Trends in Parasitology

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Review

Zoonotic dirofilariases: one, no one, or more than one parasite

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Dirofilaria **spp. are vector-borne filarial nematodes that affect a variety of animal species, including humans. *Dirofilaria immitis* and *Dirofilaria repens* are the two main zoonotic species, but also other wildlife-associated *Dirofilaria* species are occasionally reported as causative agents of human dirofilariasis, including *Dirofilaria striata*, *Dirofilaria tenuis*, *Dirofilaria ursi*, *Dirofilaria spectans*, and *Dirofilaria magnilarvata*. Since the etiological identity of most of the species mentioned here is arguable, we summarized and critically discussed data concerning infections in humans, focusing on the reliability of *Dirofilaria* species identification. We advocate the importance of combined morphological and genomic approaches to provide unequivocal evidence for their zoonotic potential and pathogenicity.**

Highlights

Several *Dirofilaria* spp. infect humans, although their identification is often insufficient.

Many cases of human dirofilariases are diagnosed based on histopathological examination and/or morphological analysis of nematodes recovered, without molecular confirmation.

Molecular data are scant for human cases, in contrast to those available for dogs.

'*Dirofilaria hongkongensis*' is a *nomen nudum*, though it is erroneously considered a proper species.

Combining morphological and molecular approaches is pivotal to confirm the identity of the species responsible for human cases.

The hidden diversity of zoonotic *Dirofilaria* spp.

Members of the family Onchocercidae are vector-borne filarial nematodes that affect many animal species, including humans. Amongst them, *Onchocerca volvulus*, the causative agent of river blindness, poses a severe burden on human health, with more than 20.9 million cases recorded in 2017, 99% of which occurred in African countries [1]. In addition, as of 2018, 51 million people were infected with *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori,* the causative agents of lymphatic filariasis[i]. Besides the aforementioned filarial parasites, which have a typical anthroponotic life cycle, many others are primarily associated with wild and domestic animals but may occasionally infect humans [2,3]. The genus *Dirofilaria* currently includes 27 described species [2], of which *D. immitis* [4], *D. repens* [5], *D. striata* [6], *D. tenuis* [7], *D. ursi* [8], *D. spectans* [9], and *D. magnilarvata* [10] have also been reported from humans. Among these, *D. repens* and *D. immitis* are by far the most frequently involved in human cases [3]. In dogs, *D. immitis* causes a severe cardiopulmonary disease, whereas *D. repens* usually produces a mild subcutaneous infection [5]. While less pathogenic to dogs, *D. repens* is the most common agent of human dirofilariasis, with a wide distribution in the Old World [3]. Conversely, *D. immitis* is the second most frequent causative agent of human dirofilariasis from a global perspective, being the principal agent of the disease in the New World [2].

Data concerning other *Dirofilaria* spp. infecting humans are scant and often questionable, due to limitations in terms of diagnostics and species identification. We summarize and critically discuss scientific information about *Dirofilaria* spp. in humans, with particular emphasis on the reliability of their identification. We also advocate the use of proper morphological and DNA data for future reports of human dirofilariasis to provide unequivocal evidence on the species involved in each case.

Dirofilaria spp., hosts, and distribution

D. repens is the most common agent of human dirofilariasis in the Old World [3,5,11]. It infects mainly domestic dogs [5,11], and to a lesser extent, cats [12,13] and several species of wild

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carnivores [11,14,15] (Table 1). The infection is often subclinical, inducing the formation of subcutaneous nodules where adult nematodes are embedded [5]. *D. repens* is endemic in many countries in Europe, Africa, the Middle East, and Asia [3,5] and has recently been found in Colombia [16]. Other studies reporting the detection of nematodes genetically related to *D. repens* in the New World (e.g., [17,18]) require confirmation, either because of the low nucleotide identity with *D. repens* or because of the small size (i.e., 247–250 bp) of the DNA fragments analyzed. Several mosquito species are involved in the transmission of *D. repens* (Table 1), such as *Culex pipiens* [19], *Aedes geniculatus* [20], *Aedes aegypti* [21], *Aedes albopictus* [19,22], and *Aedes japonicus* [20].

D. immitis (canine heartworm) has a worldwide distribution and infects mainly domestic dogs, being also responsible for several cases of pulmonary dirofilariasis in humans [2,3]. In dogs, the disease is characterized by respiratory distress, epistaxis, ascites, exercise intolerance, and anorexia [23]. A plethora of hosts, including domestic cats [13,14] and wild carnivores, have been found positive for *D. immitis*, and many mosquito species (Table 1) were demonstrated to be competent vectors [2].

D. tenuis, the third most frequent *Dirofilaria* sp. reported in humans [3], was described from raccoons (*Procyon lotor*) in North America [24], with a high prevalence in southeastern states [25]. In raccoons, adults are found in the subcutaneous tissues, around head and neck, and masseter muscles [26]. Of the 16 mosquito species experimentally infected with *D. tenuis*, *Psorophora confinnis*, *Aedes taeniorhynchus*, and *Aedes sollicitans* displayed active third-stage larvae (L3) in the proboscis and hemocoel [26]. Of the aforementioned, *A. taeniorhynchus* was able to inoculate *D. tenuis* L3 by blood feeding into receptive raccoons, therefore demonstrating its vectorial competence [26] (Table 1).

D. ursi was initially described from bears in Japan [27] and is known also to occasionally infect humans [28]. It was reported in different species of bears from the Old World (e.g., Russia,

Glossary

Cryptic species: organisms that appear identical or nearly identical to each other morphologically but are, in fact, distinct species with genetic and evolutionary differences.

Development units (DUs): in parasitology, DUs quantify the degree of development or maturation of immature parasites, particularly in the context of disease vectors. DUs are also used to measure the progress of larval stages of insects (e.g., mosquito species or other insect vectors).

Growing degree–day model (GDD): the model used to quantify the accumulation of heat units (i.e., degree-days) required for the growth and development of organisms. The GDD model is applied to predict the development and activity of parasites, helping to predict the life cycle and transmission dynamics of parasites.

***Nomen nudum*:** a proposed taxonomic name that is invalid because the group designated is not adequately described or illustrated sufficiently for recognition.

Table 1. Species, definitive hosts, geographical distribution, and vectors of *Dirofilaria* spp. infecting humans

Dirofilaria sp.	Host	Geographical distribution	Vectors	Refs
Dirofilaria repens	Domestic dog, wolf (*Canis lupus*), red fox (*Vulpes vulpes*), Eurasian badger (*Meles meles*), human	Old World (e.g., Europe, Africa, Middle East, and Asia), Colombia	*Aedes aegypti*, *Aedes albopictus*, *Aedes japonicus*, *Aedes geniculatus*, *Culex pipiens*, *Anopheles* spp., *Ochlerotatus* spp., *Coquillettidia* spp., and *Mansonia* spp.	[3,5,12–16,19–21,51, 52,59,68–72,90,92]
Dirofilaria tenuis	Racoon (*Procyon lotor*), human	North America	*Aedes taeniorhynchus*	[24–26,79–82]
Dirofilaria ursi	Kamchatka brown bear (*Ursus arctos beringianus*), brown bear (*Ursus arctos*), Japanese black bear (*Ursus thibetanus japonicus*), and American black bear (*Ursus americanus*), human	Russia, Finland, Japan, USA, and Canada	Black flies (Simuliidae), such as *Simulium venustum*	[27–34,47,48,56]
Dirofilaria striata	Cougar (*Puma concolor*), margay (*Leopardus wiedii*), ocelot (*Leopardus pardalis*), bobcat (*Lynx rufus*), and Florida panther (*Puma concolor coryi*), domestic dog and cat, human	Brazil, Venezuela, USA	Not known	[34–36,93]
Dirofilaria immitis	Domestic dog and cat, brown bear, jackal (*Canis aureus*), Iberian wolf (*Canis lupus signatus*), human	Worldwide	*Aedes* spp., *Anopheles* spp., *Culex* spp., and *Ochlerotatus* spp.	[2,13,14,20,22, 52,54,90,92,93]
Dirofilaria spectans	Giant otter (*Pteronura brasiliensis*), tayra (*Eira barbara*), Neotropical otter (*Lontra longicaudis*), human	Brazil	Not known	[9,35–40,93]

Finland, and Japan) and in North America (Table 1), where it is transmitted by black flies (Simuliidae), such as *Simulium venustum* [29]. Adults can be found in bears' esophageal and tracheal connective tissues [29,30], perirenal adipose tissue [30–33], thoracic cavity [34], and connective tissues of other parts of the body [32].

D. striata was described in the subcutaneous tissues of wild felids from Brazil, Venezuela, and the USA (Table 1) [35]. This species was extracted from the eyelid of a young human patient from the USA [6]. Microfilariae morphologically identified as *D. striata* have also been reported in dogs [36]. More recently, fragments of *D. striata* adults were recovered from skin nodules of a domestic cat, with the species identification confirmed by morphological and molecular methods [37]. The vector of *D. striata* is still unknown and though experimental infections demonstrated that microfilariae develop to L3 in *Anopheles quadrimaculatus*, they do not complete the biological life cycle in kittens after the inoculation [35].

D. spectans is a parasite geographically restricted to Brazil, which seems to have little importance in human infections, with only one reported case [9]. It was originally described in the circulatory system of the giant otter (*Pteronura brasiliensis*) [38] and later in the tayra (*Eira barbara*) [39] and in the Neotropical otter (*Lontra longicaudis*) [40] (Table 1). So far, *D. spectans* has not been extensively morphologically and molecularly studied, and the geographical range, host distribution, and potential vectors remain to be better defined.

Another species described in humans is *D. magnilarvata* (reported in [10]), a parasite of non-human primates in Malaysia, whose name derives from the large size (up to 580 µm long) of its microfilariae [41]. Nonetheless, the original description in human patients is not available, impairing the assessment of data reliability for species identification. In a similar manner, other species like *D. subdermata* and *Dirofilaria corynodes* have been previously listed as infecting humans (reported in [10]), but there is no reliable information on the case reports, resulting in literature confusion about the actual number of zoonotic *Dirofilaria* spp.

The diagnosis of *Dirofilaria* spp. infection relies on the finding of microfilariae in blood smears or the Knott test, on the molecular detection of DNA of the parasite in blood samples [5,12,15,16], or on the extraction of adult worms from the nodules [5,13]. Serological commercial tests detecting a circulating antigen are widely used for the diagnosis of *D. immitis* in dogs, and although there are no commercial tests for *D. repens* diagnosis, diagnostic markers have been selected with the use of phage display technology [42]. There are no serological tests standardized for other *Dirofilaria* spp., and their diagnosis relies mainly on their morphology or molecular detection of DNA. In the case of *D. immitis*, diagnosis may also rely on visualization of adults in the dog's heart by echography [23].

Due to climate change and global warming, it should be expected that *Dirofilaria* spp. will expand their geographic range, as suggested for *D. repens* and *D. immitis* [43–45]. Indeed, the geographical distribution of *Dirofilaria* spp. depends on a combination of several factors, including the availability of proper definitive and intermediate hosts, as well as temperature. For instance, the development of *D. immitis* in its intermediate host requires temperatures higher than 14°C, within the lifespan of a mosquito (~30 days) [43,44] according to the **growing degree–day model (GDD)** (see Glossary). This model, based on wide or local scale temperature data, may predict the occurrence and seasonality of *Dirofilaria* spp. in different parts of the world given an overall requirement of 130 **development units (DUs)** for larvae to reach infectivity [43]. Using ecological niche modeling in Spain, the presence of the *Cx. pipiens* vector was combined with the number of *Dirofilaria* spp. generations (obtained from the GDD) coupled with the presence

of animals infected with *D. immitis*, thus resulting in risk maps with less than 1 km^2 precise resolution for *Dirofilaria* spp. on the Iberian peninsula [45] and Canary Islands [46]. This model suggested a modification in the distribution of *D. immitis* rather than an increase in the extension of suitable areas for *Culex* spp.

Although temperature requirements for *D. immitis* and *D. repens* development in the mosquito vectors were found to be similar [43,44], the known GDD model is not applicable for all *Dirofilaria* spp. since some species, such as *D. ursi*, are well adapted to colder temperatures and are transmitted in a subarctic climate by black flies [47,48]. In any case, the geographical distribution of *D. repens* and *D. immitis* has changed in Europe in the past decades. For instance, *D. repens* is now widespread in the Baltic states [49,50], Northerneastern Europe [51], and it even occurs in Scandinavia [52]. The transmission of *Dirofilaria* spp. in areas with short warm summers [43,44] is only possible due to the long patency period (~7 years for *D. immitis*) observed in the definitive host [53].

Genetic diversity of *Dirofilaria* spp.

Over 4000 human dirofilariasis cases are described in the literature [3,11,12,50,54], but genetic data on the diversity of *Dirofilaria* spp. detected from humans are limited. For instance, a search for '*Dirofilaria*' in GenBank revealed 27 253 entries (i.e., gene fragments, mRNA) (as of 6 November 2023), with partial sequences originated from humans assigned to *D. repens* (*n* = 112), *D. immitis* (*n* = 11), '*Candidatus* Dirofilaria hongkongensis' (*n* = 7), and *Dirofilaria* spp. (*n* = 12). While for complete mitochondrial genomes, *D. repens* (*n* = 1) and '*Candidatus* D. hongkongensis' (*n* = 2) were listed from humans [55].

Most sequences are relatively short and thus not informative for in-depth sequence analyses and phylogenetic inferences. By contrast, there is a relatively large number of sequences generated from *D. immitis* and *D. repens* detected in animals. Genetic data of other zoonotic *Dirofilaria* spp. are scant. For example, *D. ursi* sequences available in GenBank (*n* = 22) come from bears in Finland [*cox*1 (*n* = 3), of 12S rRNA (*n* = 2)] [52] and Japan [5S rRNA (*n* = 6) and 18S rRNA-ITS (*n* = 11)] [56]. Only one small fragment (i.e., 90 bp) was amplified from a single human case, but it is not available in GenBank [28]. For *D. striata*, only three nucleotide sequences (i.e., *cox*1, 12S, and 18S genes) are available in GenBank, originating from a cat sampled in Florida [37], whereas no sequences are available for the remaining other *Dirofilaria* spp. (i.e., *D. tenuis*, *D. spectans*, and *D. magnilarvata*).

Several studies investigated the diversity of *D. immitis* and *D. repens* haplotypes using *cox*1, 12S rRNA, and NADH dehydrogenase subunit 1 genes in different animal hosts and countries [57–59]. Overall, a higher haplotype diversity was detected in *D. repens* isolates when compared with *D. immitis* [58]. This higher complexity in haplotype composition was suggested to reflect a faster spread of *D. repens* in endemic areas, such as reported in central and northeastern Europe [58,59].

Assessing the nucleotide distance (intra- and inter-species), different gene targets should be a precondition for understanding species definition within the genus *Dirofilaria* and, consequently, for inferring the identification of potential new species [60]. Based on a fragment of *cox*1 gene for barcoding [60], the mean intraspecific nucleotide divergence (Kimura two-parameter – K2P) was about 0.5% within *D. immitis* sequences, and interspecific mean distances were about 15.5%, reaching up to 27.8%, from 46 spirurid species including non-onchocercids such as *Thelazia* spp. and *Spirocerca lupi*.

A paradigmatic example of using intraspecific and interspecific genetic distances for defining the existence of different species within the genus is represented by a *Dirofilaria* sp. originally

detected in Hong Kong, China, in 2012, in a patient presenting subcutaneous nodules [61]. The nucleotide difference of *cox*1 sequences from this species was 3.8% when compared with *D. repens* and 10.7% compared with *D. immitis*. For the 18S–ITS1–5.8S rRNA region, the nucleotide identity was 94% and 94.9% when compared with *D. repens* and *D. immitis*, respectively [61]. Based on the aforementioned, this species was named as '*Candidatus* Dirofilaria hongkongensis', which was further identified in subcutaneous nodules in patients from India and Thailand [62–64], and in patients from Germany and Austria, after traveling to India [65,66]. The pairwise comparisons of complete mitochondrial genomes of *D. repens* and '*Candidatus* D. hongkongensis' revealed nucleotide identities from 94% (between *D. repens* vs. '*Candidatus* D. hongkongensis') up to 99% among those of *D. repens* [55]. Therefore, it was proposed that '*D. hongkongensis*' could be a **cryptic species** with *D. repens* [55]. To date, a proper morphological description of '*Candidatus* D. hongkongensis' is still lacking, and this name is a ***nomen nudum*** [67].

Analogously, despite the similar morphology of *D. immitis* with a *Dirofilaria* sp. collected from the eye of a 16-year-old boy in Pará (Brazil), DNA sequence analyses revealed a genetic divergence of 5% and 6% for 12S rDNA and *cox*1, respectively [68]. Indeed, morphological identification of the specimen from Brazil showed similar characteristics to *D. immitis* (i.e., ventral ornamentation of the posterior rugose region and arrangement and number of caudal papillae) as well as distinct features (e.g., absence of pre-esophageal cuticular ring and deirids more anterior than from *D. immitis*, as in *D. spectans*) [68]. Further genetic data from filarial nematodes circulating in wildlife in Brazil may help in elucidating the identity of this *Dirofilaria* sp. found in Pará.

Human cases and their diagnosis

The localization of *Dirofilaria* spp. in humans is unpredictable, although some species have been more frequently found in certain sites. For instance, *D. repens* is found in the subcutaneous tissue in various parts of the body, most commonly head/neck, trunk, and upper limbs, as well as in the subconjunctival tissue [3,5,11,14,69–71].

Humans have historically been considered aberrant hosts for *D. repens*, due to the low chance of adults to fully mature and copulate in humans, as well as to the presence of both male and female. However, microfilariae have been detected in a considerable number of cases both in the blood and in the local tissue (local microfilariasis) [72–74] whereas, in other human cases, the modified Knott's test was not performed [75]. In addition, the lack of microfilaremia could be due to the presence of immature nematodes which are difficult to differentiate from fully developed adults in the histology of nodules. Infections by *D. repens* have been reported in humans from all age groups (e.g., 4 months to 100 years), with a higher percentage in the age range of 20–69 years, and an overall higher number of cases in women than in men [3,70], except for one study in Austria where the male:female ratio was similar [76].

D. immitis has predominately a pulmonary location in humans [3,11,77], with fewer reports in the eye [3,69]. The nematodes often develop in branches of the pulmonary arterial tree where they induce vasculitis and are eventually killed by the patient's immune response, resulting in granuloma formation, recognized as the typical 'coin' lesion on a chest radiogram or computed tomography (CT) scan, which is often erroneously diagnosed as lung cancer [77]. Most human cases are subclinical, but when symptomatic, patients may present with chronic coughing and chest pain, among other less common signs [77]. In contrast to *D. repens*, infection by *D. immitis* was more frequently reported in men than in women (62.50% vs. 37.50%) and typically in patients aged between 40 and 79 years [3]. Recent statistics recorded an average of 28.80 human dirofilariosis cases annually [3]. Since 1975, dirofilariasis has been included in the national

surveillance system for notifiable diseases in Ukraine, allowing the identification of a noticeable increase in human cases after 2011, resulting from the spread of the parasite in eastern European countries [70]. The increase in human cases of *D. repens* was also observed in recent decades in Balkan Peninsula countries [50]. Conversely, in Sri Lanka, the highest number of cases of *D. repens* was reported in 2010–2012, followed by a decrease, probably due to the decreasing number of reports [71].

The geographical distribution of human cases with *D. ursi*, *D. tenuis*, *D. spectans*, and *D. striata* infection is summarized in Figure 1 and Table 2. Although cases of bears infected by *D. ursi* are

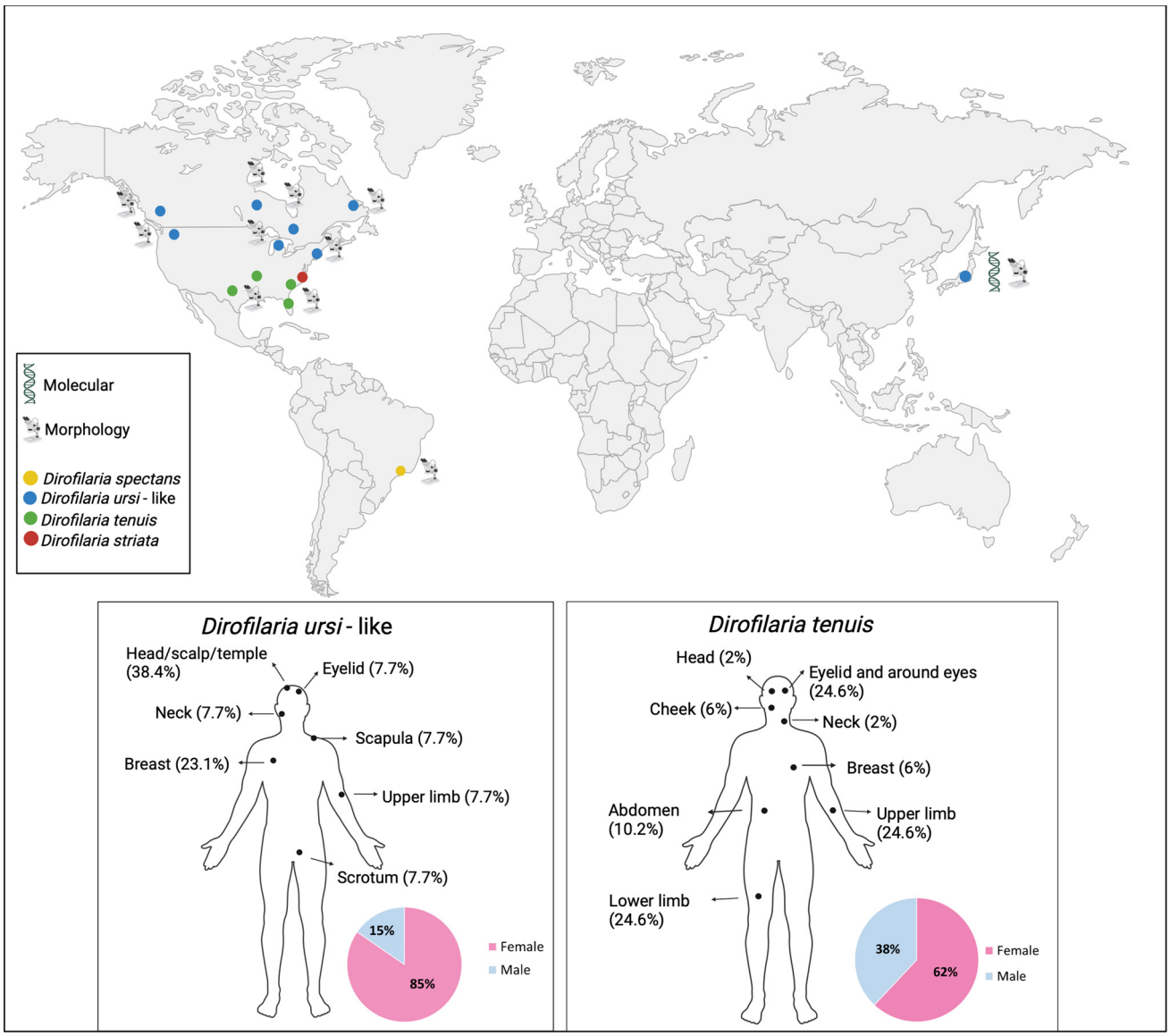

Figure 1. Distribution map of human cases of *Dirofilaria* spp. molecularly and morphologically identified. Anatomical localization of *Dirofilaria ursi*-like and *Dirofilaria tenuis* in human cases is reported in boxes.

Table 2. Geographical location, patient sex/age, localization of the helminth, and diagnostic methodologies of human cases of *Dirofilaria spectans, Dirofilaria striata, Dirofilaria tenuis*, and *Dirofilaria ursi*-like

Dirofilaria spp.	Geographical location	Patient sex	Age	Localization of the helminth	Diagnostic	Refs
D. spectans	Rio de Janeiro, Brazil	Woman	?	Finger	Morphological	[9]
D. striata	North Carolina, USA	Man	6	Eyelid	Morphological	[6]
D. tenuis	Florida, USA	Man	66	Cheek	Morphological	[25]
D. tenuis	Florida, USA	Woman	40	Forearm	Morphological	[25]
D. tenuis	Florida, USA	Woman	70	Arm	Morphological	[25]
D. tenuis	Florida, USA	Man	50	Forehead	Morphological	[25]
D. tenuis	Florida, USA	Man	45	Waist	Morphological	[25]
D. tenuis	Florida, USA	Man	30	Abdomen	Morphological	[25]
D. tenuis	Florida, USA	Woman	53	Abdomen	Morphological	[25]
D. tenuis	Florida, USA	Woman	67	Arm	Morphological	[25]
D. tenuis	Florida, USA	Woman	64	Calf	Morphological	[25]
D. tenuis	Florida, USA	Woman	61	Thigh	Morphological	[25]
D. tenuis	Texas, USA	Man	29	Leg	Morphological	[79]
D. tenuis	South Carolina, USA	Man	32	Abdomen	Morphological	[80]
D. tenuis	Florida, USA	Woman	35	Leg	Morphological	[81]
D. tenuis	Florida, USA	Man	37	Eyelid	Morphological	[7]
D. tenuis	Florida, USA	Woman	19	Eyelid	Morphological	[100]
D. tenuis	Florida, USA	Woman	49	Hand	Morphological	[100]
D. tenuis	Florida, USA	Woman	60	Arm, breast	Morphological	[101]
D. tenuis	Florida, USA	Woman	43	Cheek	Morphological	[101]
D. tenuis	Florida, USA	Woman	65	Breast	Morphological	[7]
D. tenuis	Florida, USA	Man	25	Arm	Morphological	[102]
D. tenuis	Florida, USA	Man	58	Neck	Morphological	[103]
D. tenuis	Florida, USA	Woman	42	Arm	Morphological	[103]
D. tenuis	Florida, USA	Woman	28	Forearm	Morphological	[7]
D. tenuis	Florida, USA	Woman	29	Forearm	Morphological	[7]
D. tenuis	Florida, USA	Woman	60	Eyelid	Morphological	[7]
D. tenuis	Missouri, USA	Man	30	Forearm	Morphological	[7]
D. tenuis	Oklahoma, USA	Woman	Not informed	Leg	Morphological	[7]
D. tenuis	Not informed	Woman	36	Thigh	Morphological	[7]
D. tenuis	Florida, USA	Woman	60	Not informed	Morphological	[7]
D. tenuis	Florida, USA	Woman	39	Upper arm	Morphological	[7]
D. tenuis	Florida, USA	Woman	47	Conjunctiva	Morphological	[7]
D. tenuis	Florida, USA	Woman	36	Thigh	Morphological	[7]
D. tenuis	Florida, USA	Man	46	Wrist	Morphological	[7]
D. tenuis	Florida, USA	Woman	51	Thigh	Morphological	[7]
D. tenuis	Florida, USA	Man	26	Ankle	Morphological	[104]
D. tenuis	Florida, USA	Woman	77	Periorbital	Morphological	[105]
D. tenuis	Florida, USA	Man	56	Cheek	Morphological	[106]
D. tenuis	South Carolina, USA	Woman	42	Periorbital	Morphological	[107]
D. tenuis	Mississippi, USA	Man	39	Thigh	Morphological	[108]

(continued on next page)

Table 2. (continued)

Dirofilaria spp.	Geographical location	Patient sex	Age	Localization of the helminth	Diagnostic	Refs
D. tenuis	Florida, USA	Man	Not informed	Abdomen	Morphological	[109]
D. tenuis	Florida, USA	Man	37	Eyelid	Morphological	[110]
D. tenuis	Mississippi, USA	Man	69	Eyelid	Morphological	[110]
D. tenuis	Mississippi, USA	Woman	63	Periorbital	Morphological	[110]
D. tenuis	North Carolina, USA	Man	67	Conjunctiva	Morphological	[110]
D. tenuis	North Carolina, USA	Woman	27	Eyelid	Morphological	[110]
D. tenuis	Florida, USA	Man	53	Infraorbital	Morphological	[110]
D. tenuis	Florida, USA	Woman	63	Breast	Morphological	[111]
D. tenuis	Florida, USA	Woman	72	Forearm	Morphological	[112]
D. tenuis	Not informed	Woman	15	Wrist	Morphological	[113]
D. tenuis	South Carolina, USA	Woman	60	Elbow	Morphological	[114]
D. ursi-like	Washington, USA	Woman	63	Breast	Morphological	[8]
D. ursi-like	New York, USA	Man	27	Scrotum	Morphological	[25]
D. ursi-like	Fukushima Prefecture, Japan	Woman	83	Scapula	Morphological and molecular	[28]
D. ursi-like	Ontario, Canada	Woman	54	Scalp	Morphological	[78]
D. ursi-like	New York, USA	Woman	23	Temple	Morphological	[78]
D. ursi-like	Manotiba, Canada	Woman	40	Breast	Morphological	[78]
D. ursi-like	Massachusetts, USA	Woman	21	Scalp	Morphological	[78]
D. ursi-like	Vermont, USA	Woman	66	Eyelid	Morphological	[78]
D. ursi-like	Michigan, USA	Woman	29	Scalp, sternum	Morphological	[78]
D. ursi-like	Ontario, Canada	Woman	46	Neck	Morphological	[78]
D. ursi-like	New Brunswick, Canada	Man	4	Head	Morphological	[78]
D. ursi-like	Quebec, Canada	Woman	43	Breast	Morphological	[115]
D. ursi-like	British Columbia, Canada	Woman	Not informed	Upper arm	Morphological	[116]

widely reported in many geographical regions, human cases are scarce. The first report of *D. ursi* in humans was from Washington, USA, in a 63-year-old woman who had an inflamed nodule in the pectoralis muscle [8]. Later, it was proposed that *D. ursi* recovered from humans should be designated as '*D. ursi*-like', a group that also includes *D. subdermata*, a parasite of porcupines [78]. In fact, *D. ursi* and *D. subdermata* are similar morphologically and cannot be distinguished based on the cuticle pattern only [78], nor genetically, given that molecular data are not available for the latter species. A distinguishing morphological feature of the *D. ursi*-like group is the presence of distinct longitudinal cuticular ridges regularly and widely spaced on the outer surface that are usually evident in histopathological examination, and also in deteriorate worms [78]. At least ten human cases of infection have been referred to *D. ursi*-like species in the northern USA and Canada based on morphology [78], and one case in Japan, based on histopathology and molecular sequencing (Table 2) [28]. *D. ursi*-like infections have been reported in humans from 4 to 83 years, being more prevalent in women than in men (i.e., 85% vs. 15%), with nematodes commonly localized in the head/neck (e.g., temple, eyelid) and upper parts of the body (i.e., breast, scapula, and upper arm) (Table 2 and Figure 1).

Human cases of *D. tenuis* infection have been described in the USA, particularly in the southern states of Florida [7,25], Texas [79], Missouri [7], South Carolina [80], and Oklahoma [7] (Table 2). The first case of *D. tenuis* in humans was reported from Florida in a 35-year-old woman who had

a nodule in her leg [81]. Almost 80% of all *D. tenuis* infections in humans have been reported in South Florida or were traced to a recent visit to that region [82], probably due to both the high prevalence of this parasite in raccoons in Florida and the exposure of humans to infected vectors (i.e., *A. taeniorhynchus*) [82]. Infections with *D. tenuis* have been reported in humans from 15 to 77 years, more in women than in men (62% vs. 38%), with nematodes found in various parts of the body (Table 2 and Figure 1). However, none of the human reports of *D. tenuis* mentioned earlier was confirmed molecularly. Finally, two ocular cases attributed to *D. tenuis* were reported from India [83,84], though both reports are incomplete. In the absence of molecular data, and because raccoons do not occur in India, these cases may represent misidentifications.

Singular cases of human infections have been reported for *D. spectans* and *D. striata*. *D. spectans* was found in the digital artery of a human patient from Rio de Janeiro, causing a condition known as Raynaud's syndrome [9], while an adult female identified as *D. striata* was removed from the orbit of a 6-year-old child from North Carolina, USA [6]. Both cases remained unconfirmed by molecular methods, and the real importance of *D. spectans* and *D. striata* as zoonotic agents still needs to be corroborated.

In most human cases described earlier, diagnosis was based on histopathological examination of nodules and morphological analysis of nematodes recovered [85], without molecular data. The presence of external longitudinal cuticular striations suggests a species belonging to the subgenus *Nochtiella* (Figure 2A and Box 1), whereas their absence (except for the ventral side of the caudal end in males) indicates a species of the subgenus *Dirofilaria* (Figure 2B and Box 1); however, both characteristics are not sufficient for species identification [86,87]. Although histological tissue sections are the most frequently used samples for laboratory diagnostics of subcutaneous nodules [88] (Figure 2C), key morphological features for the species identification of pre-adult (immature) or adult *Dirofilaria* spp. are usually not visible [85] (Box 1). Therefore, when possible, it is advisable to extract the intact nematode (Figure 2D) to perform a complete morphological and molecular examination.

Different molecular tools have been employed to diagnose *Dirofilaria* spp. DNA in humans. Conventional or nested-PCR are the most common techniques applied, *cox*1 and 12S rRNA genes being the most reliable molecular markers used to differentiate *Dirofilaria* species [54,60,89]. Also, duplex-qPCR (based on ITS and *cox*1 molecular markers) has been used to diagnose and differentiate between *D. repens* and *D. immitis* without the need for sequencing [90]. In addition, next-generation sequencing (NGS) was useful for whole-genome sequencing of *D. repens* and '*Candidatus* D. hongkongensis' in humans [55], but is limited to the research field.

Serology can help in diagnosing human cases, but no specific test is commercially available, either for *D. repens* or for *D. immitis*. A commonly used commercially available ELISA kit (Bordier Affinity Products SA, Crissier, Switzerland) is based on antigens of *Acanthocheilonema viteae*, a filarial parasite of rodents; it detects IgG antibodies against various genera of filarial nematodes, including *Wuchereria*, *Brugia*, *Mansonella*, *Loa*, *Onchocerca*, and *Dirofilaria*. The test has a sensitivity of 95% but does not allow for identification of the genus involved (#9400 *Acanthocheilonema viteae* IgG ELISA test kit, CE registration: H-CH/CA01/IVD/01755[ii]). Non-commercial serological tests for the detection of IgG against somatic antigens of adult *D. immitis* and *D. repens* have also been developed [91]. Serology has its shortcomings, as it may produce false negatives if the parasite localizes in immune-privileged sites, such as the eye [65], and the test may remain positive for a long time even after extraction of the worm [76]. Nevertheless, serology may be useful for a first assessment of a suspected case and for

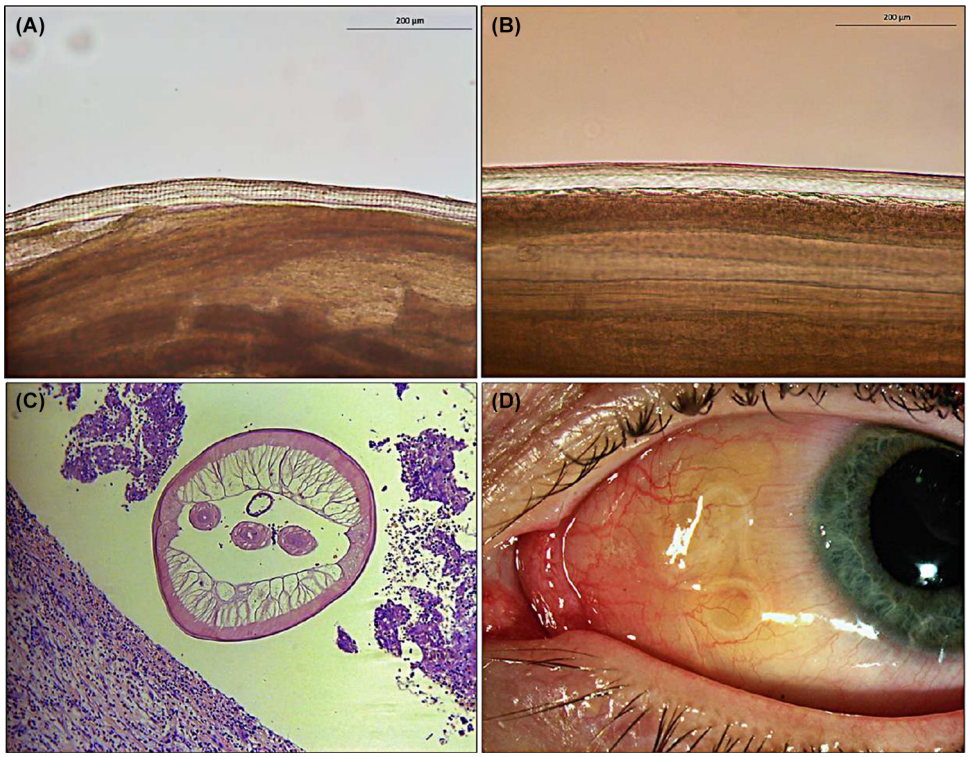

Figure 2. Photomicrography of the cuticle of *Dirofilaria* spp., striated for *Nochtiella* (A) or smooth for *Dirofilaria* (B) subgenus. (C) Histological section from an epididymal nodule with an adult specimen of *Dirofilaria repens* stained with hematoxylin and eosin. Morphologic features include muscle cells, coiled vagina, coiled intestine, lateral chords, and internal lateral ridges. Photo: Dr Johannes Esterbauer. (D) Human ocular dirofilariasis caused by '*Candidatus* Dirofilaria hongkongensis' coiled in the subconjunctiva of a patient (photograph from [65]).

Outstanding questions

Should serology be used as an additional method in suspected cases of human dirofilariasis?

Given that microfilariae are detected in *D. repens*-infected patients, should we consider humans as definitive hosts for *D. repens*?

Should the microfilaria test be routinely performed in suspected cases of humans *D. repens* infection?

What is the zoonotic potential of other *Dirofilaria* spp. (including those infecting non-human primates)?

epidemiological screening. For example, one-third of the total inhabitants of the small Italian island of Linosa (Sicily, Italy) presented anti-*D. immitis* IgG [90]. These results overlapped with the high prevalence of heartworm infection in the dog population from the same island (i.e., 58.9%), which is so far the southernmost hyperendemic European focus of *D. immitis* [92].

A recent study tested 397 individuals living on two islands where *D. immitis* and *D. repens* are endemic. The seroprevalence varied from 4% to 19.9%, depending on the antigen used and the island [90]. By qPCR, only four (1%) individuals were positive (three for *D. immitis* and one for *D. repens*) [90]. Interestingly, the authors also found six (1.5%) individuals positive for *Wolbachia* supergroup C, which includes mutualist symbionts of filarial nematodes of the genera *Onchocerca* and *Dirofilaria* [90]. The authors suggested the detection of *Wolbachia* endosymbionts as an additional diagnostic method for dirofilariasis.

Further properly designed studies are needed to assess the accuracy and validate serological and PCR-based methods for the diagnosis of human dirofilariasis.

Concluding remarks

Since the first reports (Box 2), cases of human dirofilariasis are increasing worldwide and they are mainly attributed to *D. repens* and *D. immitis*, the two species primarily infecting dogs. However, the existence of species that morphologically resemble *D. immitis* and *D. repens*, but are genetically

Box 1. The conundrum of morphology

Dirofilaria spp. are whitish filarial nematodes which present narrow hypodermal lateral chords and two-lateral internal cuticular crests [85]. They present a smooth or striated cuticle in species belonging to *Dirofilaria* or *Nochtiella* subgenera, respectively [87]. In *D. immitis*, the cuticle is smooth, with ridges and striae present only on the ventral surface of the male caudal extremity [87]. Principal differential features among males, females, and microfilariae in the genus are summarized in Table I. Males are 40–200 mm long, with 5–15 caudal papillae and two unequal spicules, the left always being longer (210–547 μm) than the right (100–299 μm) (Table I). Females are 80–360 mm in length, with the position of the vulva relative to the esophageal–intestinal junction varying according to species (Table I). *Dirofilaria* spp. nematodes recovered from human patients may be often not fully matured [69] and morphometric data should thus be interpreted with caution. In the same way, nematodes may be deteriorated, impairing a proper morphological identification to species level.

Table I. Morphological features of males, females, and microfilariae of *Dirofilaria* spp. with zoonotic importance

Life stage	Features	*Nochtiella*				*Dirofilaria*	
Male		*Dirofilaria repens* [93]	*Dirofilaria tenuis* [24,94]	*Dirofilaria ursi* [33,34,52]	*Dirofilaria striata* [36,93]	*Dirofilaria immitis* [93,95]	*Dirofilaria spectans* [38,93]
	Body length	50–70 mm	40–48 mm	63–93 mm	80–120 mm	120–200 mm	96–110 mm
	Body width	0.37–0.45 mm	0.19–0.26 mm	0.41–0.63 mm	0.34–0.40 mm	0.7–0.9 mm	0.47–0.74 mm
	Caudal papillae	Five or six preanal papillae and five post anal papillae	Up to 15 (six to nine preanal, four or five post anal and one at midventral line behind cloaca)	Vary from seven to ten at the left side and six to nine at the right side. Single transversely elongated central papilla just before cloaca	Six pedunculated preanal papillae	One pre-cloacal and four to five post-cloacal papillae	Not reported
	Spicules	Left: 530–547 μm and right: 181–189 μm	Left: 210–270 μm and right: 100–130 μm	Left: 450–610 μm and right: 150–190 μm	Left: 390–420 μm and right: 170–200 μm	Left: 300–375 μm and right: 175–299 μm	Left: 350–400 μm and right: 180–210 μm
Female	Body length	140–150 mm	80–130 mm	160–210 mm	250–360 mm	150–300 mm	140–190 mm
	Body width	0.44–0.55 mm	0.26–0.36 mm	0.44–0.84 mm	0.44–0.50 mm	1–1.3 mm	0.6–0.74 mm
	Vulva	Situated 1.84–1.92 mm from cephalic end and it is encircled by slightly projecting labia	Vulva 0.98–1.60 mm from the anterior end	Near esophageal–intestinal junction level	Not reported	Posterior to the junction of the esophagus and intestine	Not reported
Microfilaria	Length	300–360 μm	305–390 μm	189.8–242 μm	235–371 μm	290–330 μm	Not reported
	Width	6–8 μm	5–7 μm	4.7–6.5 μm	4–5 μm	5–7 μm	Not reported
	Anterior end	Obtuse	Slightly tapered	Head space with two nuclei	Two prominent nuclei	Tapered	Not reported
	Posterior end	Thin and pointed ending curved in form of an umbrella handle	Drawn out into long attenuated tail characteristically terminating in a pronounced hook	Thin anucleated caudal filament	Not reported	Pointed	Not reported

distinct, challenges their identification [61,68]. This highlights the importance of utilizing molecular techniques to uncover and understand the hidden diversity of *Dirofilaria* spp. The high molecular diversity of *Dirofilaria* spp. in carnivores in Asia suggests that there are multiple species circulating among these animals, but further combined morphological and molecular analyses are needed to better delineate possible new species. Accordingly, the zoonotic role of other *Dirofilaria* spp. (e.g., *D. magnilarvata*, *D. spectans*, *D. striata*, *D. tenuis*, *D. ursi*, and *D. subdermata*) needs further

Box 2. *D. repens* and *D. immitis* in humans: a long story

The first confirmed case of ocular dirofilariasis caused by *D. repens* (at that time, referred to as *Filaria conjunctivae*) was described at 1885 in [96]. However, reports in [97,98] described a probable first case of ocular *D. repens* infection in a 3-year-old girl from France. In the seventh edition of his *Curationum Medicinalium Centuriae*, Lusitanus stated '*...per oculi internam partem, quam angulum magnum appellamus, a jumbrici cuius dam caput appere coepis…*'. translated as '…in a 3-year-old girl, in the area we call big angle of the eye, suddenly it started to appear the tip of one worm which sometimes is sited in the eye making its opacity' [5]. This very short description does not allow us to unequivocally conclude that this was a case of ocular dirofilariasis. Based on this description, one could argue that [98] was probably observing another zoonotic worm, *Thelazia callipaeda*, which lives on the surface of the eyes, mostly under the third eyelid. In turn, *D. repens* usually localizes in the subconjunctiva (see Figure 2D in main text) [5,69]. Two years after Addario's report in Italy, the first case of *D. immitis* heartworm was recorded in humans [4]. The latter report found two filarial nematodes in the heart of a child from Rio de Janeiro, Brazil. This parasite was later described as *D. magalhaesi* in [99], but it is currently considered to be a synonym of *D. immitis*.

investigation since morphological and molecular data are scant or nonexistent (see Outstanding questions). A database of reference sequences derived from adult specimens recovered from natural hosts would be valuable for future studies, allowing a more accurate species identification in human cases. Meanwhile, the Knott test should be used also for diagnoses of human cases, as microfilaremia may occur in human patients (see Outstanding questions). Again, serological tests should be improved in terms of sensitivity and specificity, and validated by well-designed clinical studies, also to assess their positive and negative predictive values (see Outstanding questions).

Finally, physicians, veterinarians, and parasitologists play an important role in the diagnosis of dirofilariases by correctly sampling and preserving biological specimens for morphological and molecular species identification. The above synergistic efforts may contribute to an improved diagnosis of human dirofilariases.

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Declaration of interests

The authors declare no competing interests.

Resources

[i]www.who.int/news-room/fact-sheets/detail/lymphatic-filariasis

[ii]www.bordier.ch/9400%20Acanthocheilonema%20viteae/

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