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Detecting and Identifying *Wolbachia pipientis* in Occult Dirofilariasis Using a High Resolution Melting Real- time PCR in Stray Dogs from Gilan, Mazandaran and Qazvin province in Iran

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Abstract

BACKGROUND: Iran is one of the endemic areas of *Dirofilaria immitis* (*D. immitis*). The modified Knott test has been the common test for diagnosis, based on the detection and identification of microfilaria in blood samples but the results may be compromised by *D. immitis* occult infection (*D. immitis* infection without microfilariae). *Wolbachia pipientis* (*W. pipientis*) in a dog's blood is an endosymbiont of *D. immitis* as well as an indicator of dirofilariasis.

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

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

RESULTS: The results showed that 17.4% of the samples were positive by the modified Knott method, while 47.8% of the samples were positive by the molecular methods. The results of HRM Real-time PCR showed that the melting temperature of cox1 gene amplicons of *D. immitis* and *Acanthocheilonema reconditum* (*A. reconditum*) were obtained 76.67°C± 0.03°C and 81.38° C± 0.05° C respectively as well as it was achieved 80.30°C± 0.04°C for FtsZ gene of *W.*

pipientis. The results of the present study showed that it is necessary to use molecular methods for the accurate diagnosis of Dirofilariasis occult infection.

CONCLUSIONS: The HRM Real-Time PCR analysis is a simple post-PCR step that exploits the thermal characteristics of the amplicons for detecting the occult infection of dirofilariasis based on *W. pipientis* DNA tracking and Identification.

Keywords: *Acanthocheilonema reconditum, Dirofilaria immitis,* HRM-real time PCR, Nematodes, Stray dogs.

Introduction

Animals in healthcare facilities, including dogs, can serve as sources of zoonotic pathogens that could potentially infect human(Ola-Fadunsin et al., 2023)

Filariasis is a common term for a group of 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 blood, lymphatic vessels, and connective tissues of the host.

Different species of *Culicidae* mosquitoes belonging to the genera Culex, Anopheles and *Aedes* play a role in the transmission of filariasis in dogs in Iran. The most commonly reported species

from dogs in Iran are *D. immitis*, *Dirofilaria repens* (D. repens), and *A. reconditum*. The location of adult *D. immitis* worms is 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 it is often diagnosed after the death of the animal, which is not recorded due to the lack of accurate reporting systems. This leads to inaccurate epidemiology statements of the disease. Correct diagnostic methods with acceptable sensitivity and specificity can help to correct the data related to the disease (Hosseini etal., 2022). Postmortem examination plays an important role in diagnosing and finding the causes of animal death. The purpose of investigating and diagnosing 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).

In some cases, mature parasites are present without circulating microfilariae identified as an occult infection. 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%- 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 they 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, *W. pipientis*, which affects the fertility and survival of the parasites while also contributing to their pathogenicity.

W. pipientis may be applied in the diagnosis of nematode parasites. Administration of antimicrobial therapy against *Wolbachia spp*. may be effective in the treatment of 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 are being 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 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 Gilan, Mazandaran, and Qazvin provinces of Iran, The sampling method was a random sampling technique with a

predefined prevalence estimation of 10% and a 95% confidence interval with a 5% margin of error. The samples were treated with 10% sodium-ethylene diamine tetra acetic acid anticoagulant and tested for microfilariae using the modified Knott's test. A male worm of *D. immitis* retrieved from a dog in Gilan 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 ScientificTM NanoDropTM spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1.00% agarose gel electrophoresis (Fanavaran Akhtarian, Iran). Prior to 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. (Borhani zarandi et al., 2017).

The specific primers amplified a 267bp fragment of the FtsZ gene of *W. pipientis* in dogs blood samples, Wol1-Forward-5'- CCTGTACTATATCCAAGAATTACTG-3', Wol1-Reverse-5'-

ACTATCCTTTATATGTTCCATAATTTC-3' (Turb *et al.*, 2012). Mitochondrial cytochrome c oxidase subunit 1(cox1) gene of *D. immitis* in dogs blood samples were amplified with specific primers targeting a 256bp fragment, dirHRM-Forward-5'- AGTATGTTTGTTTGAACTTC-3', dirHRM-Reverse-5'-AACGATCCTTATCAGTCAA-3' (Albonico *et al.*, 2014), As well as, *A. reconditum*- specific primers to amplify a 200bp fragment of cox1gene AgTgTTgAgggACAgCCAgAATTg-3′, ARCOI- Forward-5′-

-CCAAAACTggAACAgACAAAACAAgC-3' (Rojas *et al.*, 2015). ARCOI-Reverse-5'

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 programs of the partial sequences of the genes were Performed with an initial denaturation at 95.00 ˚C for 240seconds (sec) followed by 45 cycles of denaturation at 95.00 ˚C for 5 sec, annealing at 58˚C for 15 sec for Wol1 and dirHRM primers as well as 58˚C for 15 sec for ARCOI primers, extension at 72.00 ˚C for 10 sec, and final extension at 72.00 ˚C for 240 sec. 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/sec. All samples were tested in duplicates and their melting profiles using Rotor-Gene 1.7.27 software and the HRM algorithm provided.

Conventional polymerase chain reaction (cPCR). The study used cPCR to detect *W. pipientis* DNA, amplifying a 267bp 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 256bp for D. immitis and 200bp for A. reconditum.The thermocycler (Bio-Rad, USA) program for cPCR procedures was: predenaturation at 94.00 ˚C for 240seconds(sec) followed by 40 cycles of denaturation at 94.00 ˚C for 25 sec, annealing at 57.5 ˚C for 30 sec for *W. pipientis* and *D. immitis*, extension at 72.00 ˚C for 40 sec, and final extension at 72.00 ˚C for 420 sec.

A. *reconditum* cox1 amplification was performed based on the thermocycler (Bio-Rad, USA) program prepared for *W. pipientis* and *D. immitis* except 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(2).

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; Technelysium PTY Ltd., Queensland, Australia). A phylogenetic tree was constructed using the Chromas platform version 3.1.

Statistical analyses. For proportions 95% confidence intervals (CIs) were found. Cohen's kappa (κ) was calculated to compare the agreement between modified Knott test and cPCR and HRM-Real time PCR assays. The Chi-square test were used to compare proportions, with a probability *P*-value<0.05 regarded as statistically significant. Analyses were performed using SPSS version 20 software for Windows 10 (SPSS Inc., Chicago, IL, USA).

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 Gilan, Mazandaran, and Qazvin is also provided in the table1. The sample donated from Iran parasitology museum was a thin male *D. immitis* with 18 cm long, white in color and the tail has the typical spiral that can be 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±0.43µm and *A. reconditum* microfilariae was 228±12.1 × 5.8±0.43µm, that *computed* using the digital camera (IDS uEye UI-2250SE USB 2.0 camera, Obersulm, Germany) *under 40X magnification (Figure 2 and 3).*The results of melting curve analysis of *W. pipientis* FtsZ gene amplicons shown in figure 2 that are the *outcome of* some samples assessed using HRM real-time PCR.

HRM real-time PCR results

Table 1. The frequency of infection between three provinces including Gilan, Mazandaran and Qazvin using HRM-Real time PCR

Discussion

W. pipientis as an intracellular endosymbiont bacteria of *D. immitis* plays an important role in the pathogenesis, treatment, and also diagnosis of dirofilariasis infection In the present study, dirofilariasis was diagnosed through *W. pipientis,* and *D. immitis* DNA was found in all samples which had presented positive results with *W. pipientis*. While, The modified Knott's test presented the following results: Gilan 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, Chi-square test, *P*-value<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*-value<0.05) and *A. reconditum* (κ = 0.5 Chi-square test, *P*-value<0.05).

The results of conventional PCR and HRM-real-time PCR showed 47.8% (66:138) of dog's blood samples were infected, furthermore, the rate of infection in Gilan, Mazandaran, and Qazvin was 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. As well as, the results of *A. reconditum* illustrated 3.2% (95%CI 2.88- 3.5) in Gilan and 4.4% in Mazandaran(95%CI 3.96- 4.83) in many endemic regions including Iran(Hosseini *et al.*, 2022). 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).

The Modified Knott test has been used as one of the most common methods for dirofilariasis diagnosis 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 , but 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 the presence of helminths in the dog pulmonary artery, microfilariae are not released in the peripheral blood. The following are the most important reasons for occult infection: The presence of male worms without females, the host's immune system responses to adults, during the first six months after infected mosquito bites, and also 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, it was no microfilaria could be detected in the microscopic examination of blood samples (Landum *et al.*, 2014).

ELISA and SNAP Heartworm RT Test are the preferred detection method for canine heartworm diagnosis (a disease is caused by *D. immitis*) due to its simplicity and efficiency, however, these kits are not available in Iran and expensive since they are imported.

While *W. pipientis* and *D. immitis* genes become available in GenBank and 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 be able to detect *W. pipientis*. If HRM curve analysis is added to the real-time PCR, it will possible to differentiate *W. pipientis*.

In the present study 47.8% of samples suffered from infection with *W. pipientis* and *D. immitis* while microfilariae were not shown by modified Knott test in the 30.4% of samples, microfilaria was reported in the 17.4% of blood samples. In 30.4% of the samples, microfilariae were not observed and they probably were 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 Cytochrome oxidase subunit 1(cox1) gene and serological method .The partial DNA sequencing of cox1 gene of adult parasites showed considerable homology and close 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* (Turb *et al.*, 2012). *W. pipientis* detection could be a reflection of dirofilariasis infection was the principal hypothesis of this study which was verified(Turb *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 as well as saving time to get results. The HRM-real-time PCR technique is rapid with 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(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 dog's genomic DNA isolated from 138 blood samples of dogs in Gilan, Mazandaran, and Qazvin provinces in Iran and it was found that molecular methods detected more infected dogs in comparison with modified Knott's test (i.e., 66 vs 24 for *D. immitis*) this difference was statistically significant (Chi-square test, $\chi 2 = 77.7$, df = 3, P = 0.001).

Occult infection can be caused by low parasitemia(Panarese *et al*., 2020), and the first six months after mosquito bites or the presence of only adult male worms alone.(Panarese *et al*., 2020). In the present study, both 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. Although, samples that were infected with *A. reconditum* showed negative results with *W. pipientis.*

Conflict of interest

The authors declare that they have no conflict of interest to report.

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Fig. 1. From left to right: microfilariae of *Dirofilaria immitis* and microfilariae of *Acanthocheilonema reconditum* isolated by modified Knott test from dog blood in Gilan under the light microscope at 40X magnification.

Fig. 2. Melting curves analysis of amplicons in dog blood DNA by *Rotor-Gene* (Qiagen, Hilden, Germany). Melting curves of *Wolbachia pipientis (W. pipientis)* FtsZ gene PCR products (267bp) with Tm: 80.30°C± 0.04°C; the dark red color upper curve was positive control with 80.29° C melting temperature (Tm); the brown curves was an infected dog blood DNA with 80.26° C Tm; the blue curve was an infected dog blood DNA with 80.34° C Tm; the red and yellow-green color curves were DNA of infected dog drops of blood with 80.3° C Tm. The pink straight line was negative control (the sample without template DNA).

تشخیص و شناسایی *وولباشیا پایپینتیس* درعفونت مخفی دیروفیلاریازیس با استفاده از روش HRM Real-time PCR درسگ‌های بی‌صاحب‌استان‌های گیلان، مازندران‌و قزوین

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چکیده

زمینه مطالعه : بررسی آلودگی های‌انگلی سگ های ولگرد با توجه به اهمیت بیماری های مشترک قابل انتقال از سگ به انسان است. حائز اهمیت فراوان است و می تواند تهدیدی برای بهداشت جامعه باشد. ایران یکی از مناطق بومی دیروفیلاریا ایمیتیس نات اصلاح شده رایج ترین آزمایش برای تشخیص فیلاریازیس است که اساس آن مشاهده و شناسایی میکروفیلرها در تست نمونه‌های خون است، اما نتایج این آزمایش در موارد عفونت مخفی بطورکاذب منفی می‌شود. *وولباشیا پایپینتیس* بعنوان همزیست ، نشان دهنده دیروفیلاریازیس درخون سگ است. اجباری *دیروفیلاریا ایمیتیس*

هدف: مطالعه حاضربا هدف بررسی عفونت مخفی دیروفیلاریا ایمیتیس در سگ های ولگرد استان های گیلان، قزوین و مازندران انجام شد. Real-Time PCR (HRM) بر اساس ردیابی دنای *وولباشیا پایپینتیس* با استفاده از روش ذوب با وضوح بالا

روش کار: تعداد 138 نمونه خون از سگ‌های ولگرد در استان‌های گیلان، مازندران و قزوین جمع‌آوری شد که با دو روش نات بطول 267جفت باز ftsz *وولباشیا پایپینتیس* با تکثیر بخشی از ژن اصلاح شده و مولکولی مورد ارزیابی قرار گرفتند. حضور بررسی شد.

نتایج: نتایج این مطالعه نشان داد که 17.4 درصد از نمونه ها با روش نات اصلاح شده مثبت و 47.8 درصد از نمونه ها با روش نشان داد که دمای ذوب توالی های تکثیر شده ژن HRM Real-time PCR مولکولی از نظر آلودگی مثبت بودند. نتایج (81/38±0/05 *رکوندیتوم* (76/67±0/03 درجه سانتیگراد) و *آکانتوکیلونما* سیتوکروم اکسیداز یک برای *دیروفیلاریا ایمیتیس* بدست (80.30°C±0.04°C) *وولباشیا پایپینتیس* ftsz درجه سانتی گراد) حاصل شد، دمای ذوب توالی های تکثیر شده ژن آمد که براین اساس روش قابلیت تشخیص تفریقی عفونت را داردو تفاوت در میانگین دماهای ذوب از نظر آماری معنی‌دار)، تغییرات دمای ذوب بر اساس تفاوت در توالی‌های نوکلئوتیدها است.<*P*-value بود(0/05

نتیجه گیری نهایی: لازم است این نکته مورد توجه قرارگیرد که استفاده از روش های مولکولی برای تشخیص دقیق عفونت است یک مرحله ساده پس ازواکنش زنجیره ای پلیمراز HRM Real-Time PCR مخفی دیروفیلاریازیس ضروری است. آنالیز دنای که از ویژگی های حرارتی قطعات تکثیر شده، برای تشخیص عفونت دیروفیلاریازیس دیروفیلاریازیس بر اساس ردیابی *وولباشیا* پایپینتیس استفاده می کند.

کلمات کلیدی: *وولباشیا پایپینتیس*، *دیروفیلاریا ایمیتیس*، *رکوندیتوم*، سگ های ولگرد، نماتودها، *آکانتوکیلونما* ،

HRM Real-time PCR

شکل 1:از چپ به راست: میکروفیلر *دیروفیلاریا ایمیتیس* و *آکانتوکیلونمارکوندیتوم* با آزمایش نات اصلاح شده از خون سگ در گیلان در با میکروسکوپ نوری با بزرگنمایی 40× جدا شدند.

محصول پی سی آر از دنای خون سگ توسط هیلدن ، روتورژن کیاژن، آلمان). منحنی های ذوب **اشکل2**: تجزیه و تحلیل منحنی های ذوب 80.30±0.04 درجه سانتی گراد. منحنی بالایی رنگ قرمز :بطول 267 جفت باز با(دمای ذوب) FtsZ محصولات وولباشیا پایپینتیس ژن تیره کنترل مثبت با دمای ذوب 80.29 درجه سانتیگراد (دمای ذوب) بود. منحنی های قهوه ای یک دنای خون سگ آلوده با 80.26 درجه سانتی گراد (دمای ذوب). منحنی آبی یک دنای خون سگ آلوده با 80.34 درجه سانتی گراد(دمای ذوب). منحنی های رنگ قرمز و زرد-سبز دنای قطرات خون سگ آلوده با دمای 80.3 درجه سانتی گراد (دمای ذوب)بود. خط مستقیم صورتی، شاهد منفی بود (نمونه بدون دنای الگو).