Annals of Parasitology 2026, 72, 00–00
doi: 10.17420/ap72.549

Original paper

Serological and molecular detection of *Dirofilaria immitis* in pet dogs of Lahore, Pakistan

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ABSTRACT. Heartworms, parasitic nematodes, are responsible to cause a potentially life threatening condition, heartworm disease in mammals particularly dogs and cats. The disease is prevalent in warm, humid areas with mosquitoes as intermediate hosts, increases the chances of transmission. This study aimed to investigate heartworm infection (*Dirofilaria immitis*) in pet dogs for the first time in Lahore using morphological, serological and molecular techniques including microscopy, PCR and rapid diagnostic kit tests. Blood samples were tested for antigens, with positive cases further analyzed using microscopy, Polymerase Chain Reaction, X-ray imaging, and echocardiography to detect heart-related changes. Modified Knott's Test, remained a valuable tool for detecting and differentiating microfilariae, particularly when used alongside other diagnostic techniques. The microfilariae was confirmed morphologically through wet mount method but had low sensitivity and requires expertise, while microscopy showed high sensitivity but yielded more false positives compared to PCR. Thoracic radiography revealed characteristic signs of *D. immitis* infection, but may not show early-stage changes. PCR detected *D. immitis* in 10% of samples, with positive bands (at 203 bp for specific primers and 450 bp for general primers) observed for both species-specific and general primers. Using these diagnostic techniques improves early detection and treatment of heartworm disease in dogs, setting a new standard for veterinary care in Pakistan.

Keywords: heartworm disease, *D. immitis*, parasitic nematodes, mosquitoes, dogs, microfilariae

Introduction

Many zoonotic pathogens can be effectively stored in dogs, and many blood-feeding arthropods can quickly obtain food from them [1]. Public health is facing new challenges as a result of the growing number of dogs and their growing proximity to people in both urban and rural regions [2]. Dogs worldwide are afflicted with a significant group of disorders known as canine vector-borne diseases (CVBDs). These illnesses are brought on by a wide variety of pathogens that are spread by various arthropod vectors, including insects (such as phlebotomine sandflies, fleas, and mosquitoes) and ticks. Some of the CVBDs are of significant zoonotic concern in addition to their veterinary significance, as dogs may act as sentinels and reservoirs for human illnesses [1]. The most common filarial nematode is *D. immitis*, which is spread by mosquitoes. Dogs, cats, and other wild animals are the main mammals that are afflicted by these worms. Dirofilariasis, also referred to as heartworm disease, is a dangerous and potentially lethal illness caused by *D. immitis*. In regions with warm, humid weather, where a large number of mosquitoes serve as intermediate hosts, the disease

has become endemic [3]. *Dirofilaria's* life cycle is divided into multiple stages. In the definitive host, such as dogs and other canines, adult worms typically reside in the heart and the pulmonary arteries. Female worms produce and release microfilariae into the bloodstream. During the process of feeding on an infected host, mosquitoes ingest these microfilariae. The microfilariae develop within the mosquito, transforming into third-stage larvae that possess infective capabilities. In the course of a later blood meal, the infected mosquito transmits these infectious larvae to various susceptible hosts. Upon entering the new host, the larvae undergo a developmental process that spans several months before maturing into adult worms. Untreated, these worms pose a significant risk to the heart, lungs, and other vital organs, potentially resulting in severe health complications and even mortality [4]. Heartworm is a newly emerging disease because mosquitoes, the primary vectors of disease transmission, thrive in warm, humid environments. Standing water significantly enhances the likelihood of heartworm transmission, as it creates ideal conditions for mosquito breeding. Heartworm prevalence is notable in regions characterized by mild winters and tropical or subtropical climates, as these environments favor the year-round survival of mosquitoes [5].

Patent infections can affect a wide variety of wild and companion animal species. Wild canids and perhaps other carnivore species in Europe are considered to be part of wild animal reservoirs [3]. In companion animals, heartworm disease is most commonly diagnosed in dogs; however, it is less common in cats and ferrets. Differences in diagnostic techniques and the parasite's lifespan in various species are the causes of this variation [2]. The main cause of heartworm infection is adult heartworms and their antigenic products, which can cause serious and even fatal cardiovascular disease [6]. According to [3], a wide range of mosquito species can serve as intermediate hosts worldwide. Although infected mosquitoes can spread the parasite to humans, the infection is still not protected by a patent. The infectious larvae move to the lungs, where they encapsulate and eventually die, and causing granulomatous reactions in humans called „coin lesions„. These lesions are medically significant because they radiologically resemble metastatic lung cancer. This illness has been reported in several nations with tropical, semitropical, or temperate climates [3]. According to [2], the majority of *D. immitis* infections in Europe occur in southern nations, including Spain, Portugal, France, Italy, Greece, and Turkey. It is believed that these nations represent the continent's historically endemic region. According to recent findings from several European countries, the parasite is more widely distributed [2]. With occasional autochthonous instances being documented in the Czech Republic, Slovakia, Hungary, Bulgaria, Croatia, Romania, and Serbia are currently acknowledged as endemic locations in Eastern Europe [6]. To reduce parasite infections in both humans and pets, it is essential to diagnose parasitism in dogs. Numerous tests are used in the diagnosis process, such as PCR-based identification, X-rays, echocardiography, ELISA, rapid test kits, and microscopic techniques, including wet mount and Modified Knotts's Test. Because of its great sensitivity and specificity, ELISA is the most recommended technique. To monitor heartworm disease and to ensure prompt and efficient treatment, X-rays and echocardiography are crucial. Microfilariae in blood samples and mosquito vectors carrying larvae can be reliably identified via PCR-based identification. Dirofilariasis spreads more easily when dogs with heartworms travel across areas, which highlights how crucial it is to regulate animal mobility to prevent and treat the illness. This study aims to conduct morphological, serological, and molecular analyses to detect *D. immitis* infection in pet dogs in Lahore, Pakistan.

Materials and Methods

Collection of samples

Blood samples were collected from 30 dogs at the UVAS Pet Center, Lahore, based on clinical signs and symptoms, primarily cough, exhaustion, and difficulty breathing, with or without anemia, weight loss, and swelling in the abdomen.

Examination and analysis

The suspected dogs underwent evaluation for microfilariae through wet smear examination of blood samples, Knott's method, SNAP® 4Dx® Plus Test (IDEXX Laboratories, Westbrook, Maine, United States), PCR, sequencing, and radiographic assessment of the heart's major blood vessels. The wet smear and Knott's method were applied to anticoagulated whole blood, while the SNAP® 4Dx® Plus Test was conducted on serum. The

SNAP® 4Dx® Plus Test demonstrates higher accuracy in the detection of *D. immitis* antigens. Microscopy refers to the use of microscopes to observe objects that are too small to be seen by the naked eye. It encompasses various techniques and applications across multiple scientific disciplines.

Modified Knott's Test

The Modified Knott's Test was conducted on the blood samples to detect circulating microfilariae. A milliliter of blood containing EDTA was mixed with nine ml of 2% formalin in a 15 ml tube, followed by centrifugation for 15 minutes at 447.2 g [7]. Following centrifugation, the supernatant was removed, and the pellet was subsequently combined with methylene blue stain. A single drop of sediment was placed on the slide, covered with a coverslip, and examined with an Olympus microscope at low power magnification (100× and 400×).

Wet mount

RBCs were allowed to precipitate at the bottom of the EDTA tube by avoiding any movement of the tube for about fifteen to twenty minutes, a process that enhances the formation of the buffy coat layer. A droplet was then taken from the buffy coat and placed right in the middle of the slide. The sample was then examined under an Olympus microscope at low magnification (100× and 400×) after placing a coverslip to minimize the drying effect.

DNA extraction and PCR analysis

Total genomic DNA was isolated from those dogs' blood samples, which were suspected of having heartworm using a molecular biology extraction kit Quick-DNA™ Miniprep plus Kit USA following the manufacturer's instructions and also using the technique of [8]. Whole blood, 60 µl, was mixed with 540 µl of DNA lysis buffer and 10 µl. Proteinase K and allowed to incubate at 55°C for 12 hours. After the addition of phenol-chloroform-isoamyl alcohol (PCI), the system was subjected to centrifugation and the liquid phase of the aqueous layer was extracted. Afterwards, the chloroform treatment was given repeatedly till the sample was subjected to further centrifugation. Samples were treated with sodium chloride and ethanol and then spun through precipitation, incubated at –20°C, and then spun again. The DNA was washed twice with 1 ml 70% ethanol before being dried and eluted in 50 µl of double distilled water, and then stored at –20°C. The amount of DNA obtained was measured using a NanoDrop 2000 spectrophotometer, and the reading obtained, which is OD260, was used as the quantification point. The DNA samples were kept at –20□ for PCR amplification, and reaction was carried out in a Thermal cycler (Applied Biosystems, USA). The sample was subjected to PCR using species-specific primers (*D. immitis*) as described by [9] and a general primer reported by [10] as shown in Table 1. In this case, the PCR reaction mixture (20 µl) was used according to the conditions mentioned above. After PCR amplification, the amplicon was analyzed through a 1.5% agarose gel and photographed using a Gel Documentation System from Biobase, USA. All amplified PCR products were purified by Gel Purification Kit (WizPrep, Korea Ref No: W70150-300).

Sequencing and phylogenetic analysis

Positive PCR products were dispatched to a recognized sequencing laboratory. The sequences obtained were analyzed with NCBI's BLAST to evaluate their similarity to recognized *D. immitis* species globally. Phylogenetic analysis was conducted utilizing MegaX software version 10. A phylogenetic tree was constructed using MEGA-X software [12] through maximum likelihood methods and 1000 bootstrap replications, illustrating the evolutionary relationships among study isolates and reported sequences.

X-ray/radiography characterization

X-ray/radiography is an essential imaging technique utilized in medical diagnostics. They enable visualization of internal structures, aiding in the identification of various conditions and guiding treatment decisions.

Radiography was carried out in the UVAS Pet Centre X-ray room for the detection of pulmonary and cardiac changes associated with heartworm infection in dogs. During the procedure, two views were acquired: dorsolateral and ventral or a 45° [13] angled view, as well as a left lateral view. The radiographic findings of right ventricular enlargement, dilated pulmonary artery or changes in interstitial or alveolar patterns were sought from the radiographs [14]. The result was compared with the other tests to determine the seriousness and the spread of heartworm disease among dogs.

Statistical analysis

Validation parameters of diagnostic methods:

sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), were calculated according to below formulas in three combinations:

Calculations for SNAP test, considering PCR as the gold standard

Calculations for microscopy, considering PCR as the gold standard

Calculations for microscopy, considering SNAP test as the gold standard

$$Specificity = \left[\frac{d}{b+d}\right] \times 100 \qquad Sensitivity = \left[\frac{a}{a+c}\right] \times 100$$

$$PPV = \left[\frac{a}{a+b}\right] \times 100 \qquad NPV = \left[\frac{d}{c+d}\right] \times 100$$

a (true positive), **b** (false positive), **c** (false negative), d (true negative)

Ethics statement

The animals were handled as per the guidelines of the Animal Ethical Review Committee (No. DR/621, dated 20-10-2022).

Results

Out of thirty dogs, one dog had higher clinical severity: significant decrease in physical activity and appetite refusal, shortness of breath, obviously swollen belly, and minimal desire to move as shown in the Figure 1. In the present research, the dog in Figure 1 was considered to undergo analysis, providing more details on it.

This illness is highly associated with a clinical indication, manifesting key symptoms such as fever, chills, vomiting, fatigue, headache, cough, sore throat, muscle aches, and joint pain all the time. The

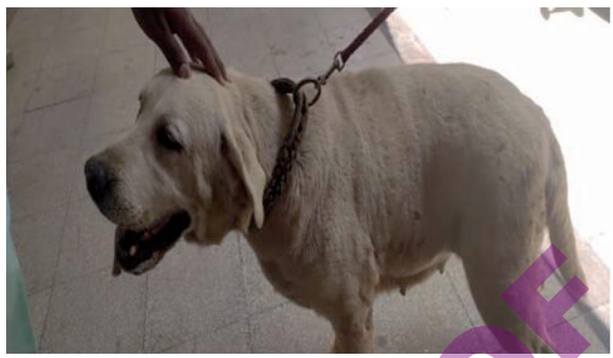

Figure 1. A dog showing signs of lethargy, labored breathing, distended abdomen and reluctance to movement/exercise was presented to the UVAS pet center, Lahore

dog presented to the UVAS Pet Centre has a history of lethargy, marked dyspnea, and mild ascites and was not willing to put on any physical activities.

Diagnostic tools

Out of the 30 dogs that were analyzed, 4 tested positive for *D. immitis* antigen using the SNAP® 4Dx® Plus Test, as can be seen in Figure 2. This test detects active heartworm infection from the presence of heartworm antigen in the sera of the dogs. The stark results coincide with observed clinical signs such as: lethargy, dyspnea, abdominal enlargement and reluctance to exercise, which clinically establish heartworm disease to warrant treatment.

Microscopic examination

Modified Knott's Test

Five samples had *D. immitis,* according to the results obtained by the modified Knott's method.

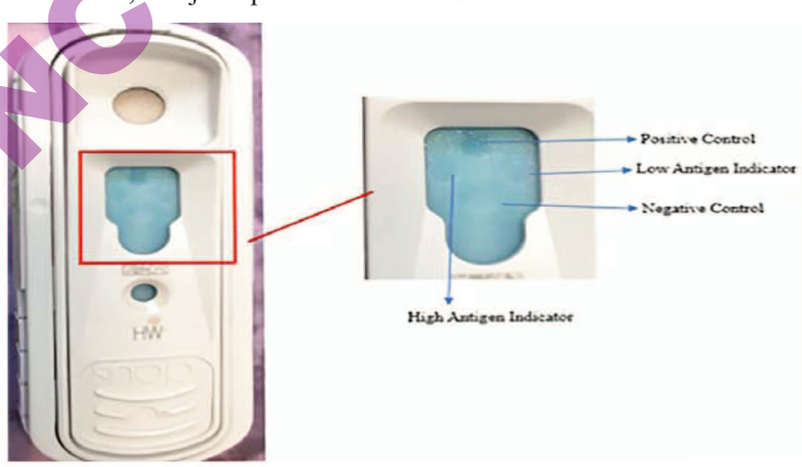

Figure 2. Positive SNAP® 4Dx® Plus Test result indicating D. immitis infection in a dog

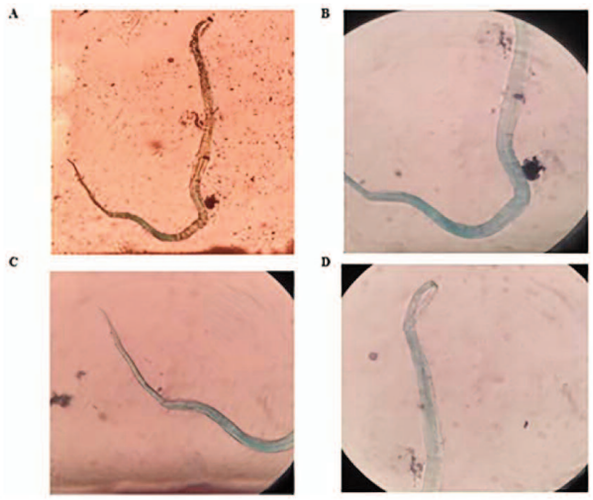

Figure 3. Microscopic image of microfilariae detected in a canine blood sample using Knott's method

The test confirmed the presence of microfilariae, characterized by a tubular body, tapered tail, and rounded head, aligning with the morphological characteristics of *D. immitis* as shown in Figure 3.

Wet mount

The plasma of the dog was processed via the wet mount technique and analyzed microscopically. This method demonstrated the presence of motile microfilariae, which are characterized by an elongated, undulating shape, resembling to *D. immitis* parasite, as can be seen in Figure 4.

Radiographic examination

Radiographic examination using oblique dorso-ventral and left lateral views revealed signs consistent with *D. immitis* infection, including right ventricular enlargement, prominent pulmonary arteries, and mild perihilar opacity. The findings

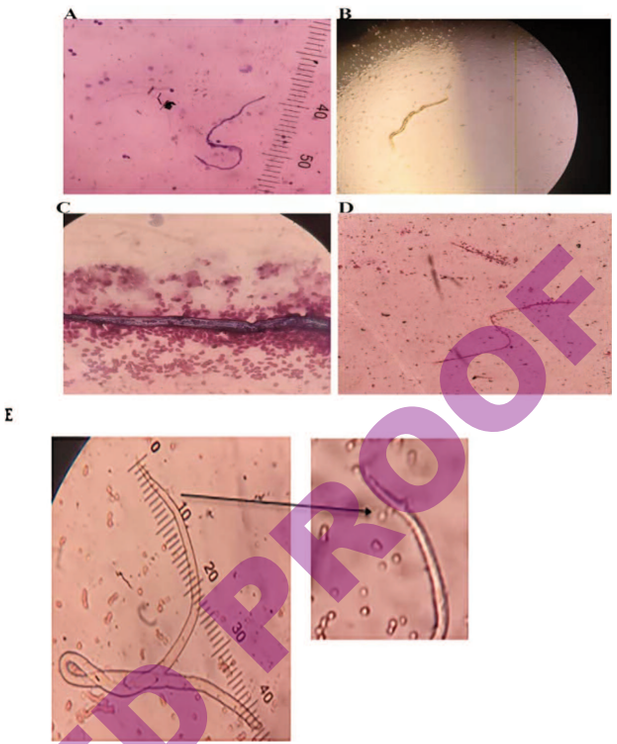

Figure 4. Wet mount images of D. immitis at 400× under an Olympus microscope. Images A–D show an adult worm with a tubular body, tapered tail and rounded head. Image E shows another male worm, the arrow shows a pre-anal supplementary organ (reproductive structure)

strongly support a positive diagnosis of heartworm disease, as shown in the Figure 5.

PCR analysis

This study analyzed 30 samples using PCR with general primers for the confirmation of *Dirofilaria* parasite and specific primers for the confirmation of the *D. immitis* parasite. The anticipated band sizes

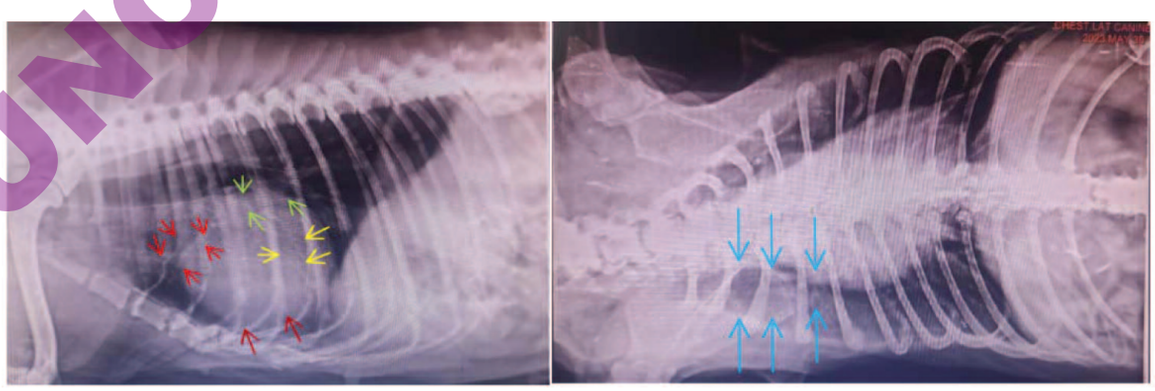

Figure 5. Radiographic examination using oblique dorso-ventral and left lateral views revealed signs consistent with D. immitis infection, including right ventricular enlargement, prominent pulmonary arteries, and mild perihilar opacity. The findings strongly support a positive diagnosis of heartworm disease

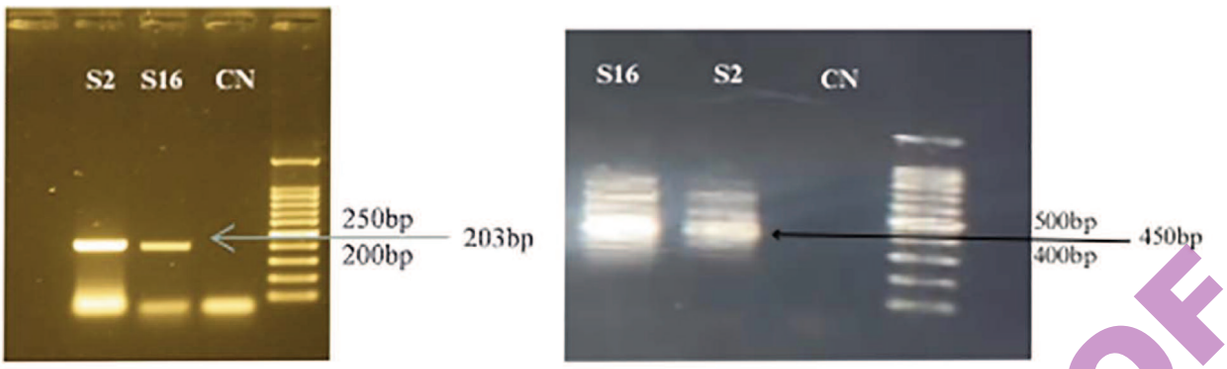

Figure 6. Shows results of heartworm infected female dog sample with specific primers (on left) of D. immitis, DNA Ladder 50bp (Cat # SM0373, Thermo Fisher), CN: control negative, S2: (Sample 2), S16: (Sample 16) and of general primer (on right) DNA Ladder 100bp (Cat # SM0373, Thermo Fisher), CN: control negative, S2: (Sample 2), S16: (Sample 16)

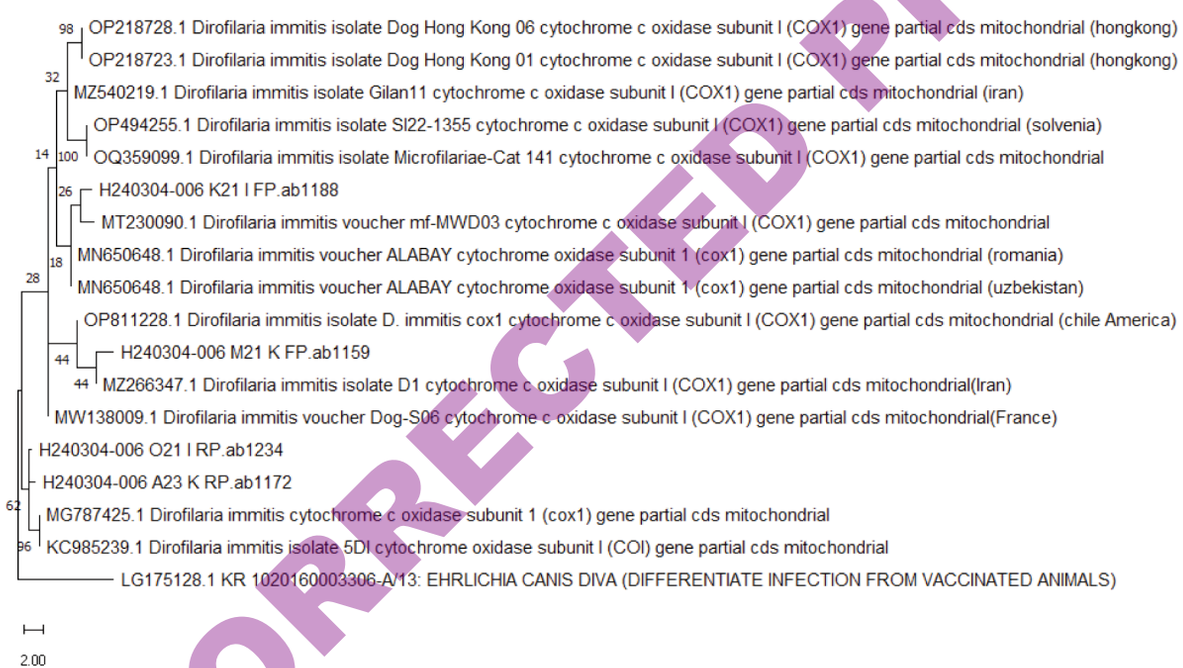

Figure 7. Phylogenetic study of D. immitis using 18S rRNA sequences. Mega 7.0 software was used for sequence alignment and phylogenetic sequence building, while the remaining sequences were obtained from the (NCBI) Genbank database

for the PCR products were 203 bp for the specific primers and 450 bp for the general primers. Based on the results shown by gel electrophoresis of the PCR products, 10% of the samples, specifically 3 out of 30, exhibited positive bands for both the specific and general primers as can be seen in Figure 6. This signifies the occurrence of *D. immitis* in these samples. The remaining 27 samples did not exhibit the anticipated bands, indicating either the absence of the pathogen or an infection level too low to be detected by these methods.

The Sanger sequencing method was used by [15] to sequence the PCR product. The chromatograms were analyzed with Chromas Pro software (version 1.7.4), and the sequence was compared to the GenBank database to assess nucleotide sequence homology. The data search utilized the BLAST algorithm on the NCBI network server. Alignment was performed using CLUSTAL W [16]. The alignment of the sequence was performed using Mega 7.0 software. A phylogenetic tree was constructed employing a maximum-likelihood method alongside a Tamura-Nei model, utilizing Mega 7.0 software. The PCR results were validated through sequencing. The BLAST analysis demonstrated significant similarity with the 18S

Table 1. General and specific primers for *D. immitis* in blood samples collected from suspected dogs, primer sequences, targeted genes, product sizes and PCR conditions for both general and specific primers

Primers	Primer's sequence	Target gene	PCR condition	Product size	Reference
D. immitis specific primers	DI-COI-F1 (5AGTGTAGAGGGTCA GCCTGAGTTA3') DI-COI-R1: (5ACAGGCACTGAC AATACCAAT3)	Cytochrome C Oxidase Subunit-1 (Cox1)	Initial denaturation 94ºC (2 min) (32 cycles) Denaturation (94ºC/30 sec) Annealing (63ºC/1 min) Extension (72ºC/30 sec) Final extension (72ºC/7 min)	203 bp	[11]
Dirofilaria general primers	**12SF:** (5GTTCCAGAATAATC GGCTA3 **12SR:** (5ATTGACGGATGA GTTTGTACC3)	12S rDNA	Initial denaturation 94ºC (2 min) (40 cycles) Denaturation (94ºC/45 sec) Annealing (50ºC/45 sec) Extension (72ºC/30 sec) Final extension (72ºC/90 sec)	About 450 bp	[10]

Table 2. Comparison of overall samples

	SNAP® 4Dx® Plus Test	Microscopy	PCR
Positive	4 (13.33%)	5 (16.67%)	3 (10%)
Negative	26 (86.67%)	25 (83.3%)	27 (90%)
Total	30	30	30

Table 3. Comparison of results of PCR and SNAP® 4Dx® Plus Test

	PCR positive	PCR negative	Predictive values
SNAP positive	(a) 3	(b) 1	PPV (75%)
SNAP negative	(c) 0	(d) 26	NPV (100%)
Sensitivity & specificity	Sensitivity (100%)	Specificity (96.29%)	

Table 4. Comparison of results of PCR and microscopy

	PCR positive	PCR negative	Predictive values
Microscopy positive	(a) 3	(b) 2	PPV (60%)
Microscopy negative	(c) 0	(d) 25	NPV (100%)
Sensitivity & specificity	Sensitivity (100%)	Specificity (92.59%)	

rRNA DNA of *D. immitis*, along with sequences from various global regions, as shown in Figure 7.

Table 5. Comparison of microscopy and SNAP® 4Dx® Plus Test

	SNAP positive	SNAP negative	Predictive evalues
Microscopy positive	(a) 4	(b) 1	PPV (13.79%)
Microscopy negative	(c) 0	(d) 25	NPV (100%)
Sensitivity & specificity	Sensitivity (100%)	Specificity (96.15%)	

The positive and negative results of microscopy, SNAP® 4Dx® Plus Test and PCR of overall samples are compared in Table 2.

The results of PCR and SNAP® 4Dx® Plus Test are compared in Table 3, keeping PCR as gold standard in this case.

The results of PCR and microscopy are compared in Table 4, keeping PCR as gold standard in this case.

The results of microscopy and SNAP® 4Dx® Plus Test are compared in Table 5, keeping SNAP® 4Dx® Plus Test as gold standard in this case.

Discussion

Dirofilariasis (heartworm disease), caused by *D. immitis*, is an increasingly significant parasitic infection that affects dogs, cats, and, in rare cases, humans. Several factors like rising environmental temperature, climate change and the movement of the infected animals aid in the spread of this disease over a wide range [18]. Moreover, lack of awareness about the disease and improper preventive strategies

also contribute to the increasing risk of the disease. Compared to *Dirofilaria repens*, *D. immitis* has a shorter transmission period, which allows it to spread more rapidly and cause severe health complications, such as pulmonary hypertension and right-sided heart failure [2], [19]. The risk of *D. immitis* infection increases particularly in warm and humid conditions, where mosquito vectors thrive, and in areas with a large population of stray dogs that serve as reservoirs for infection. Given these conditions, Southeast Asia is considered the most susceptible region for the continued emergence and persistence of this parasitic disease [20]. Regular screening, vector management and improved awareness among pet owners and veterinarians are important strategies for limiting and controlling the spread of dirofilariasis and mitigating its impact on both animal and human health.

This study is the first in Pakistan to assess the sero-occurrence and molecular detection of *D. immitis* in pet dogs in Lahore. The study included 30 blood samples from the dogs having a history of clinical signs like, cough, fatigue, labored breathing, weight loss, anemia, and abdominal swelling. Similar signs have been reported in previous studies [21], highlighting the importance of early recognition for timely diagnosis and treatment.

Microscopy, the SNAP® 4Dx® Plus Test, radiography, and PCR were used as diagnostic tools in this study, aligning with previous studies conducted by [22], that combined these techniques for accurate detection of *D. immitis* in canines.

The SNAP® 4Dx® Plus Test detected *Dirofilaria immitis* in 4 of 30 dogs (13.33%), highlighting its effectiveness in diagnosing heartworm infections. Previously, the study reported by [23], showed 99% sensitivity and 99.3% specificity of the SNAP® 4Dx® Plus Test. Since the test detects antigens from adult female heartworms, it may produce false negatives during the initial 5 to 8 months of infection or in all-male infections [24]. Compared to PCR, which is considered as the gold standard, the SNAP® 4Dx® Plus Test showed 100% sensitivity, 96.29% specificity, a 75% positive predictive value (PPV), and a 100% negative predictive value (NPV). The diagnostic accuracy of the SNAP® 4Dx® Plus Test is high; the slightly reduced specificity in this study could be influenced by differences in sample populations or testing environments.

The Modified Knott's Test remains a valuable diagnostic tool for detecting and differentiating microfilariae, especially when combined with other diagnostic methods. In this study, it successfully identified *D. immitis* in 5 out of 30 samples (16.67%), highlighting its reliability in diagnosing microfilariae based on morphological characteristics. However, its limitations must be acknowledged, as false negatives may arise in occult infections or when blood sample volume is insufficient, potentially leading to undetected microfilariae even in heartworm-positive animals. Researches conducted by [25] and [26] also supports these findings, emphasizing the test's effectiveness in distinguishing *D. immitis* from other filarial species. Despite these challenges, the Modified Knott's Test continues to be a useful technique for microfilariae identification, reinforcing its role in comprehensive heartworm diagnostics.

Microscopy proved highly effective in detecting positive cases, exhibiting 100% sensitivity, a specificity of 92.59%, a PPV of 60%, and an NPV of 100%, though it showed a higher rate of false positives compared to PCR. The wet mount method also provided clear visual confirmation of microfilariae and their morphological characteristics, reinforcing its role in diagnosing *D. immitis* infection. Its reliability was further supported by [22], who found strong agreement between wet mount findings and other diagnostic techniques. However, this method has certain drawbacks, including reduced sensitivity in cases with low microfilarial counts and the necessity for skilled personnel, particularly in non-endemic areas. Despite these limitations, both wet mount and microscopy remain valuable diagnostic tools, with their effectiveness improving when used alongside complementary testing methods.

Advanced cases of *D. immitis* infection have been effectively detected by thoracic radiography, revealing characteristic signs such as right ventricular enlargement and prominent pulmonary arteries. The role of thoracic radiography in assessing heartworm disease severity is supported by [27], while [28] emphasized its diagnostic limitations in early-stage infections. Since radiographic changes may not always be evident in the initial phases of the disease, relying solely on this method can be insufficient. Therefore, integrating radiography with other diagnostic approaches is essential for achieving a more comprehensive and accurate diagnosis.

PCR identified 10% of the tested samples as positive, demonstrating its reliability in detecting *D. immitis* infection. Both species-specific primers,

targeting a 203 bp region, and general primers, targeting a 450 bp region, produced positive bands, allowing for cross-validation and ensuring diagnostic accuracy. Among the 30 canine blood samples analyzed, three tested positive for *D. immitis* using species-specific primers, with the same samples also yielding positive results with general primers. This confirms the ability of general primers to amplify conserved filarial DNA regions across multiple species, including *D. immitis*, further emphasizing PCR's effectiveness in diagnosing infections. Similar findings were observed in studies on filarial species detection by [26] and [29], confirming PCR's superiority in diagnosing *D. immitis* infection accurately and distinguishing it from other filarial infections.

To achieve a comprehensive diagnosis of *D. immitis* infections, relying solely on PCR is not sufficient. While PCR is highly effective, integrating additional diagnostic tools such as the SNAP® 4Dx® Plus Test and microscopy enhances accuracy and minimizes the risk of false negatives. A comprehensive approach enhances accuracy, leading to more effective detection and evaluation of diarofilariasis.

The genetic diversity of *D. immitis* isolates from Hong Kong, Iran, Slovenia, and Pakistan was revealed through phylogenetic analysis of COX1 gene sequences. Distinct clustering of Pakistani isolates highlights the uniqueness of regional strains, shaped by local evolutionary pressures and transmission cycles. The effectiveness of molecular techniques in studying the parasite's evolutionary patterns and geographic distribution is reinforced by the use of both specific and general primers for *D. immitis* detection. These findings further validate COX1 as a reliable molecular marker for exploring genetic variations among isolates.

By utilizing a range of diagnostic tools with high sensitivity and specificity, this study provides valuable insights into *D. immitis* infection in dogs in Lahore, Pakistan. The integration of multiple diagnostic methods enhances detection accuracy, contributing to a more comprehensive understanding of the disease. These findings play a crucial role in guiding future prevention and treatment strategies, particularly in both endemic and emerging regions.

In conclusion, this research is the first in Pakistan to diagnose *D. immitis* infection in dogs using a combination of PCR, radiography, microscopy, and the SNAP® 4Dx® Plus Test, indicating the importance of a multifaceted diagnostic approach. Integrating these methods enhances the accuracy of dirofilariasis detection, facilitating early diagnosis and treatment. By setting a new standard for veterinary care, this study emphasizes the value of combining multiple diagnostic techniques to improve disease management in dogs.

Acknowledgements

I would express my sense of gratitude to all researchers who supported us in handling the experiment. The study was conducted as per the guidelines of the Animal Ethics Committee of UVAS, Lahore, Pakistan.

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Received 13 September 2025
Accepted 02 March 2026