

Detection of *Dirofilaria* DNA and host blood-meal identification in *Culicoides paolae* biting midges

Research Article

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Abstract

The vectorial role of blood-sucking arthropods, other than mosquitoes, has been explored for *Dirofilaria immitis* and *Dirofilaria repens* which are filarioids of increasing interest due to their zoonotic potential. Given the expansion of canine heartworm infection in southern Europe due to *D. immitis* and the report of the infection in Linosa Island (Sicily, Italy), we investigated and focused on the potential involvement of *Culicoides* spp. in *Dirofilaria* spp. transmission as well as the blood-meal source in *Dirofilaria* spp. molecularly positive biting midges. Out of 1791 specimens collected 1773 were morphologically and molecularly identified as *Culicoides paolae* and 18 as *Culicoides obsoletus* complex. Of the 1596 females, 4 (0.26%) parous *C. paolae* tested positive for *D. immitis* and *D. repens* (i.e. 2 *Culicoides* specimens for each filarial species), of which *D. immitis*-positive samples fed on both humans and dogs and *D. repens*-positive samples fed only on humans. The detection of *D. immitis* and *D. repens* DNA in *Culicoides* midges suggests their potential involvement in the epidemiology of the filarial nematodes, which may be of medical and veterinary importance in hyper-endemic areas. Findings of this study shed new light on the epidemiology of *Dirofilaria* spp. infections indicating that further investigations are needed.

Introduction

Dirofilaria immitis and *Dirofilaria repens* (Spirurida, Onchocercidae) are among the most common canine vector-borne pathogens in Europe, being a real threat for animal and human health (Otranto *et al.*, 2009; Baneth *et al.*, 2016). *Dirofilaria immitis* is the causative agent of heartworm disease (HWD), whereas *D. repens* causes subcutaneous dirofilariasis (Otranto *et al.*, 2009). While the former parasite is widely distributed in the New World, the latter is more commonly diagnosed in the Old World (Dantas-Torres and Otranto, 2020). Similarly, reports of canine and human dirofilariasis (i.e. pulmonary dirofilariasis and subcutaneous/ocular dirofilariasis due to *D. immitis* and *D. repens*, respectively) overlap the geographic distribution of the two filarial species (Dantas-Torres and Otranto, 2020). Indeed, a strong relationship occurs among the occurrence of human cases, the prevalence of the infection in dogs as well as the presence of competent vectors (Otranto *et al.*, 2011).

During the past few decades, changes in the geographical distribution of *Dirofilaria* spp. have been observed in Europe (Genchi and Kramer, 2020). For instance, the prevalence of HWD in the dog populations has increased in the southern regions of Italy, previously considered as non-endemic (Mendoza-Roldan *et al.*, 2020), resulting in new hot spots of HW infection (i.e. Apulia region, Panarese *et al.*, 2020; Linosa Island, Sicily, Brianti *et al.*, 2021). Noteworthy, Linosa is the southernmost European focus of *D. immitis* infection, arriving at a prevalence of 58.9% in the dog population (Brianti *et al.*, 2021) and a seroprevalence of 7.9% in the human population (Mendoza-Roldan *et al.*, 2021).

To date, more than 70 mosquito species (i.e. genera *Aedes/Ochlerotatus*, *Anopheles* and *Culex*) have been recognized as competent or putative vectors for *Dirofilaria* spp. (Eldridge and Edman, 2000). However, other nematodes, belonging to the family Onchocercidae, can also be transmitted by diverse arthropod species, including *Acanthocheilonema reconditum* transmitted by the cat flea *Ctenocephalides felis* (Brianti *et al.*, 2012; Napoli *et al.*, 2014) and *Mansonella perstans* and *Mansonella streptocerca* vectored by *Culicoides* biting midge (Shelley and Coscaro'n, 2001; Ta-Tang *et al.*, 2018). For their haematophagous behaviour and catholic host-seeking preferences (Kirkeby, 2018), *Culicoides* spp. are responsible for transmitting several pathogens, such as arboviruses (Borkent, 2004; Veiga *et al.*, 2018), protozoa (Atkinson, 1988) and filarial nematodes (Khanzadeh *et al.*, 2020). Of about 1400 *Culicoides* species described worldwide (Mellor *et al.*, 2000), 23 species have been identified, so far, in the Mediterranean basin (Blanda *et al.*, 2018). A few *Culicoides* spp. have been demonstrated to act as vectors of pathogens, such as filarial worms (e.g. the avian filaroid *Chandlerella quiscali*), is limited (Mellor *et al.*, 2000; Shelley and Coscaro'n, 2001; Ta-Tang



Fig. 1. Schematic representation of the island of Linosa and position of trapping sites for the entomological survey. In orange the CDC light traps ($n=4$) and in blue BG sentinel mosquito traps ($n=2$) equipped with light and BG-Lure, respectively.

et al., 2018) whereas no studies have focused on *D. immitis* and *D. repens* transmission though these insects are prevalent in endemic areas for dirofilariases (Blanda *et al.*, 2018).

Based on the peculiar epidemiological context of the island of Linosa where a high number of *D. immitis*-infected dogs (Brianti *et al.*, 2021) coupled with a large population of *Culicoides* spp., we investigated the potential involvement of these blood-sucking insects in *Dirofilaria* spp. transmission and epidemiology.

Materials and methods

Study area and insect collection

Linosa is a small island (5.4 km^2) located in the middle of the channel of Sicily, almost equidistant from Sicilian and Tunisian coasts ($35^{\circ}51'33.05''\text{N}$, $12^{\circ}51'45.79''\text{E}$). The Island is of volcanic origins and is characterized by a semi-arid and windy climate, moderate rainfall during the mild winters and hot and humid summers. Linosa is populated with nearly 350 inhabitants, a total of 59 dogs and 240 cats (no stray dogs or wild canids), whereas the presence of livestock is negligible (i.e. 10 sheep, 4 cattle and 3 horses). In the island, moreover, a large population of wild rabbit is present (*Oryctolagus cuniculus*), but no wild carnivore species have been recorded (Ozella *et al.*, 2016). Recently, an epidemiological survey conducted on the island described one of the highest prevalence of *D. immitis* infection (i.e. 58.9%), ever reported in dogs in Europe. In the framework of this epidemiological study, 8 sites in different parts of the island (Fig. 1) were selected for an entomological survey. Trapping was performed daily between 5.30 p.m. and 9.00 a.m., from July to November 2020 using Centers for Disease Control and Prevention (CDC) light traps ($n=4$) and BG sentinel (Biogents GmbH, Regensburg, Germany) mosquito traps ($n=2$) equipped with light and BG-lure, respectively. CDC light traps were placed outdoors, while the BG sentinel traps were placed indoors or next to dog kennels; CDC light traps were set approximately 50 cm above the ground. Trapping was not performed in rainy and windy days.

After each sampling, the collection bags were stored at -4°C , until transferred to the laboratory at the end of the sampling period. *Culicoides* spp. were divided from other insects under a stereomicroscope, and thereafter identified by the characteristic wing patterns and spots using morphological keys (Rawlings,

1996; Goffredo and Meinswinkel, 2004). Some of the specimens (i.e. 10%) were molecularly processed to confirm the species identification (see later).

Finally, all the collected females were grouped as unfed, engorged or parous according to the absence, the presence of fresh or digested blood meal, respectively, and then, molecularly analysed for the detection of *Dirofilaria* spp. DNA (see the next section).

Molecular analyses

Genomic DNA was extracted from each female specimen using a phenol/chloroform extraction method followed by ethanol precipitation (Sangioni *et al.*, 2005). Before the DNA extraction, the specimens were freeze-thawed in $50 \mu\text{L}$ of TE buffer (Tris-HCl 10 mmol L^{-1} , EDTA 1 mmol L^{-1} , pH 7.4), cycling twice for 15 min between 100 and -80°C and then, homogenized and extracted.

For the molecular confirmation of the *Culicoides* identification, a 523 bp fragment was amplified by polymerase chain reaction (PCR) using the C1-J-1718 and C1-N-2191 primers, targeting the mitochondrial *cox1* gene (Nolan *et al.*, 2007).

Furthermore, all DNA samples were processed by duplex real-time PCR for the detection and differentiation of *Dirofilaria* spp., using two species-specific primers targeting *cox1* and internal transcribed spacer-2, set for *D. immitis* and *D. repens*, respectively (Latrofa *et al.*, 2020). All DNA samples were tested in duplicate, and positive and negative controls were included in each quantitative PCR (qPCR) run. The specificity of the qPCR assay was established by melting curve analysis as described elsewhere (Latrofa *et al.*, 2012).

For the blood-meal identification, conventional PCR was conducted on *Culicoides* positive for *Dirofilaria* spp. DNA ($n=4$) using primers cyto 1 (5'-CCATCAAACATCTCAGCATGAA A-3') and cyto 2 (5'-CCCCTCAGAATGATATTTG TCTC-3') for the amplification of a fragment of 359 bp of vertebrate cytochrome b gene, and then sequenced (Gonzalez *et al.*, 2017).

Results

A total of 1791 *Culicoides* biting midges were captured in the study, of which 1596 were females (i.e. 1551 parous, 39 unfed



Fig. 2. Parous female specimen of *Culicoides paolae* (A) and *Culicoides obsoletus* complex (B).

and 6 engorged) and 195 were males. All the specimens were morphologically identified as *Culicoides paolae* (Fig. 2A) and only 18 parous females as *Culicoides obsoletus* complex (Fig. 2B). The sequencing analysis of COI gene amplicons, conducted in 20 and 5 specimens classified as *C. paolae* and *C. obsoletus* complex, respectively, confirmed the morphological identification. Four parous females (i.e. 0.26%) of the 1596 collected, tested positive for *Dirofilaria* spp. DNA presence. In particular, two specimens of *C. paolae* were positive for *D. immitis* (i.e. 0.13%) and two *C. paolae* were positive for *D. repens* (i.e. 0.13%) by qPCR. The *D. immitis* positive *C. paolae* were also positive for humans (i.e. $n = 1$; 99.7% nucleotide identity with the GenBank sequence, accession number MK617272) and dog DNA (i.e. $n = 1$; 100% nucleotide identity with the GenBank sequence, accession number, MW435612). The positive specimens for *D. repens* were collected in July in site 5 and were positive for human DNA ($n = 2$; 99.3 and 98.9% nucleotide identity, accession numbers MN503463 and LS997794).

Light traps were more effective at capturing biting midges compared to those baited with BG-lure; in fact, all the specimens, but one, have been collected with CDC light traps. During the same sampling period, the traps collected also 262 mosquitoes (i.e. 107 males and 155 females) belonging to six species (i.e. *Aedes mariaae*, *Culex pipiens*, *Aedes albopictus*, *Culex laticinctus*, *Culiseta annulate* and *Aedes caspius*) as reported in Brianti et al. (2021).

Discussion

Dirofilaria immitis and *D. repens* have been herein molecularly identified for the first time in *C. paolae* biting midges collected from the island of Linosa, with a focus on HW infection. To date, *Culicoides* spp. have been mainly studied for their vectorial role in the transmission of bluetongue disease virus and African horse sickness virus (Borkent, 2004; Veiga et al., 2018). However, they are, along with simuliids, the vectors of mansonellosis (Khanzadeh et al., 2020), and are also considered as competent vectors of onchocercidae worms (Mellor et al., 2000; Shelley and Coscaro'n, 2001; Ta-Tang et al., 2018; Khanzadeh et al., 2020), such as *M. perstans* and *M. streptocerca* (Shelley and Coscaro'n, 2001; Ta-Tang et al., 2018). Therefore, the presence of *Dirofilaria* spp. DNA in *C. paolae* biting midges may represent an additional clue in understanding the filarial vector competence, although the molecular detection alone is not sufficient to demonstrate this. On the other hand, the detection of filarial DNA in parous females indicates a potential survival of the

nematode to the digestion process and, eventually, its development to further stages.

The species diversity of *Culicoides* spp. observed in Linosa is significantly lower compared to those observed either in the closest Sicily (i.e. 23 species; Blanda et al., 2018) or in North Africa (i.e. 14 species in Algeria, Hakima et al., 2020; 54 species in Morocco, Bourquia et al., 2019). The low species diversity observed in Linosa could be related to the dimension of the island, the paucity of ecotypes and microhabitats present as well as the limited presence of animals. Indeed, several factors may influence the presence, the abundance and composition of the *Culicoides* fauna, including both biotic (e.g. vegetation, human presence, other animal presence) and abiotic (light, soil, water, air, climatic factors, etc.) factors. The two species of *Culicoides*, *C. paolae*, the most representative species, and *C. obsoletus* complex, herein described, are widespread in the Mediterranean basin (Blanda et al., 2018; Bourquia et al., 2019; Hakima et al., 2020).

The molecular prevalence of *Dirofilaria* spp. in the *Culicoides* specimens collected in Linosa is lower compared to that of the mosquito population collected in the same island (0.6%; Brianti et al., 2021). Interestingly, in the same period none of the mosquitoes analysed tested positive for *D. repens*, as well as any of the sampled dogs in the island scored positive for *D. repens* (Brianti et al., 2021). Noteworthy, the two *C. paolae* positive for *D. repens* were positive for human DNA and, considering the low seroprevalence in humans in the island (i.e. 3.96%) (Mendoza-Roldan et al., 2021), the positive *C. paolae* may have acquired the *D. repens* microfilariae elsewhere, for instance in the island of Lampedusa where *D. repens* infects both dogs and humans (Brianti et al., 2021; Mendoza-Roldan et al., 2021). Considering that mosquitoes are competent and recognized vectors of *Dirofilaria* spp., the epidemiological importance of *Culicoides* species should be carefully assessed in future experimental studies and by detecting further developmental stages in these insects. During the past few decades, other blood-sucking arthropods have been investigated as potential vectors of HW infection. For instance, fleas of the genus *Ctenocephalides* could develop the filarial nematode in their Malpighian tubules with the infective stage larvae living in the haemocoelic cavity of the arthropod (Summers, 1940). Also, blackflies, belonging to the *Simulium turgaicum* complex, have been regarded as potential vectors of *Dirofilaria* spp. (Khanzadeh et al., 2020) even if they exist a definitive proof of their involvement in the life cycle of *Dirofilaria* spp. is yet to be assessed.

This study confirmed the general feeding habits of *C. paolae*, which can adapt to any type of hosts (e.g. humans and dogs).

In the present survey, the majority of positive specimens fed on humans and/or dogs. This may be related to host availability in the island since only 10 cows and less than 20 sheep are present in Linosa (Brianti *et al.*, 2021). Considering this lack of hosts the probability that the infection could be transmitted from dogs to humans through *Culicoides* spp. bites will increase. Accordingly, the seroprevalences of *D. immitis* and *D. repens* in humans were, respectively, 7.7 and 3.96% in Linosa and 7.9 and 19.93% in Lampedusa (Mendoza-Roldan *et al.*, 2021), being slightly higher compared to those recorded in other similar surveys conducted in the human populations (e.g. 10.8 and 0.2% of *D. immitis* and *D. repens* prevalence, respectively, in Romania-Moldova, Ciuca *et al.*, 2018; 6.4% of *D. immitis* prevalence in the Canary Islands, Cabrera *et al.*, 2018). This high seropositivity for *D. immitis* in humans was most likely associated with the hyperendemicity of *D. immitis* in dogs.

This study represents the first evidence, even if circumstantial, of the role of *Culicoides* spp. in the life cycle of *Dirofilaria* spp. The evaluation of the epidemiological role of other blood-sucking arthropods, different from mosquitoes, is still of importance and should be further investigated to better control the *Dirofilaria* spp. transmission to both human and animal hosts, mainly in geographically confined epidemiological contexts such as Linosa Island.

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