Infection, Genetics and Evolution 67 (2019) 121–125

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Whether *Dirofilaria repens* parasites from South India belong to zoonotic *Candidatus Dirofilaria hongkongensis* (*Dirofilaria* sp. *hongkongensis*)?

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ARTICLE INFO

Keywords:
Dirofilariosis
Candidatus Dirofilaria hongkongensis
Cytochrome oxidase subunit I (*COI*) gene
South India
Zoonosis

ABSTRACT

The canine and zoonotic dirofilarioses are arthropod-borne parasitic infections caused by nematodes of the genus *Dirofilaria*, infecting canines, felines and humans throughout the world. *Dirofilaria repens* was considered as the most common cause of human dirofilariosis in Kerala. In the present study, molecular characterization of *Dirofilaria* isolates causing dirofilariosis in humans, dogs and jackal from Kerala, South India was undertaken by performing sequence and phylogenetic analysis based on cytochrome oxidase subunit I (*COI*) gene. The live worms from swellings/ nodules in subconjunctiva or subcutaneous tissue or scrotum were recovered from humans ($n = 3$), dogs ($n = 4$) and one jackal. The PCRs targeting a repetitive fragment, 18S rRNA and *COI* genes yielded products of ~246 bp, ~875 bp and ~350 bp respectively in all the samples. The sequence analysis of 18S rRNA gene revealed the closest identity (98 to 99%) with an already published sequence of *D. repens* isolated from a human in Japan. However, based on the sequence and phylogenetic analysis of partial sequences of *COI* gene, the *Dirofilaria* infecting both animals (dogs, jackal) and humans native to Kerala, South India were identified as genetically conserved and closely related to *Dirofilaria* sp. *hongkongensis*. Hence, the results of the present study suggested the existence of *Candidatus Dirofilaria hongkongensis* (*Dirofilaria* sp. *hongkongensis*) in Kerala, South India causing zoonotic filariosis in canines and humans.

1. Introduction

Dirofilariosis is a mosquito-borne parasitic infection caused by nematodes of the genus *Dirofilaria* Railliet and Henry, 1911, taxonomically positioned under the phylum Nemathelminthes, class Nematoda, subclass Secernentea, order Spirurida, superfamily Filarioidea, family Onchocercidae. These parasites are transmitted through mosquitoes of the genera *Culex, Anopheles, Aedes, Armigeres, Stegomyia*, *Coquillettidia, Ochlerotatus* and *Mansonia* (Anyanwu et al., 2000; Otranto et al., 2013). To date, about 27 valid species of *Dirofilaria* are reported affecting canines, felines and other mammals throughout the world and 15 species of questionable validity (Canestri et al., 1997). However, only a few species are known to cause zoonotic dirofilariosis, viz., *Dirofilaria repens, D. immitis* (dogs, cats, fox, other wild canids), *D. tenuis* (raccoons), *D. ursi* (bears), *D. striata* (panther, bobcats) and *D. subdermata* (porcupines) (Beaver et al., 1987; Horst, 2003; Warthan et al., 2007; Chandy et al., 2011; Simón et al., 2012). Of these filarial nematodes, *D. immitis* and *D. repens* are the two important species causing canine dirofilariosis throughout the world; which are also identified as the etiological agents of zoonotic human dirofilariosis in which humans are the dead-end host (Dissanaike, 1979).

Dirofilaria repens infection in dogs is asymptomatic, moderately pathogenic and adult worms reside in painless subcutaneous or subconjunctival nodules (Bredal et al., 1998). Presence of adult worms and/or microfilariae in the skin produces apparent dermatological signs in dogs (Živičnjak et al., 2006). *D. repens* infection in humans results in the subcutaneous lump in the affected area which most commonly includes; face and conjunctiva of the eye and sometimes chest wall, upper arms, thighs, abdominal wall and male genitalia (Simón et al., 2012). Sometimes it may complicate to nodules, painful inflammatory swellings and abscess formation in subcutaneous tissue. Most of the human ocular infections included scleritis, subconjunctival cysts, eyelid

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https://doi.org/10.1016/j.meegid.2018.10.019
Received 23 May 2018; Received in revised form 16 October 2018; Accepted 25 October 2018

Table 1
Filarid worms collected from humans and animals.

Sl. No.	Place	Sample details		
		Host	Site of collection	Length (cm)
1	Wayanad, Kerala	Jackal	Subcutaneous abscess (Skin)	8.6
2	Wayanad, Kerala	Dog	Subcutaneous nodule (skin)	11.2
3	Wayanad, Kerala	Dog	Sub-conjunctival space (eye)	12
4	Wayanad, Kerala	Dog	Sub-conjunctival space (eye)	28
5	Thiruvananthapuram, Kerala	Dog	Scrotum	14
6	Thiruvananthapuram, Kerala	Human	Sub-conjunctival space (eye)	5.8
7	Thiruvananthapuram, Kerala	Human	Subcutaneous nodule on leg	9
8	Thiruvananthapuram, Kerala	Human	Sub-conjunctival space (eye)	13.8

swelling, involvement of the anterior chamber and motile swelling on the conjunctiva. In addition, scrotal dirofilariosis is also reported from both humans (Pampiglione et al., 2001; Soussi et al., 2004; Fleck et al., 2009; Singh et al., 2010) and dogs (Demiaszkiewicz et al., 2009; Ravindran et al., 2016).

The canine and human dirofilarial infections caused by *D. repens* are most prevalent in zones with moderate climate viz., Europe, Africa, Middle Eastern and Asian countries including India. In Europe, over 1500 cases of human dirofilariosis due to *D. repens* were described, of which most cases were reported from Italy (Pampiglione and Rivasi, 2000; Genchi et al., 2011; Cielecka et al., 2012). In Asia, Southern countries like India and Sri Lanka are endemic for the canine and zoonotic dirofilariosis caused by *D. repens* (Rani et al., 2010). Generally, it is believed that *D. immitis* is predominantly prevalent and restricted to north-eastern states of India (Bortharkur et al., 2015) and *D. repens* is confined to southern parts of the country (Gogoi, 2002; Ananda et al., 2006; Rani et al., 2010). In India, the first case of human ocular dirofilariosis (Joseph et al., 1976) and subcutaneous dirofilariosis caused by *D. repens* (Senthilvel and Pillai, 1999) were recorded from Kerala. In the recent years, human dirofilariosis due to *D. repens* is considered as a fast emerging zoonotic disease in India (Bhat et al., 2003; Reddy, 2013), especially in Kerala (Sekhar et al., 2000; Sabu et al., 2005; Singh et al., 2010; Sanjeev et al., 2011). However, few cases were reported from other states like Assam (Nath et al., 2010; Singh et al., 2010), Orissa (Reddy, 2013), Karnataka (Nadgir et al., 2001; Kotigadde et al., 2012), Gujarat (Patel et al., 2014), Maharastra (Choure et al., 2015; Kombade et al., 2015) and Tamilnadu (Sathyan et al., 2006). Moreover, the prevalence rate of microfilariae in domestic dogs at different regions of Kerala ranged between 7 and 42% in different studies (Saseendranath et al., 1986; Radhika, 1997; Sabu et al., 2005; Ravindran et al., 2014).

Few reports of cases caused by *D. tenuis* (Bhat et al., 2003) and *D. conjunctivae* (Joseph et al., 1976) in humans in India were also available in literature. *D. tenuis* is principally a parasite infecting subcutaneous tissues of racoons and believed to be restricted only to USA (Orihel and Beaver, 1965). Recently, from Hong Kong, China a novel *Dirofilaria* species, *Candidatus Dirofilaria hongkongensis* (*Dirofilaria* sp. *hongkongensis*) was proposed as the causative agent of subcutaneous or subconjunctival dirofilariosis in humans and dogs based on the phylogenetic analysis of the cytochrome oxidase subunit 1 (*COI*), 12S rRNA and 18S-ITS1–5.8S gene (To et al., 2012; Kwok et al., 2016). *Dirofilaria* species with similar ITS-1 sequences were reported from humans and dogs in Europe (Suzuki et al., 2015; Liesner et al., 2016).

Even though Kerala is a hot-spot for human filariosis due to *D. repens*, very few studies were undertaken to characterize the worms (Nazar et al., 2017). Hence, in the present study, we describe the occurrence and molecular confirmation of *Dirofilaria* genotypes similar to that of the newly proposed species *Dirofilaria* sp. *hongkongensis* from dogs, jackal and humans in Kerala; a South Indian state, based on the sequence and phylogenetic analysis of cytochrome oxidase subunit I (*COI*) gene.

2. Materials and methods

2.1. Ethics statement

All the worm samples used in the present study were recovered from humans/dogs/jackal during the surgical procedures. They were sent to Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookode, Kerala for species identification. Therefore, no approval of ethical committee was obtained. All the samples were used after anonymization of patient data and obtaining patient/owner's consent.

2.2. Dirofilaria sp.

Live worms of *Dirofilaria* sp. were recovered surgically from the human ($n = 3$), dog ($n = 4$) and one jackal. Swellings/nodules in subconjunctiva or subcutaneous tissue or scrotum were observed in all the cases. Details of the worms collected from the animal/human patients are given in the Table 1.

2.3. Polymerase chain reaction (PCR)

Genomic DNA from the whole worms was isolated by the conventional phenol chloroform isoamyl alcohol method (Sambrook and Green, 2012). Initially, amplification of *D. repens* specific 246 bp repetitive fragment was performed for confirmation of the species (Vakalis et al., 1999). Further, molecular characterization of these worms was performed by polymerase chain reaction (PCR) amplification and sequencing of 18S rRNA and cytochrome oxidase subunit I (*COI*) genes (Suzuki et al., 2015) specific for the genus *Dirofilaria* sp. The PCR assay was carried out in 25 μL volume containing 2.5 μL 10 × PCR buffer (Genaxy, New Delhi), 0.25 mM dNTP, 20 pmol of each primer, 1.5 U *Taq* polymerase (Genaxy, New Delhi) and 5 μL of template DNA. The primer details and cycling conditions for all PCR amplifications are given in Table 2. The PCR products were visualized after electrophoresis using 2% agarose gel.

2.4. Sequencing

The PCR products were purified using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel, Germany) and were sent for sequencing (SciGenom Labs, Kochi, Kerala). After sequencing of the 18S rRNA and *COI* gene products, the homology search was performed using NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST). The partial nucleotide sequences of 18S rRNA and *COI* genes were deposited in GenBank sequence database under the accession numbers (18S rRNA gene: MF776625, MF776631, MF776665, MF776709, MF776710, MG657262, MG657263, MG780293 and *COI* gene: MF784454, MF784455, MF784456, MF784457, MF784458, MG676448, MG676449, MG783342).

Table 2
Primers and cycling conditions for polymerase chain reactions.

Regions amplified	Sequence (5′-3′)	Cycling conditions	Reference
246 bp repetitive fragment	F-CCGGTAGACCATGGCATTAT R-CGGTCTTGG ACGTTTGGTTA	94° for 5 min 40 cycles of 94° for 30 s 55° for 30 s 72° for 25 s 72° for 5 min	Vakalis et al., 1999
18S rRNA	F-CCATGCATGTCTAAGTTCAA R-TCGCTACGGTCCAAG AATTT	94° for 5 min 35 cycles of 94° for 30 s 60° for 30 s 72° for 1 min 72° for 5 min	Suzuki et al., 2015
COI gene	F-GCTTTGTCTTTTTGGTTTACTTTT R-TCAAACCTCCAATAGTAAAAAGAA	94° for 5 min 35 cycles of 94° for 30 s 54° for 30 s 72° for 1 min 72° for 5 min	Suzuki et al., 2015

2.5. Phylogenetic analysis

The partial sequences of the *COI* gene of *Dirofilaria* species in the present study were subjected to multiple sequence alignment with previously published sequences from GenBank using the Clustal W programme in MEGA7 (Kumar et al., 2016). The following sequences were used for making phylogenetic tree: *Dirofilaria* sp. *hongkongensis* (KX265050, KY750548, JX187591, JX187592, JX187593, JX1875954, JX187595, JX187596, JX187597, JX187598, JX187599), *D. repens* (AB973225, AM749230, AM749231, AM749232, AM749233, KR998259, KR780980), *D. immitis* (AJ271613, DQ358815, AB973227, AM749226, AM749227, AM749228, AM749229, NC005305), sequences from the present study and *Onchocerca volvulus* (AM749285) as outgroup. The evolutionary history of *COI* gene sequences was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Support for the topologies was tested by boot strapping over 1000 replications.

3. Results

All the eight worms collected were morphologically identified as *Dirofilaria repens*. The 246 bp repetitive fragment specific for *D. repens* was amplified in all eight worm DNA samples. The PCR targeting 18S rRNA and *COI* genes yielded single bands of ~875 bp and ~350 bp products. The NCBI BLAST analysis of 18S rRNA gene of all *Dirofilaria* isolates revealed the closest identity (98 to 100%) with an already published sequence (AB973229) of *D. repens* isolated from a woman who travelled in Europe. However, the partial sequences of *COI* gene of all *Dirofilaria* isolates used in the present study showed maximum identity (98 to 99%) to *Dirofilaria* sp. *hongkongensis* (KX265050/ KY750548) isolated from humans who travelled in India, followed by sequence identity of 95 to 96% for *D. repens*, up to 92% for *Onchocerca* spp. and $< 90\%$ for *D. immitis*.

The phylogenetic analysis of *COI* gene sequences of *Dirofilaria* spp. revealed that all the *Dirofilaria* isolates from Kerala, South India were genetically conserved and were clustered in clade 1 comprised of *Dirofilaria* sp. *hongkongensis* from Hong Kong and India. They were distinctly separated from *D. repens* isolates (clade 2) from Italy, Estonia, Czech Republic and Japan. It was also inferred that *Dirofilaria* sp. *hongkongensis* group (Clade 1) formed a sister clade with *D. repens* (Clade 2). All the *D. immitis* isolates were clustered in Clade 3 and *O. volvulus* formed an out group. The phylogenetic analysis of *COI* gene sequences is depicted in Fig. 1.

4. Discussion

Species identification of filarid worms based on morphology is difficult especially in case of immature worms. Very often, misidentification of *Dirofilaria* species may occur due to the limited expertise of technicians especially under poor sampling conditions like alteration in parasite structures and decomposition of worms inside nodules (Suzuki et al., 2015). Specificity of the serological methods is also low when compared to DNA based molecular techniques (Rishniw et al., 2006). Hence, in the present study, molecular characterization of *Dirofilaria* isolates of humans, dogs and a jackal from Kerala was undertaken by performing the phylogenetic analysis of partial nucleotide sequences of cytochrome oxidase subunit 1 (*COI*) gene. The *COI* gene sequence used for genetic characterization in the present study, was used previously for the differentiation of filarial species (Dang et al., 2010; Ondriska et al., 2010; Ferri et al., 2009; Hrckova et al., 2013; To et al., 2012; Yilmaz et al., 2016).

Poppert et al. (2009) described *D. repens* infection with subcutaneous nodule and concomitant meningoencephalitis in a German lady who travelled in India and Sri Lanka, and speculated that the *D. repens* was a different strain (GQ292761) showing 3% deviation from other *D. repens* sequences based on the genetic analysis of highly conserved 12S rRNA gene sequence. Later, a new zoonotic species, *Candidatus Dirofilaria hongkongensis* (*Dirofilaria* sp. *hongkongensis*) infecting humans and dogs was described (To et al., 2012) based on the sequence homology of the *COI* and 18S-ITS 1–5.8S genes in Hong Kong, China. Interestingly, Yilmaz et al. (2016) based on phylogeny of the *COI* gene sequence, confirmed the presence of *Candidatus Dirofilaria hongkongensis* in a person native of Germany who travelled in South India. Recently, Winkler et al. (2017) reported *Candidatus Dirofilaria hongkongensis* causing ocular dirofilariosis in a person native of Austria after traveling to India. Nazar et al. (2017) described the genetic relationship of human *Dirofilaria* isolates of Kerala with *Dirofilaria* sp. *hongkongensis* based on the sequence and phylogenetic analysis of *COI* gene. However, human and canine *Dirofilaria* isolates from Kerala were not previously compared.

In the present study, all the *Dirofilaria* worms (from animals and humans) were identified as genetically conserved within the geographical region and were closely related to *Dirofilaria* sp. *hongkongensis* group in clade 1 (Fig.1) based on the sequence and phylogenetic analysis of partial sequences of *COI* gene. This might suggest the existence of *Dirofilaria* sp. *hongkongensis* in Kerala, South India in addition to *D. repens*. It can, therefore be concluded that *Dirofilaria* sp. from Kerala in the present study and *Dirofilaria* sp. *hongkongensis* (from Hong Kong) were clearly distinct origin from *D. repens* of Italy, Estonia and Japan. There is a hypothesis that both *Dirofilaria* sp. *hongkongensis* and *D.*

Fig. 1. Phylogenetic analysis of *Dirofilaria* sp. using partial sequences of cytochrome oxidase subunit I (*COI*) genes. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model, MEGA7. The analysis involved 35 nucleotide sequences. The *Dirofilaria* isolates from the present study are indicated in red dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

repens are independent valid species (To et al., 2012; Yilmaz et al., 2016) since both are placed as separate clades in the phylogenetic tree. Similarly, the results of the present study further strengthened this hypothesis. Hence, zoonotic *Dirofilaria* sp. hongkongensis type population is prevalent in Kerala, South India and all the *Dirofilaria* sp. causing canine and human dirofilariosis tested under the study were *Candidatus Dirofilaria hongkongensis* type.

The NCBI BLAST analysis of 18S rRNA gene of all *Dirofilaria* isolates in the present study revealed the closest identity (98 to 100%) with an already published sequence of *D. repens* (AB973229) isolated from a human in Tokyo, Japan who travelled in Europe (Suzuki et al., 2015). However, the sequence of the *COI* gene of *D. repens* isolated from the human in Japan (AB973225) showed 99% identity with *D. repens* isolated from a human in Italy (AM749233) (Suzuki et al., 2015). Hence, it was difficult to differentiate *D. repens* from *Dirofilaria* sp. *hongkongensis* based on the sequence analysis of 18S rRNA gene. Therefore, 18S rRNA based sequence analysis and phylogeny may not help in differentiating zoonotic from non-zoonotic *D. repens*. Moreover, there are only very few sequences of 18S rRNA specific for *D. repens* and *Dirofilaria* sp. *hongkongensis*.

In conclusion, the present study proved that the *Dirofilaria* sp. causing filariosis in both animals (dogs, jackal) and humans in Kerala, South India are genetically similar and also closely related to *Candidatus Dirofilaria hongkongensis* (*Dirofilaria* sp. *hongkongensis*). They are distinct from *D. repens* isolates of Italy, Estonia and Japan based on the *COI* gene sequences.

Acknowledgements

This work was supported financially by the Kerala State Council for Science, Technology and Environment (020/SRSAGR/2006/CSTE, 022/YIPB/KBC/2013/CSTE, 010-14/SARD/13/CSTE), Indian Council of Agricultural Research (NAIP/C2066, NFBSFARA/BSA-4004/2013-14, NASF/ABA-6015/2016-17) and Department of Animal Husbandry, Kerala [B2.3858/04/Plg(3) dt.2/2/07, B2.8401/08/Plg dt.19/18/2008].

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