Whole-genome reference of *Dirofilaria immitis* from Australia to determine single nucleotide polymorphisms associated with macrocyclic lactone resistance in the USA

Daisy Ching-Wai Lau, Stephanie McLeod, Sara Collaery, Selina Peou, Andy Truc Tran, Michelle Liang, Jan Slapeta *

Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, NSW, 2006, Australia

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**ABSTRACT**

For the past 30 years, chemoprophylaxis with macrocyclic lactone (ML) anthelmintics has been the primary strategy for canine heartworm (*Dirofilaria immitis*) control in both the USA and Australia. ML-resistant *D. immitis* isolates have been confirmed to exist in the USA and studies have shown that 42 single nucleotide polymorphisms (SNPs) are associated with phenotypic ML-resistance. Currently, ML-resistance has not been reported in any Australian clinical cases of canine heartworm. The aim of the study is to determine whether the 42 SNPs associated with resistance to MLs in the isolates from the USA are present in adult heartworms from a clinical case in Australia. Five adult *D. immitis* obtained from a dog at post-mortem (Sydney, Australia) were sequenced using the Illumina sequencing technology. The genomic analyses revealed 6 out of the 42 SNPs associated with ML-resistance to be present in our samples, 3 out of the 6 SNPs identified were nonsynonymous SNPs but not in candidate genes for ML-resistance. ML-susceptibility profile was mixed using the 42-SNP and 10-SNP models, but the 5-SNP, 3-SNP and 2-SNP models demonstrated ML-susceptibility for all five individuals. In this study, the first whole-genome reference of *D. immitis* from Australia establishes a new baseline for comparative studies and will be valuable for tracking ML-resistance emergence.

**1. Introduction**

*Dirofilaria immitis* (canine heartworm) are mosquito-borne filarial nematodes capable of causing cardiopulmonary disease. Canine heartworm disease affects dogs in Australia each year, particularly in Queensland (Nguyen et al., 2016). Chemoprophylaxis with macrocyclic lactone (ML) anthelmintics is the primary strategy for heartworm prevention with preventative products approved to be 100% efficacious (Hampshire, 2005). Within 20 years after the introduction of ML preventative anthelmintics, loss of efficacy (LOE) reports from the Mississippi Delta region in the USA have led to the speculation that *D. immitis* has developed ML-resistance (Atkins et al., 2014). Initially, distinguishing whether LOE cases are truly due to ML-resistance has proven challenging due to frequent owner non-compliance (Bowman, 2012). As the number of LOE cases continued rising, controlled efficacy studies were conducted to investigate ML-resistance of *D. immitis* and clinical isolates have been confirmed to be ML-resistant (Pulaski et al., 2014).

Genome comparison of ML-resistant isolates show they are distinct signatures from the ML-susceptible populations of *D. immitis*. Single nucleotide polymorphisms (SNPs) associated with failure to reduce microfilariae following ML prophylaxis have been identified (Bourguinat et al., 2011c, 2015). The initial genome comparison between susceptible and LOE *D. immitis* populations identified 186 SNPs that segregated with ML-resistance (Bourguinat et al., 2015). However, some of the 186 SNPs could be random mutations unrelated to ML-resistance (Bourguinat et al., 2015). Instead, by assessing the allele frequencies between well characterised ML-susceptible and ML-resistant isolates obtained from controlled efficacy studies, a subset of 42 ML-resistant markers was identified (Bourguinat et al., 2015). These appeared to better differentiate the ML-susceptible phenotype from the LOE and ML-resistant phenotype (Bourguinat et al., 2015).

ML anthelmintics were introduced into Australia in the 1990s and have been used in the USA and Australia for over 30 years (Hampshire, 2005; Orr et al., 2020). Research published from the USA demonstrated...
that ML-resistance is emerging; however, it is unknown whether the SNPs associated with ML-resistance in the isolates from the USA are present in Australian canine heartworms. The objective of this study is to determine whether any of the 42 SNPs associated with ML-resistance in the isolates from the USA are present in *D. immitis* adults from a clinical case in Australia.

2. Materials and methods

2.1. Sample processing and DNA extraction

Samples tested include five adult *D. immitis* JS5873 to JS5877 (P6/20 A:C female and D-E: male) obtained at necropsy from a deceased dog donated to the Sydney School of Veterinary Science, The University of Sydney, in September 2020, as part of the Sydney School of Veterinary Sciences Animal Donation Programme. The adult dog came from Sydney, New South Wales, Australia, but the history of travel and rigorous treatment with ML products is unknown. Individually extracted nematodes from the heart were washed 3 times in phosphate-buffered saline (pH 7.4) and 1 cm from between the first and second third of the anterior portion of the body was excised using a sterile scalpel blade for DNA isolation. The genomic DNA from samples JS5873 to JS5876 (n = 5) was extracted with a Monarch® Genomic DNA Purification Kit (New England Biolabs, Australia) as per the manufacturer’s protocol.

2.2. Whole-genome sequencing

Extracted *D. immitis* DNA samples (A-C: 4.72–90.15 ng/μl and D-E: 0.27–1.06 ng/μl) were sent to Novogene (HK) Co., Ltd for indexing, library construction and whole-genome sequencing; JS5873 to JS5875 (A-D) were sequences at an expected depth of 10G while JS5876 and JS5877 were sequenced at a depth of 1G. Sequencing was performed with the HiSeq platform from Illumina® with PE150 paired-end reads.

2.3. Genome analysis and identification of SNP markers

Based on previous work, the 42 SNPs that appeared to better differentiate the ML-susceptible phenotype from the LOE and ML-resistance phenotype were selected for analysis (Bourguinat et al., 2015). The list of 42 SNPs evaluated in this study are available at LabArchives: https://doi.org/10.25833/8gx-j055. From the 42 SNPs, a subset of SNP loci (10-SNP, 5-SNP, 3-SNP and 2-SNP model) which appears to be promising markers for predicting ML resistance are used to predict the phenotypic response of JS5873 to JS5877 to ML treatment (Ballesteros et al., 2015). The list of 42 SNPs were found in JS5873, JS5874 and JS5875, compared with 11–12× for JS5876 and JS5877. Across all JS5873 to JS5877 *D. immitis* samples, total number of variations across the alignment against the reference genome varied from 178,977 to 207,665 (Table 1).

Five *D. immitis* were individually sequenced and 90.21%–97.91% of the reads from the JS5873 to JS5877 samples could be aligned to the reference genome of *D. immitis* nDi.2.2. (88 Mb) (Table 1). Average coverage was 101–126× for JS5873, JS5874 and JS5875, compared with 11–12× for JS5876 and JS5877. Across all JS5873 to JS5877 *D. immitis* samples, total number of variations across the alignment against the reference genome varied from 178,977 to 207,665 (Table 1).

From the list of 42 SNPs evaluated in this study, 6 of the proposed SNPs which appear to better differentiate the ML-susceptible phenotype from the LOE and ML-resistance *D. immitis* samples were found in JS5873 to JS5877 samples (Table 2). All 5 samples had the same SNP at each of the 6 markers, apart from JS5875 to JS5877 at nDi.2.2. Wolbachia reference sequence.

### Table 1

| Sample ID  | No. of reads | Alignment against nDi.2.2. D. immitis (No. of reads (% of total reads)) | Average coverage (average no. of reads aligning at the specific position of the 42 SNPs) | Variants (total no. of variants across alignment against nDi.2.2. D. immitis) |
|------------|--------------|--------------------------------------------------------------------------|------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| JS5873     | 71,971,763   | 68,875,509 (95.70)                                                       | 98,914 (96.72)                                                                           | 101                                                                             |
| JS5874     | 73,578,378   | 72,039,266 (97.91)                                                       | 101                                                                                     | 126                                                                             |
| JS5875     | 67,774,096   | 65,465,532 (96.59)                                                       | 101                                                                                     | 206,681                                                                        |
| JS5876     | 8,704,246    | 8,419,566 (96.72)                                                        | 11                                                                                      | 176,559                                                                        |
| JS5877     | 8,778,535    | 7,919,458 (90.21)                                                        | 12                                                                                      | 178,977                                                                        |
and JS5875 to JS5876 at nDi.2.2.scaf00597_12915 where a heterozygous SNP is present. For the remaining 36 SNP markers, JS5873 to JS5877 samples are not part of the 5-SNP, 3-SNP or 2-SNP model. All other ML-resistance markers found in the reference genome. The nDi.2.2.scaf00597_12915 ML-resistance marker JS5877 samples are homozygous reference to the nDi.2.2.scaf00597_12915 A G G R a (60:58) R a (13:10) G change.

Based on the 42-SNP and 10-SNP models, the ML phenotypic response of JS5873 to JS5877 samples was considered mixed since both ML-resistance and ML-susceptible SNP markers are present (Table 3). For the 5-SNP, 3-SNP and 2-SNP model, the nucleotides at all SNP loci were homozygous reference to the nDi.2.2. D. immitis reference genome and the ML phenotypic response of JS5873 to JS5877 samples would be predicted to be ML-susceptible (Table 3).

Table 2
Distinct SNPs found within 42 SNPs Dirofilaria immitis reference

| SNP Position              | Reference Allele | JS5873 | JS5874 | JS5875 | JS5876 | JS5877 |
|---------------------------|------------------|--------|--------|--------|--------|--------|
| nDi.2.2.sca00021_25243    | T                | C      | C      | C      | C      | C      |
| nDi.2.2.sca00021_212599    | A                | G      | C      | G      | G      | G      |
| nDi.2.2.sca00238_29165     | C                | T      | T      | T      | T      | T      |
| nDi.2.2.sca00597_15334     | A                | G      | G      | R³ (48:125) | R³ (7:9) | R³ (8:8) |
| nDi.2.2.sca00597_12915     | A                | G      | G      | R³ (60:58) | R³ (13:10) | G      |
| nDi.2.2.sca01422_4176     | G                | A      | A      | A      | A      | A      |

* R stands for purine (adenine or guanine), proportion of A:G given in parentheses.

Table 3
Predictive Dirofilaria immitis SNP models predicting ML resistance

| Predictive model | JS5873 | JS5874 | JS5875 | JS5876 | JS5877 |
|------------------|--------|--------|--------|--------|--------|
| 42 SNP           | Mixed  | Mixed  | Mixed  | Mixed  | Mixed  |
| 10 SNP           | Mixed  | Mixed  | Mixed  | Mixed  | Mixed  |
| 5 SNP            | Susceptible | Susceptible | Susceptible | Susceptible | Susceptible |
| 3 SNP            | Susceptible | Susceptible | Susceptible | Susceptible | Susceptible |
| 2 SNP            | Susceptible | Susceptible | Susceptible | Susceptible | Susceptible |

Table 4
Synonymous SNPs and nonsynonymous SNPs at the 6 ML resistance marker sites for Dirofilaria immitis

| SNP position        | Amino acid change | Protein annotation |
|---------------------|-------------------|--------------------|
| nDi.2.2.sca00021_25243 | CCT Pro            | GSK3B-interacting protein |
| nDi.2.2.sca00021_212599 | ATG Met           |                     |
| nDi.2.2.sca00238_29165 | CCA Pro           | Unannotated         |
| nDi.2.2.sca00597_15334 | TCA Ser           |                     |
| nDi.2.2.sca00597_12915 | ATG Val           |                     |
| nDi.2.2.sca01422_4176 | GAT Asp           | Protein kinase domain |

Table 5
Wolbachia endosymbiont of Dirofilaria immitis from Australia

| Sample ID | No. of reads aligned against Wolbachia endosymbiont of D. immitis wDi.2.2 | Variants (total no. of variants across alignment against wDi.2.2) |
|-----------|---------------------------------------------------------------------------|-----------------------------------------------------------------|
| JS5873    | 3,725,184                                                                 | 7,407                                                          |
| JS5874    | 1,597,092                                                                 | 7,962                                                          |
| JS5875    | 2,034,077                                                                 | 7,570                                                          |
| JS5876    | 50,985                                                                   | 7,751                                                          |
| JS5877    | 1,016,571                                                                 | 4,924                                                          |

In recent years, NGS technologies have been used for high-throughput whole-genome sequencing for genetic marker discovery (Davey et al., 2011). Read coverage is a crucial parameter to evaluate variant calling accuracy and comparison of whole-genome sequencing data showed that isolates have only been recovered in the USA (Bourguinat et al., 2015; Orr et al., 2020; Pulaski et al., 2014). With no studies published from Australia, there is a gap in scientific knowledge regarding any potential LOE or ML-resistant cases in the Australian canine heartworm population. In studies from the USA, phenotypic characterisation of clinically infected dogs and controlled efficacy studies utilising animal models enabled them to establish SNPs as predictors of ML-resistance (Bourguinat et al., 2015; Pulaski et al., 2014). With the recent re-emergence of D. immitis in Queensland, genetic variants previously found associated with ML-resistance in the isolates from the USA may also be present in clinical cases of canine heartworm in Australia (Nguyen et al., 2016). In this study, the first whole-genome reference sequences of D. immitis from Australia have been sequenced using modern NGS techniques. These reference sequences will become a valuable tool to refine the genetic identity of ML-resistance in comparative studies.

4. Discussion

This study utilised a whole-genome sequencing approach to characterise the ML-susceptibility profiles of D. immitis in Sydney, Australia, based on the genotyping data available from the USA. For nearly three decades, ML anthelmintics have been used as the primary strategy for canine heartworm prevention in the USA and Australia but ML-resistant
SNPs achieved > 95% of concordance at 17.6× coverage (Kishikawa et al., 2019). Following this standard, the accuracy of the 6 SNPs found in JS5873 to JS5875 *D. immitis* samples is > 95%. In this study, ~16 million sequencing reads were required to achieve a coverage of 17.6×. This number of sequencing reads should be the minimum level of data acquired in future studies to obtain accurate genomic data for subsequent analyses of SNPs associated with ML-resistance.

In a previous study, a whole-genome sequencing approach between ML-susceptible, LOE and ML-resistant clinical isolates of *D. immitis* from the USA identified a set of 42 SNPs to differentiate between the different ML-susceptibility profiles (Bourguinat et al., 2015). Using a similar approach, 6 out of the 42 ML-susceptance SNP markers were found in JS5873 to JS5877 *D. immitis* samples obtained from a clinical case of canine heartworm from Australia. These adult canine heartworm samples did not show a similar genetic profile to previous phenotypically well-characterised *D. immitis* ML-susceptible and ML-resistant isolates, which suggests that the 42 SNPs may be geographically specific and therefore cannot be used globally (Bourguinat et al., 2015, 2017). In Canenorhabditis elegans, a SNP is expected to appear by chance in the nuclear genome at an average rate of $2.7 \times 10^{-9}$ per-generation (Denver et al., 2009). If this mutation rate is representative of random base substitutions in *D. immitis*, then ~96,000 mutations is expected to occur by chance across the genome (Denver et al., 2009). In our study, 178, 977–207,665 genetic variants were found between the JS5873 to JS5877 *D. immitis* samples and the nD1.2.2. *D. immitis* reference genome. Using the mutation rate of *C. elegans*, ~46% of the genetic variants seen across the genomes are not due to chance. This level of non-random genetic variability could be due to population structure (geographical separation, genetic bottleneck, selective sweep, or selection by ML anthelmintics), which could affect the viability of the SNP markers previously identified to be promising in detecting ML-resistant cases (Gilleard & Beech, 2007).

5. Conclusion

This is the first study to analyse whole-genome reference sequences of adult *D. immitis* originating from Australia. While finding evidence of ML-resistance in *D. immitis* is still pending, whole-genome sequencing and bioinformatics provide an affordable and scalable approach for future studies. Efforts to design reliable and sensitive in vitro tests for resistance remains unsuccessful (Evans et al., 2013; Maclean et al., 2017). Therefore, identifying reliable genetic markers to predict ML response may be the only way to monitor the extent and spread of ML-resistant isolates in epidemiological studies. Much remains unknown about the distribution of ML-resistant heartworm populations in Australia and the extent to which the efficacy of ML anthelmintics is threatened. This creates a need for further research into whether ML-resistance is present in the canine heartworm population in Australia. Improvements to the approach used in this study combined with the 7-day suppression test will be essential to refine ML-resistant genetic profiles using Australian strains of *D. immitis*.

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Daisy Ching-Wai Lau: Methodology, Validation, Formal analysis, Data curation, Writing - Original Draft, Writing - Review & Editing. Sara Colihaery: Methodology, Formal analysis, Writing - Original Draft. Michelle Liang: Methodology, Formal analysis, Writing - Original Draft. Stephanie Mcleod: Methodology, Formal analysis, Writing - Original Draft. Selina Perng: Methodology, Formal analysis, Writing - Original Draft. Andy Tran: Methodology, Formal analysis, Writing - Original Draft. Jan Slapeta: Conceptualization, Validation, Investigation, Resources, Data Curation, Writing - Review & Editing, Supervision, Funding acquisition. All authors read and approved the final manuscript.

Data availability

Raw fastq sequence data was deposited at SRA NCBI BioProject PRJNA681066. SNP data tables and additional data are available at LabArchives: https://doi.org/10.25833/8eqx-j055.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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