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Molecular detection of *Dirofilaria immitis* and its *Wolbachia* endosymbionts in dogs from Myanmar

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ABSTRACT

Heartworm disease in dogs and cats caused by *Dirofilaria immitis* continues to be a major clinical issue globally. This study focused on dogs suspicious of having tick-borne diseases (TBD) brought to a clinic and a veterinary teaching hospital in Myanmar. Blood samples were collected and initially screened using SNAP® 4Dx® Plus test kit. All dog blood samples were subjected to conventional PCR to detect both *Dirofilaria* spp. (*cox*1 gene) and *Wolbachia* spp. (16S rDNA) infections. Infection with *D. immitis* was detected in 14 (28.0%) of 50 examined samples, while the detection rate of TBD causative agents, including *Anaplasma phagocytophilum* and *Ehrlichia canis*, was 26.0% (13/50) and 26.0% (13/50), respectively, as determined by ELISA rapid test. In this study, *D. immitis* infection was moderately but significantly correlated with TBD infections (Pearson's $r = 0.397$, $P = 0.008$). Comparative sequence and phylogenetic analyses provided molecular identification of *D. immitis* in Myanmar and confirmed the identity of its *Wolbachia* endosymbiont with *Wolbachia* endosymbionts isolated from *D. immitis*, *Rhipicephalus sanguineus* and *Aedes aegypti*. The present study contributes to our understanding of the coexistence of *D. immitis* and *Wolbachia* endosymbiosis in dogs, and the findings may benefit the future prevention and control of dirofilariasis in dogs.

1. Introduction

Heartworm disease in dogs and cats caused by *Dirofilaria immitis* continues to be a global clinical concern. Infection with *D. immitis* is most common in members of the family Canidae, which includes domestic dogs, wolves, foxes, and coyotes; however, cats, ferrets, muskrats, and humans may also be affected (Atkins, 2005). *Wolbachia* spp. are endosymbiont alphaproteobacteria that live inside the cells of insects and nematodes and are vital for the development, reproduction, and survival of nematodes, as well as for providing critical metabolites to the filarial nematodes (Pfarr and Hoerauf, 2007; Werren et al., 2008; Ichimori et al., 2014). It has been demonstrated that some filarioids of the subfamilies Onchocercinae and Dirofilariinae are associated with an intracellular bacterium of the genus *Wolbachia* (Martin and Gavotte, 2010). Furthermore, the *Wolbachia*-nematode symbiosis has suggested treatment options for controlling and eradicating filarial infection in the host by employing *Wolbachia* as an antibiotic target (Pfarr and Hoerauf, 2006; Slatko et al., 2010).

Filarial infections are currently diagnosed using various approaches, including microscopy, serology, and molecular methods (Irwin and Jefferies, 2004). To detect microfilariae, the basic conventional technique generally employed is microscopic examination. Additionally, to identify *D. immitis* molecularly, species-specific PCR targeting the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene has been used (Casiraghi et al., 2001; Satjawongvanit et al., 2019).

Data on *Dirofilaria* spp. infections in dogs in Myanmar are virtually inexistent (Nguyen et al., 2021). No molecular information on *Dirofilaria* spp. or their *Wolbachia* endosymbionts in dogs is available for Myanmar. Here, we used molecular approaches to confirm and characterize the species of *Dirofilaria* and its *Wolbachia* endosymbionts in blood samples from dogs brought to a clinic in Yangon and a veterinary teaching hospital in Nay Pyi Taw, Myanmar, because they were suspected of

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having tick-borne diseases (TBD).

2. Materials and methods

2.1. Animals and sampling sites

The study was conducted between August 2020 and November 2021. Blood samples were taken from 50 dogs (24 males and 26 females) aged between 6 months and 7 years, that were brought to a clinic in Yangon (16.51°N, 96.11°E) ($n = 34$) and a veterinary teaching hospital of the University of Veterinary Science (UVS) in Nay Pyi Taw (19.62°N, 96.02°E) ($n = 16$), Myanmar. The animals involved in the study displayed clinical signs consistent with TBD, including fever, prostration, weight loss, vomiting, diarrhea, anorexia, jaundice, dehydration, pale mucous membranes, and unexplained lameness (Hii et al., 2015). Following the owners' verbal approval, blood samples were collected. Blood samples (2 ml) were obtained from each dog *via* a cephalic vein in EDTA tubes and stored at 4 °C before being transported to the laboratory. The clinical characteristics, history, and blood examinations of the dogs were documented. Clinical signs of heartworm disease such as mild cough, fatigue, decreased appetite, and weight loss, were also recorded. Complete cell counts were determined using an automated blood analyzer (Merilyzer CelQuant 360™, Meril Diagnostics Pvt. Ltd., India). The severity of anemia was categorized as mild (30% ≤ hematocrit (HCT) < 37%), moderate (20% ≤ HCT < 30%), severe (13% ≤ HCT < 20%), or very severe (HCT < 13%) (Tvedten and Weiss, 2000).

2.2. Determination of antigens and antibodies

The canine SNAP® 4Dx® Plus test kit (IDEXX Laboratory Inc., USA) was used to test all collected samples for *D. immitis*-specific antigen, and antibodies of canine *Borrelia burgdorferi*, *Ehrlichia canis* and *Anaplasma phagocytophilum*, according to the manufacturer's instructions.

2.3. Canine blood smears

A thin blood smear was made immediately following blood collection. The slides were then air-dried, methanol-fixed, and stained with Giemsa stain. Microfilariae were detected on slides using light microscopy at magnifications of 100× and 400× (Rosenblatt, 2009).

2.4. DNA extraction and molecular identification of D. immitis

The DNA from 200 μl of canine blood samples was extracted using a QIAamp DNA Mini Kit® (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was eluted in 50 μl of elution buffer and stored at −80 °C until use.

To amplify the *cox*1 gene of *Dirofilaria* spp., a primer set consisting of the forward primer COIintF (5′-TGA TTG GTG GTT TTG GTA A-3′) and the reverse primer COIintR (5′-ATA AGT ACG AGT ATC AAT ATC-3′) was employed (Casiraghi et al., 2001). Each reaction contained 100–200 ng DNA, 10 μM forward and reverse primers, and TksGflex DNA polymerase (1.25 U/μl) (TaKaRa Bio Inc., Shiga, Japan). Thermal cycling commenced with denaturation at 94 °C for 1 min, followed by 40 cycles (98 °C for 10 s, 52 °C for 15 s, 68 °C for 1 min) and a final extension step at 68 °C for 5 min.

2.5. Molecular identification of Wolbachia endosymbionts of D. immitis

For amplification of the 16S ribosomal RNA gene (16S rDNA) of *Wolbachia* spp., the primer set 16FwolbF (5′-GAA GAT AAT GAC GGT ACT CAC-3′) and 16FwolbR3 (5′-GTC ACT GAT CCC ACT TTA AAT AAC-3′) was used (Casiraghi et al., 2001). The preparation for the PCR reaction was the same as in the previous section. Thermal cycling was initiated with denaturation at 94 °C for 1 min, followed by 5 cycles (98 °C for 10 s, 60 °C for 15 s, 68 °C for 1 min), 35 cycles (98 °C for 10 s, 55 °C for 15 s, 68 °C for 1 min), and a final extension step at 68 °C for 5 min.

2.6. Sequencing and phylogenetic analyses

The PCR products were visualized using a 1.5% agarose gel stained with RedSafe DNA Loading Dye; electrophoresis was performed for 30 min at 100 V. The PCR products of five randomly selected positive samples for each primer were excised from the gel and purified using the NucleoSpin® Gel and PCR Cleanup Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The purified PCR products were sequenced directly using an Applied Biosystems 3130 Genetic Analyzer with a Big Dye v3.1 Terminator cycle sequencing kit (Applied Biosystems, Inc., Carlsbad, CA, USA). Multiple sequence alignment was performed using the sequence analysis software package ATGC version 7 (Genetyx Co., Tokyo, Japan). To identify highly similar sequences among the isolated nucleotide sequences, the NCBI BLAST program was used (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed using the sequences from this study and those accessible in the GenBank database (https://www.ncbi.nlm.nih.gov/) (Supplementary Table S1). MEGA X was used to explore the phylogenetic relationships using the Maximum Likelihood (ML) method (Tamura-Nei model) (Kumar et al., 2018). The bootstrap analysis was performed with 1000 replicates. Sequences for *Dracunculus insignis* and *Rickettsia conorii* were used as the outgroup in phylogenetic analyses of *Dirofilaria* spp. and *Wolbachia* spp., respectively.

2.7. Haplotype analysis of D. immitis cox1 sequences and statistical analysis

A haplotype network was generated using PopART 1.7 with the median-joining network method (MJ) based on *cox*1 sequences from *D. immitis* in the present study and reference haplotypes (Leigh and Bryant, 2015). The haplotype diversity (Hd), nucleotide diversity (π), and the number of variable sites (S) were evaluated. The correlation between *D. immitis* infection and tick-borne diseases was assessed using the statistical software Jamovi (ver. 2.3.21) (The Jamovi project, 2021).

3. Results

3.1. Infection rates of D. immitis and tick-borne diseases

Infection with *D. immitis* was found in 14 (28.0%) of 50 examined samples, while the detection rate of the TBD causative agents *A. phagocytophilum* and *E. canis* was 26.0% (13/50) and 26.0% (13/50), respectively, as determined by the ELISA rapid test. Out of 12 samples showing *D. immitis* infection, 7 samples were found to be co-infected with TBD causative agents and 22 of the 50 dogs scored positive for more than one vector-borne disease. Microscopic examination revealed that *D. immitis* and *Babesia* spp. were present in 12.0% (6/50) and 48.0% (24/50) of the collected samples, respectively. Using the PCR method, 28.0% (14/50) and 18.0% (9/50) of the samples were found to be positive for *D. immitis* and *Wolbachia* endosymbionts, respectively (Supplementary Tables S1 and S2). In this study, regardless of the diagnostic method, dogs positive for at least one TBD causative agent were presumed to be TBD-infected. Infection with *D. immitis* was moderately but significantly correlated with TBD infection (Pearson's $r = 0.397$, $P = 0.008$).

Only three of the 14 dogs infected with *D. immitis* showed mild cough, tiredness, and emaciation, while the remainder were asymptomatic. Although all of the dogs in the study had comparable clinical symptoms, 19 of them tested negative for *Dirofilaria* and/or TBD infection. Due to the limitations of our laboratory, we were unable to analyze by PCR or serological tests other TBD causative agents such as *Babesia* spp. or *Theileria* spp.

Table 1
Hematological parameters of examined dogs.

Parameter/unit	Mean ± SD	Range	Reference[a]
Red blood cells (× 10^6 cells/μl)	3.66 ± 0.95	1.9–4.8	5.5–8.5
Hemoglobin (g/dl)	9.33 ± 2.72	4.6–12.5	12.0–18.0
Packed cell volume (%)	26.15 ± 6.8	14.5–35.6	37.0–55.0
Platelets (× 10^3 cells/μl)	170.27 ± 87.07	41–284	200–500
White blood cells (× 10^3 cells/μl)	15.96 ± 8.47	6.4–34.8	6.0–17.0
Neutrophils (%)	56.87 ± 10.92	44.5–78.5	60.0–77.0
Lymphocytes (%)	32.86 ± 10.06	14.7–49.8	12.0–30.0

[a] Larry and Francis (2016).

3.2. Haematological parameters of examined blood samples

The mean values of the examined blood samples shown in Table 1 indicate low red blood cell counts, Hb concentration, and PCV. Among the examined samples, 47 (94.0%) of infected dogs showed moderate anemia (20% ≤ HCT < 30%), and 3 (6.0%) showed severe anemia (13% ≤ HCT < 20%).

3.3. PCR detection of D. immitis and Wolbachia spp.

Clinical findings by rapid test kit, microscopic examination, and PCR test are shown in Table 2. The PCR test revealed a positive rate of 28.0% (14/50) for *D. immitis* and 18.0% (9/50) for *Wolbachia* endosymbionts, respectively. The PCR products for the *cox*1 gene of *D. immitis* and the 16S rDNA of *Wolbachia* spp. were ~650 bp and 1010 bp in size, respectively. The results of the NCBI BLAST search are summarised in Supplementary Table S2. Ten sequences obtained in this investigation (5 for *D. immitis* and 5 for *Wolbachia* spp.) are available in the GenBank database under the accession numbers ON259769-ON259773 and ON259763-ON259767, respectively. The *cox*1 sequences of 5 isolates of *Dirofilaria* (ND-1, ND-3, ND-12, YD-9 and YD-15) were found to be identical. These sequences were also found to be identical to *D. immitis* *cox*1 gene sequences deposited in GenBank derived from dogs in different countries, including Bangladesh (KC107805), France (KP760184 and MT230079), French Guiana (MT193088), Iran (KT318126, MZ266347 and KR870344), Japan (AB973226), and Thailand (MK250715, MK250759, MK250760 and MK250742), Italy (AM749228), as well as a golden jackal in Iran (MZ266360). Additionally, the sequences were determined to be identical to *D. immitis* sequences generated from mosquitoes in Myanmar (OL721653 and OL721654). The sequences were also found to be 99.8% identical to *D. immitis* sequences derived from dogs in Algeria (MW138019) and Iran (MZ509546) deposited in GenBank. The ML trees based on the *Dirofilaria* spp. *cox*1 gene (Fig. 1) revealed that all sequences generated in the present study clustered together with the sequences for *D. immitis* deposited in GenBank with high support (99%); the *D. immitis* clade was resolved as a sister clade to *D. repens*.

A total of five haplotypes were found in the nucleotide dataset for *D. immitis* (Table 3). Three were unique, while the remaining two were shared by two or more populations. Only one *cox*1 haplotype (H1) was detected in the sequenced isolates of *D. immitis* in this study. This most common haplotype was shared with isolates reported from Bangladesh, France, French Guiana, Iran, Iraq, Italy, Japan, and Thailand (Fig. 2). Single isolates from Iraq, New Caledonia, and Thailand, and 2 isolates from Algeria were found to belong to five different haplotypes (H2–H5;

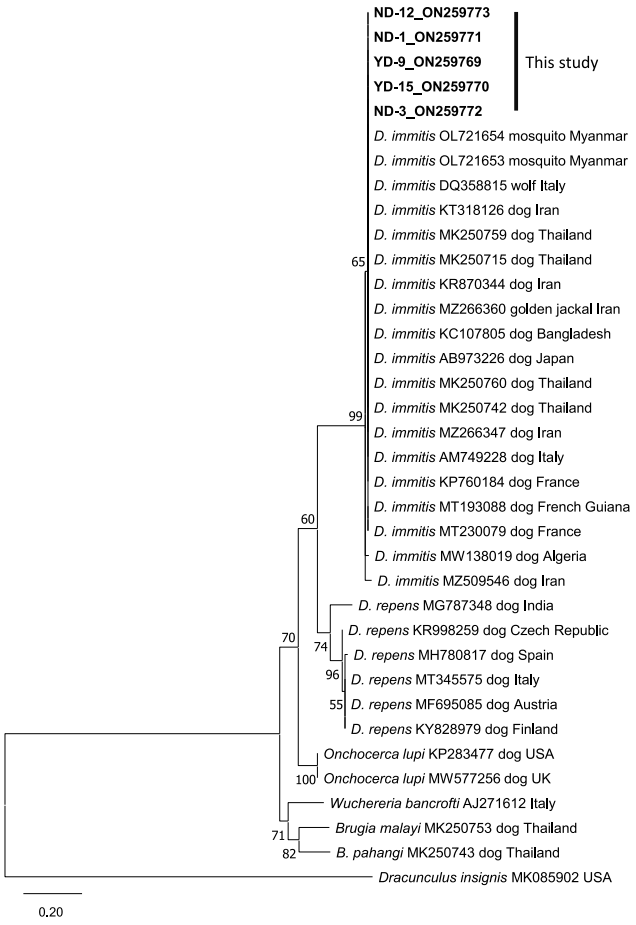

Fig. 1. Maximum Likelihood phylogenetic tree (Tamura-Nei model) based on partial *cox*1 sequences for *Dirofilaria immitis* generated from dogs in Myanmar and reference sequences for *Dirofilaria* spp. and closely related species retrieved from GenBank. The bootstrap support is based on 1000 replicates.

Table 3
Haplotype distribution in the *cox*1 dataset of *Dirofilaria immitis* studied.

Location	GenBank ID	Haplotype and no. of isolates				
		1	2	3	4	5
Algeria	MW138019	–	–	–	–	2
Bangladesh	KC107805	1	–	–	–	–
France	KP760184	2	–	–	–	–
French Guiana	MT193088	1	–	–	–	–
Iran	MZ509546	7	–	–	–	–
Iraq	MZ619051	1	1	–	–	–
Italy	AM749228	2	–	–	–	–
Japan	AB973226	1	–	–	–	–
Myanmar	ON259772	5	–	–	–	–
New Caledonia	KY347824	–	–	–	1	–
Thailand	MK250759	5	–	1	–	–

Table 2
Summary data for the detection of TBD causative agents and *Wolbachia* spp. by ELISA, microscopy, and PCR test (see Supplementary Table S1 for details).

Location	ELISA				Microscopy		PCR	
	D. immitis Ag	*A. phagocytophilum* Ab	*B. burgdorferi* Ab	*E. canis* Ab	*D. immitis*	*Babesia* spp.	*D. immitis*	*Wolbachia* sp.
Yangon (n = 34)	10 (29.4%)	10 (29.4%)	0	7 (20.6%)	4 (11.8%)	16 (47.1%)	11 (32.4%)	6 (17.6%)
Nay Pyi Taw (n = 16)	4 (25%)	3 (18.7%)	0	6 (37.5%)	2 (12.5%)	8 (50.0%)	3 (18.8%)	3 (18.8%)
Total	14 (28.0%)	13 (26.0%)	0	13 (26.0)	6 (12.0%)	24 (48.0%)	14 (28.0%)	9 (18.0%)

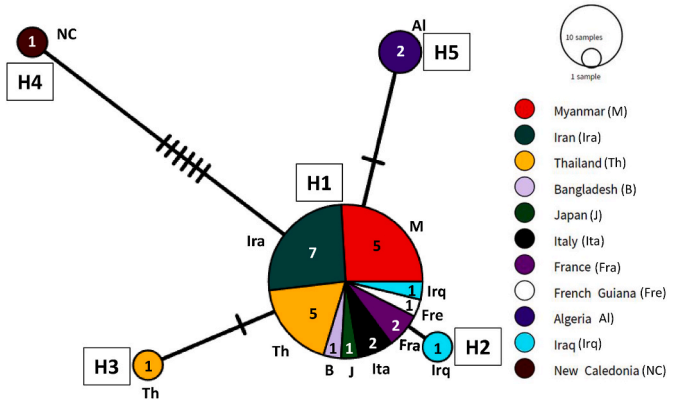

Fig. 2. The haplotype network of *Dirofilaria immitis* based on sequences of a fragment of the *cox*1 gene from nematodes of dogs in Algeria, Bangladesh, France, French Guiana, Iran, Iraq, Italy, Japan, Myanmar, New Caledonia, and Thailand. The circle size is scaled to the frequency of each haplotype. The hash marks indicate nucleotide substitutions among adjacent haplotypes.

Fig. 2). The haplotype diversity (Hd) estimated for the entire *D. immitis* dataset examined was 0.2903 and the nucleotide diversity (π) was 0.00024.

Of the 9 PCR-positive samples, 16S rDNA sequences were generated from 5 isolates of *Wolbachia* spp. (ND-1W, ND-3W, ND-12W, YD-9W and YD-15W); these were all identical. These sequences were also identical to 16S rDNA sequences for *Wolbachia* endosymbionts isolated from *D. immitis* in various countries, including France (KU255236), Italy (Z49261), Russia (MN200331), and the USA (AF088187). These sequences also exhibited a 100% similarity with *Wolbachia* endosymbionts obtained from *Rhipicephalus sanguineus* in Japan (AF304445) and *Aedes aegypti* in the Philippines (MN046719 and MN046788) deposited in GenBank. The phylogenetic analysis based on 16S rDNA sequences for *Wolbachia* spp. revealed that the sequences from the present study clustered with sequences for *Wolbachia* endosymbionts of *D. immitis*, *R. sanguineus* and *Ae. aegypti* in a strongly supported clade (94%) (Fig. 3), well differentiated from the clades comprising *Wolbachia pipientis* and *Wolbachia* spp. from *D. repens*, *Cinara* spp., *Loxodontifilaria* sp., *Onchocerca* spp. and *Tuberolachnus* spp.

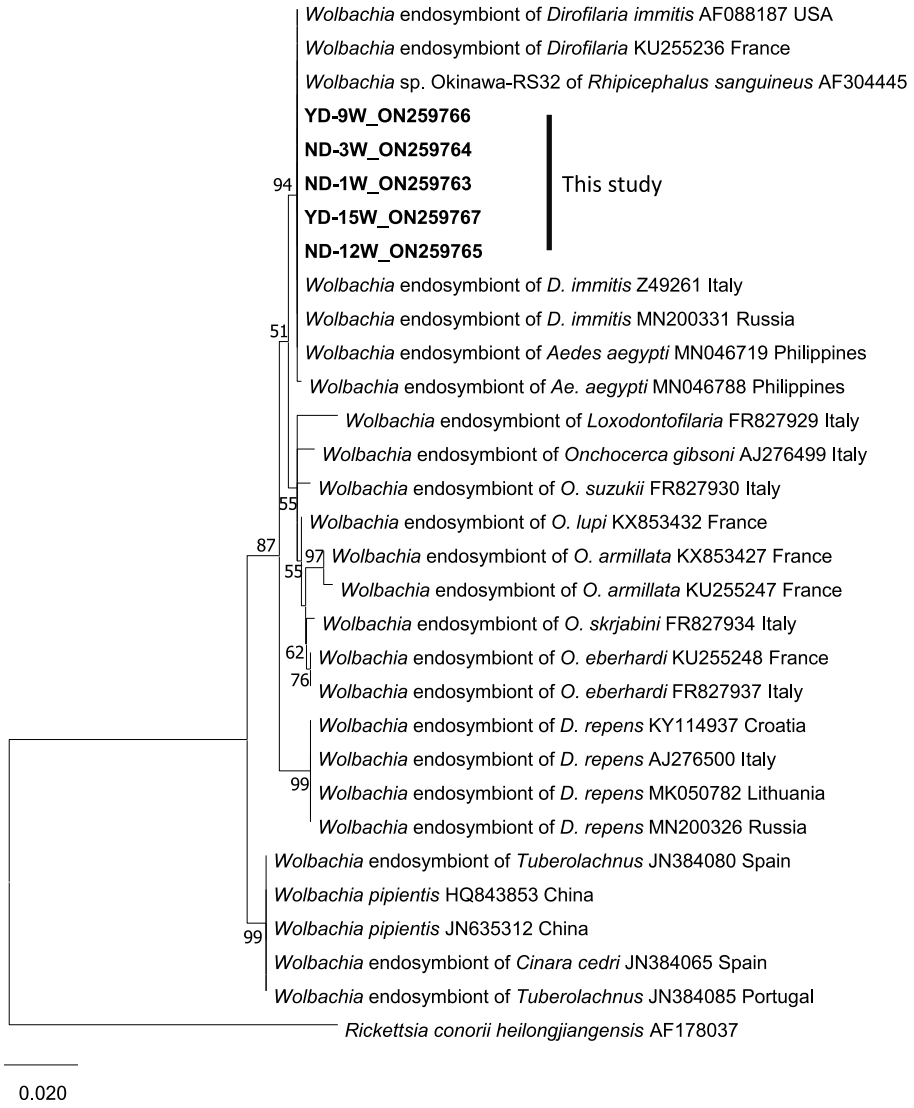

Fig. 3. Maximum Likelihood phylogenetic tree (Tamura-Nei model) based on partial 16S rDNA sequences for *Wolbachia* spp. generated from dog blood samples collected in Myanmar and reference sequences retrieved from GenBank. The bootstrap support is based on 1000 replicates.

4. Discussion

In this study, dogs suspected of having TBD infections were initially tested using ELISA rapid test. All dog blood samples were also subjected to conventional PCR for detection of *Dirofilaria* spp. and *Wolbachia* spp. and the positive samples were sequenced. The present investigation demonstrated the existence of *D. immitis* infection in dogs in addition to TBD. *Dirofilaria immitis* antigen tests are the most extensively used assays in Myanmar, both in the field and in veterinary clinics. These tests can detect adult *D. immitis* antigens from both occult and non-occult infections (Duran-Struuck et al., 2005). The SNAP® 4Dx® Plus test kit indicated the presence of *D. immitis* antigens in 14 dogs in this study; of these, seven were also infected with TBD causative agents. Reports on lower prevalence of *D. immitis* infection in dogs than our findings have been published from various countries, including China (Wang et al., 2016), Korea (Byeon et al., 2007), Iran (Khedri et al., 2014), Portugal (Alho et al., 2014), and Thailand (Tiawsirisup et al., 2015), with the infection rates of 13%, 10.2%, 24.2%, 15.1% and 10%, respectively, using rapid test kits.

Co-infections were also identified in the present study and infection with *D. immitis* was significantly correlated with TBD infections. Causative agents of TBD such as *Theileria orientalis*, *Theileria buffeli*, *Theileria* cf. *velifera*, *Theileria luwenshuni*, *Theileria* sp., *Babesia vogeli*, *Babesia gibsoni*, *Ehrlichia canis* and *Anaplasma platys* have been recorded in dogs in Myanmar (Bawm et al., 2021; Hmoon et al., 2021). Likewise, co-infection with multiple TBDs has also been documented in Southeast Asia; infection with *Ehrlichia* spp., *Rickettsia* spp., *Anaplasma* spp., *Babesia* spp. etc., in dogs and vectors in Thailand and Lao PDR, neighbouring countries of Myanmar (Suksawat et al., 2001; Liu et al., 2016; Nguyen et al., 2021). Depending on the existence and abundance of arthropod vectors in places where canine vector-borne diseases are common, dogs can be infected with many vector-borne agents at the same time or sequentially (Otranto et al., 2009). In the present study, 22 of the 50 dogs scored positive for more than one vector-borne disease, being diagnosed by serological and microscopic examinations, or PCR.

Molecular diagnosis, in comparison to parasitological assays, remains the most effective approach for studying the diversity of different parasites (de Argôlo et al., 2018). Furthermore, the *cox*1-based method was found to be more appropriate for diagnosing canine filariasis (Satjawongvanit et al., 2019); Oh et al. (2017) also proposed the *cox*1 as the "barcode" gene for identifying filarial species diversity. Five sequences were successfully generated among the *cox*1 PCR-positive samples. In the ML trees constructed using the *cox*1 sequences for *Dirofilaria* spp. the isolates identified in the present study clustered together with isolates of *D. immitis* and were distinct from those of *D. repens*. The *cox*1 haplotype identified for *D. immitis* in this study was the most common haplotype shared with isolates from Bangladesh, France, French Guiana, Iran, Iraq, Italy, Japan, and Thailand.

Recent studies have explored the connection between the molecular detection and identification of *Wolbachia* spp. in onchocercid nematodes in general (Manoj et al., 2021), and the diagnosis of canine filariasis in particular (Satjawongvanit et al., 2019; Laidoudi et al., 2020). In the present study, five 16S rDNA sequences for *Wolbachia* spp. were isolated from nine PCR-positive samples. The phylogenetic analysis showed that the five 16S rDNA sequences generated during this study clustered with *Wolbachia* endosymbionts of *D. immitis*, *Rhipicephalus sanguineus*, and *Aedes aegypti*. Because *Wolbachia* has a detrimental effect on the physical state of canine tissues infected with *D. immitis* (Kramer et al., 2008), antimicrobial therapy may be beneficial in treating heartworm disease in cats and dogs. Using this therapeutic strategy not only stops *D. immitis* growth and reproduction but also reduces the inflammatory responses associated with infection (Frank and Heald, 2010).

5. Conclusions

To the best of our knowledge, this is the first molecular detection of *D. immitis* and its *Wolbachia* endosymbionts from dogs in Myanmar. *Dirofilaria immitis* has recently been detected in mosquitoes of the *Culex pipiens* complex (Aung et al., 2023), indicating that these mosquitoes are potential vectors for this parasite in Myanmar. Therefore, regular and appropriate use of preventive medications, as well as avoiding vector contact with dogs and employing appropriate diagnostic tools are critical for the control of the vector-borne *Dirofilaria* spp. Veterinarians are also responsible for providing proper heartworm-preventive education to their clients. Finally, surveillance of all types of filarial parasites in dogs should be explored in the future using a large sample size. The present study contributes to our understanding of the coexistence of *D. immitis* and *Wolbachia* endosymbiosis in dogs, and the findings may benefit future prevention and control of dirofilariasis in dogs.

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Ethical approval

The collection of samples from dogs was approved by the Research Ethics Review Committee, University of Veterinary Science, Nay Pyi Taw, Myanmar (Approval no: RERC/Recom/2020-1).

CRediT authorship contribution statement

Saw Bawm: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Yadanar Khaing:** Conceptualization, Investigation, Data curation, Formal analysis, Writing – review & editing. **Hla Myet Chel:** Conceptualization, Investigation, Data curation, Formal analysis, Writing – review & editing. **Myint Myint Hmoon:** Conceptualization, Investigation, Data curation, Formal analysis, Writing – review & editing. **Shwe Yee Win:** Conceptualization, Investigation, Data curation, Formal analysis, Writing – review & editing. **Min Bo:** Data curation, Formal analysis, Writing – review & editing. **Tint Naing:** Data curation, Formal analysis, Writing – review & editing, All authors read and approved the final manuscript. **Lat Lat Htun:** Conceptualization, Data curation, Formal analysis, Writing – review & editing.

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.

Data availability

The data supporting the conclusions of this article are included in this published article and its supplementary files. The newly generated sequences were submitted to the GenBank database under the accession numbers ON259769-ON259773 (*D. immitis*) and ON259763-ON259767 (*Wolbachia*).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2023.100148.

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