Candidatus *Dirofilaria Hongkongensis* Infections in Humans During 2005 to 2020, in Kerala, India

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Abstract. We tried to determine the epidemiology and species of human dirofilariasis observed at two tertiary care hospitals in Kerala. We searched the hospital database to identify cases of dirofilariasis from January 2005 to March 2020. Along with human isolates, one dog *Dirofilaria* isolate was also subjected to PCR and sequencing of pan filarial primers cytochrome oxidase subunits 1 and 12S rDNA. We documented 78 cases of human dirofilariasis. The orbit, eyelid, and conjunctiva were the most commonly affected sites. Molecular characterization identified one dog and five human isolates as *Candidatus* *Dirofilaria Hongkongensis*. A rare case of subconjunctival infestation by *B. malayi* was also documented. Human dirofilariasis is a public health problem in the state of Kerala in India, and it is mostly caused by *Candidatus* *Dirofilaria Hongkongensis*. We propose that all dirofilaria isolates are subjected to sequencing for identification.

INTRODUCTION

Human dirofilariasis is caused by two species of zoonotic filarial nematodes that naturally infect dogs, cats, and wild animals and are transmitted by mosquito species that belong to the Culicidae family.^{1,2} Humans are accidental hosts where the parasite meets a dead end because it is not usually able to attain sexual maturity.^{1–3} The infection is characterized by the presence of pre-adult worms and, rarely, microfilaraemia.^{2,3} *Dirofilaria repens* and *Dirofilaria immitis* are of canine origin and are the most common species that cause human infections.^{1,2} Human infections are increasingly being reported, mostly in the form of subcutaneous nodules, and commonly involve the eyelids or conjunctiva.^{2,4} However, the involvement of the thigh, chest, male genitalia, breast, cheek, abdomen, and upper arm has also been reported.^{2,4} Most reports of *D. repens* infections are from Europe and Asia, but *D. immitis* is more prevalent in North America and Southern Europe.^{1,2} In southern Europe, canine *D. immitis* infections are highly prevalent.^{1,2} The first cases of human ocular (1975) and subcutaneous (1999) dirofilariasis caused by *D. repens* in India were reported in the state of Kerala.⁵ Since then, cases of *D. repens* infection have been reported in eight other Indian states, and all were identified based on morphological features like the presence of longitudinal ridges on the cuticle.^{4,5} Studies of microfilaremia in dogs in Kerala have reported incidence rates of 13.4% in Calicut, 24.2% in Thrissur, and 7.59% in Ernakulam districts.⁵ In 2012, a new species, *Candidatus* *Dirofilaria Hongkongensis*, was proposed as a causative agent for dirofilariasis in dogs and humans.⁶ A recent study performed in Kerala has also identified *Candidatus* *Dirofilaria Hongkongensis* in humans and dogs.⁷ We describe the clinical and molecular characteristics

of human dirofilariasis observed at two tertiary care centers in Kerala.

METHODS

This study was approved by the institutional review board of both participating hospitals (IRB-AIMS-2018-176; 2018/89/IEC/LF). We searched the hospital database to identify cases of dirofilariasis from January 2005 to March 2020. Cases from 2005 to 2017 were identified based on morphological worm features in histopathological sections. From 2018 to 2020, PCR and sequencing were used to identify the parasite in clinical specimens using pan filarial primers targeting cytochrome oxidase subunit 1 (COX1) (Figure 1A) and 12S rDNA (Figure 1B), as described previously.⁸ All COX1 PCR-positive samples were subjected to 12S rDNA-based multiplex PCR that could simultaneously identify *D. repens* and *D. immitis* (Figure 1C).⁹ Seven random human isolates and one dog *Dirofilaria* isolate were sequenced and analyzed using the NCBI BLASTn program. The 12S rRNA and COX1 sequences, along with the sequences retrieved from the GenBank, were subjected to the phylogenetic analysis. *Ascaris lumbricoides* was used as the outgroup for phylogenetic analysis. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Mega X Version 10.1.8). The accuracy of the tree topology was assessed using 100 bootstrap replications.¹⁰ Scanning electron microscopy (SEM) examinations of the surgically removed worm were performed by fixing 1-cm sections of the worm in 2.5% glutaraldehyde using JEOL JSM 6490LA microscope.

RESULTS

During the 15-year period, we identified 78 cases of dirofilariasis. The median age was 45.5 years (9 months–81 years); 24.35% ($N = 19$) of patients were of pediatric age (younger than 14 years). The youngest patient was 9 months old and presented with a nodule on the dorsum of

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FIGURE 1. (A) A 100-bp DNA ladder. (B) PCR products of the Cox1 gene: lane 1, 100-bp DNA ladder; lane 2, DNA from canine (DNA from dog heartworm); lanes 3 and 4, DNA from human eye specimens. The 321-bp PCR products seen in lanes 2, 3, and 4 indicate the presence of *Dirofilaria* sp. (C) PCR product of 12S rDNA gene: lane 1, PCR-amplified product from dog heartworm DNA; lanes 2 to 5, PCR-amplified product from roundworm DNA obtained from a human eye; lane 6, 100-bp DNA ladder. The 506-bp PCR products seen in lanes 1 to 5 indicate the presence of *Dirofilaria* sp. (D) Multiplex PCR analysis: lane 1, PCR-amplified product using 12S rDNA forward and 12S rDNA reverse primer pairs; lane 2, PCR-amplified product using 12S rDNA forward and *D. repens* reverse primer; lane 3, multiplex PCR showed a PCR-amplified product using 12S rDNA forward, 12S rDNA reverse, *D. repens* reverse, and *D. immitis* forward primers; lane 4, 100-bp DNA ladder. The 506-bp length fragment in lane 1 represents the *Dirofilaria* genome. The 327-bp length fragment in lane 2 represents the *Dirofilaria repens*. The 506-bp genome-specific band and 327-bp species-specific band are seen in lane 3.

the penis. The most commonly affected sites were the eyelid/conjunctiva/orbit ($N = 47$); 59.6% of cases involved the right side. The second commonest sites were the head/neck/face ($N = 11$), followed by the breast ($N = 6$), lower limb ($N = 3$), upper limb ($N = 2$), abdomen ($N = 2$), axilla ($N = 2$), and scrotum ($N = 2$). Solitary cases involving the penis, supraclavicular region, and paraspinal regions were also observed. Of the 30 cases with available eosinophil counts, 11 had eosinophilia ($> 5\%$). The worm was extracted from 20 cases and in the remaining biopsy specimens ($N = 58$) were evaluated to determine the diagnosis. Only 38 specimens collected between 2018 and 2020 were available for PCR; the remainder were morphologically identified as *Dirofilaria* spp. or *D. repens*. Sequencing of COX1 and 12S rRNA sequences identified five human isolates (Accession number: MT808309, MT800754, and MT800755) and one dog isolate (Accession number: MT808310) as *Candidatus* *Dirofilaria* Honkongensis. However, the multiplex PCR of one slender live worm extracted from the eye of a 1-year-old girl provided a PCR product only for the 12S rDNA PCR that was negative for species-specific PCR and had a sequence that showed 98.65% similarity with *Brugia malayi* (Accession number: MT808311). The COX1 and 12S rRNA sequences of the *Dirofilaria* species obtained from human and dog isolates were found to be highly conserved. Although the COX1 sequences were 100% identical, the sequences of 12S rRNA were 99.71% identical with the existing sequences of the *Dirofilaria* sp. 'hongkongensis' in the NCBI database. The phylogenetic analysis of COX1 and 12S rRNA sequences of *Dirofilaria* species also showed that the *Candidatus* *Dirofilaria* Honkongensis clinical isolates from humans and dogs belong to the same clade and are separated from the other *Dirofilaria* species, *D. immitis*, *D. repens*, and *D. ursi* (Figure 2). The outgroup *Ascaris lumbricoides* was clearly separated from the clade for both 12S rDNA and COX1 sequences of the *Dirofilaria* species. The SEM images of the *Candidatus* *Dirofilaria* Honkongensis surface at different magnifications showed the

longitudinal cuticular ridges, which were similar to *D. repens* (Figure 3).

DISCUSSION

Our study describes the largest series of human dirofilariasis from the state of Kerala and from India. We observed a high (45%) occurrence of dirofilariasis in active patients (age, 20-49 years). Sequencing of randomly selected isolates in our study identified *Candidatus* *Dirofilaria* Honkongensis in five cases of human dirofilariasis and one case of dog dirofilariasis. We believe that the remaining 33 PCR-positive cases would be identified by sequencing as *Candidatus* *Dirofilaria* Honkongensis. Because morphological features like cuticular ridges are observed in both species, the 40 cases that were morphologically identified as *D. repens* were also likely to be *Candidatus* *Dirofilaria* Honkongensis. *D. repens* can be differentiated from *Candidatus* *Dirofilaria* Honkongensis only by sequencing. A recent study by Pradeep et al. identified *Candidatus* *Dirofilaria* Honkongensis in three humans and four dogs in Kerala.⁷ Another sequencing study by Nazar et al. performed in Kerala identified human isolates as *D. repens*, albeit, it was closely related to *Candidatus* *Dirofilaria* Honkongensis.¹¹ Therefore, we concluded that human dirofilariasis in the state of Kerala in India is exclusively caused by *Candidatus* *Dirofilaria* Hongkongensis, and not by *D. repens*, as reported by previous studies. Subcutaneous filariasis caused by *B. malayi* has also been noted. Because of its high literacy rate and better access to quality health care in the state of Kerala, more cases are being diagnosing there than in the other states of India. Although the findings of our study may be basic in nature, the data are crucial to stimulating discussions about policy changes and making human dirofilariasis a notifiable disease so that its true burden can be determined and remedial measures can be suggested. Our study had limitations. The generated data are from two tertiary care referral hospitals and do not reflect the true prevalence of human dirofilariasis because most patients may seek treatment at a

FIGURE 2. Phylogenetic analysis using the Maximum Likelihood method and Kimura 2-parameter model with 1000 bootstrap replications using MEGA X version 10.1.8. (A) Phylogenetic analysis of the 12S rRNA gene sequences of the *Dirofilaria* species. (B) Phylogenetic analysis of COX1 gene sequences of *Dirofilaria* species. *Ascaris lumbricoides* was used as an outgroup for the analysis. *Dirofilaria* isolates from the present study are indicated by the green dot (human isolate) and red dot (dog isolate). The *B. malayi* isolate is indicated by the black dot. GenBank accession numbers and species names of the sequences used for the study are shown on the phylogenetic trees. Boot strap values are shown on branches. This figure appears in color at www.ajtmh.org.

local hospital or standalone ophthalmology center. Sequencing of all PCR-positive cases could not be performed because of a paucity of funds. Kerala is endemic to Brugian filariasis, and recent reports have identified dogs as a reservoir host for *B. malayi*.¹² Therefore, it was not surprising that one of the worms extracted from the eye of a patient was identified as *Brugia malayi*.

CONCLUSION

Dirofilariasis in the state of Kerala in India is exclusively caused by *Candidatus* *Dirofilaria Hongkongensis*. It is not caused by *D. repens*. We propose that all dirofilaria isolates should be subjected to PCR and sequencing for identification. Efforts should also concentrate on eliminating the reservoir of infection in the canine population by active case detection and treatment. The vector density needs to be controlled to achieve

effective elimination. The increasing trend of human dirofilariasis is worrisome. Without intervention, it may become a significant public health problem.

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FIGURE 3. Scanning electron microscopy images of the surface of *Dirofilaria* sp. hongkongensis worm showing longitudinal cuticular ridges in 250 $\times$ and 500 $\times$ magnifications, respectively.

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