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BRIEF REPORT

Molecular detection of *Leishmania donovani*, *Dirofilaria* sp. "hongkongensis," and *Wolbachia* spp. in the dog population from tribal settlements of Western Ghats, Kerala, India

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Abstract
Leishmaniasis and dirofilariasis are significant emerging zoonotic diseases. This study aimed to assess the prevalence of single and co-infections involving *Leishmania donovani*, *Dirofilaria* sp. "hongkongensis", and *Wolbachia* spp. among dogs from the Kani tribal settlements of Western Ghats, Kerala. In July 2023, blood samples from 75 dogs were collected and analyzed using microscopy, serology, and molecular diagnostics. Molecular analysis using kDNA-PCR and ITS1 PCR identified leishmaniasis in 39 out of 75 dog samples (52.0% [95% CI, 40.2 to 63.7], with phylogenetic analysis confirming the species as *L. donovani*. Additionally, 19 out of 75 dog samples (25.3% [95% CI, 16.0 to 36.7]) produced a ~ 720 bp band for nematode-specific COI amplification, which was confirmed as *Dirofilaria* sp. "hongkongensis". Among the 19 *Dirofilaria*-positive samples, 14 (73.7% [95% CI, 48.8 to 90.9]) tested positive for *Wolbachia* using the *wsp* primer. Among the 75 dogs screened, 12 (16% [95% CI, 08.6 to 26.3]) were co-infected with *Leishmania* and *Dirofilaria*, while 9 (12% [95% CI, 05.6 to 21.6]) were co-infected with all three parasites: *L. donovani*, *Dirofilaria* sp. "hongkongensis", and *Wolbachia* spp. The findings suggest that Kerala is gradually becoming more vulnerable to zoonoses like leishmaniasis and dirofilariasis emphasizing the need for prompt intervention and prevention measures. These results underscore the critical need for continued development of multi-pathogen detection methods, not only in regions where multiple diseases are endemic but also in areas where such diseases may emerge.

Keywords Leishmaniasis · Dirofilariasis · *Wolbachia* · Dogs · Co-infection · Molecular analysis · Kerala

Abbreviations

VL	Visceral leishmaniasis
CL	Cutaneous leishmaniasis
kDNA	Kinetoplast deoxyribonucleic acid
ITS1	Internal transcribed spacer1
EDTA	Ethylenediamine tetra acetic acid
COI	Cytochrome oxidase subunit I
PCR	Polymerase chain reaction
UV	Ultraviolet
NCBI	National centre for biotechnology information
BLAST	Basic alignment search tool
MEGA	Molecular evolutionary genetic analysis

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Introduction

Leishmaniasis and dirofilariasis are two groups of emerging arthropod-borne diseases caused by flagellated protozoans and filarial worms, respectively. Leishmaniasis is transmitted through the bites of female phlebotomine sandflies (Steverding 2017), while dirofilariasis is spread by mosquitoes from the Culicidae family, which serve as biological vectors for the filarial worms, respectively (Simón et al., 2012).

Leishmaniasis is a notable neglected tropical disease that causes fatal and serious implications in humans. It is maintained in multiple hosts including wild, synanthropic, and domestic host reservoirs. India occupied 18% of the global burden of visceral leishmaniasis (VL) in 2020 (Dutta et al. 2023) and is also endemic for cutaneous leishmaniasis (CL). In the past 10 years, Kerala has documented about 50 human

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cases of leishmaniasis which is genetically characterized as *L. donovani* zymodeme Mon-37 in the Western Ghats (Saini et al. 2024a, b). This zoonosis can be symptomatic or asymptomatic depending on the parasite species involved and the host's immune responses. Likewise, some dogs can keep the infection at bay for a long time without displaying any clinical signs, and in some instances, they might even recover on their own without treatment. The clinical symptoms in affected dogs may vary such as generalized non-pruritic alopecia or other dermatological abnormalities, ulcerative lesions, and lymphadenomegaly, but some manifest a variety of clinical spectra that involve any organ (Ribeiro et al. 2013). Leishmaniasis can be transmitted from mother to offspring (transplacental) and through sexual contact (venereal) in dogs. This mode of transmission might play a crucial role in sustaining the disease, particularly when the insect vector responsible for spreading is not present (Svobodova et al. 2017).

Dirofilariasis is a group of zoonotic parasitic infections that affect humans by causing ocular or subcutaneous diseases, and, rarely, pulmonary infection. These infections are caused by helminth nematodes and are vector-borne zoonoses. This disease system primarily exists in European and Asian countries. Over the 5-year duration, the incidence of human cases has increased dramatically from a thousand cases to four thousand cases globally (Simón et al., 2012, Simón et al., 2017). Although humans are considered accidental hosts, there have been at least 100 cases of subcutaneous/ocular dirofilariasis reported in India (Simón et al., 2017). Notably, literature reviews state that Kerala accounts for approximately 55% of cases in the country (Thilakarathne et al. 2023). The coexisting species *Dirofilaria immitis* and *D.repens* are found in many regions, including India. The prevalence rate of both species ranges between 15-60% in animal reservoirs. These reservoirs include animals such as foxes, jackals, or raccoon dogs, which can harbor infections and increase the risk for domestic animals and humans. Among these mammalian hosts, domesticated and wild dogs are the most suitable for adaptation (Simón et al., 2012). Female mosquitoes from different genera act as the primary vectors for dirofilariasis transmission (Fuehrer et al., 2016).

Wolbachia is a genus of endosymbiotic gram-negative bacteria from the order Rickettsiales seen either in groups or alone. These bacteria are found in numerous pathogenic filarial worms of onchocerciasis family that help in the biological development of filaria and are found in all stages of filarial nematode (Latrofa et al., 2023), including those of the genus *Dirofilaria*, which infect both humans and animals. These bacteria are thought to have a mutualistic relationship with their hosts, providing essential metabolites crucial for the nematode survival (Bouchery et al. 2013). Compelling evidence suggests that a symbiotic association exists between these bacteria and their filarial hosts. Persuasive findings suggest that there is an intensification of symptom severity by Wolbachia (Kramer et al. 2008). The symbiosis between *Wolbachia* and *Wuchereria bancrofti*, the primary causative agent of lymphatic filariasis in India, illustrates the nematode's dependence on *Wolbachia* for its survival and reproductive functions (Gayen et al., 2010). This symbiotic dependence provides multiple therapeutic applications including antibiotic depletion of *Wolbachia* as a strategy for treating filarial infections (Aljayyoussi et al., 2017, Quek S et al., 2022). Although morphological methods remain the gold standard for clinical parasitology diagnosis, molecular methods have gained considerable attention for diagnosing parasitic diseases due to their exceptional sensitivity, specificity, and capacity to detect trace amounts of parasitic DNA or RNA (Wong et al. 2014) which is especially useful in detecting co-infections.

Co-infections can result in atypical clinical presentations, making diagnosis, treatment, and prognosis more challenging. The current study focuses on parasite detection and co-infection involving *L. donovani*, *Dirofilaria* sp. "hongkongensis", and *Wolbachia*, and molecular characterization of parasites among the dog population of the Kani tribal settlement, Western Ghats, Kerala.

Methodology

Study area

The study area comprised 14 of the 28 Kani tribal settlements, Western Ghats, Kerala, which collectively house 1477 individuals as per the Census 2011. These settlements are located in the Western Ghats region of Thiruvananthapuram district, Kerala, and were selected based on prior reports of VL/CL human cases (Simi et al. 2010; Kumar et al. 2015). According to the local Panchayat census of 2022, these settlements house 1396 domesticated dogs and 491 stray dogs. The 14 settlements were selected based on feasibility and logistical considerations, using data from a cross-sectional survey conducted in all 28 Kani tribe settlements (Srinivasan et al. 2015). The tribes live in scattered settlements at varying altitudes, ranging from 267 to 2425 ft, with each settlement covering 1.5–2 km^2 and located 5–10 km apart. The terrain is extremely challenging with no transport or communication facilities (Srinivasan et al. 2015), spanning from coordinates 08° 36′ 51.2″ N 077° 09′ 54.9″ E; 08° 37′ 49.7″ N 077° 11′ 29.7″ E (Saini et al. 2024a, b) (Fig. 1). The average minimum temperature recorded was 24.09 °C (ranging from 22.2 °C in January to 30.2 °C in September), and the maximum temperature was 32.1 °C (ranging from 31.1 °C in August to 33.8 °C in March). Monthly rainfall ranged from 15.2 mm in February to 386.9 mm in October, with

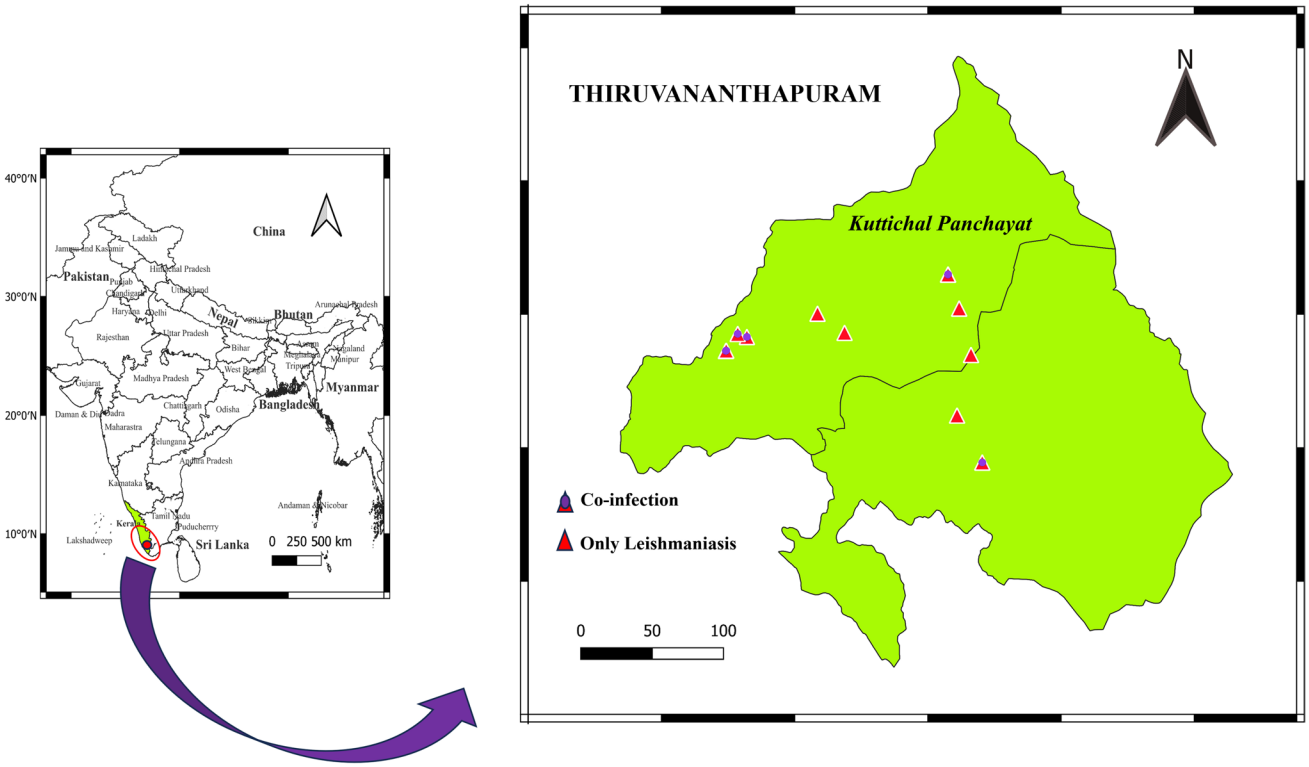

Fig. 1 Settlements of Kani tribes; the red triangle indicates infection with *Leishmania*, and the red triangle along with blue dots indicates co-infection of *Dirofilaria* sp. "hongkongensis", *Leishmania donovani*, and *Wolbachia* spp

an annual average of 2102.8 mm, while relative humidity varied from 79.1 to 88.5% in the morning and 62.9 to 79.1% in the evening (Srinivasan et al. 2015).

Sample collection

In July 2023, venous blood samples were collected from 75 dogs in the chosen settlements through a cross-sectional survey. The sample group consisted of domesticated/stray dogs, aged approximately 3 months to 12 years of both sexes. Detailed information on the geographic location of these settlements was recorded. Additionally, data on the dogs' gender, approximate weight, habitat, and health status, including skin coat condition, age, and owner details, were collected. Around 2 mL of venous blood was drawn from each animal and transferred into EDTA-treated vacutainers for molecular diagnostics. Additionally, 1 to 2 mL of blood was collected in clot-activated tubes for serological testing for leishmaniasis. A fresh smearing of each blood sample was performed on microscopic slides and fixed with methanol soon after the blood collection. The slides were stained with Giemsa (1/10 dilution) and examined for the presence of *Leishmania* amastigotes or nematodes using a compound microscope before proceeding with the molecular diagnosis.

Serological diagnosis

After collection, the samples were transported to the laboratory where serum separation was done by centrifuging at 3000 × *g* for 15 min. For preliminary screening, 20 µL of serum was used to perform the rK39 rapid test (Kalazar Detect™ Rapid Test, InBios, Washington), an immunochromatographic dipstick assay designed for the qualitative detection of antibodies against *L. donovani*, aiding in the presumptive diagnosis of VL.

DNA extraction and molecular characterization of parasites

The genomic DNA was extracted from EDTA-containing blood samples using the DNeasy Blood and Tissue kit (Qiagen, Germany). The DNA was eluted in nuclease-free water and stored in deep freezers until further use. The extracted DNA samples were utilized as templates for the diagnostic PCR for detecting *Leishmania* infection in the samples. A PCR was carried out to amplify the mini-circle kinetoplast DNA (kDNA) in the samples, using a primer set already described by Aransay et al. (2000)—LINR4 (F) – 5′ GGG GTT GGT GTA AAA TAG GG-3′ and the LIN19 (R) – 5′ CAG AAC GCC CCT ACC CG-3′. Each reaction was performed in a

total volume of 50 µL with the following thermal cycling program: an initial denaturation of 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 50 s, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min 20 s. This was followed by a final extension of 72 °C for 7 min. For species characterization, another PCR was performed using the primer set LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′). These primers were used to amplify ITS1 sequences in a 50 µL reaction volume. The thermal cycling program included an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 50 s, annealing at 53 °C for 50 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Subsequently, a PCR–RFLP assay targeting the ITS1 region was conducted (Schonian et al., 2003). The products were digested with the *Hae*III restriction enzyme and visualized in 2.5% agarose gel. *Dirofilaria* sp. "hongkongensis" was already characterized and reported from the aforementioned tribal settlements of Thiruvananthapuram (Saini et al. 2024a, b). The COI barcoding PCR was conducted using a 50 µL reaction mixture with COIF1 and COIR2 primers. The PCR program began with an initial denaturation of 95 °C for 5 min, followed by 38 cycles consisting of denaturation at 95 °C for 50 s, annealing at 51 °C for 1 min, and elongation at 72 °C for 2 min. A final extension step was performed at 72 °C for 7 min (Saini et al. 2024a, b). The samples positives for *Dirofilaria* nematode were screened for the presence of *Wolbachia* using non-strain specific primer pair *wsp*81F and 691 R (Zhou et al. 1998). The PCR amplification, consisting of a 50 µL reaction mixture, was carried out with the following program: Pre-heating at 95 °C for 4 min, followed by 38 cycles of 94 °C denaturation for 1 min, annealing at 55 °C for 1 min 30 s and extension at 72 °C for 1 min, and ends with the final extension at 72 °C for 7 min.

The PCR products were visualized using 1.5% agarose stained with ethidium bromide in a UV transilluminator. The bands were extracted and purified using the gel extraction kit (QIAGEN). The purified products were lyophilized and custom-sequenced by Sanger sequencing. The sequences were submitted to GenBank and phylogenetic analysis was done using MEGA 11. The phylogenetic analysis of ITS1 sequences was conducted by the Neighbor-Joining method using the Kimura-2-parameter algorithm with 1000 bootstrap, which are expressed in units of base substitutions per site.

Results

Microscopy and serology

Giemsa staining of blood smears was performed on a total of 75 dogs. Among these, amastigotes were observed in 28 slides and microfilariae were observed in 9 slides. *Leishmania* amastigotes were 2–3 µm in diameter, with round ovoid bodies inside and near ruptured macrophages (Fig. 2). The microfilariae were initially identified as *Dirofilaria* (Levine 1968) but species-level identification proved difficult due to the similarity in their morphological structures with other related species of *Dirofilaria*. However, the microfilariae were eventually identified and characterized at the molecular level (Saini et al. 2024a, b). Twenty-three out of 75 tests yielded positive results for the rK-39 dip strip.

Molecular diagnosis

Molecular analysis using kDNA-PCR and ITS1 PCR revealed that 39/75 (52%) dog samples were positive for leishmaniasis. The species were confirmed using ITS1 RFLP with *L. donovani* positive patient samples as the positive control. Five samples that tested positive with Giemsa staining were negative for rk-39. However, all samples that were positive for rk-39 also showed positive results in the slide. Separated bands having sizes of 190 bp, 80 bp, and 60 bp were visualized in 2.5% gel (Fig. 3). A few samples were sequenced for ITS1, and the results were analyzed using NCBI BLAST. These sequences were submitted to the GenBank with accession numbers PP754046 to PP754048 and PQ394656 to PQ394658. Phylogenetic trees were created using MEGA 11. The Neighbor-Joining analysis of the ITS1 gene sequences of *L. donovani* from domesticated animals revealed that they are closely related to *L. donovani* from atypical CL and sandflies (*P. argentipes*) (Fig. 4).

A total of 19 out of 75 dog samples (25.3%) yielded a band of interest (~ 720 bp) for the nematode-specific COI amplification. Upon sequencing, the amplicon was confirmed as *Dirofilaria* sp. "hongkongensis" and phylogenetic analysis identified the same species to be closely related to

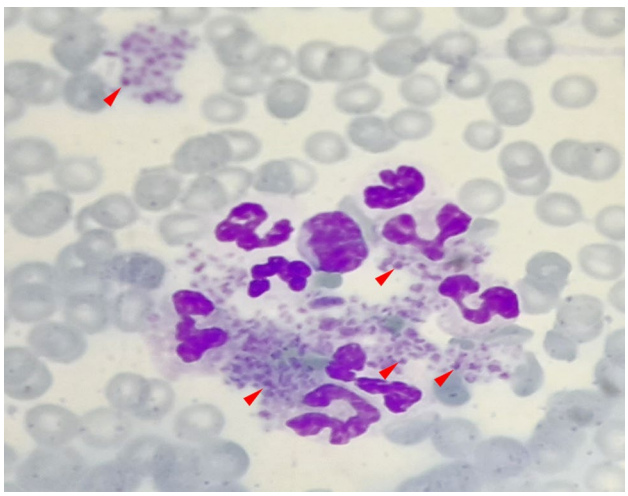

Fig. 2 Array showing amastigotes (100×) ruptured from macrophages after Giemsa staining of blood smear

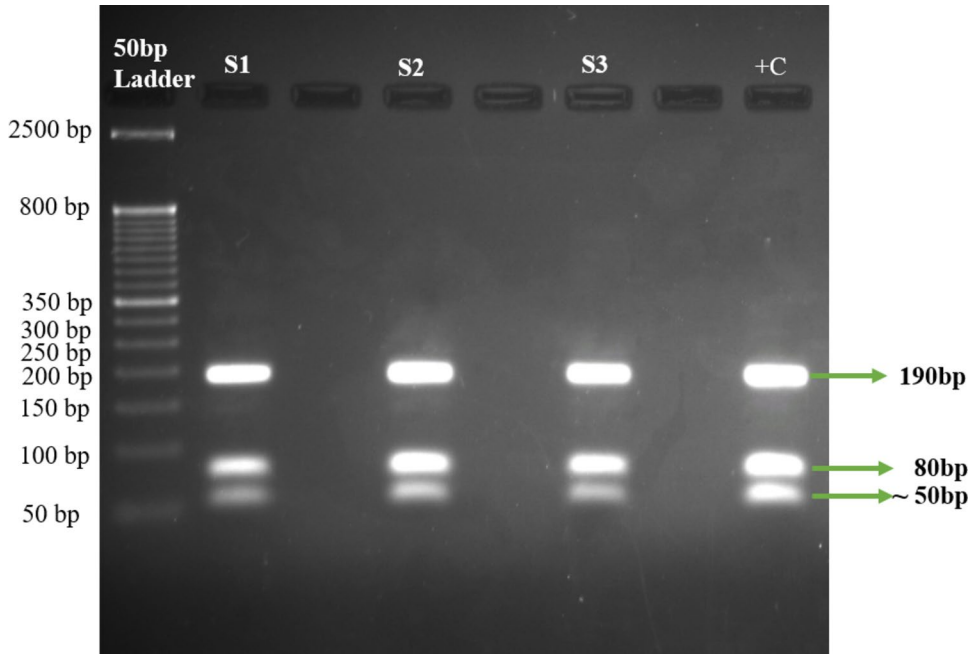

Fig. 3 *Hae*III restricted fragments of ITS1 gene with separated bands size of 190 bp, 80 bp, and 60 bp

D. hongkongensis from both humans and dogs in Kerala (Saini et al. 2024a, b). The 19 *Dirofilaria*-positive samples were tested for *Wolbachia* with the *wsp* primer. Fourteen of these samples yielded the ~ 600 bp band (73.6%), confirming a positive result for *Wolbachia* spp. Out of the 75 dogs screened, 12 dogs (16%) were co-infected with *Leishmania* and *Dirofilaria*, while 9 dogs (12%) were co-infected with all three parasites: *Leishmania donovani, Dirofilaria* sp. "hongkongensis", and *Wolbachia* spp. (Table 1).

Discussion

Kerala is an emerging hotspot for leishmaniasis and an endemic region for dirofilariasis. The present study identified co-infection of *L. donovani*, *Dirofilaria* sp. "hongkongensis", and Wolb*achia* spp. for the first time among the dog population in the Western Ghats using a combination of microscopic, serological, and molecular techniques. In developing countries like India, diagnosis of leishmaniasis primarily relies on microscopic smear examination and serological immunochromatographic tests like the Kala-azar Rapid Test Kit. Although this rapid diagnostic test (RDT) detects human VL antibodies with 100% sensitivity and 97% specificity (NVBDCP, Government of India), this study found that its effectiveness reduced when applied to canine samples.

In this study, nearly half of the surveyed dog population tested positive for *Leishmania* (39/75) using PCR, despite the absence of amastigotes on Giemsa-stained slides (28/39) or detection by the rapid test kit (23/39). These findings highlight the superior accuracy of PCR over traditional methods like morphology and serology, with microscopy showing a sensitivity of 67.7% and a specificity of 87.8%, while the rK-39 rapid test kit demonstrated a sensitivity of 58.9% and a specificity of 100% in comparison to PCR results.

Although microscopic and serological methods are commonly used for diagnosing parasitic infections, it has certain limitations in sensitivity and specificity in canine samples (Gontijo and Melo 2004). The discrepancies observed between these traditional diagnostic techniques and molecular diagnostics in this study further highlight the challenges associated with accurate diagnosis. PCR, however, has demonstrated superior sensitivity and specificity for identifying *Leishmania* species and subspecies, especially in cases with low parasite loads, outperforming conventional methods like direct microscopy and culture (Andrade et al. 2005). This study reinforces PCR as the most reliable diagnostic tool for detecting *Leishmania* in dogs.

In 2010, an incidence rate of 57.14% (4/7) for human CL was reported in the tribal population residing in the Agasthyamala Biosphere Reserve in Kuttichal Panchayat of Thiruvananthapuram (Simi et al. 2010). A subsequent study by Kumar et al. (2015) identified 13 more cases of CL among the same population, identifying *L. donovani* as the causative species. Genetic analysis revealed a close relationship between the local *L. donovani* isolates and those from Sri Lanka, highlighting local endemism within this bio-geographic region. Further entomological studies revealed the natural infection of *L. donovani* in the wild-caught *P. argentipes* (Srinivasan et al. 2016). Additionally,

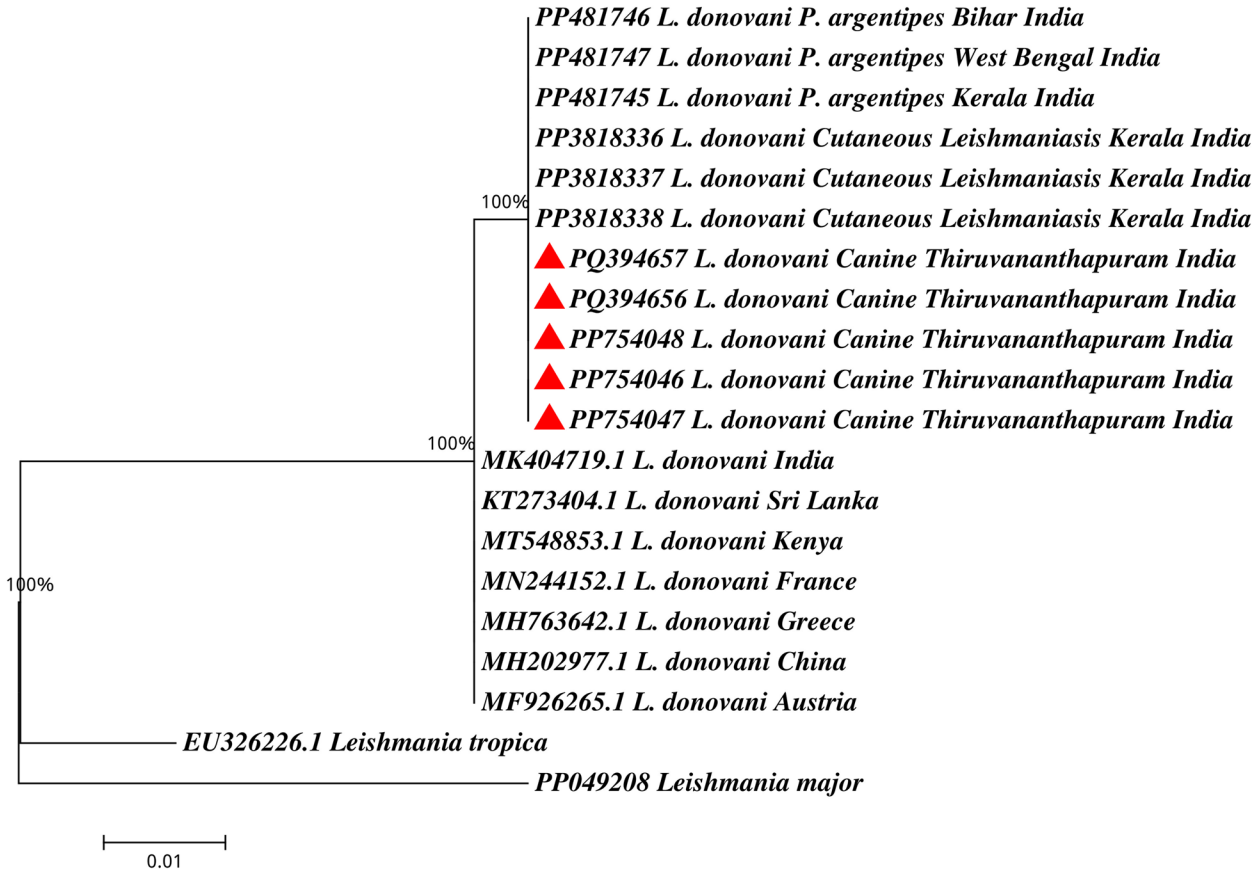

Fig. 4 Phylogenetic tree showing ITS1 gene sequences of *L. donovani* of domesticated animals (red triangle) neighbor-joins with the *L. donovani* from cutaneous leishmaniasis cases (atypical) and sandflies (*P. argentipes*) using Neighbor-Joining methods

the possible zoonotic transmission was identified by Jambulingam et al. (2017) by reporting the *L. donovani* infection in domesticated dogs in the Kani tribal settlements. A study on autochthonous transmission of human CL and VL found that both diseases were caused by Zymodeme MON 37 of *L. donovani* in the Western Ghats region, Kerala (Saini et al. 2020, 2024a). These studies collectively demonstrate the circulation of *L. donovani* across multiple hosts, including the vector, reservoir hosts, and humans, highlighting the interconnected transmission dynamics of the parasite within the ecosystem. In the current study, a blood survey conducted in the same bio-geographic unit in July 2023 revealed the presence of *L. donovani* DNA in 52% (39/75) of the dog population, indicating a significant increase in the prevalence of leishmaniasis among dogs.

Human dirofilariasis is a significant public health concern in Kerala with 78 confirmed cases recorded from January 2005 to March 2020. Orbit, eyelids, and conjunctiva are the most frequently affected body sites. Molecular analysis of the isolates identified *Dirofilaria* sp. "hongkongensis" as the primary cause of human dirofilariasis (Kumar et al. 2021). In addition, various studies have reported the prevalence of microfilariae in domestic dogs across different regions of Kerala, ranging from 7 to 42% (Pradeep et al. 2019). Furthermore, the study by Bhairavi et al. (2025) is the first to report natural infection of *Dirofilaria* sp. among field-collected mosquitoes in India, specifically identifying *Dirofilaria* sp. "hongkongensis" in the Kani tribal settlements of Kerala. Additionally, this is the first instance reporting the presence of *Wolbachia* in *Dirofilaria* sp. "hongkongensis", as *D. immitis* and *D. repens* are the only two species of *Dirofilaria* reported for the presence of endosymbiont bacteria (Maia et al. 2016). Out of 14 settlements studied, 8 settlements were co-infected with all three parasites (*L. donovani*, *Dirofilaria* sp. "hongkongensis", and Wolb*achia* spp.).

The findings of this study underscore the increasing risk of zoonotic diseases, specifically leishmaniasis and dirofilariasis, in the Western Ghats region of Kerala, with co-infections involving *Wolbachia* spp. adding complexity to disease dynamics. Co-infection of *L. infantum*, *D. immitis*, and *Wolbachia* spp. has been reported in Greece, Italy, and Portugal (Maia et al. 2016; Ntais et al. 2016, Latrofa et al., 2023). While co-infection of *Leishmania* sp., *Dirofilaria* sp., and *Wolbachia* sp. has been reported in the literature

Table 1 Details of infection among dog population across various settlements

Area	No. of dogs surveyed	*Leishmania* positives	*Dirofilaria* positives	*Wolbachia* positives (out of the tested 19 *Dirofilaria* positive samples)	Co-infection of *Leishmania* and *Dirofilaria*	Co-infection of *Leishmania*, *Dirofilaria* and *Wolbachia*
Ayiramkal	4	1	-	-	-	-
Cherumankal	9	4	1	1	1	1
Chonampara	6	3	3	2	1	-
Kaithode	3	2	1	1	-	1
Kamalakam	6	3	3	2	3	2
Keezheamala	4	1	1	1	-	-
Komba	2	2	1	1	1	1
Kombidi	5	3	1	-	1	-
Kunnadi	13	9	5	4	3	2
Melaamala	2	1	-	-	-	-
Mukothivayal	5	3	-	-	-	-
Podium	11	5	3	2	2	2
Thannipara	2	1	-	-	-	-
Vlavila	3	1	-	-	-	-
Total	75	39	19	14	12	9
Infection rate (95% confidence interval)		52% (40.2–63.7)	25.3% (16.0–36.7)	73.7% (48.8–90.9)	16% (08.6–26.3)	12% (05.6–21.6)

before, the mechanism of their interaction remains poorly explored. Latrofa et al. (2023) suggested that the presence of *D. immitis* and *Wolbachia* may influence the development of patent leishmaniasis since co-infected dogs had lower antibody titers of *L. infantum*. Detailed studies in this regard may reveal valuable information on host specificity, intensity of infection, display and severity of symptoms, and potential methods for concomitant treatment. Efficient strategies can be devised for the simultaneous eradication of multiple parasites. By improving our ability to simultaneously detect and identify various pathogens, we can enhance surveillance, improve response times, and better manage outbreaks, ultimately safeguarding public health across diverse settings. Apart from the obvious veterinary consequences, the prevalence of *Leishmania* and *Dirofilaria* poses a serious threat to the human populace and demands urgent action. Despite the high prevalence of infection, most dogs remain asymptomatic for either zoonosis. This is concerning, as unnoticed infections can persist for extended periods, heightening the risk of transmission to humans. Furthermore, the detection of these zoonoses emphasizes the need for detecting pathogens and reporting cases in the human population of these settlements. Given that these settlements are isolated from the general population, these findings emphasize the critical need for detecting pathogens and uncovering concealed cases among people.

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Author contribution Prasanta Saini: Conceptualization, design of the Study, Project administration, Supervision, Investigation, funding acquisition, Writing- review and editing. Sivalaxmi B and Haritha HA: Methodology, data curation, Writing original draft, Validation. Harish Kumar Shah and Fathima P A: Software, Writing – review and editing. Ajithlal P M: Methodology, Investigation, Software, Formal analysis, Validation, writing- review and editing. Manju Rahi: Resources and Writing – review and editing.

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Data availability The data can be obtained from the corresponding author upon a request.

Declarations

Ethical approval The ethical clearance for the study was obtained from the Indian Council of Medical Research-Vector Control Research Centre, Puducherry (approval no. ICMR-VCRC/IAEC/2021-A/3/2022-B/6).

Consent to participate Not applicable.

Consent for publication All authors have read the final version of the manuscript and gave their consent for publication.

Competing interests The authors declare no competing interests.

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