadic | ProHeart SR-12 | HW6/HW12 | 2,856/2,217 | -22 | +/+ | +/+ | 22.0/22.9 | 26.6/32.3 |
| 3 | Deeragun, QLD | Yes | ProHeart SR-12 | HW5/HW14 | 9,540/8,208 | -14 | +/+ | +/+ | 22.7/24.4 | 26.1/35.1 |
| 43 | Deeragun, QLD | n/a | ProHeart SR-12 | HW74/HW78 | 2,590/2,590 | 0 | +/+ | +/+ | 24.7/22.7 | 30.6/33.1 |
| 19 | Tugun, QLD | Yes | HeartGard Plus | HW29/HW35 | 0/0 | n/a | +/- | +/+ | 33.1/- | -/- |
| Increase in microfilariae (mf) | | | | | | | | | | |
| 7 | Townsville, QLD | n/a | ProHeart SR-12 | HW9/HW16 | 38/565 | +1,386 | +/+ | +/+ | 28.7/24.0 | 31.8/34.3 |
| 2 | Thursday Is, QLD | No | HeartGard Plus | HW3/HW4 | 510/1436 | +182 | +/+ | +/+ | 21.8/22.9 | 27.0/31.9 |
| 25 | Deeragun, QLD | Yes | ProHeart SR-12 | HW39/HW41 | 24,800/6,2160 | +151 | +/+ | +/+ | 21.3/24.1 | 26.1/28.8 |

Note: n/a – not available, + (DiroCHEK) – positive, - (DiroCHEK) – negative, - (qPCR) – negative (>40), ML – macrocyclic lactone, mf – microfilariae, HeartGard Plus (ivermectin/pyrantel; Boehringer-Ingelheim), ProHeart SR-12 (moxidectin; Zoetis).

Fig. 1

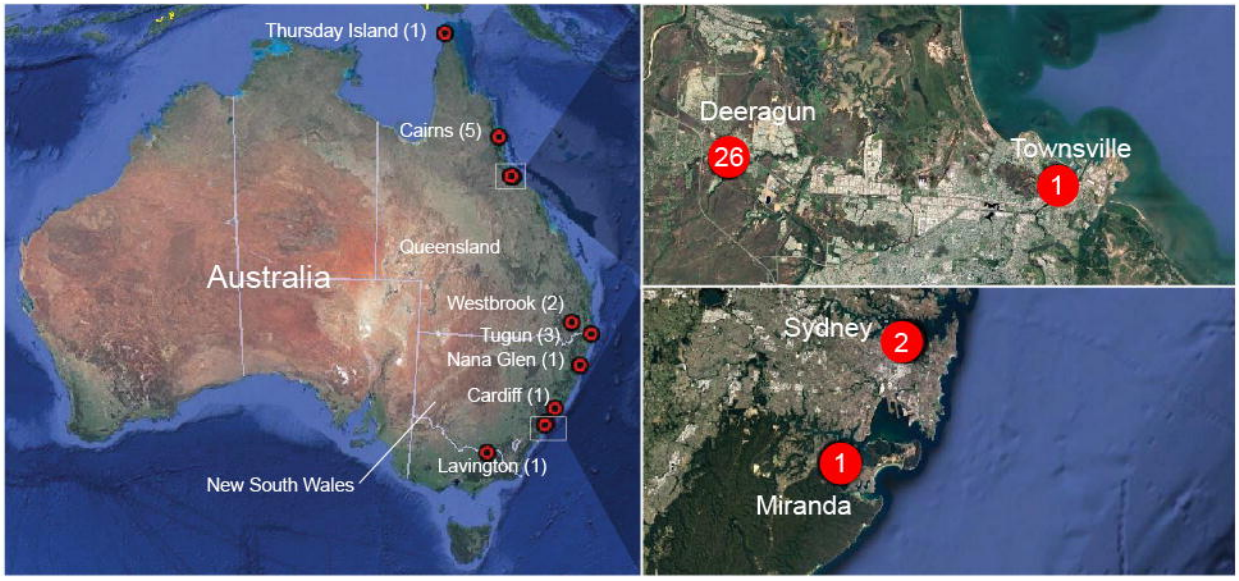

Thursday Island (1)
Cairns (5)
Queensland
Australia
Westbrook (2)
Tugun (3)
Nana Glen (1)
Cardiff (1)
Lavington (1)
New South Wales
Deeragun
26
Townsville
1
Sydney
2
1
Miranda

Fig. 2

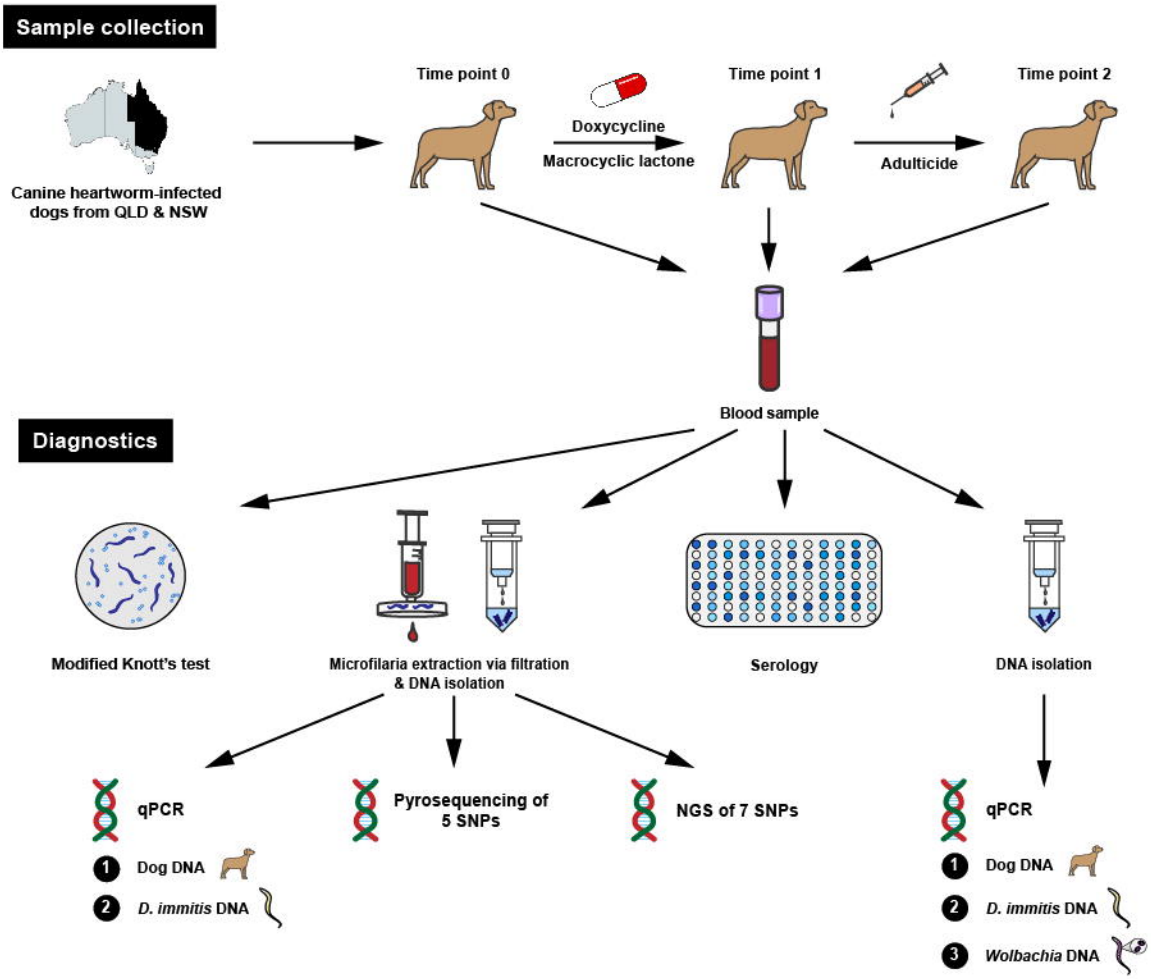

Sample collection
Canine heartworm-infected dogs from QLD & NSW
Time point 0
Doxycycline
Macrocyclic lactone
Time point 1
Adulticide
Time point 2
Blood sample
Diagnostics
Modified Knott's test
Microfilaria extraction via filtration & DNA isolation
Serology
DNA isolation
qPCR
1 Dog DNA
2 D. immitis DNA
Pyrosequencing of 5 SNPs
NGS of 7 SNPs
qPCR
1 Dog DNA
2 D. immitis DNA
3 Wolbachia DNA

Fig. 3

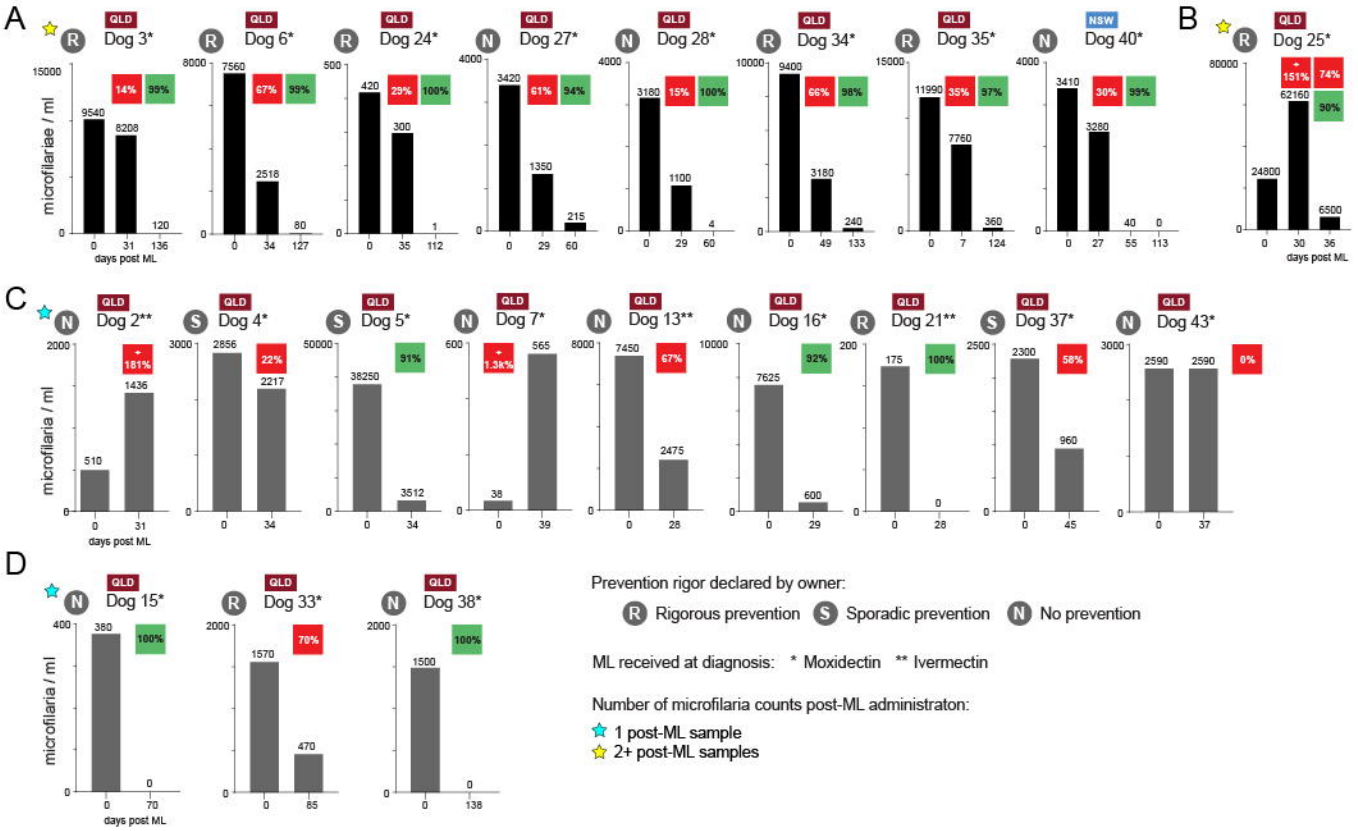
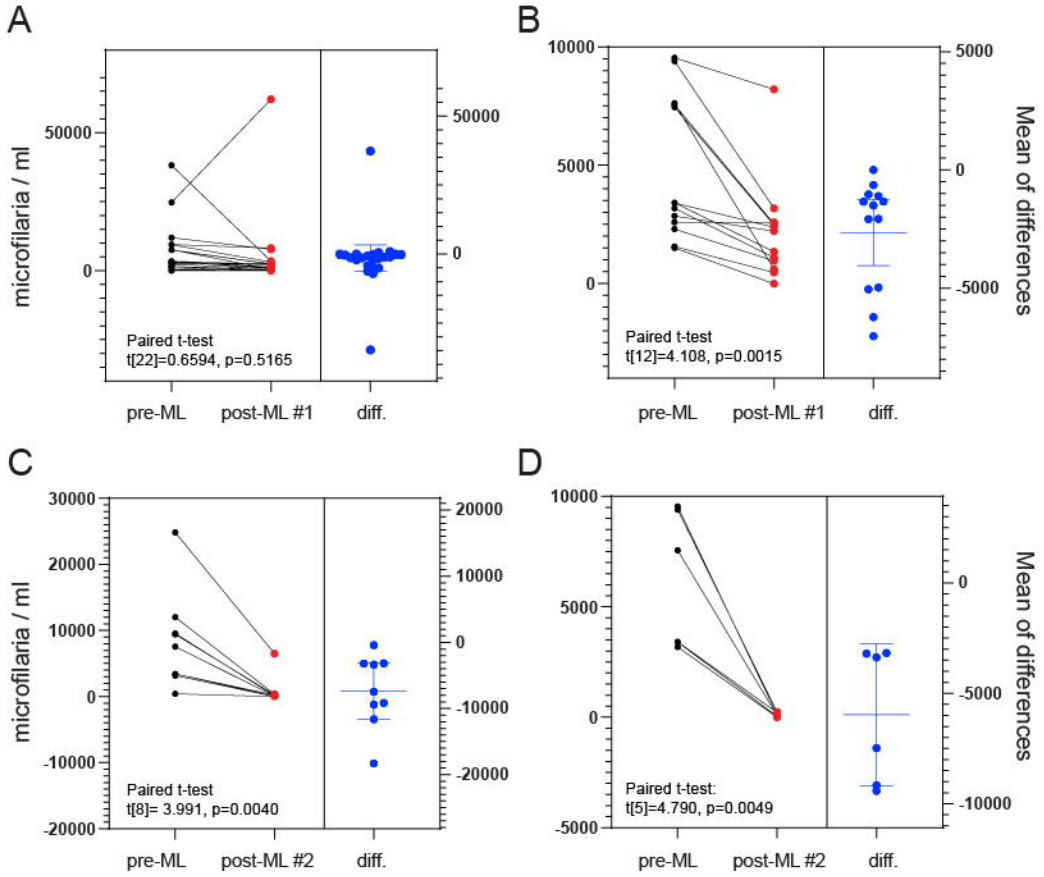

A
B
C
D
QLD
NSW
Dog 3*
Dog 6*
Dog 24*
Dog 27*
Dog 28*
Dog 34*
Dog 35*
Dog 40*
Dog 25*
Dog 2**
Dog 4*
Dog 5*
Dog 7*
Dog 13**
Dog 16*
Dog 21**
Dog 37*
Dog 43*
Dog 15*
Dog 33*
Dog 38*
microfilariae / ml
microfilaria / ml
days post ML
Prevention rigor declared by owner:
R Rigorous prevention
S Sporadic prevention
N No prevention
ML received at diagnosis: * Moxidectin ** Ivermectin
Number of microfilaria counts post-ML administraton:
1 post-ML sample
2+ post-ML samples

Fig. 4


A
microfilaria / ml
50000
0
Paired t-test
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pre-ML
post-ML #1
diff.
B
10000
5000
0
Paired t-test
t[12]=4.108, p=0.0015
pre-ML
post-ML #1
diff.
Mean of differences
C
microfilaria / ml
30000
20000
10000
0
-10000
-20000
Paired t-test
t[8]= 3.991, p=0.0040
pre-ML
post-ML #2
diff.
D
10000
5000
0
-5000
Paired t-test:
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pre-ML
post-ML #2
diff.
Mean of differences

Fig. 5

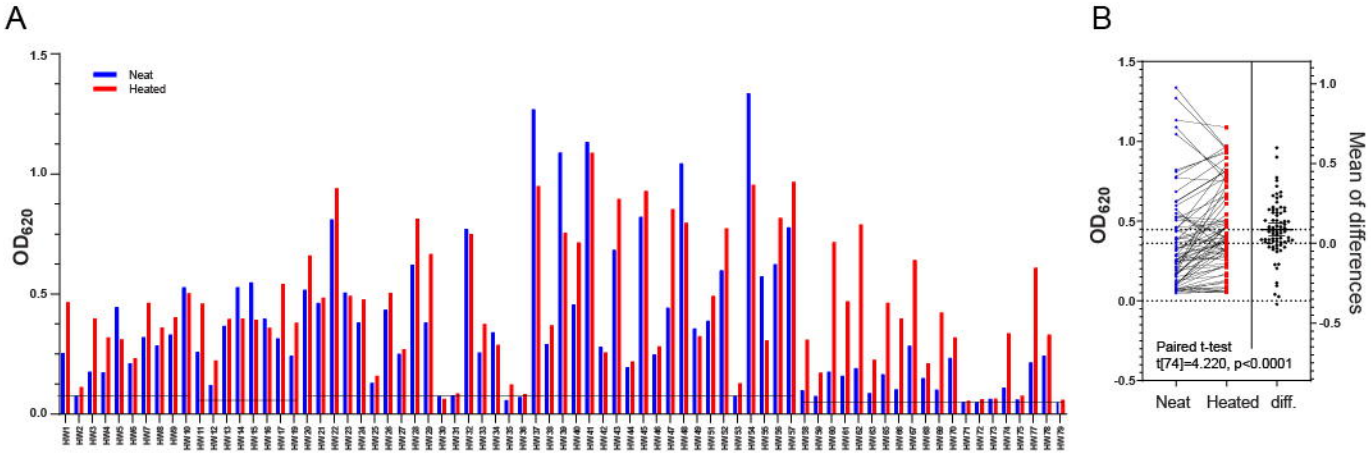

A
Neat
Heated
OD620
1.5
1.0
0.5
0.0
B
OD620
Mean of differences
Paired t-test
t[74]=4.220, p<0.0001
Neat
Heated
diff.

Fig. 6

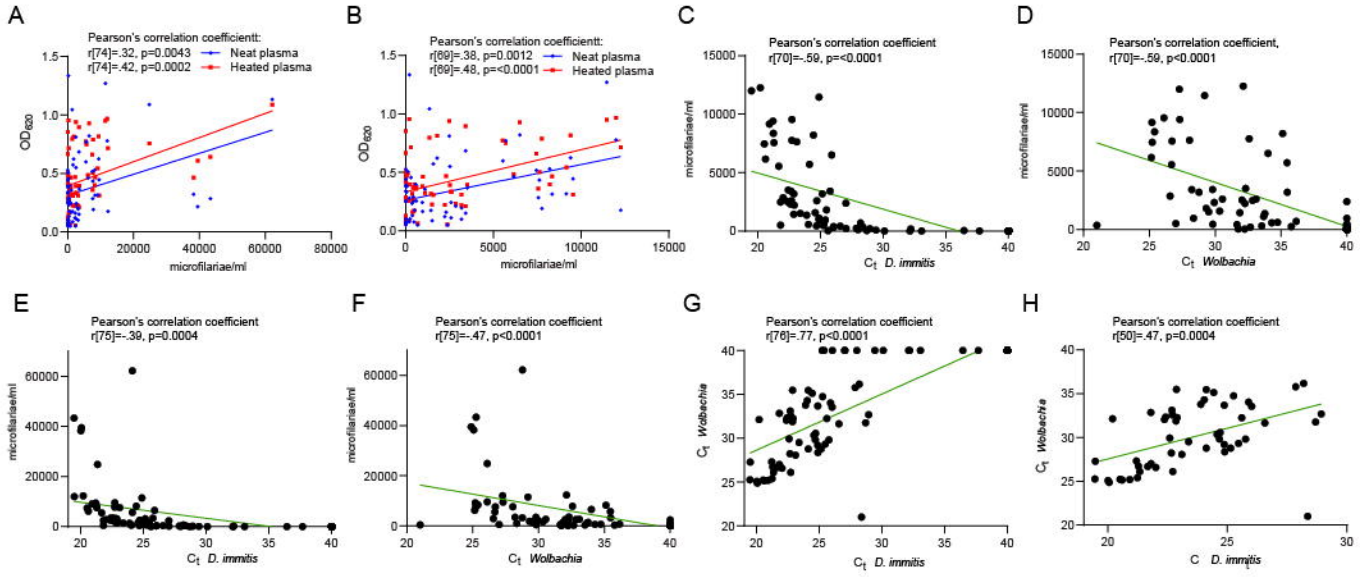
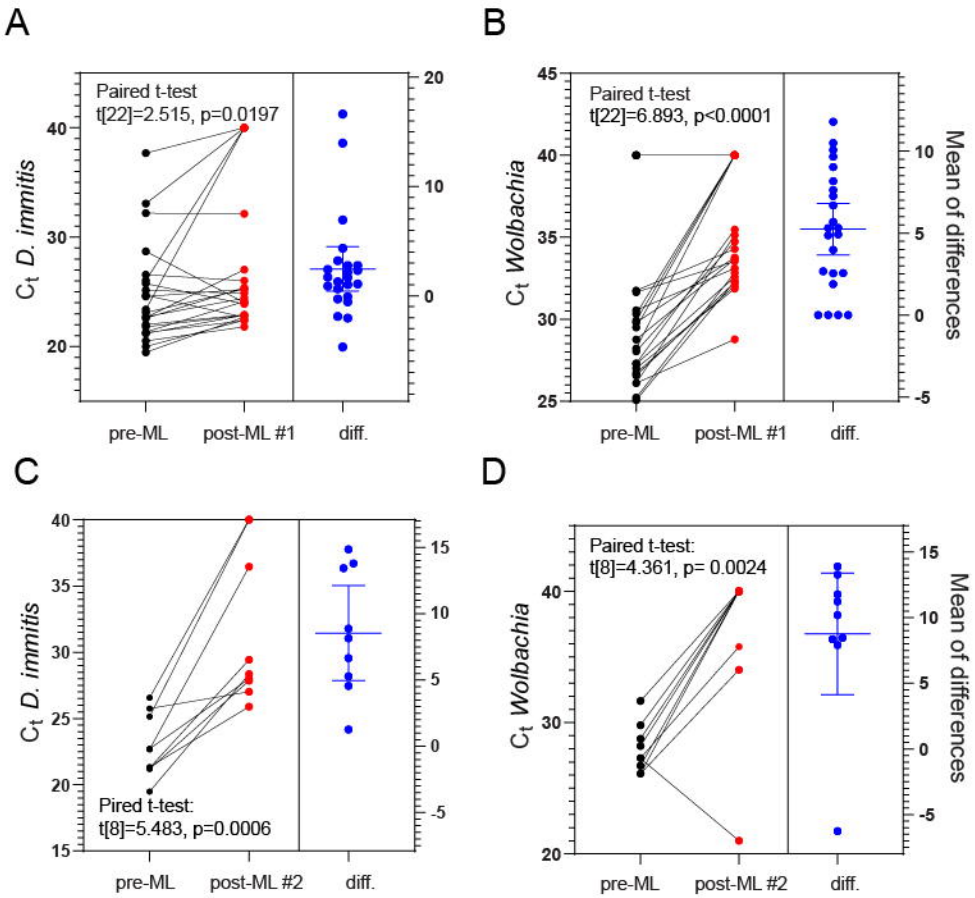

A
Pearson's correlation coefficientt:
r[74]=.32, p=0.0043
r[74]=.42, p=0.0002
Neat plasma
Heated plasma
OD620
microfilariae/ml
B
Pearson's correlation coefficientt:
r[69]=.38, p=0.0012
r[69]=.48, p=<0.0001
Neat plasma
Heated plasma
OD620
microfilariae/ml
C
Pearson's correlation coefficient
r[70]=-.59, p=<0.0001
microfilariae/ml
Ct D. immitis
D
Pearson's correlation coefficient,
r[70]=-.59, p<0.0001
microfilariae/ml
Ct Wolbachia
E
Pearson's correlation coefficient
r[75]=-.39, p=0.0004
microfilariae/ml
Ct D. immitis
F
Pearson's correlation coefficient
r[75]=-.47, p<0.0001
microfilariae/ml
Ct Wolbachia
G
Pearson's correlation coefficient
r[76]=.77, p<0.0001
Ct Wolbachia
Ct D. immitis
H
Pearson's correlation coefficient
r[50]=.47, p=0.0004
Ct Wolbachia
Ct D. immitis

Fig. 7


A
Paired t-test
t[22]=2.515, p=0.0197
$C_t$ D. immitis
pre-ML
post-ML #1
diff.
B
Paired t-test
t[22]=6.893, p<0.0001
$C_t$ Wolbachia
Mean of differences
pre-ML
post-ML #1
diff.
C
Pired t-test:
t[8]=5.483, p=0.0006
$C_t$ D. immitis
pre-ML
post-ML #2
diff.
D
Paired t-test:
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$C_t$ Wolbachia
Mean of differences
pre-ML
post-ML #2
diff.

Fig. 8

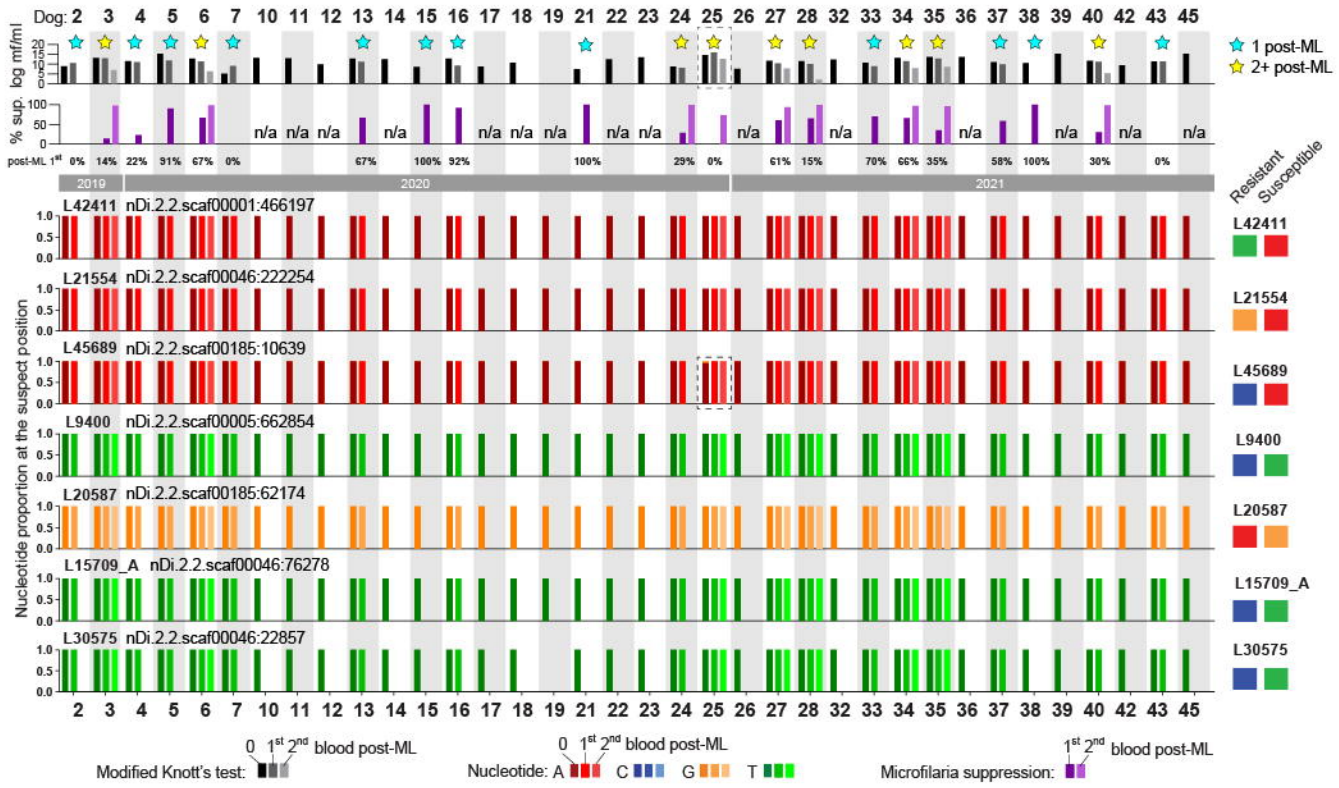
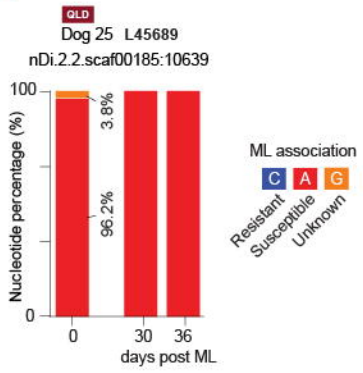

Dog: 2 3 4 5 6 7 10 11 12 13 14 15 16 17 18 19 21 22 23 24 25 26 27 28 32 33 34 35 36 37 38 39 40 42 43 45
log mf/ml
% sup.
post-ML 1st 0% 14% 22% 91% 67% 0% 67% 100% 92% 100% 29% 0% 61% 15% 70% 66% 35% 58% 100% 30% 0%
n/a
2019
2020
2021
1 post-ML
2+ post-ML
Resistant
Susceptible
Nucleotide proportion at the suspect position
L42411 nDi.2.2.scaf00001:466197
L21554 nDi.2.2.scaf00046:222254
L45689 nDi.2.2.scaf00185:10639
L9400 nDi.2.2.scaf00005:662854
L20587 nDi.2.2.scaf00185:62174
L15709_A nDi.2.2.scaf00046:76278
L30575 nDi.2.2.scaf00046:22857
Modified Knott's test: 0 1st 2nd blood post-ML
Nucleotide: A C G T 0 1st 2nd blood post-ML
Microfilaria suppression: 1st 2nd blood post-ML

Fig. 9


QLD
Dog 25 L45689
nDi.2.2.scaf00185:10639
100
0
Nucleotide percentage (%)
3.8%
96.2%
0
30
36
days post ML
ML association
C
A
G
Resistant
Susceptible
Unknown