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Prevalence and zoonotic implications of Dirofilariasis in shelter dogs in Gilan province, Iran

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Abstract

Dirofilariasis, caused by *Dirofilaria immitis* (*D. immitis*), is a zoonotic disease. Iran is an endemic country, and shelter dogs are particularly susceptible due to limited veterinary care, high-density housing, and increased exposure to mosquito vectors. This study aimed to determine the prevalence of *D. immitis* in shelter dogs in Gilan province, northern Iran, using a combined parasitological and molecular approach. A total of 271 blood samples were collected from shelter dogs across seven cities in Gilan province. All samples were initially screened for microfilariae using the modified Knott's test. Subsequently, they were analyzed by a species-specific PCR targeting the 155 bp partial sequence of the ITS1 gene of *D. immitis*, with the beta-actin gene amplified as an internal control. The modified Knott's test identified microfilariae in 32 dogs (11.80% prevalence), while PCR analysis detected *D. immitis* DNA in 143 dogs, corresponding to a significantly higher prevalence of 52.8%. Measurements taken with Axiovision software (v4.1) yielded average microfilarial dimensions of 301.89 µm in length (±13.351) and 6.392 µm in width (±0.5). This discrepancy highlights a substantial number of occult (amicrofilaremic) infections, and the hyper endemic status of *D. immitis* in shelter dogs in Gilan province. Within the shelter environment, the high-density housing of susceptible dogs creates an amplification hotspot. In Gilan province, the hyperendemicity is likely driven by environmental and entomological factors. These findings necessitate the development of integrated control strategies, including routine molecular screening, preventive chemotherapy, and formal shelter management protocols, to mitigate animal suffering and reduce zoonotic risk.

Introduction

Dirofilariasis, primarily caused by the filarial nematode *D. immitis* (heartworm), is a significant vector-borne zoonosis with a global distribution that is expanding into new regions, partly due to climate change (1). The disease poses a serious health threat to canines, affecting the cardiopulmonary system and leading to fatal

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complications such as heart failure and caval syndrome (2, 3), while in humans, it can cause pulmonary and ocular nodules that mimic malignancies (4, 5).

In endemic countries like Iran, where infection has been reported in 22 of 32 provinces, the disease cycle is maintained between canine reservoirs and mosquito vectors (6). The transmission dynamics are heavily influenced by the presence of competent mosquito vectors, which can include various species of *Aedes*, *Culex*, and *Anopheles* (7). Within this ecosystem, animal shelters represent critical hotspots for transmission. The high-density, confined populations of shelter dogs facilitate rapid parasite spread, presenting a pronounced challenge for veterinary management and posing a heightened- though often unquantified- zoonotic risk to surrounding communities (8).

While previous studies in Iran have established the country's endemicity, including in regions like Iranshahr (9), they have primarily focused on owned dogs or specific regional outbreaks. A significant gap remains in the systematic surveillance of shelter populations, which are likely to carry the highest disease burden. Furthermore, many existing studies rely on a single diagnostic method, which can be limiting. Microscopic techniques, such as the modified Knott's test, can miss low-grade or amicrofilaremic (occult) infections, where adult worms are present but microfilariae are not circulating (10, 11). Serological antigen tests, while valuable, can also yield false negatives in single-sex or immature worm infections (12).

Antigen testing serves as the primary and most accurate diagnostic method for detecting canine heartworm (*D. immitis*) infections, as recommended by international guidelines for its high sensitivity and specificity in identifying adult female worm antigens (12). In comparison, the modified Knott's test is less reliable, particularly for occult infections, low worm burdens, or cases involving only male worms, as it primarily detects microfilariae. Molecular techniques, such as PCR, provide excellent specificity for species identification and are useful in resolving ambiguous results, but are not ideal for routine screening and do not surpass antigen tests in detecting hidden infections (13). To address this gap, the present study provides the first comprehensive assessment of dirofilariasis in shelter dogs in Gilan province, a high-risk region in northern Iran, by integrating parasitological (modified Knott's test) and molecular (PCR) techniques. We hypothesized that the shelter environment would harbor a high prevalence of *D. immitis* and that molecular tools would reveal a burden of infection underestimated by microscopy alone. The management of animal shelters carries significant public health importance. In Gilan province, a region endemic and in some areas hyperendemic for canine *Dirofilaria immitis*, determining the prevalence of this infection in shelters is therefore a priority (6). Despite the necessity to monitor this parasite in shelter environments, no epidemiological studies have been conducted on the shelter dog population within the province, representing a critical gap in the literature. The aim of this study was **to determine** the baseline prevalence of dirofilariasis in this vulnerable, high-risk canine population.

Materials and Methods

Ethical Statement

All procedures involving animals were conducted in accordance with relevant guidelines and regulations. The study protocol was approved by the Ethics Committee of Vice Chancellor of research and technology in faculty of veterinary medicine at University of Tehran, (Reference No. IR.UT.REC.7502001/6/23).

Study Area and Sampling

This study was conducted in seven government-run dog shelters across Gilan province, Iran. Blood samples were collected from these animals between 15 April and 30 December 2024. The total dog population across these shelters averaged approximately 350 animals. The sample size was determined using the formula developed by Krejcie and Morgan (1970) to minimize sampling error (14). A total of 271 shelter dogs (aged ≥2 years) were systematically selected for the study. Dogs were sampled from each shelter proportionally to the

facility's population size, distributed across seven cities: Lakanshahr (n=80/95), Siahkal (n=36/41), Rasht (n=38/40), Talesh (n=32/33), Astara (n=19/20), Roudbar (n=19/21), and Anzali (n=47/50).

Canine blood samples (2-4 mL) were obtained from the cephalic vein following restraint and disinfection of the site. Two aliquots were collected per subject: a) One aliquot was placed in a tube pre-treated with 10% EDTA for general parasitological and molecular examination, and b) The second aliquot was added to a tube containing 9 mL of 2% formalin for the modified Knott's test for microfilariae.

Modified Knott's Test

Microfilariae were detected using the modified Knott's test. The blood sediment (about 1 mL) was stained with methylene blue (1%) and examined microscopically. Detected microfilariae were analyzed and measured with Axiovision software (version 4.1).

Polymerase chain reaction (PCR)

A polymerase chain reaction (PCR) assay was performed to amplify a fragment of the ITS1 gene. Genomic DNA was first extracted using a commercial MBST kit (MBST, Tehran, Iran), and its quality was assessed by horizontal gel electrophoresis. For this, a 1% agarose gel was prepared by dissolving 0.25 g of agarose in 25 mL of 0.5x TBE buffer. For electrophoresis, 8 µL of each DNA sample was mixed with 2 µl of loading buffer and loaded into the wells. Following electrophoresis, the gel was visualized and photographed using a micro DOC Compact Gel Documentation System with UV Transilluminator (UVT312, Merck KGaA, Darmstadt, Germany). The beta-actin gene was employed as an internal control in this study; a 300-bp fragment was amplified using the specific primers (beta-actin-F: 5′-ACCCACACGGTGCCCATCTA-3′; beta-actin-R: 5′-CGGAACCGCTCATTGCC-3′). For the target pathogen, PCR was carried out using species- specific primers for *D. immitis* (Forward: 5′-GCTTAATTGATGATGATTGC-3′; Reverse: 5′-CAAGTGATCCACCGCTAAGAGT-3′) synthesized by Sinaclon (Sinaclon, Tehran, Iran), which target a 155-bp fragment of the ITS1 gene (GenBank Accession: 964684.1). The thermocycling (Bio-Rad Laboratories, Inc., USA) protocol consisted of an initial denaturation at 95°C for 10 minuts, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 51.5°C for 45 seconds, and extension at 72°C for 45 seconds, with a final extension step at 72°C for 600 seconds. The positive control was DNA from a *D. immitis* specimen (obtained from the Iran Parasitology Museum), and its identity was verified by DNA sequencing.

Results

Among 271 shelter dogs from seven cities in Gilan province, the overall prevalence of microfilariae detected by the modified Knott's test was 11.8% (32: 271). Morphometric measurements of these microfilariae, performed with Axiovision software (v4.0), averaged 301.89 ± 13.351 µm in length and 6.392 ± 0.5 µm in width (Figure1). Confirmation of *D. immitis* as the causative agent was achieved through conventional PCR 52.8% (143:271), which successfully amplified a species-specific 155 bp fragment of the ITS1 gene in samples from three shelters (Figure 2) and (Table 1). The number and percentage of dogs positive for *D.immitis*, as identified by the modified Knott's test and PCR, are summarized in Table 1. The level of agreement between the two diagnostic techniques was assessed using Cohen's kappa. The resulting kappa value of <0.2 indicates poor agreement (Table1), reinforcing previous observations on the limited concordance between microscopy and molecular assays for canine dirofilariasis.

Fig. 1. (A) Microfilaria of *Dirofilaria immitis* observed in a peripheral blood smear from a shelter dog in Gilan Province, Iran, stained with 1% methylene blue. (B) The microfilaria was captured and measured using Axiovision software (version 4.1) via light microscopy at 400× magnification.

Table 1. Prevalence and agreement of *Dirofilaria immitis* detection methods in shelter dogs from Gilan province

Shelters Located	The number of samples	Number of positive samples			Cohens *Kappa*
		Knott test	Mean of *mf/mL (min, max)	PCR	Value of ***K**** ** *X^2, p-value and 95%CI*
Lakan Shahr	80	17(21.25%)	8.7 (1, 47)	53(38.4%)	
Rasht	36	0	0	10(27.7%)	< 0.20
Siahkal	38	0	0	20(52.6%)	χ^2=23.57
Talesh	32	3(9.37%)	14(4, 29)	15(46.9%)	*p-value*<0.05 95%CI (30.6-56.3)
Astara	19	2(10.5%)	14.5(11, 18)	10(52.6%)	
Roudbar	19	8(42.1%)	18.6(2, 95)	14(73.7%)	
Anzali	47	2(4.2%)	5(1, 9)	21(44.7%)	
Total	271	32(11.8%)	60.8(1, 95)	143(52.8%)	

*mf/mL: microfilaria count per milliliter of peripheral blood

**The agreement between diagnostic methods was quantified using Cohen's kappa coefficient, following Altman (1991). The kappa value was interpreted as follows: <0.20, poor; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, good; and 0.81-1.00, very good (15).

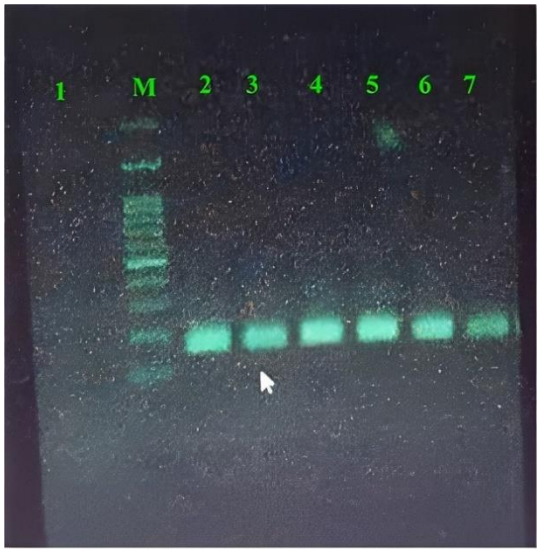

Fig. 2. Agarose gel electrophoresis (2.5%) of PCR products for *Dirofilaria immitis* detection. A specific 155 bp fragment from the ITS1 gene was amplified. Lanes from left to right: negative control (no-template DNA); 100 bp DNA marker (Sinaclon, Tehran, Iran); positive samples (Lanes 2-7) exhibiting the specific 155 bp amplicon. The gel was stained with DNASafe Stain (Sinaclon, Tehran, Iran).

Discussion

Heartworm disease, caused by the parasite *D. immitis*, is a significant zoonotic infection primarily affecting dogs (16). With a global distribution that is prominent in tropical and subtropical regions, its range is expanding (17). This spread into previously non-endemic areas is largely driven by factors such as climate change and the dispersal and emergence of mosquito vector populations (18). The purpose of this study was to determine the prevalence of *D. immitis* infection in dogs housed in shelters in Gilan province, since previously the status of dirofilariasis in shelter dogs of Iran has not been investigated. The risk of developing parasitic infections appears to be greater in shelter animals than pets because stray and shelter animals are less likely to have received veterinary care or preventive medications (19). Dogs in shelters are more exposed to mosquitoes carrying microfilariae and, as a result, are more at risk of contracting dirofilariasis due to their crowded living conditions and being outdoors (19).

This study focused on dogs in municipal shelters, as these facilities often face greater challenges in maintaining animal welfare and health compared to private shelters, potentially increasing disease susceptibility, a common issue in resource-scarce environments. The prevalence of *D. immitis* infection among 271 shelter dogs across seven cities in Gilan province was 52.8%, as determined by combined Knott's and molecular methods. An average of 18.6 microfilariae of *Dirofilaria immitis* per milliliter of peripheral blood, with maximum 95 mf/mL, in shelter dogs in Roudbar, compared to a significantly lower average of 5 microfilariae per milliliter in Anzali shelter, with maximum 9 mf/mL, using the modified Knott's test, indicate this difference can be attributed to risk factors such as the population of dogs in each shelter, shelter management practices (e.g., major risk factors: location in high-risk zones and the use of local dogs without preventive treatment), and also geographic location (e.g., coastal or rural areas with high densities of vector mosquitoes like *Anopheles* or *Culex*). In similar studies, higher prevalence has been observed in shelters in southern Italy (up to 17% overall, with a focus on *D. immitis* at 11.4%) (6) and Portugal (15.1%, with higher rates in coastal areas such as Setúbal at 24.8%), correlating with higher microfilarial loads, while lower rates (such as 5-7% in Larissa or Attica, Greece) have been reported in

central or northern regions (17). This high rate is consistent with the province's status as a hyperendemic region. Previous studies in Gilan have reported a wide range of prevalence rates in dogs, depending on the diagnostic method: up to 15.2% by microscopic blood examination, 60.9% by necropsy, and 78.6% by molecular survey (20). The American Heartworm Society recommends parasitological methods like the modified Knott's test and serological antigen tests (e.g., ELISA) for diagnosing dirofilariasis (12). While the Knott's test is highly specific for microfilariae, its accuracy depends on the technician's skill (13). Our findings reveal variable infection rates across shelters, indicating that localized transmission is driven by specific environmental and management factors, necessitating tailored, shelter-specific control strategies. Epidemiologically, this research advances theoretical understanding by refining the disease profile in an understudied and high-risk subpopulation, providing critical data that underscores the severity of the issue in shelter environments. The implications of these findings extend beyond veterinary medicine. Given the confirmed zoonotic potential of dirofilariasis, which can lead to serious pulmonary and other systemic presentations in humans (21), this work reinforces the necessity of a One Health approach. It stresses that controlling the disease in animal reservoirs, such as shelter dog populations, has direct benefits for public health by reducing the risk of human transmission. Monitoring infection rates in various environments is vital for both veterinary and public health (22). Shelters with high infection rates act as significant reservoirs for disease spread, elevating the risk of human transmission in endemic areas, especially for nearby communities. This underscores the need for public education and emphasizes the critical role of veterinarians in promoting prevention, accurate diagnosis, and prophylaxis (22). In low-prevalence areas, implementing diagnostic testing, preventive treatments, mosquito management, and population control of infected dogs is key to preventing the establishment of an endemic cycle (23).

The study emphasizes the need for targeted shelter interventions, including mosquito control, preventive medication, and routine PCR screening to curb heartworm transmission. However, it does not establish specific correlations between management practices and infection rates, highlighting the need for future research that quantitatively analyzes shelter protocols to determine the most effective measures.

Conclusion

While this study identifies canine shelters as key epidemiological reservoirs for dirofilariasis, supporting a One Health approach, it acknowledges limitations including the lack of established causal links to management practices and potential sampling variability. These findings nonetheless underscore the urgent need for standardized control protocols in endemic regions.

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Conflict of Interest

No conflicts of interest are reported between the authors.

Ethical Approval

This study was conducted in strict accordance with all relevant ethical guidelines, animal welfare protocols, and data confidentiality standards established by the Animal Research Ethics Committee of the Faculty of Veterinary Medicine at the University of Tehran, under approval number IR.UT.REC.7502001/6/23.

Artificial Intelligence Statement

In the course of preparing this manuscript, the authors utilized the GPT-4o AI model exclusively as a tool for linguistic refinement and grammatical correction. All research data, intellectual contributions, and scientific

conclusions are the sole responsibility of the authors. We affirm that no AI-generated content was used for any factual or analytical components of this work.

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