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Prevalence and risk factors associated with *Dirofilaria immitis* infection in dogs using practical methods in hospitals in Thailand

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Abstract

To date, routine diagnosis of canine heartworm disease relies on detecting *Dirofilaria immitis* antigens in blood or the microscopic examination of blood smears. However, each method has limitations, potentially leading to life-threatening situations for infected dogs. This study aimed to determine the prevalence of filarial infection, risk factors, and appropriate detection methods in practical clinics. A total of 113 dog blood samples from two provinces in Thailand (Chonburi = 73 and Nakhon Nayok = 40) were analyzed for *D. immitis* infection using buffy coat smears, commercial immunochromatographic tests (SNAP 4Dx Plus), and polymerase chain reaction. Overall prevalence was 51.53% (58/113) across all methods. The positivity rates were 15.38% (12/78) for buffy coat smears, 8.00% (4/50) for SNAP 4Dx Plus, and 45.43% (51/113) for polymerase chain reaction. All positives from the test kits correlated with other methods. A significantly high prevalence was observed in dogs under 2 years old. Accessibility to pet care services in urban areas appeared to have a protective effect. Positive commercial test results could confirm *D. immitis* infection. However, selecting more than one diagnostic technique in clinics, including morphological examination, immunochromatography, or molecular methods, is recommended for early and more accurate detection, along with the promotion of heartworm prevention strategies.

Introduction

Canine heartworm disease (CHD), primarily caused by *Dirofilaria immitis*, is commonly encountered in veterinary practice and a potential zoonotic disease in human medicine.[1] Mosquitoes carrying infective larvae transmit the disease to other dogs through bites. Once infected, larvae migrate and develop in the host dog, maturing into adult worms in the right ventricle and pulmonary artery, obstructing blood flow, and leading to right-sided heart failure. Clinical signs include depression, coughing, ascites, and breathing difficulty.[2] Many cases require time to manifest clinical signs, potentially leading to misdiagnosis with other cardiovascular disorders.

In diagnosing CHD, a history of mosquito exposure without monthly heartworm prevention is helpful. Humans are less suitable hosts for *D. immitis* compared to the dogs,[1] but cases in humans correlate with high canine infection rates in particular areas. Standard diagnostic techniques include buffy coat smear and lateral flow antigen immunoreactivity tests; although, low microfilaria or antigen levels in bloodstream may limit diagnosis. Polymerase chain reaction (PCR) is one of the available molecular techniques used to detect small amounts of antigen.[3,4]

Heartworm disease is a significant public health concern in tropical and subtropical regions, especially in Southeast Asia. In Thailand, studies show varying prevalence rates of *D. immitis* in dogs, with 24.10% in Songkhla, 13.90% in Bangkok, and 18.20% in Chiangmai.[2] Despite the known endemic nature of *D. immitis* in Thailand, information is still limited in some areas.

This study aimed to provide practical diagnostic guidelines for veterinary clinics to detect *D. immitis* before severe CHD signs develop, facilitating timely treatment. Specifically, it sought to determine the prevalence and risk factors of *D. immitis* infection in dogs in Chonburi and Nakhon Nayok provinces, located in eastern and central parts of Thailand, using broadly applicable detection methods.

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Materials and Methods

Sample collection. A total of 113 dogs were selected using stratified random sampling from two provinces (Chonburi = 73 and Nakhon Nayok = 40; Fig. 1), between February 2020 and April 2021. Sample size was influenced by community distribution, residents' willingness, and fieldwork safety. Expected samples ranged from 7 to 165, based on a 0.0043 to 0.1220 prevalence from previous studies,[5,6] with a 0.05 margin of error and 95.00% confidence interval (CI), calculated using Epitools Epidemiological Calculators.[7] Detailed clinical histories were recorded, and blood samples (minimum 5.00 mL) were taken from cephalic veins, stored in ethylene-diaminetetraacetic acid-contained tubes, and transferred on ice for analysis.

Fig. 1. Map of Thailand indicating Chonburi and Nakhon Nayok provinces.

Microscopic examination. Seventy-eight blood samples were examined for microfilaria using the buffy coat smear method as described in previous study.[8]

Immunochromatographic assay. The rapid SNAP 4Dx Plus Test (IDEXX Laboratories, Westbrook, USA) was conducted following the manufacturer's protocols to test 50 fresh blood samples.

DNA extraction and PCR. The DNA extraction was performed on all 113 blood samples using the E.Z.N.A.® blood DNA Mini Kit (Omega Bio-tek, Norcross, USA) following the manufacturer's instructions. Primers (forward: ATTGGGTGGCCCTGAAATGG and reverse: CCCT CTACACTCAAAGGAGGA) targeting conserved regions of the *COI* gene specific to *D. immitis* were used,[4] with an expected size of 150 base pairs (bp). The PCR protocol followed the methods outlined in a previous study.[4] The PCR products were assessed by electrophoresis on a 1.00% agarose gel. Additionally, the types of blood samples (plasma and whole blood) related to the concentration of nucleic acid from DNA extraction were measured using NanoDrop® ND-1000 (Thermo Fisher Scientific, Waltham, USA) and compared by *t*-test at a significance level less than 0.05 using Epitools Epidemiological Calculators.[7]

Statistical analysis. The χ^2 test was used to analyze statistical relationships among proportions, with a *p*-value < 0.05 considered significant. Potential risk factors were defined as geographic areas (Chonburi and Nakhon Nayok provinces), age (classified into three groups, [9] including dogs aged < 2, 2 - 7, and > 7 years), sex (male and female), breed (purebred and mixed breeds), rearing management (indoor and outdoor; stray and owned dogs), and experience with heartworm prevention. Results were reported as odds ratios (OR) with 95.00% CIs. Epitools Epidemiological Calculators[7] were utilized for analysis.

Results

The overall prevalence of filariasis was 51.53% (58/113; Table 1). A sample was diagnosed as positive when the infection was detected by at least one method. Out of the 113 blood samples, 51 samples (45.13%) tested positive for *D. immitis* by PCR assay. Seventy-eight blood samples were examined by buffy coat smear, and 12 samples (15.38%) were found to be positive. Four samples (8.00%) from 50 fresh blood samples tested positive on commercial test kits (SNAP 4Dx Plus), which were also sensitive by the other methods. It was found that 46 and 7 samples were positive only by PCR and buffy coat smear methods, respectively, but no sample was positive only by the commercial test kit.

Both whole blood and plasma were extracted for PCR assay. The mean nucleic acid concentration of whole blood (23.40) was significantly higher than that of plasma (6.04) with $p < 0.001$.

The prevalence of dirofilarial infection in Nakhon Nayok province was significantly higher (70.00%) than Chonburi (41.10%) with OR of 3.34, 95.00% CI = 1.47 - 7.60, and *p*-value = 0.00. Dogs less than 2 years old had the highest prevalence of infection (74.07%; OR of 3.74, 95.00% CI = 1.37 - 10.16, and *p*-value = 0.01), followed by dogs older than 7 years (53.33%), and dogs aged 2 - 7 years (40.00%). Dogs mostly living outside had a prevalence of 54.55% with OR of 3.00, while dogs staying inside houses had a lower prevalence of 28.57%.

Table 1. Overall prevalence and univariate analysis of risk factors associated with *Dirofilaria imitis* infection in dogs in Chonburi and Nakhon Nayok provinces, Thailand, during 2020-2021.

Risk factors		No. samples	Prevalence (%)	Odds ratio	95.00% CI	*p*-value*
Geographic location (n = 113)	Nakhon Nayok province	40	70.00	3.34	1.47 - 7.60	0.00*
	Chonburi province	73	41.10			
Sex (n = 113)	Male	40	60.00	1.72	0.79 - 3.76	0.17
	Female	73	46.58			
Breed (n = 113)	Purebred	19	42.11	0.64	0.24 - 1.73	0.529
	Mixed breeds	94	53.19			
Age (n = 87; unknown = 26)	< 2 years	27	74.07	3.74	1.37 - 10.16	0.01*
	2 – 7 years	45	40.00	0.33	0.14 - 0.80	0.01*
	> 7 years	15	53.33	1.02	0.60 - 1.70	0.969
Rearing management (n = 113)	Outdoor	99	54.55	3.00	0.88 - 10.21	0.125
	Indoor	14	28.57			
	Stray dogs	23	39.13	0.54	0.21 - 1.37	0.19
	Owned dogs	90	54.44			
Heartworm prevention (n = 58; no data = 55)	Used to	41	58.54	0.77	0.24 - 2.49	0.89
	Never	17	64.71			
Overall		113	51.33	-	-	-

CI: Confidence interval of odds ratio. *$\chi 2$ test at significant level of $p < 0.05$.

Moreover, the percentage of infection in purebred dogs (42.11%) was lower than mixed-breed dogs (53.19%) with OR of 0.64. The experience of heartworm medication showed OR of 0.77 with a prevalence of 58.54% compared to 64.71% of dogs that never used heartworm medication. Additionally, male dogs had a higher percentage of *D. immitis* infection (60.00%) than female dogs (46.58%) with OR of 1.72 (Table 1).

Discussion

Thailand, along with most countries in Southeast Asia, is an endemic area for canine filariasis. One of the most frequently detected species across Thailand is *D. immitis*.[2,10] Over the past 30 years, the prevalence of *D. immitis* in dogs has been continuously reported in several parts of Thailand, ranging from 0.43 to 58.00%.[2,3,5,6,10-12] Our study's overall prevalence (51.53%) was remarkably higher than previous reports. This could be attributed to the abundant natural reservoirs, forests, and grasslands in the two provinces studied, supporting vector breeding sites. Additionally, dogs in Nakhon Nayok province had 3.34 times higher infection than those in Chonburi province. This disparity could be explained by the fact that Chonburi province is a central hub of eastern Thailand and tends to be more urbanized than Nakhon Nayok province. Research from Portugal confirmed that a rural environment was a significant risk factor for CHD.[13] This aligns with studies from major cities in Thailand, such as Bangkok, Chiang Mai, and Songkhla, where urban dog owners may more readily access and afford pet-care services.[5,6,11]

The *D. immitis* infections were significantly higher in young dogs than those over 2 years old, contrasting with studies indicating infections are mostly found in dogs older than 2 - 3 years.[11,14] This suggests that *D. immitis* can infect dogs at a younger age than previously observed. Therefore, monitoring the young population could be considered, and heartworm prevention should be initiated as early as possible, ideally before six months. The high prevalence of infection in outdoor-housed dogs and those without chemo-prophylactic treatment was not surprising. It insisted that heartworm medication had a preventive effect on infection, consistent with previous researches.[12,13] Outdoor-living dogs showed a threefold higher risk of *D. immitis* infection; although, this was just a trend. Indoor housing may reduce exposure to mosquitoes, as supported by several former studies.[12-14] No significant difference in infection rates was observed between owned and stray dogs. In Thailand, many owned dogs live outdoors, increasing exposure risk; while, some stray dogs, especially near schools, frequently receive vaccinations and preventive treatments, a common practice in the country.

The first case involved positive results solely from the buffy coat smear. The absence of detection from the SNAP 4Dx Plus and PCR methods could be ascribed to the possibility that the parasites found in the bloodstream were not *D. immitis*.[3,15] Differentiating filarial species under a light microscope is limited and requires expertise, as well as special staining techniques.[11,16] Furthermore, both the SNAP 4Dx Plus and PCR demonstrate high specificity for *D. immitis*, with no cross-reaction with other filarial helminths that can also infect dogs, such as *Dirofilaria* spp., *Acanthocheilonema* spp., and *Brugia* spp.[3,15] Microscopic examination is one of the popular methods widely used as a screening technique because of reasonable cost and easy accessibility. However, it can lead to false negatives due to its low sensitivity and the need for a sufficient microfilarial load.[16,17]

The second case showed positive results only with the PCR method. This could be attributed to early infection

where the parasite has not fully developed or to infection with male *D. immitis* alone.[18,19] Consequently, specific antigens from the parasite's ovaries may not be detected in commercial immunochromatographic tests.[20] Negativity from buffy coat smears can occur due to the low sensitivity of this method and the need for high load of microfilaria infection.[16] Moreover, *COI*-PCR is specific for *D. immitis* identification,[3,4] ensuring that other filarial species are not detected.

The last case was positive across all methods, confirming that the dog was indeed infected with *D. immitis*. A positive result on the buffy coat smear indicated the presence of microfilariae in the dog's bloodstream, while SNAP 4Dx Plus test suggested the presence of adult female *D. immitis*, as the test specifically targets proteins from the reproductive tract of this species.[18,20] Finally, a positive PCR result confirmed the infection by detecting the genetic material of *D. immitis* in the dog. However, no definitive evidence could confirm that the dogs were free of filarial infection.

Commercial test kits are popular in practical clinics due to their accuracy, availability, and user-friendliness. A positive result from this kit can confirm *D. immitis* infection, but a negative result does not necessarily mean being free from disease. There are several other factors that can interfere with antigen detection on test kits, such as early doxycycline or heartworm preventive treatments, the age of infected dogs, and the duration of infection.[19] Hence, a negative result on either a commercial test kit or a buffy coat smear does not confirm the absence of infection and should not be used alone for diagnostic purposes in clinics.

The PCR has become a viable option for early detection, monitoring, and confirmation of *D. immitis* infection. Moreover, the PCR method can clarify the similar morphology of microfilariae under microscopic examination and help to differentiate among filarial species.[3,15] We recommend incorporating PCR as a parallel technique for diagnosing heartworm infection.

A limitation of this study was the unequal number of samples for each diagnostic method. Some fresh blood samples could not be processed due to the transportation delays or insufficient volume. Additionally, safety concerns when working with untamed or stray dogs affected the total number and volume of samples collected.

The type of blood collection is important for the diagnostic process. Whole blood samples have a significantly higher concentration of nucleic acid than plasma samples. However, both types of samples could be processed using molecular techniques. Considering the convenience of transportation in remote areas, plasma or serum may be more appropriate than whole blood. Applying blood samples to filter paper or dried blood spots is recommended for easy shipping.[15]

In conclusion, our study indicates a high prevalence of *D. immitis* infection in dog population across Chonburi and Nakhon Nayok provinces, reaffirming Thailand's status as an endemic area. We recommend employing more than one detecting method to confirm infections in practical clinics. These findings can serve as guidelines for clinicians in the field and support the development of effective heartworm control strategies.

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Conflict of interest

The authors declare no conflict of interest.

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