Original Article

Detecting and Identifying *Wolbachia pipientis* in Occult Dirofilariasis Using a High-resolution Melting Real-time PCR in Stray Dogs

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ABSTRACT

Background: Iran is one of the endemic areas of *Dirofilaria immitis*. The modified Knott test is the common diagnostic test based on detecting and identifying microfilaria in blood samples. Still, the results may be compromised by *D. immitis* occult infection (*D. immitis* infection without microfilariae). *Wolbachia pipientis* in a dog's blood is an endosymbiont of *D. immitis* and an indicator of dirofilariasis.

Objectives: This study aimed to investigate the occult infection of *D. immitis* in stray dogs in Guilan, Qazvin, and Mazandaran provinces, Iran, based on *W. pipientis* DNA tracking.

Methods: Blood samples (n=138) were collected, and the presence of *W. pipientis* was evaluated by amplifying a partial sequence of the *FtsZ* gene (267 bp).

Results: The results showed that 17.4% of the samples were positive by the modified Knott method, while 47.8% were positive by the molecular methods. The results of high-resolution melting (HRM) real-time PCR showed that the melting temperature of *cox1* gene amplicons of *D. immitis* and *Acanthocheilonema reconditum* were obtained at 76.67±0.03 ˚C and 81.38±0.05 ˚C, respectively as well as it was achieved 80.30±0.04 ˚C for *FtsZ* gene of *W. pipientis*. The results of the present study showed that it is necessary to use molecular methods to diagnose dirofilariasis occult infection accurately.

Conclusion: The HRM real-time PCR analysis is a simple post-PCR step that exploits the thermal characteristics of the amplicons to detect the occult infection of dirofilariasis based on *W. pipientis* DNA tracking and identification.

Keywords: *Acanthocheilonema reconditum*, *Dirofilaria immitis*, High-resolution melting (HRM) real-time PCR, Nematodes, Stray dogs

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Introduction

Animals in healthcare facilities, including dogs, can be sources of zoonotic pathogens that could infect humans (Ola-Fadunsin et al., 2023) Filariasis is a common term for diseases caused by parasitic nematodes belonging to the superfamily Filarioidea. These parasites are transmitted through blood-sucking insects and adult worms typically live in the host's blood, lymphatic vessels and connective tissues. Different species of Culicidae mosquitoes belonging to the genera *Culex*, Anopheles and Aedes play a role in transmitting filariasis in dogs in Iran. The most commonly reported species from dogs in Iran are *Dirofilaria immitis*, *Dirofilaria repens* and *Acanthocheilonema reconditum*. Adult *D. immitis* worms are located in the pulmonary arteries and the right ventricle of the dog, causing congestive heart failure in severe cases.

In some cases, heartworm disease may not present with clinical signs and is often diagnosed after the animal's death, which is not recorded due to the lack of accurate reporting systems. This condition leads to inaccurate epidemiology statements of the disease. Correct diagnostic methods with acceptable sensitivity and specificity can help correct the data related to the disease (Hosseini et al., 2022). A postmortem examination is essential in diagnosing and finding the cause of animal death. The purpose of investigating and analyzing the disease after death is to confirm the suspected diagnosis and clarify the conditions that led to the death of the animal. The imaging methods used in veterinary medicine after death are called VetVirtopsy (Molazem et al., 2022).

Some mature parasites are present without circulating microfilariae and have been identified as occult infections. The accurate determination of the prevalence of dirofilariasis is subject to occult infection detection. The frequency of other filariasis has been reported between 10.16% and 63.3%, including *A. reconditum*, *Dipetalonema evansi*, *Onchocerca cervicalis* in horses and *Onchocerca fasciata* in camels.

D. immitis and *D. repens* are parasitic nematodes that can infect humans globally and are commonly found in Mediterranean countries of Europe. These species have also been reported in Central and Eastern European countries. A notable aspect of these nematodes is their endosymbiont, *Wolbachia pipientis*, which affects the parasites' fertility and survival while contributing to their pathogenicity.

W. pipientis may be applied to diagnose nematode parasites. Antimicrobial therapy against *Wolbachia* spp. may effectively treat heartworm disease in dogs. Serological diagnostic tests based on the detection of antibodies against *Wolbachia* or female nematode antigens are conventionally used. Various PCR methods have been developed to detect *W. pipientis* in the blood of infected dogs. The present study aimed to evaluate the diagnosis of *D. immitis* based on *W. pipientis FtsZ* gene tracking by the high-resolution melting (HRM)-real-time PCR and compare it with the conventional PCR method.

Materials and Methods

Samples

Blood samples (2-3 mL) were collected from 138 stray dogs in Iran's Guilan, Mazandaran, and Qazvin provinces. The sampling method was random, with a predefined prevalence estimation of 10% and a 95% confidence interval (CI) with a 5% margin of error. The samples were treated with 10% sodium-ethylenediaminetetraacetic acid anticoagulant and tested for microfilariae using the modified Knott's test. A male worm of *D. immitis* retrieved from a dog in Guilan Province was used as a positive control. All samples were preserved in 95% ethanol until further analysis.

Extraction of blood DNA

Genomic DNA was extracted from 500 µL of each blood sample using a commercial kit (MBST, Tehran, Iran) following the manufacturer's instructions. The DNA was quantified and qualified using a Thermo Scientific™ NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1.00% agarose gel electrophoresis (Fanavaran Akhtarian, Iran). Before extraction, the blood samples were evaporated at room temperature with an open door until the ethanol completely evaporated.

High-resolution melting real-time PCR

Real-time PCR was performed using a thermocycler (Rotor-Gene Q; Qiagen, Hilden, Germany) and SYTO 9 dye (Thermo Fisher Scientific, USA). The method was optimized and set up based on the methodology of Borhani et al. (2017). The specific primers amplified a 267-bp fragment of the *FtsZ* gene of *W. pipientis* in dogs blood samples, Wol1-Forward-5'-CCTGTACTATATCCAAGAATTACTG-3', Wol1-Reverse-5'- ACTATCCTTTATATGTTCCATAATTTC-3' (Turba et al., 2012). The cytochrome oxidase subunit 1 (*cox1*) gene

of *D. immitis* was amplified from blood samples of dogs using specific primers designed to target a 256-bp fragment. The primers used were: Forward primer: dirHRM-forward: 5'-AGTATGTTTGTTTGAACTTC-3', reverse primer: dirHRM-Reverse: 5'-AACGATCCT-TATCAGTCAA-3' (Albonico et al., 2014). Additionally, primers specific to *A. reconditum* were employed to amplify a 200-bp fragment of the *cox1* gene: Forward primer: ARCOI-Forward: 5'CCAAAACTGGAA-CAGACAAAACAA-3', reverse primer: AgTgTT-gAgggACAgCCAgAATTg: 5'-3' (Rojas et al., 2015).

The PCR amplification was performed in a 20 µL reaction mixture containing 4.0 µL extracted DNA, 2 µM of each primer, 10 µL Master mix (Amplicon, USA), 4.4 µL of double-distilled water, and 0.6 µl of SYTO 9 dye (Thermo Fisher Scientific, USA).

The amplification of the partial sequences of the genes was performed with an initial denaturation at 95.00 ˚C for 240 s followed by 45 cycles of denaturation at 95.00 ˚C for 5 s, annealing at 58 ˚C for 15 s for Wol1 and dirHRM primers as well as 58 ˚C for 15 s for ARCOI primers, extension at 72.00 ˚C for 10 s, and final extension at 72.00 ˚C for 240 s. After real-time PCR, all PCR products were submitted to high-resolution melting analysis (HRM) in a Rotor-Gene 6000 (Qiagen, Hilden, Germany) with the melting profile from 70 ˚C to 85 ˚C using a ramping degree of 0.2 ˚C/s. All samples were tested in duplicates, and their melting profiles were tested using Rotor-Gene software, version 1.7.27 and the HRM algorithm provided.

Conventional polymerase chain reaction (cPCR)

This study used cPCR to detect *W. pipientis* DNA, amplifying a 267-bp length with the same primers as real-time PCR. To determine *D. immitis* and *A. reconditum*, a primer pair was used for the mitochondrial cytochrome C subunit (*cox1*), amplifying a PCR product of 256 bp for *D. immitis* and 200 bp for *A. reconditum*. The thermocycler (Bio-Rad, USA) program for cPCR procedures included predenaturation at 94.00 ˚C for 240 s followed by 40 cycles of denaturation at 94.00 ˚C for 25 s, annealing at 57.5 ˚C for 30 s for *W. pipientis* and *D. immitis*, extension at 72.00 ˚C for 40 s, and final extension at 72.00 ˚C for 420 s.

Reconditum *cox1* amplification was performed based on the thermocycler (Bio-Rad, USA) program prepared for *W. pipientis* and *D. immitis*, except for the annealing temperature, which was 58.5 ˚C for *A. reconditum*. In this study, β-actin-specific primers were applied to amplify a highly conserved 300 bp fragment of dog β-actin gene as an internal control, including β-actin-Forward-5´-ACCCACACGGTGCCCATCTA-3´ and β-actin-Reverse-5'-CGGAACCGCTCATTGCC-3´ primers.

The cPCR programs for β-actin primers were the same as those for *FtsZ* and *cox1* genes, except for the annealing temperature, which was 55 ˚C. PCR products were visualized using 1.5% agarose gel electrophoresis stained with safe-red and the microDOC-Compact Gel Documentation System with UV Transilluminator. Positive and negative controls were used, with extracted DNA from *D. immitis*, an adult male, as a positive control and samples with no-template DNA as a negative control for all reactions.

Sequencing

PCR products were purified using a quick PCR products purification kit (MBST, Iran) and sent for sequencing (Bioneer, Korea). The sequence chromatograms were analyzed using Chromas software, versions 2.4.1 and 3.1. A phylogenetic tree was constructed using the Chromas platform software, version 3.1.

Statistical analyses

For proportions, 95% CIs were found. Cohen's kappa (κ) was calculated to compare the agreement between the modified Knott test and cPCR and HRM-real-time PCR assays. The chi-square test was used to compare proportions, with a $P \leq 0.05$ regarded as statistically significant. Analyses were performed using SPSS software, version 20.

Results

The results of the present study showed out of the 138 samples, 17.4% (n=24; 17.4%, 95% CI, 15.69%, 19.10%) were positive by the modified Knott test, while 47.8% (n=66; 47.8%, 95% CI, 43.11%, 52.48%) were positive with the molecular methods. The frequency of infection in the three provinces of Guilan, Mazandaran and Qazvin is also provided in Table 1. The sample donated from the Iran Parasitology Museum was a thin male *D. immitis* 18 cm long, with a white tail with the typical spiral seen in Figure 1. The measuring results of microfilaria using AxioVision® software, version 4.1 (Zeiss, Göttingen, Germany) illustrated that the length and width of *D. immitis* microfilariae occurred 295.13±14.9×5.8±0.43 µm and *A. reconditum* microfilariae was 228±12.1×5.8±0.43 µm, that computed using the digital camera (IDS uEye UI-2250SE USB 2.0 camera, Obersulm, Germany) under

40× magnification (Figure 2). The results of the melting curve analysis of *W. pipientis FtsZ* gene amplicons shown in Figure 3 are the outcome of some samples assessed using HRM real-time PCR.

HRM real-time PCR results

Table 1 presents the frequency of infected dogs in three provinces, Guilan, Mazandaran and Qazvin, using HRM-real-time PCR.

Discussion

W. pipientis, as an intracellular endosymbiont bacteria of *D. immitis*, plays an essential role in the pathogenesis, treatment, and diagnosis of dirofilariasis infection. In the present study, dirofilariasis was diagnosed through *W. pipientis* and *D. immitis* DNA was found in all samples that had presented positive results with *W. pipientis.* Meanwhile, the modified Knott's test presented the following results: Guilan 38.7%, Mazandaran 16.1%, and Qazvin 2.1%. The sensitivity of Knott's test was significantly lower than molecular methods for *D. immitis* diagnosis (κ=0.3, the chi-square test P≤0.05). Cohen's kappa showed poor agreement between two tests, modified Knott's test and molecular methods, for both *D. immitis* (κ=0.3, chi-square test P≤0.05) and *A. reconditum* (κ=0.5, the chi-square test P≤0.05).

The results of conventional PCR and HRM-real-time PCR showed that 47.8% (66:138) of dog blood samples were infected. Furthermore, the rates of infection in Guilan, Mazandaran and Qazvin provinces were 61.3% (95% CI, 55.29%, 67.30%), 52.9% (95% CI, 47.71%, 58.08%) and 28.2% (95% CI, 25.26%, 31.14%), respectively. Also, the results of *A. reconditum* illustrated 3.2% (95% CI, 2.88%, 3.5%) in Guilan and 4.4% in Mazandaran (95% CI, 3.96%, 4.83%) in many endemic regions, including Iran (Hosseini et al., 2022) information about the distribution of those nematodes is scant in several regions. Therefore, we investigated the prevalence of these filarial parasites in stray dogs from five Iranian provinces where no information about these parasites is available. Methods: Blood samples were collected from 344 stray dogs in five provinces of Iran (i.e. Mazandaran, Guilan, Esfahan, Qazvin and Loresan. The study collected whole blood samples from dogs in different counties in East Azerbaijan Province. The results indicated an infection prevalence of 37.5% with microscopic evaluation and 45.8% with PCR test. The most common filarial parasite identified was *D. immitis*, followed by *Acanthocheilonema* species (Razmaraii et al., 2013).

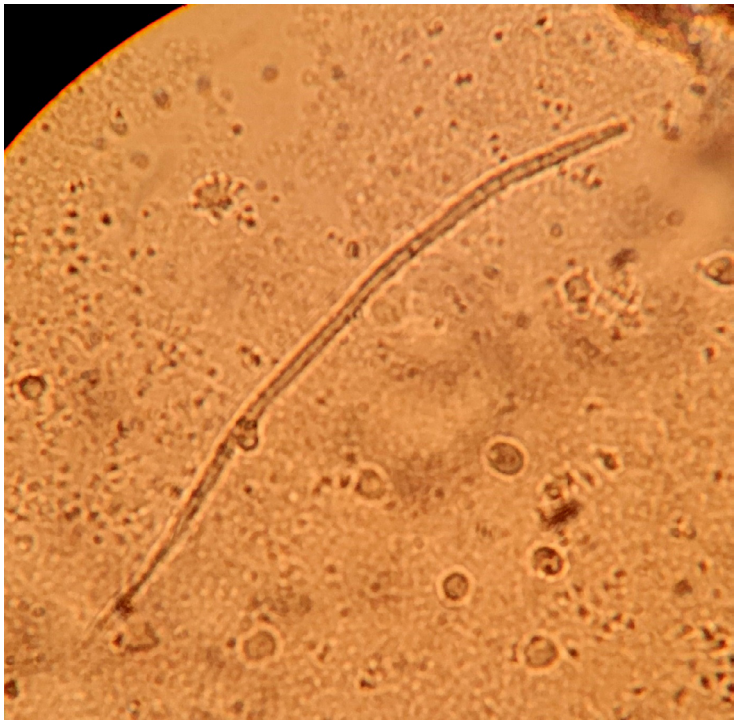

Figure 1. The microfilariae of *D. immitis*, in dog blood in Guilan Province through the use of the modified knott's test (seen under a light microscope at x40 magnification)

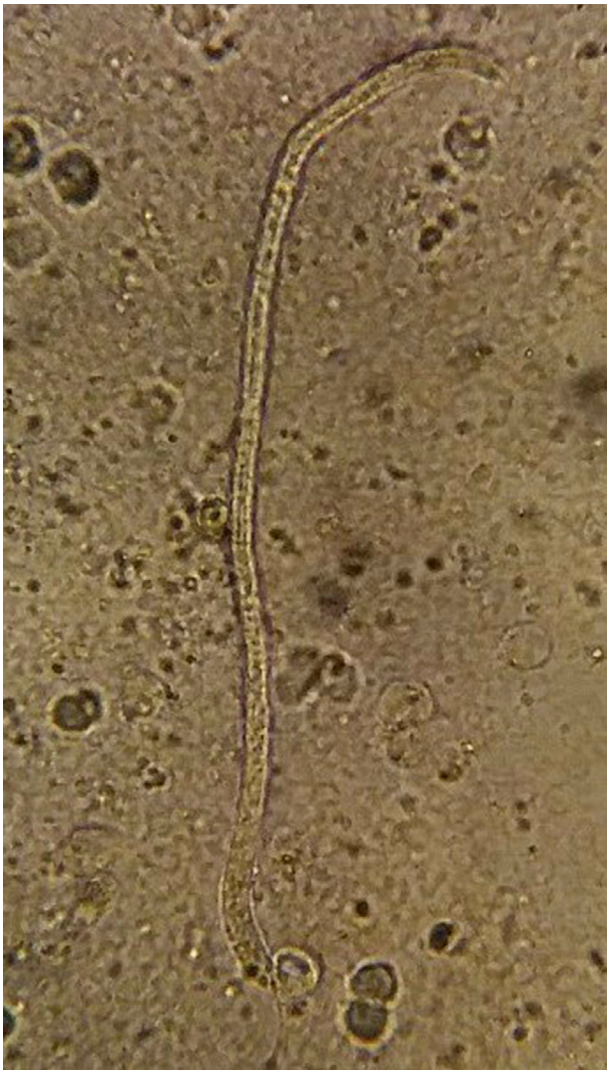

Figure 2. The microfilariae of *A. reconditum* in dog blood from Guilan Province characterized by distinct features, elongated form, and hook-like structure at the tail (light microscope at x40 magnification)

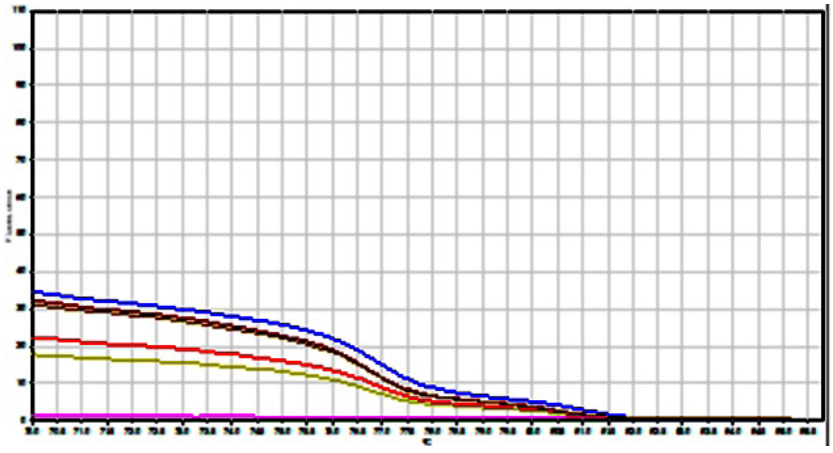

Figure 3. The melting curves of *W. pipientis FtsZ* gene amplicons (267 bp) from DNA extracted from infected dogs' blood using the rotor-gene (Qiagen, Hilden, Germany) real-time PCR instrument

Note: The blue, dark and light brown, as well as yellow-green curves, represent DNA samples from infected dogs' blood with melting temperatures (Tm) of 80.34 ˚C, 80.26 ˚C and 80.3 ˚C, respectively. The red curve represents the positive control, with a Tm of 80.29 ˚C, while the pink straight line represents the negative control, which involved the sample without template DNA.

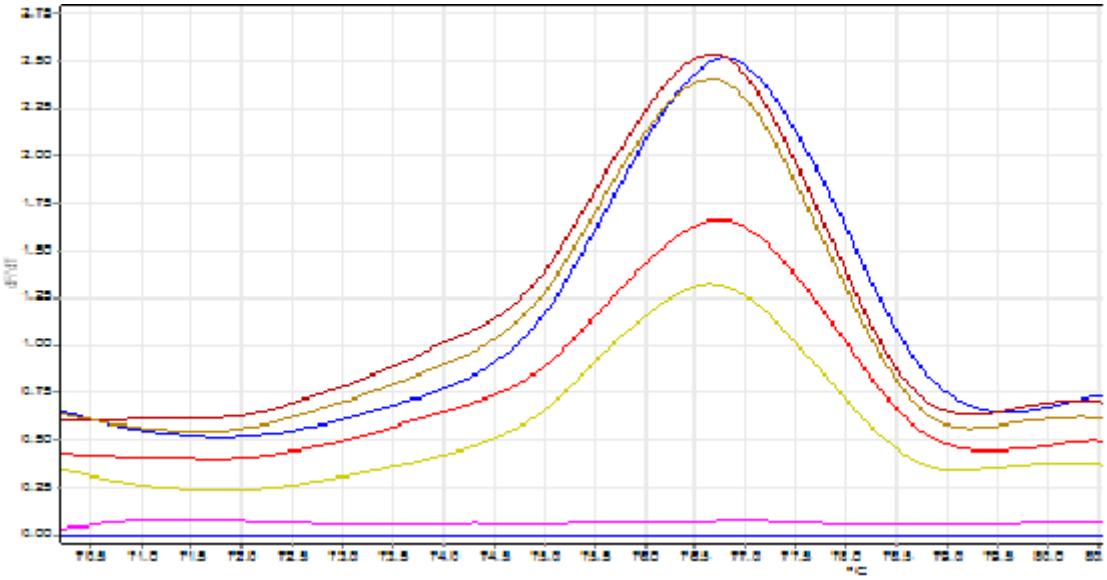

Figure 4. The ΔF/ΔT vs temperature (˚C) of DNA amplicons from infected dogs' blood, obtained using the Rotor-Gene (Qiagen, Hilden, Germany) real-time PCR instrument

Notes: The melting curves of *W. pipientis FtsZ* gene PCR products (267 bp) are depicted, with a mean melting temperature (Tm) of 80.30±0.04 ˚C. The red curve represents the positive control, exhibiting a melting temperature (Tm) of 80.29 ˚C. The straight pink line corresponds to the negative control, which involves the sample without template DNA.

The modified Knott test has been used as one of the most common methods for diagnosing dirofilariasis in dogs. It is a recommended test for detecting microfilariae in peripheral blood or other body fluids as a concentrated method with relatively rapidity and cost-effectiveness. However, the challenge is still faced when the samples have a low number of microfilaremia and samples reveal an occult infection (Hoseini et al., 2020). Other assays, such as molecular methods, have been developed for dirofilariasis diagnosis and can be used for epidemiologic investigations in endemic areas; moreover, accurate recognition of dirofilariasis is demanded in occult infection (Mathison et al., 2019).

Occult infection is one of the issues of dirofilariasis diagnosis; in occult dirofilariasis, despite helminths in the dog pulmonary artery, microfilariae are not released in the peripheral blood. The following are the most important reasons for occult infection. In the presence of male worms without females, the host's immune system responds to adults during the first six months after infected mosquito bites and after treatment with antiparasitic drugs (Wysmołek et al., 2022). In the present study, stray dogs have not been administered any anthelmintic. A molecular detection based on *W. pipientis* DNA tracking is more suitable than *D. immitis* DNA since *W. pipientis* delivered enormously either by adult worms or microfilariae (Panarese et al., 2020). In this issue, *Wolbachia* was recognized in 52.6% of blood samples with occult infection, and no microfilaria could be detected in the microscopic examination of blood samples (Landum et al., 2014). ELISA and SNAP heartworm RT tests are the preferred detection methods for canine heartworm diagnosis (*D. immitis* causes a disease) due to their simplicity and efficiency. However, these kits are not available in Iran and are expensive since they are imported.

Table 1. Percentage and 95% confidence interval of infected cases by city

Province	No. Samples	No. (%), 95% CI	
		Positive Samples Infected	
		W. pipientis* and *D. immitis	***A. recoditum***
Guilan	31	19(61.3), 55.29-67.30	1(3.2), 2.88-3.5
Mazandaran	68	36(52.9), 47.71-58.08	3(4.4), 3.96-4.83
Qazvin	39	11(28.2), 25.26-31.14	0

While *W. pipientis* and *D. immitis* genes become available in GenBank, their specific primers can be designed to be extended in HRM-real-time PCR. In this study, an HRM-real-time PCR was designed to detect *W. pipientis*. If HRM curve analysis is added to the real-time PCR, it will be possible to differentiate *W. pipientis*.

In the present study, 47.8% of samples suffered from infection with *W. pipientis* and *D. immitis*, while the modified Knott test did not show microfilariae in 30.4% of samples. Microfilaria was reported in the 17.4% of blood samples. In 30.4% of the samples, microfilariae were not observed, probably due to the occult infection.

The results of investigating *D. immitis* in Meshkinshahr, northwest Iran, showed 62.8% infection in dogs using molecular characterization based on the cytochrome oxidase subunit 1 (*cox1*) gene and serological method. The partial DNA sequencing of the *cox1* gene of adult parasites showed considerable homology and proximity to the previously isolated from Meshkinshahr (Khanmohammadi et al., 2020).

In cats, a massive release of *Wolbachia* occurs during 90 days after entering L1 microfilaria and during L5 migration, then remained in host cells after the disappearance of the adult nematodes as well as other sources than *D. immitis* could have released *Wolbachia* (Turba et al., 2012). *W. pipientis* detection could reflect dirofilariasis infection, which was the principal hypothesis of this study and was verified (Turba et al., 2012).

Indeed, the other sources of *Wolbachia* organisms should be further investigated; it was noteworthy that its most common cases encouraged *D. repens* to cause cutaneous dirofilariasis. The HRM-real-time PCR could distinguish *W. pipientis*. Wherever the HRM-real-time PCR is available, this technique is preferred due to the high sensitivity and specificity achieved and the time saved in getting results. The HRM-real-time PCR technique is rapid and has a reliable melting temperature. Real-time RT PCR is a powerful tool that can update existing knowledge on the phenotypic and genetic relationships and diversity of the new targeted gene of the IBV strains. Infectious bronchitis virus (IBV) is a highly diverse RNA virus that causes significant economic losses in the global poultry industry (Hajijafari Anaraki et al., 2022).

The conventional PCR and the HRM-real-time PCR were used for assessing the detection of both filarial and *Wolbachia* DNA in stray dogs' genomic DNA isolated from 138 blood samples of dogs in Guilan, Mazandaran, and Qazvin provinces in Iran. It was found that molecular methods detected more infected dogs than modified Knott's test (i.e. 66 vs 24 for *D. immitis*); this difference was statistically significant (χ^2=77.7, df=3, P=0.001).

Occult infection can be caused by low parasitemia in the first six months after mosquito bites or the presence of only adult male worms alone (Panarese et al., 2020). In the present study, conventional and HRM-real-time PCR techniques were designed to amplify the partial sequence of *FtsZ*, the gene of *W. pipientis* endosymbiont of *D. immitis*. In Iran, molecular methods are more cost-effective than serological tests. However, samples infected with *A. reconditum* showed negative results with *W. pipientis*.

Conclusion

In conclusion, this study emphasizes the necessity of integrating molecular diagnostic techniques into routine screening for *D. immitis* in endemic regions to ensure accurate detection of both symptomatic and occult infections. The findings advocate for improved diagnostic protocols that can better inform treatment strategies and control measures, ultimately contributing to better management of canine dirofilariasis in Iran.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualizations: Fateme Manshori, and Seyed Hossein Hosseini; Laboratory tests: Minoo Soltani; Field works and initial draft preparation: Fateme Jalousian; Review and editing: Parviz Shayan; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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مطالعه پژوهشی

تشخیص و شناسایی وولباشیا پایپینتیس در عفونت مخفی دیروفیلاریازیس با استفاده از روش HRM Real-time PCR در سگ‌های بی‌صاحب

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چکیده

زمینه مطالعه: بررسی آلودگی هایانگلی سگ‌های ولگرد باتوجه‌به اهمیت بیماری‌های مشترک قابل‌انتقال از سگ به انسان حائز اهمیت بسیار است و می‌تواند تهدیدی برای بهداشت جامعه باشد. ایران یکی از مناطق بومی دیروفیلاریا ایمیتیس است. تست نات اصلاح‌شده، رایج‌ترین آزمایش برای تشخیص فیلاریازیس است که اساس آن مشاهده و شناسایی میکروفیلرها در نمونه‌های خون است، اما نتایج این آزمایش در موارد عفونت مخفی به‌طورکاذب منفی می‌شود. وولباشیا پایپینتیس به‌عنوان هم‌زیست اجباری دیروفیلاریا ایمیتیس، نشان‌دهنده دیروفیلاریازیس در خون سگ است.

هدف: مطالعه حاضر با هدف بررسی عفونت مخفی دیروفیلاریا ایمیتیس در سگ‌های ولگرد استان‌های گیلان، قزوین و مازندران براساس ردیابی دنای وولباشیا پایپینتیس با استفاده از روش ذوب با وضوح بالا Real-Time PCR (HRM) انجام شد.

روش کار: تعداد ۱۳۸ نمونه خون از سگ‌های ولگرد در استان‌های گیلان، مازندران و قزوین جمع‌آوری شد که با دو روش نات اصلاح‌شده و مولکولی ارزیابی شدند. حضور وولباشیا پایپینتیس با تکثیر بخشی از ژن ftsz به طول ۲۶۷جفت‌باز بررسی شد.

نتایج: مطابق یافته‌های این مطالعه، ۱۷/۴ درصد از نمونه‌ها با روش نات اصلاح‌شده مثبت و ۴۷/۸ درصد از نمونه‌ها با روش مولکولی ازنظر آلودگی مثبت بودند. نتایج HRM Real-time PCR نشان داد دمای ذوب توالی‌های تکثیرشده ژن سیتوکروم اکسیدازیک برای دیروفیلاریا ایمیتیس (۰/۰۳±۷۶/۶۷ درجه سانتی‌گراد) و آکانتوکیلونما رکوندیتوم (۰/۰۵±۸۱/۳۸ درجه سانتی‌گراد) حاصل شد، دمای ذوب توالی‌های تکثیرشده ژن ftsz و ولباشیا پایپینتیس (۰/۰۴±۸۰/۳۰) به دست آمد که براین‌اساس روش قابلیت تشخیص تفریقی عفونت را دارد و تفاوت در میانگین دماهای ذوب ازنظر آماری معنی‌دار بود ($P\leq 0/05$)، تغییرات دمای ذوب براساس تفاوت در توالی‌های نوکلئوتیدهااست.

نتیجه‌گیری نهایی: لازم است به این نکته توجه شود که استفاده از روش‌های مولکولی برای تشخیص دقیق عفونت مخفی دیروفیلاریازیس ضروری است. آنالیز HRM Real-Time PCR یک مرحله ساده پس از واکنش زنجیره‌ای پلیمراز است که از ویژگی‌های حرارتی قطعات تکثیرشده برای تشخیص عفونت دیروفیلاریازیس براساس ردیابی دنای وولباشیا پایپینتیس استفاده می‌کند.

کلیدواژه‌ها: وولباشیا پایپینتیس، دیروفیلاریا ایمیتیس، آکانتوکیلونما رکوندیتوم، سگ‌های ولگرد، نماتودها، HRM Real-time PCR

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