



Original article

***Dirofilaria hongkongensis* – A first report of potential zoonotic dirofilariasis infection in dogs from Tamil Nadu**S. Gowrishankar^{a,*}, M. Aravind^a, Sushmita Sastya^a, Bhaskaran Ravi Latha^a, P. Azhahianambi^a, S Vairamuthu^b, C. Jayanthi^c^a Department of Veterinary Parasitology, Tamil Nadu Veterinary and Animal Sciences University, Chennai 600007, India^b Centralised Clinical Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai 600007, India^c Department Of Clinics, Madras Veterinary College, Tamil Nadu Veterinary And Animal Sciences University, Chennai 600007, India

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ABSTRACT

Dirofilariasis, is a mosquito borne anthro-zoonotic disease caused by the filarial nematodes of the genus *Dirofilaria*. The current paper reports a species of *Dirofilaria*, which has not been recorded in Tamil Nadu, in addition to differentiating it from the existing species of *Dirofilaria* spp. through morphological and molecular techniques. Micrometry of the microfilaria detected by Knott's technique as well as in Giemsa stained blood smears revealed that the length was on an average 280 µm to 310 µm whereas the width was 5 to 8 µm. The microfilariae were unsheathed with blunt heads and tapering tails. For molecular confirmation, the blood sample was subjected to genomic DNA isolation. PCR amplification using specific primers for *D. hongkongensis* was carried out using thermal cycler with the isolated genomic DNA as template. Molecular analyses revealed the band size of about 230 bp which confirmed the presence of *D. hongkongensis*. Phylogenetic analysis was done using UPGMA and MLM method. *Dirofilaria* is recognized as a zoonotic entity throughout the world, and dirofilariasis should be considered an emerging zoonosis in southern India. The control of ubiquitous mosquito population is essential to prevent the transmission of microfilaria from animals to arthropods and from arthropods to humans.

1. Introduction

Dirofilariasis, which is caused by filarial nematodes of the genus *Dirofilaria*, is an anthro-zoonotic disease transmitted through the bite of mosquitoes (Reddy, 2013). In dogs, *Dirofilaria immitis*, *Dirofilaria repens*, *Acanthocheilonema reconditum*, *Acanthocheilonema dracunculoides*, *Brugia malayi*, *Brugia ceylonensis* and *Brugia pahangi* (Irwin, 2002; Rishniw et al., 2006 and Simón et al., 2007) have been commonly reported. In India, *Dirofilaria* spp., *Acanthocheilonema* spp. and *Brugia* spp. have all been reported (Gogoi, 2002 and Ananda et al., 2006). For *D. immitis* and *D. repens*, *Culex*, *Aedes*, *Anopheles*, *Armigeres* and *Mansonia* species of mosquitoes act as a vector while the flea, *Ctenocephalides* spp. and the lice *Heterodoxus* spp. are vectors of *A. reconditum* (Nelson, 1962). The biting fly, *Hippobosca longipennis*, acts as a vector for *A. dracunculoides* (Nelson, 1963). Ticks are also presumed to act as vectors (Joseph et al., 2011).

Dirofilaria immitis (Leidy, 1856), the heartworm of dogs, is one of the most important and pathogenic filarial nematodes, inhabiting the right ventricle and pulmonary artery of dogs causing congestive heart failure

(CHF). In humans, *D. immitis* infection is very rare. If the condition occurs, it is usually associated with pulmonary lesions. Radiologically, coin like lesion in the lung can be visualized. *Dirofilaria repens* seen in subcutaneous tissues of dogs can also accidentally infect humans, causing subcutaneous dirofilariasis. Although most infections with *D. repens*, *Acanthocheilonema* spp. and *Brugia* spp. have minimal veterinary significance, they all have the potential to infect humans and thus are important from the public health point of view. In Tamil Nadu, *D. repens* has been commonly reported in dogs.

Dirofilaria hongkongensis, existing in different parts of world has not been diagnosed and reported in India till date. The current study detected *D. hongkongensis* for the first time in Tamil Nadu in addition to differentiating it from the existing species of *Dirofilaria* spp. through morphological and molecular techniques. *D. hongkongensis* is a zoonotic parasite which causes lymphadenopathy, abdominal swelling and subconjunctival mass in humans.

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2. Materials and methods

Blood samples were collected in EDTA vials during June 2017 to May 2018 from dogs ($n = 3$) attending the Small animal out-patient unit, Madras Veterinary College Teaching Hospital with anamnesis of pyrexia, lymphadenopathy, anorexia, labored breathing, nocturnal cough and exercise intolerance. Immediately after collection of blood, it was aseptically transported to the Central Clinical Laboratory, Madras Veterinary College. In the laboratory, about 20 μ l of the blood was placed on a clean grease free slide to which a drop of normal saline was added and mixed. A cover slip was placed on the slide and examined under low power (4 \times , Leica, Germany) to screen for extracellular parasites. The Knott's concentration technique was performed to study the micrometry as well as morphology of the microfilaria. Blood smears were also prepared and stained with Giemsa stain (Sigma-Aldrich, Germany) and examined under oil immersion (100 \times , Leica, Germany) to identify the extracellular microfilaria based on the morphological characters.

For molecular confirmation, the blood sample (200 μ l) was subjected to genomic DNA isolation by QiagenDNeasy kit method (Germany) as per manufacturer's protocol. The DNA was eluted in 40 μ l elution buffer and used as a template for PCR.

3. PCR based detection

Since the morphology of microfilariae corresponded to that of *D. hongkongensis*, PCR amplification was carried out for *D. hongkongensis* targeting ITS1–5.8S gene using a set of primers as given in Table 1. The PCR was performed in a final volume of 20 μ l containing 10 μ l of PCR Master-mix (Amplicon, Red dye master mix), which had all the constituents (1.5 mM of MgCl₂, 10 mM of dNTP mix and 1 U/ μ l of Tag DNA polymerase), 1.0 μ l of each 10 μ M primer, 5.0 μ l template DNA and 3 μ l Nuclease free water. The reaction was performed with the following cyclical conditions in a thermal cycler (Eppendorf, USA) as shown in Table 1. Amplicons were resolved in ethidium bromide-stained agarose gel (1.5%) and sized by comparison with Gene Ruler™ 50-bp DNA Ladder (Thermo-Scientific) as molecular marker. Gels were photographed using Gel Doc 2000 (BioRad, Hercules, CA, USA). PCR product was excised from the gel, purified using Qiagen kit (Germany) and sequenced using the Sanger's method (Eurofin, Bangalore). Their nucleotide sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (<http://www.ncbi.nlm.nih.gov>). Phylogenetic tree for nucleotides of Tamil Nadu isolate was constructed using Mega 7.0 software by Maximum likelihood method and UPGMA method to understand its relationship with other referral isolates.

4. Result

The wet film examination revealed the presence of motile extracellular microfilariae in all three animals. Knott's concentration technique revealed numerous methylene blue stained microfilariae. Micrometry of the microfilaria detected by Knott's technique as well as Giemsa stained blood smears of 23 microfilariae revealed that the length was on average 280 μ m to 310 μ m whereas the width was 5 to 8 μ m. The microfilariae were unsheathed with blunt heads and tapering tails (Fig. 1).

Table 1

PCR Primers and cyclical conditions for *D. hongkongensis*.

S. no	Parasite	Primers	PCR conditions	Product size	Reference
1.	<i>D. hongkongensis</i> (Nested)	DH ITSF, 5'AAACGGTGATATTCGTTGGTGT-3' DH ITSr, 5'GC (TAAGAGTTAAATATTTTTGTT-3'	Initial denaturation- 94 °C for 1 min Denaturation- 94 °C for 30 s Annealing- 55 °C for 1 min Extension- 72 °C for 1 min Final extension at 72 °C for 10 min	237 bp	To et al., 2012

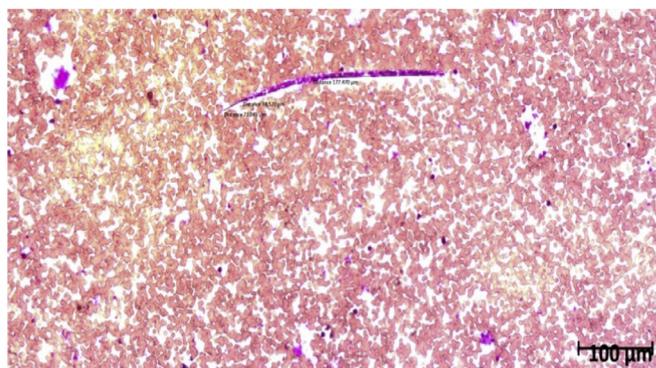
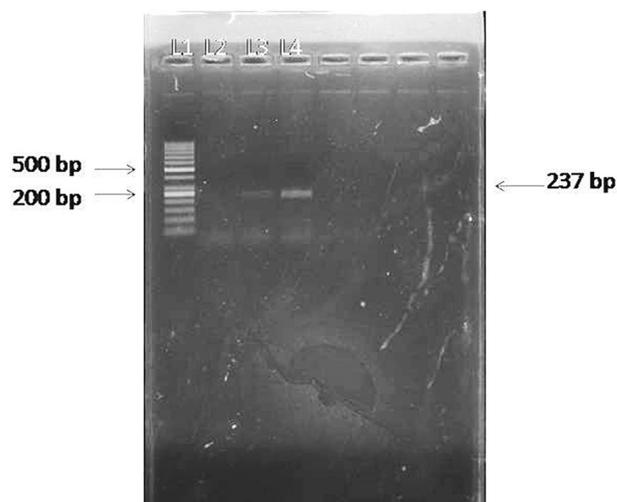


Fig. 1. Microfilaria in giemsa stained blood smear (100 \times).



L1 - 50 bp Ladder, L2 – Negative control, L3 & L4 – Positive for *D. hongkongensis*

Fig. 2. Molecular identification of *D. hongkongensis* microfilaria.

Molecular analyses revealed the band size of about 230 bp, which confirmed the presence of *D. hongkongensis* (Fig. 2). The sequence obtained showed 99% homology to *D. hongkongensis* sequence available in Genbank. The sequence obtained was submitted to Genbank and accession number (MH590660) was obtained. Results revealed that the Tamil Nadu isolate of *D. hongkongensis* was more closely related to the Hong Kong isolate (Figs. 3 and 4).

5. Discussion

The current study reports a zoonotic *D. hongkongensis* microfilaria in dogs from Tamil Nadu. The pathogenicity and pathology of *D. hongkongensis* in dogs or other animal hosts is unknown. If this species is proven to be pathogenic in animals, new antigen detection tests or molecular techniques might be required. To the best of our knowledge, this is the first study that uses gene sequencing to demonstrate the relationship between the canine reservoirs of *Dirofilaria* in the same geographical area at the molecular level. All *Dirofilaria* spp. are known

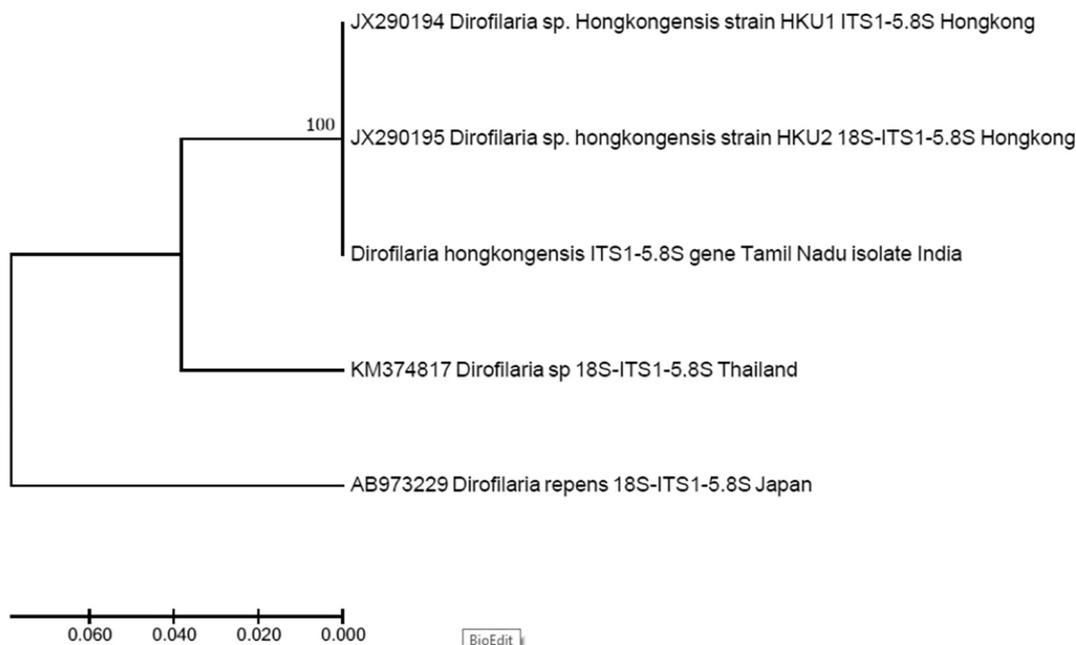


Fig. 3. Phylogenetic analysis by UPGMA method.

to require an arthropod vector for transmission; it is likely that the life cycle of *D. hongkongensis* also involves such vectors. However, we do not yet know the vector transmitting this *Dirofilaria* species (To et al., 2012). Epidemiological studies suggest that human dirofilariasis occurs in areas with high rates of canine infection (Winkler et al., 2017).

India has a wide range of climatic zones, from montane (cold, wet alpine) and semi-arid regions to the wet tropics, which make it suitable for a diverse range of vectors and pathogens of medical and veterinary importance, whose transmission and geographical distribution are closely linked to regional temperature, rainfall and humidity. The climate is conducive for parasite survival and transmission in various parts of the country. Although other species of *Dirofilaria* have been extensively studied in India, *D. hongkongensis* has not previously been reported. Borthakur et al., 2015 reported 18.03% prevalence of *D.*

immitis in Northeastern states of India. He also reported that 30% of dogs harbored heartworm infection out of 240 dogs in North East India during postmortem examination. Two recent surveys of microfilaraemic dogs in Kerala and Karnataka States in southern India found only *D. repens* at a prevalence of 7% (n = 160) and 21% (n = 400) respectively. Joy et al., 2017 reported *D. repens* in a twelve year old girl in Kerala. Dogs are considered to be the main reservoir for human *D. repens* and *D. immitis* infections because adult worms can survive in dogs for many years, resulting in high levels of microfilaremia, which allows efficient transmission to mosquito vectors.

Although the morphological and micrometry methods are available for identification of *Dirofilaria* spp. it cannot be used as an accurate or reliable method because the dimensions and morphological characters of most *Dirofilaria* spp. often overlap which potentially misleads the

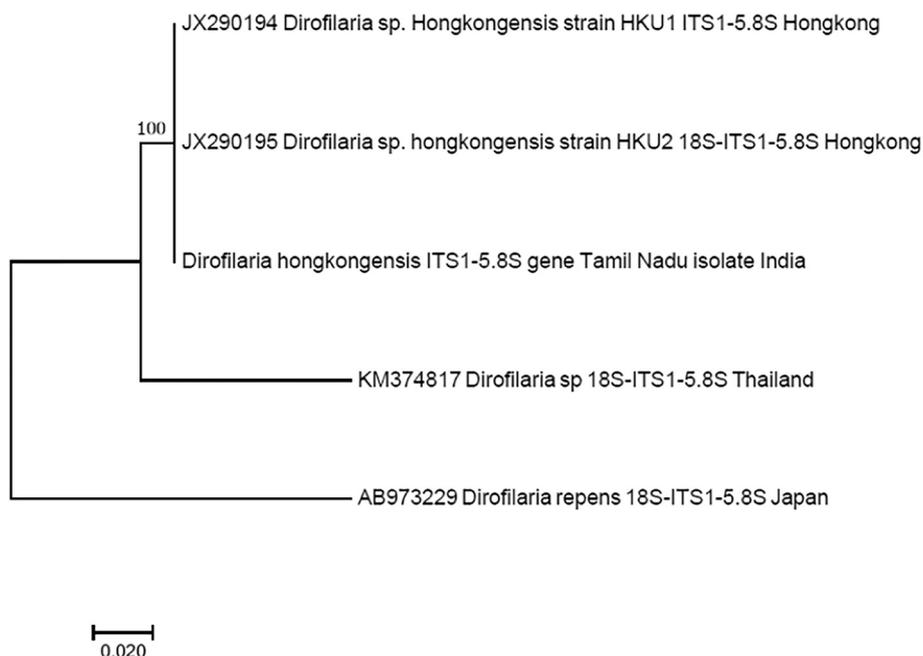


Fig. 4. Phylogenetic analysis by MLM method.

identification. Moreover, it may be difficult to detect multiple or mixed infections with more than one species of filarial worm when using morphological techniques alone. The current study used both morphological and molecular methods to aid in definitive diagnosis of the species of microfilaria. Hence, both morphological and molecular identification must be done to identify the *Dirofilaria* spp. present in infected animals. Despite the limited number of surveys performed, veterinarians in India strongly believe that *D. immitis* is confined to the northeast and *D. repens* to southern India. As a one health approach is developing, physicians must also be aware of the new species of *Dirofilaria* in Tamil Nadu apart from *Brugia* spp. and *D. repens*.

6. Conclusion

Zoonotic filariasis is increasingly recognized in different countries. Dirofilariasis should be considered as an emerging zoonosis in southern India. Climate change may contribute to the changing epidemiology and increasing incidence of the disease. As in the case of other vector-borne infections, accurate knowledge of the natural reservoir and vectors is crucial for control and prevention of the disease.

Declaration of Competing Interest

None.

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