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Dirofilaria repens infection in dogs and associated risk factors from an endemic region of Kerala: a molecular study

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Abstract

Canine dirofilariosis caused by Dirofilaria repens is an emerging zoonotic concern in Kerala, India. The present study investigated the molecular prevalence of D. repens infection and associated risk factors among dogs in and around Palakkad district, Kerala. A total of 102 blood samples from dogs presented to the District Veterinary Centre, Palakkad, were examined for microfilariae using Leishman-stained thin blood smears, and host- and management-related data were collected via a structured questionnaire. Genomic DNA was extracted and PCR-amplified targeting a 209 bp fragment of the cytochrome oxidase subunit 1 (COI) gene specific to D. repens. Microscopy detected microfilariae in 19.61% (20/102) of samples, while PCR confirmed D. repens infection in 8.82% (9/102). Majority of the host-related factors, including age, sex, breed, fever, deworming, and vaccination status, did not show a statistically significant association with D. repens infection (p>0.05). However, environmental exposure was significantly associated with positivity, with outdoor dogs showing a higher prevalence than indoor dogs (p<0.05). Sequencing and BLAST analysis confirmed species identity, and phylogenetic analysis revealed close clustering of the study isolate with Indian D. repens isolates from animals and humans, highlighting its zoonotic potential. The findings reaffirm the endemicity of D. repens in Kerala and emphasise the role of vector exposure in disease transmission.

Keywords: Dog, Microfilariae, *Dirofilaria repens*, PCR

The filarial nematodes *Dirofilaria immitis* and *Dirofilaria repens* may infect a wide variety of mammalian species (McCall et al*.,* 2008). They are particularly common in many parts of the world, including the Indian sub-continent. *Dirofilaria immitis* adult worms live in the pulmonary arteries and right ventricles and are responsible for major clinical disease with a variety of symptoms. *Dirofilaria repens* adults are found in subcutaneous tissues. Subcutaneous dirofilariosis caused by nematodes *D. repens* is often asymptomatic. Infected dogs may develop subcutaneous nodules, pruritus, dermatitis, and, in some cases, ocular lesions. It is also a cause of zoonotic human dirofilariasis, a mild disease that generally causes subcutaneous or ocular nodules in humans. Domesticated dogs (*Canis familiaris*) serve as reservoirs among mammalian hosts and are the primary means of human transmission for both the species. They have also been reported in wild carnivores, such as wolves (*Canis lupus)*, red foxes (*Vulpes vulpes*), and golden jackals (*Canis aureus*) (Manas et al*.,* 2005; Segovia et al*.,* 2001; Gortazar et al*.,*1994).

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Filarial nematodes are distinguished by their tissue tropism and reliance on blood-feeding arthropod vectors to transmit it. *Dirofilaria repens* is the causative agent of "Old World" cutaneous dirofilariosis and is found throughout Europe, areas of Asia, and Africa (Rjeibi et al*.,* 2017). It has been established that mosquitoes from the genera *Culex, Aedes, Armigeres,* and *Anopheles* are appropriate vectors for this parasite (Anyanwu et al*.,* 2000). There are five larval stages in *D. repens*' life cycle. Mosquitoes consume the L1 microfilaria while nourishing on an infected host. In the Malpighian tubules of mosquitoes, the microfilaria matures into the infectious third stage larva (L3) before passing through the body cavity to the proboscis. When a vector bites dogs or other hosts during the following blood meal, transmission occurs (McCall et al*.,* 2008).

While circulating microfilariae induce dermatological symptoms such pruritus, erythema, alopecia, scattered dermatitis, and itching, canine subcutaneous dirofilariosis caused by *D. repens* is frequently thought to be asymptomatic, albeit occasionally the parasites cause subcutaneous nodules (Tarello, 2002). Ocular sites and subcutaneous nodules are linked to the classic appearance of human dirofilariosis with parasites detected in periocular tissues or nodules in the eye in majority of the human cases of ocular dirofilariosis brought on by *D. repens* (Pampiglione and Rivasi*,* 2000). Nonetheless, human dirofilariosis has been reported in a few odd locations including lungs, scrotum, penis, spermatic cord, epididymis, and female mammary glands (Pampiglione and Rivasi*,* 2000; Genchi et al., 2011).

Dirofilariosis is diagnosed through a number of conventional microscopic techniques, including microfilarial detection in blood samples, such as the Knott's test and Giemsa or Leishman-stained blood smears. Furthermore, antibodies against *Dirofilarial* antigens in the host's blood can be detected by serological tests such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), and immunochromatographic tests (ICT), particularly when microfilariae are absent. Notwithstanding their intended use, standard diagnostic approaches for dirofilariasis are beset with intrinsic difficulties. Particularly in infections with low microfilarial densities and mild infections, microscopic procedures such as the Knott's test and blood smear examination may not be sufficiently sensitive to detect low levels of microfilariae, leading to false-negative results. Also, it can be challenging to identify distinct filarial worm species solely on the basis of microfilarial morphology, particularly within the genus *Dirofilaria* (Ravindran et al., 2016). Likewise, there is a chance of possible false-positive results and misinterpretation due to the cross-reactivity with antibodies against related parasites in serological assays like ELISA and IFA. Polymerase Chain Reaction (PCR), being a potent diagnostic tool with high sensitivity and specificity, reliably detects *Dirofilaria* spp. DNA in clinical samples. The current study investigates the molecular prevalence of *D. repens* and risk factors associated with dogs in and around the Palakkad district of Kerala, India.

Materials and methods

Sample collection

A total of 102 blood samples (in EDTA vials) were collected from dogs within and neighboring Palakkad, Kerala. Samples were aseptically drawn from dogs at random presented to District Veterinary Centre, Palakkad. During the sample collection, details on a variety of risk variables, including age, breed, gender, fever, environment, deworming, and vaccination status were collected through a structured questionnaire.

Parasitological examination

Blood samples were used to prepare thin blood smears on clean glass slides, which were subsequently stained with Leishman stain. The stained smears were then examined under a light microscope using oil immersion (100× objective), for the detection of several microfilariae.

Molecular detection of D. repens and sequencing

All the molecular techniques were performed at the Animal Disease Research Centre, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. As per the manufacturer's protocol, DNA was extracted from all the 102 blood samples using the Qiagen DNA extraction kit (Qiagen Germany). Amplification using species-specific primers for *D. repens* targeting a 209 bp region of the *cytochrome oxidase subunit 1* (*COX 1*) gene was performed (Rishniw et al*.,* 2006). The PCR assay was performed in a 25 µL reaction mixture containing 12.5 µL of 2x Taq PCR master mix (Qiagen, Germany), 4 µL of DNA template, 20 pmol of each primer, and 4.5 µL of nuclease-free water. Cycling conditions included an initial denaturation step (2 min at 94°C), followed by 32 cycles of denaturation (94°C for 30s), annealing (58°C for 30s), extension (72°C for 30s), and a final extension at 72°C for 7 min. Products were finally analyzed on 2% agarose gel.

The PCR products were purified using a PCR purification kit (HiMedia, India) and sent for nucleotide sequencing to Barcode Biosciences, Bangalore. The obtained *COX1* nucleotide sequences were analyzed through multiple sequence alignment using ClustalX and subsequently compared with existing sequences. Similarly, *COX1* nucleotide sequences were aligned using ClustalW and compared with reference sequences through the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLASTn). The partial *COI* gene *of D. repens* was downloaded from NCBI for multiple alignment and used for phylogenetic analysis using the neighbour-joining method with 1000 bootstrap values

employing programs in MEGA 12.0 software (Kumar et al., 2024).

Statistical analysis

Associations between risk factors (age, sex, breed, deworming, vaccination, fever, and environment) and *D. repens* prevalence were evaluated using the Chi-square (χ^2) test. Differences were considered significant at $p<0.05$, and analyses were performed. (https://www.icalcu.com/stat/chisqtest.html)

Results and discussion

In the present study, a microfilarial prevalence of 19.61% (20/102) was observed based on examination of Leishman-stained thin blood smears (Fig. 1). PCR amplification of a 209 bp fragment confirmed *D. repens* infection in 9 out of 102 dogs, indicating a prevalence of 8.82% (Fig. 2). The discrepancy between microscopic and PCR-based prevalence suggests that not all circulating microfilariae detected by microscopy were attributable to *D. repens* and indicates the possible presence of other canine filarial species in the study population (Ravindran et al., 2014). This finding supports the recognition of Kerala as an endemic region for canine *D. repens*, where prevalence has been shown to vary widely across districts. High prevalence rates of 39.02% have been reported in Alappuzha (Ravindran et al., 2014), while moderate prevalence levels of 7–7.59% have been recorded in dogs from Mannuthy and Thrissur (Radhika, 1997; Sabu et al., 2005). Molecular studies have further confirmed the endemic presence of *D. repens* in dogs, humans, and wild animal hosts in Kerala (Ravindran et al., 2016; Pradeep et al., 2019). Furthermore, Nazar et al. (2017) reported that phylogenetic analysis of human *Dirofilaria repens* isolates demonstrated a close evolutionary relationship with Asian isolates of *Dirofilaria hongkongensis.* Similarly, Kumar et al. (2021) molecularly characterized human and canine *Dirofilaria* isolates and identified as *Candidatus Dirofilaria hongkongensis*, indicating the circulation of the parasite in the region and its zoonotic relevance. The comparatively lower prevalence observed in the present study, compared with higher rates reported in certain districts, may be attributed to the smaller sample size and differences in the study population and management practices.

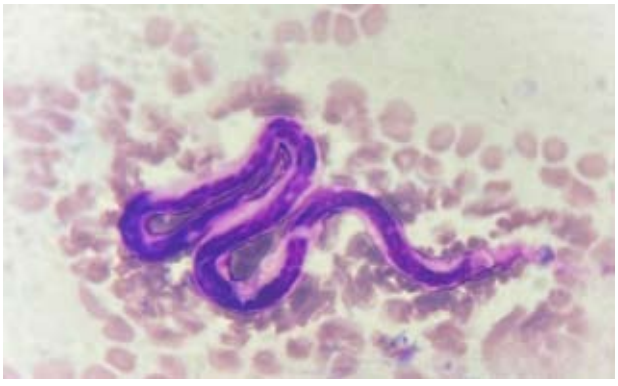

Fig. 1. Microfilaria in Leishman-stained thin blood smear from a dog

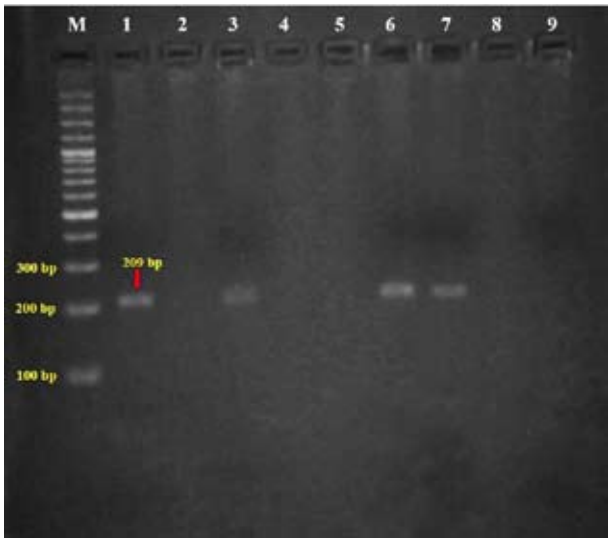

Fig. 2. PCR Analysis of *D. repens* from various samples based on the *COI* gene amplified 209bp fragment**.** (M: 100bp DNA Ladder RTU (Fermentas); 1: Positive Control; 2: Negative Control; 3-7 Collected samples; 3,6,7- Positive samples; 4,5,8,9-Negative samples)

The present study emphasizes *D. repens* as an important filarial parasite of dogs in India, with a particularly high prevalence in Kerala. As the infection is mosquito-borne and dogs act as the primary reservoir hosts, canine infection plays a key role in disease transmission and zoonotic risk. Kerala has been a major focus of human dirofilariasis in India, with early reports of ocular and subcutaneous infections (Joseph et al., 1976; Senthilvel & Pillai, 1999) and continued documentation of cases over the years, especially involving ocular and subcutaneous tissues (Sekhar et al., 2000; Sabu et al., 2005; Singh et al., 2010; Sanjeev et al., 2011). Although human cases have been reported from other regions, Kerala accounts for a disproportionately higher number of infections (Bhat et al., 2003; Reddy, 2013).

At the national level, *D. repens* has been reported across different regions of India, with marked variation in prevalence among canine populations. A relatively low prevalence of 1.79% was recorded in Guwahati, Assam (Borthakur et al., 2015), whereas much higher rates have been reported from southern India, including Mangalore, with prevalence ranging from 38.09% to 44.39% (Ananda & Placid, 2007; Malatesh et al., 2019). In contrast, moderate prevalence levels of 4.9% and 16.7% have been documented in Delhi and Mumbai, respectively (Megat Abd Rani et al., 2010).

Prevalence of *D. repens* in relation to associated risk factors is presented in Table 1. Most host-related factors such as age, sex, and breed did not show a statistically significant association with positivity ($p > 0.05$). Similar proportions of positive cases were observed in animals

less than 1 year old (8%) and more than 1 year old (9.09%), indicating that age may not play a major role in susceptibility in this population. Likewise, the absence of significant differences between males and females suggests that gender does not substantially influence infection rates. The higher prevalence of microfilariosis observed among older and male dogs has also been reported by several authors. (Radhika et al., 2001; Christopher & Abel-Danjuma, 2016; Malatesh et al., 2019).

Management-related factors, including deworming and vaccination status, also did not show significant associations. Although a higher proportion of positives was observed in non-dewormed (20%) and non-vaccinated animals (15.38%) than in dewormed (7.60%) and vaccinated (7.86%) animals. The differences were not statistically significant, possibly due to the low number of positive cases and limited sample size in these categories. Deworming, even when performed once, can reduce parasite load, while vaccination is generally associated with better veterinary care and improved immunity, together contributing to a lower incidence of microfilariosis in treated animals. The higher prevalence in descript breeds (9.30%) compared to non-descript breeds (6.25%) may be attributed to the larger representation of descript dogs in the study population and the general preference of owners to keep these breeds, resulting in more positive cases (Bhattacharjee & Sarmah, 2014; Malatesh et al., 2019). Similarly, fever was not significantly associated with positivity, suggesting that clinical signs alone may not be a reliable indicator of infection.

In contrast, environmental exposure was significantly associated with positivity. Animals kept outdoors had a significantly higher proportion ($p<0.05$) of positive cases (16%) compared to indoor animals (1.9%). The higher prevalence of microfilariosis in outdoor dogs compared to indoor dogs may be due to greater exposure to mosquito vectors, whereas keeping dogs indoors can reduce contact with disease-transmitting mosquitoes, as reported in previous studies (Walter, 1995; Theis et al., 1999; Malatesh et al., 2019). The result underscores the role of vector exposure as a key risk factor and supports the need for improved vector control, ectoparasiticides, and management practices, particularly for animals housed outdoors. Risk factors and their prevalence are summarized in Table 1.

The positive sample confirmation was obtained after sequencing and comparison with the sequence by the Basic Local Alignment Search Tool in NCBI. The sequence was submitted in NCBI, and an accession number was obtained (Accession No. PX928033). The nucleotide per cent identity ranged from 99.93 to 100%. The phylogenetic tree of the positive sample showed that the isolate from the study clustered closely with previously reported Indian *D. repens* strains from dogs, humans, and cats, particularly those from southern India (Fig. 3). This study isolate showed closer genetic relatedness to a human-derived *D. repens* isolate (KT588609.1), suggesting genetic similarity between animal and human isolates and supporting the zoonotic relevance of the parasite, consistent with previous reports describing zoonotic *Dirofilaria* species, including *D. repens* and *D. hongkongensis* (Pradeep et al., 2019).

Conclusion

In conclusion, *Dirofilaria repens* infection is prevalent among dogs in Palakkad district, Kerala, confirming its continued endemic presence and zoonotic relevance. While microscopic screening indicated a higher detection rate, PCR provided more specific

Table 1. Prevalence of *D. repens* in relation to associated risk factors

Risk Factor	Parameter	Positive samples	Chi-Square value	*P value*
Age	Less than 1 year	2/25 (8%)	0.023	0.878 NS
	More than 1 year	7/77 (9.09%)		
Gender	Male	6/59 (10.16%)	0.265	0.606 NS
	Female	3/43 (6.98%)		
Breed	Descript	8/86 (9.30%)	0.595	0.440 NS
	Non-descript	1/16 (6.25%)		
Deworming	Dewormed	7/92 (7.60%)	1.322	0.250 NS
	Non-dewormed	2/10 (20%)		
Vaccination	Vaccination	7/89 (7.86%)	0.635	0.425 NS
	Non- vaccinated	2/13 (15.38%)		
Fever	With fever	1/5 (20%)	0.623	0.429 NS
	Without fever	8/97 (8.24%)		
Environment	Outdoor	8/50 (16%)	5.269	0.021*
	Indoor	1/52 (1.9%)		

Note: **P< 0.05,* NS- Non-significant

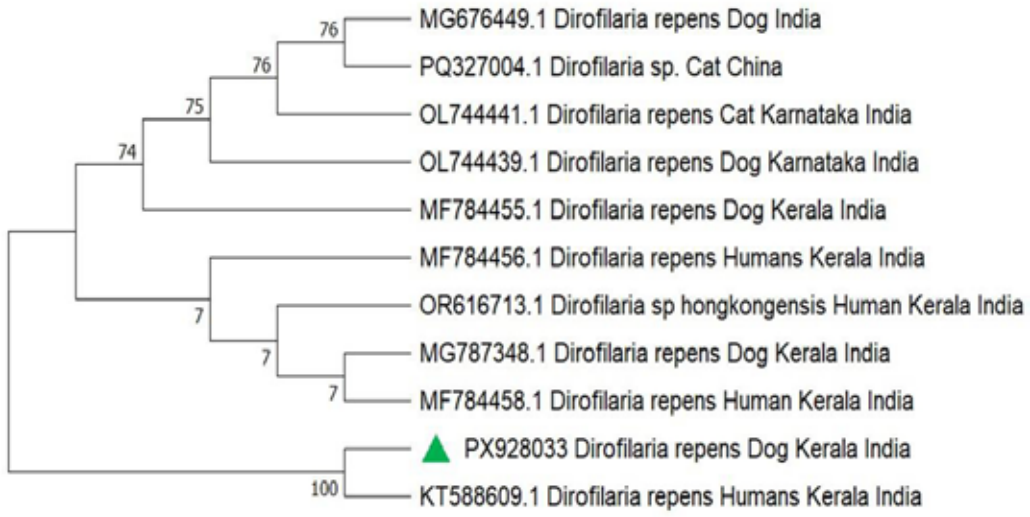

Fig. 3. Phylogenetic tree based on the partial *COI* gene of *D. repens* constructed using the neighbour-joining method using 1000 bootstrap values. The sequence generated in the present study is highlighted in green mark.

confirmation of infection. Most host-related factors were not significantly associated with infection status; however, increased exposure to outdoor environments emerged as an important risk factor, underscoring the role of vector contact in transmission. Molecular and phylogenetic analyses further validated the identity of the parasite and demonstrated close relatedness to other Indian isolates from both animal and human sources. These findings highlight the need for improved surveillance, vector control, and preventive management strategies to reduce the risk of canine and zoonotic transmission.

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Conflict of interest

The authors report no conflict of interest.

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