Research Article

Title Molecular Epidemiology of Canine Heartworm (*Dirofilaria immitis*) in *Culex pipiens* Mosquitoes Collected from Tehran.

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Abstract

Dirofilaria immitis is a nematode parasite that can inhabit and develop within the heart and pulmonary arteries of canids, felids, also in humans. The microfilariae of this parasite circulate in the blood of the host and are transmitted to new hosts through the bites of *Culex pipiens* mosquitoes. *Culex pipiens*, commonly known as the common house mosquito, is a native urban pest species. Since the early 20th century, various control campaigns have been implemented across European countries targeting this species. It exhibits high ecological plasticity, resulting in complex feeding behaviors and vector potentials. This study was conducted during the June to September of 2024 in Tehran. A total of 100 *Culex pipiens* mosquitoes were collected from different areas of the city and examined for infection with *Dirofilaria immitis* using molecular methods (PCR). The results showed that 15 of the collected mosquitoes were carriers of *D. immitis* microfilariae. Considering the results of the present study, the high potential for infection in the studied areas, and the importance of *D. immitis*-associated disease, it is expected

that maintaining hygiene and cleanliness of mosquito-prone areas and dog shelters, along with controlling mosquito populations, especially in humid regions during warm seasons, will prevent the spread of this zoonotic disease among dogs and humans.

Introduction

Dirofilariasis is a zoonotic parasitic disease caused by the filarial nematode *Dirofilaria immitis*, commonly known as the canine heartworm. This parasite primarily affects canids, where adult worms inhabit the pulmonary arteries and right side of the heart, leading to progressive cardiopulmonary disorders. Microfilariae produced by adult worms circulate in the bloodstream and, when ingested by hematophagous mosquitoes such as Aedes, Culex, and Anopheles species, develop into infective third-stage larvae. During subsequent blood feeding, infected mosquitoes transmit the parasite to new hosts [1].

In dogs, the clinical presentation depends on infection intensity, ranging from asymptomatic cases to severe disease characterized by persistent cough, exercise intolerance, anorexia, weakness, and dyspnea. Both the mechanical presence of worms and host immune responses contribute to disease pathogenesis. In humans, however, the larvae do not mature into adults; instead, immature stages elicit pulmonary dirofilariasis through inflammatory reactions to dying larvae [2].

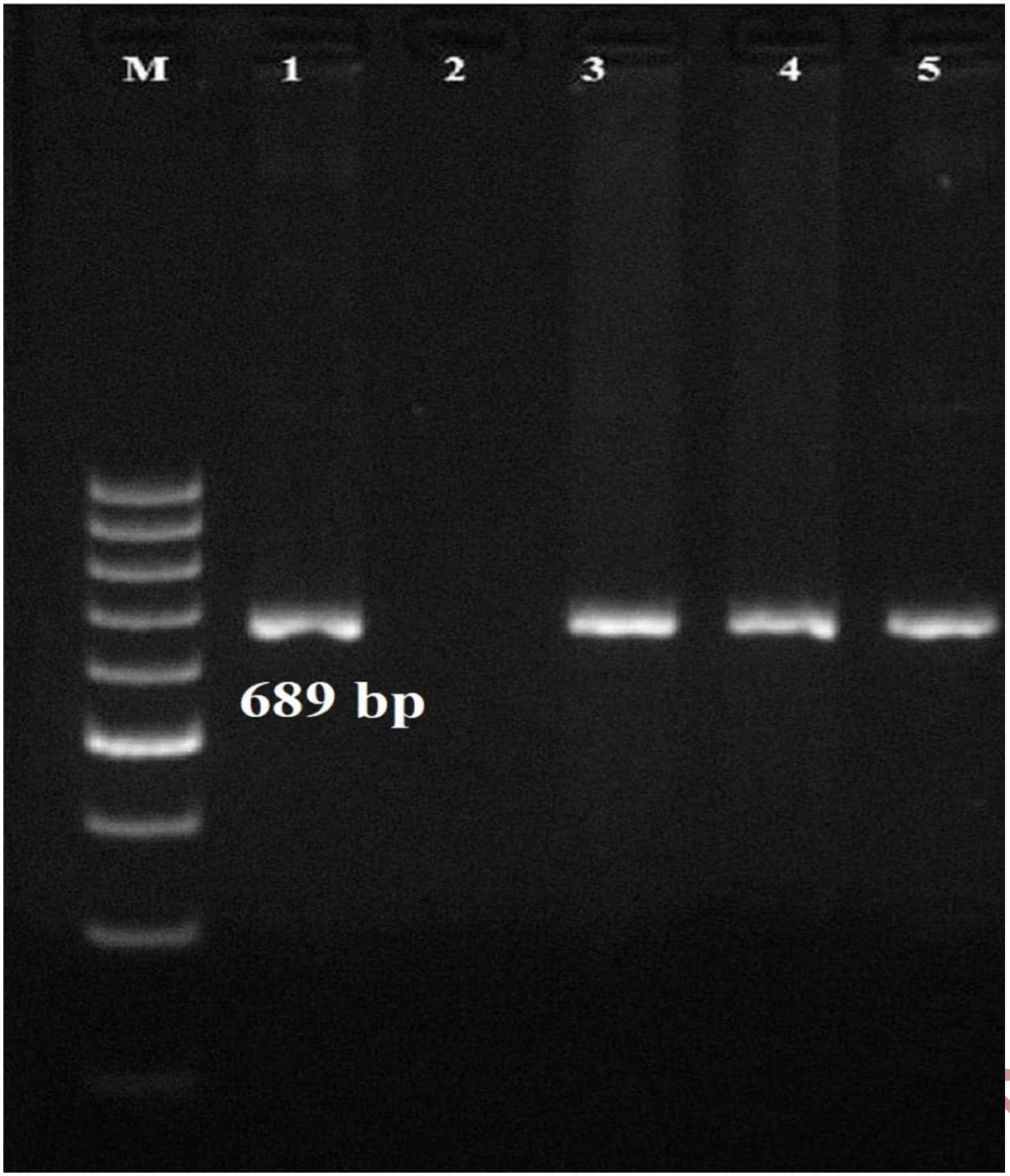
Dogs serve as the main reservoir, often harboring infections for years and producing large numbers of microfilariae. Since mosquitoes are not host-specific and feed on a wide range of species, they play a crucial role in transmitting dirofilariasis to both canids and humans in endemic areas [3]. Although more than 300 human pulmonary dirofilariasis cases had been reported worldwide by 2014 most of them in the United States no confirmed human cases have been documented in Iran. This absence may be attributed to limited serological testing and the difficulty of distinguishing pulmonary nodules caused by *D. immitis* from those of other

etiologies [4]. Given the importance of reservoir hosts and mosquito vectors in disease transmission, surveillance of mosquito populations can provide valuable insights into the circulation of D. immitis. Therefore, the present study aims to investigate the molecular epidemiology of canine heartworm (*D. immitis*) in *Culex pipiens* mosquitoes collected from Tehran.

Results

Molecular Results

The PCR amplification of the Cox1 gene using the specific primers generated a 689 bp fragment. One sample was sequenced and served as the positive control. Agarose gel electrophoresis confirmed the presence of the expected 689 bp band: lane M shows the 100 bp DNA ladder, lane 1 represents the positive control, lane 2 is the negative control (no genomic DNA), and lanes 3–5 correspond to the tested samples, all of which produced the specific 689 bp amplicon (Figure 3).



Statistical Analysis

Out of 100 collected *Culex* mosquitoes in this study, 15 were found to carry microfilariae of *Dirofilaria immitis*. This indicates that 15% of the *Culex* mosquitoes collected from the studied areas during the June to September of 2024 were vectors of this parasite. (Table 2).

Table 2.
Distribution of *Dirofilaria immitis* infection rates in *Culex pipiens* mosquitoes across different regions of Tehran

Region	Mosquitoes	Number Infected	Infection Rate
Chitgar	20	2	10%
Niyavaran	20	3	15%
Janat abad	20	4	20%
Lavasanat	20	2	10%
Kouhsar	20	4	20%
Total	100	15	15%

Discussion

A comparison of historical epidemiological data over the past years shows significant changes in the global prevalence of dirofilariasis. These changes may be related to climate change and the distribution of vector populations of *Dirofilaria* species. Microfilariae may enter peripheral blood circulation at specific times during the day or night and hide from circulation at other times [6]. Therefore, the samples in the present study were collected at night.

In this study, we focused on *Culex pipiens* because it is the predominant mosquito species in Tehran and has been consistently reported as a potential vector of *D. immitis* in Iran. The widespread presence of this mosquito in both urban and rural habitats, together with its opportunistic feeding behavior on dogs and humans, highlights its importance in the local transmission cycle. Therefore, targeting *Culex pipiens* not only reflects the most epidemiologically relevant vector in this region but also provides reliable insights into the risk of dirofilariasis transmission in Tehran. The transmission rate of dirofilariasis caused by different species depends on the presence of infected dogs as the main reservoir hosts and the availability of competent vectors for disease transmission. Thus, the transmission of dirofilariasis is influenced by two factors affecting both components of the worm's life cycle: first, human behavior with pets; second, climatic factors that enable the presence of competent vector populations and development of *Dirofilaria* larvae. Similar disease conditions in humans, such as pulmonary and cardiac diseases, have also been reported [7]. According to

serological findings, the current prevalence is lower than in other regions of Iran, but due to warm and semi-arid climatic conditions, this prevalence is considered high for this region. Previous studies have reported two species of the genus *Dirofilaria*, including *D. immitis* (canine heartworm) and *D. repens*, in several regions of Iran [8].

In the study by Azari Hamidian *et al.* (2009), the COX1 sequence of the third-stage larvae of *D. immitis* from Iranian samples was described. Similar disease conditions in humans as in dogs, including pulmonary and cardiac diseases, have also been reported [9].

Sharifdini et al. (2022), in a study on 334 dogs in Mazandaran and Gilan provinces, found that 75 dogs (21.8%) were infected with *D. immitis* by PCR. Infection was detected in all cases. In Mazandaran, infection with *D. immitis* was confirmed in 22% and *Acanthocheilonema reconditum* in 4.5% of dogs by PCR. Previous studies in this province showed infection with *D. immitis* in 15.2% of dogs by microscopy and 60.9% by necropsy [10].

Also, in studies conducted by Ranjbar-Bahadori et al. (2007, 2011), the microfilariae infection rate with *D. immitis* in stray dogs around Garmsar was reported as 12.29%. Infection rates from Tonekabon (15%), Golestan province (18.18%), and Shiraz (9.5%) were also reported. In this study, 100 herd dogs around Mashhad were examined for the presence of *D. immitis* microfilariae. Microfilariae were found in 15 dogs, but none were *D. immitis*, and these microfilariae were identified as *A. reconditum* based on morphology and morphometry [11,12].

In a study by Borthakur et al. (2015) in northeastern India on stray, domestic, and working dogs, diagnostic methods including modified Knott's test, fresh blood smear, ELISA serology, molecular PCR, and gene sequencing were used. The results showed 11.38% positive by Knott's test, 18.03% by ELISA, and 13.93% by PCR. No significant difference in infection was observed between males and females or different regions, but stray dogs were significantly more infected than other groups. ELISA revealed 22.69% occult infection in working dogs, and

the molecular method detected *D. repens* in 1% of stray dogs. The ITS region of *D. immitis* showed close homology with South Asian isolates [13].

Nguyen et al. (2016) in Australia used Real-Time PCR to differentiate *D. immitis* from *A. reconditum* in 39 dogs positive by Knott's test [14].

In the present study, 15% of samples taken from public places where many dogs live were carriers of *D. immitis* microfilariae. Therefore, it can be concluded that humans and dogs residing in these areas are at risk of infection with this parasite.

Khamesipour et al. (2020) studied 75 dogs in Mazandaran province and found a 21.8% infection rate with *Dirofilaria*, which is consistent with the present study [15].

In a study by Khodabakhsh et al. (2016) on 103 cats in Meshginshahr, Ardabil province, only 1 cat (0.96%) was a carrier of the disease, which is inconsistent with the present study [16].

In a study by Manshoori et al. (2023) on stray dogs in Mazandaran, Gilan, and Qazvin provinces, 17.4% of samples were infected with *Dirofilaria*, consistent with the present study [17].

Also, in a study by Malmasi et al. (2009) on stray dogs in Mazandaran and Golestan provinces, 25.5% of samples were carriers of *D. immitis*, aligning with the present study [18]. Khodari et al. (2014) studied 120 stray dogs in Sistan and Baluchestan Kerman provinces, with infection rates of 24.2% and 27.4%, respectively, consistent with the present study [19].

The positive rate of *D. immitis* in various provinces of Iran correlates with climatic and ecological features. The highest prevalence was recorded in Gilan (78.6%) and Mazandaran (50%) in the Caspian region, likely due to a Mediterranean temperate climate year-round. Also, in Isfahan (0.9%), Lorestan (6.9%), and Qazvin (27.3%), this parasite has been reported, possibly due to increased temperature and decreased annual rainfall. Since the first report of *D. immitis* infection in dogs in northern Iran, several studies have reported prevalence rates up to 62.8%. Although data on possible infection of dogs in 9 out of 31 provinces is lacking, reports

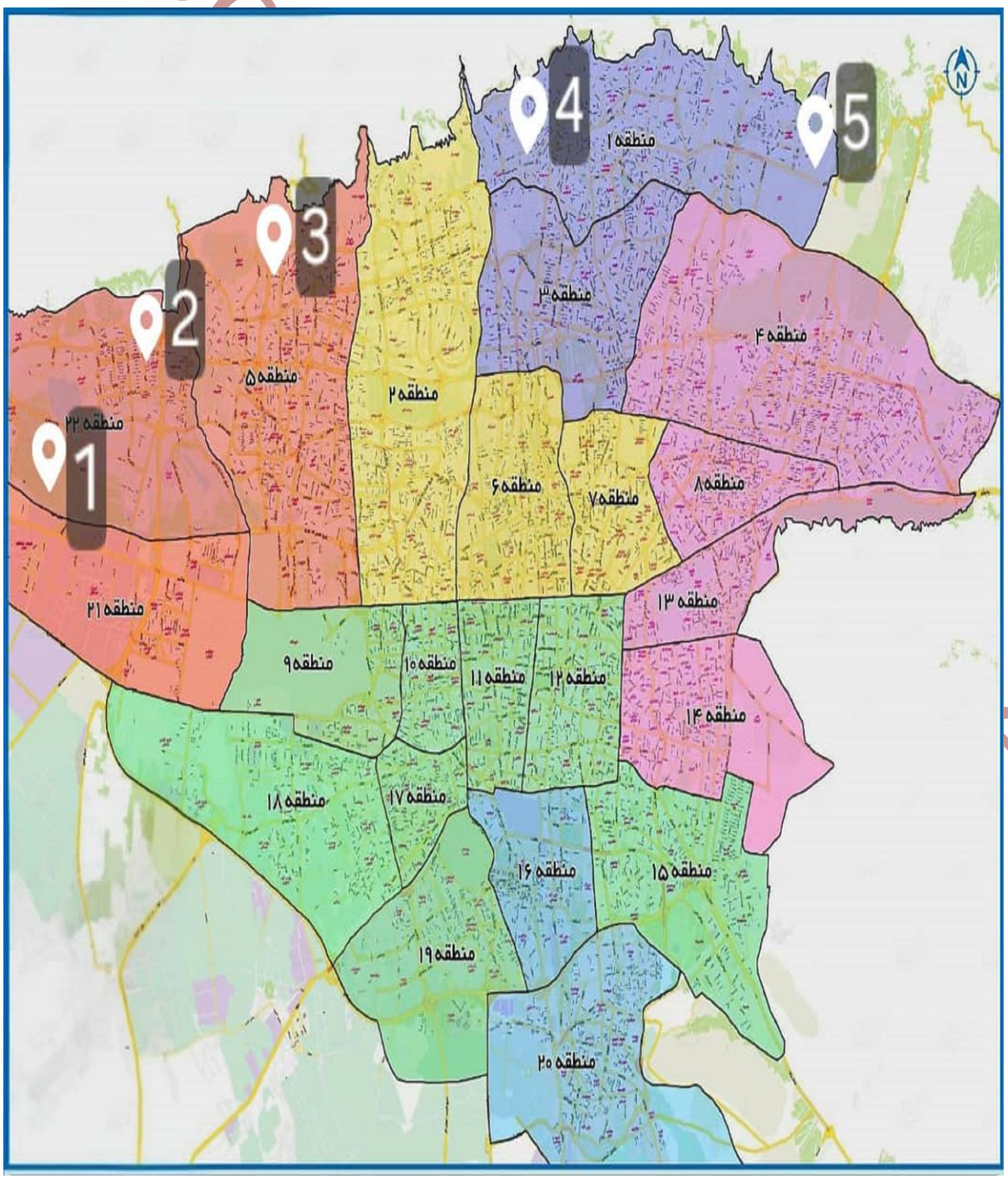
of human infection with *D. immitis* and *D. repens* in Alborz and Hormozgan provinces indicate parasite circulation in dog populations. Furthermore, since wild carnivores such as wolves, jackals, and foxes play an important role in the epidemiology of dirofilariasis, further epidemiological studies are necessary to fill knowledge gaps regarding wild reservoir hosts [20].

The most popular parasitological methods among concentration tests are serological assays for detecting antibodies against *D. immitis*, such as enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests. Antigen tests may detect occult infections like a microfilaremia case, but positive dogs may have cleared infection or only been exposed without actual infection [21,22].

Materials and Methods

Study Area

This study was conducted in the June to September of 2024 in Tehran. The foothill areas of Tehran are home to local and indigenous dogs, which can become infected by *Culex* mosquitoes in the region. Therefore, public places and veterinary clinics in Niyavaran, Lavasanat, Jannatabad, Kouhsar, and Chitgar were selected for sampling (Figure 1).



Sample Collection

Mosquito sampling was conducted at night using light traps and aspirators. In total, 100 Culex mosquitoes were collected from the designated areas and divided into five groups of 20 individuals each. Every mosquito was placed separately in an individual package and assigned a unique identification code. Subsequently, each group of 20 mosquitoes from the five regions was preserved in a separate container filled with 100% ethanol and stored in a freezer at -21 °C.

Morphological characteristics:

Culex pipiens is a medium-sized mosquito (3–6 mm) characterized by a brownish body covered with a mixture of pale and dark scales. The proboscis is uniformly dark and unbanded, females possess short palps, and both legs and wings are evenly dark. The abdomen displays dark tergites with pale basal bands, while the thorax lacks clear longitudinal stripes. This species can be distinguished from Aedes by the absence of leg and thoracic banding, and from Anopheles by the presence of short female palps and a larval siphon. Within the pipiens complex, C. pipiens differs from C. quinquefasciatus by having a longer larval siphon and being more common in temperate regions. Its wings are uniformly dark and unmarked, unlike those of Aedes, which bear white bands, or Anopheles, which show alternating dark and pale spots. The plain, dark wings therefore represent a key diagnostic feature of Culex pipiens (Figure 2).

DNA Extraction

Blood samples were first removed from the -21°C freezer, and DNA extraction was performed using the MBST DNA extraction kit (Iran) following the manufacturer's instructions. Extracted DNA samples were stored at -21°C until further use.

PCR Reaction

PCR was carried out using Colint-F and Colint-R primers targeting the ribosomal Cox1 gene, producing a 689 base pair PCR product (5) (Table 1). The total PCR reaction volume was 30 µL, containing One Time PCR Buffer, 3 µL genomic DNA, 1.25 U Taq polymerase (Ampliqon, Denmark), 10 pmol of each primer (Metabion, Korea), 200 µM of each dATP, dTTP, dCTP, and dGTP (Ampliqon, Denmark), and 1.5 mM $MgCl_2$.

Table 1.
Nucleotide Sequences of Primers Used for Amplification of Cox1 Gene Region

Reactions	Name of primer	Nucleotide sequence (5´– 3´)	PCR product
Primers	Colint-F Colint-R	TGATTGGTGGTTTTGGTAA ATAAGTAC GAGTATCAATATC	689 bp

PCR was performed using an automatic thermocycler (SimpliAmp, USA) with the following cycling conditions: initial denaturation at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 45 seconds, extension at 72°C for 60 seconds; followed by a final extension at 72°C for 7 minutes.

Negative controls without genomic DNA were included in all reactions. PCR products were analyzed on a 1.5% agarose gel in 1X TBE buffer, stained with ethidium bromide, and visualized under a UV illuminator.

Statistical Analysis

The results and infection rates according to age and sex were analyzed using the Chi-square test. Data analysis was performed using SPSS software version 24 (SPSS Inc., Chicago, USA), and a significance level of $P < 0.05$ was considered.

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عنوان مقاله: بررسی حضور میکروفیلر انگل دیروفیلاریا ایمیتیس در پشه های کولکس مناطق مختلف شهر تهران

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خلاصه فارسی: کرم قلب سگ (*دیروفیلاریا ایمیتیس*) یک نوع کرم نماتود است که می تواند در قلب و سرخرگ ششی سگ سانان، گربه سانان و انسان مستقر شده و رشد نماید. میکروفیلر این انگل در خون میزبان وجود داشته و توسط گزش پشه های *کولکس* به میزبان جدید منتقل می شود. Common house mosquito با نام علمی *Culex pipiens* نوعی پشه بومی است که به عنوان یک آفت در محیط های شهری شناخته می شود. از اوایل قرن بیستم، کمپین هایی در بسیاری از کشورهای اروپایی برای کنترل این گونه سازماندهی شده است. این گونه انعطاف پذیری اکولوژیکی بالایی را نشان می دهد که تصویر پیچیده ای از نظر رفتار تغذیه ای و ظرفیت های برداری به دست می دهد. این مطالعه در بازه زمانی فصل تابستان سال 1403 و در شهر تهران انجام شد، لذا تعداد صد عدد پشه *کولکس* از مناطق مختلف شهر برای بررسی میزان آلودگی به انگل *D.immitis* جمع آوری شد و به روش مولکولی مورد بررسی قرار گرفت. در نتیجه این مطالعه پانزده عدد از صد پشه کولکس جمع آوری شده ناقل میکروفیلر انگل *D.immitis* شناسایی شد. با توجه به نتایج مطالعه حاضر و پتانسیل بالای آلودگی در مناطق تحت مطالعه و اهمیت بیماری ناشی از *D.immitis* ، انتظار می رود با رعایت بهداشت و نظافت مکان های پشه خیز و محل زندگی سگ ها و کنترل جمعیت پشه ها به خصوص در مناطق مرطوب در فصول گرم سال از انتشار این بیماری زئونوز در بین سگ ها و انسان ها جلوگیری شود.

واژگان کلیدی: میکروفیلر، *دیروفیلاریا ایمیتیس* ، پشه کولکس ، پی سی آر