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Research paper

Culex pipiens as a potential vector for transmission of *Dirofilaria immitis* and other unclassified Filarioidea in Southwest Spain

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ABSTRACT

Dirofilaria immitis is one of the most frequently detected mosquito-transmitted zoonotic filarioid nematode in mammals in Europe, being canine dirofilariosis a major animal health problem, endemic in the Mediterranean area. This study, focused on Southwest Spain, in order to bring new insights into (i) the epidemiology of *Dirofilaria* spp., (ii) the species of Culicid vectors possibly involved in their transmission and (iii) the genetic variability of those potential vectors. A total of 881 adult female mosquitoes from 11 different species, were captured during 2012–2013, and detection of filarioid DNA was attempted by PCR using specific primers (ITS-2 and COI), followed by DNA sequencing. In a single *Culex pipiens* specimen *D. immitis* DNA was detected both in the head-thorax and abdomen sections. Filarioid nematode DNA was also detected in eight additional *Cx. pipiens* specimens also in both the thorax and the abdomen, but analysis of sequence data did not allow unambiguous assignment of any of the obtained sequences to a previously defined species. All *Cx. pipiens* with filarioid DNA were individually analysed by CQ11 to discriminate between *pipiens*, *molestus*, and hybrid forms. Besides, rDNA ITS-2 sequence analysis revealed the presence of haplotype H1 and H2 of *Cx. pipiens*. To our knowledge this study revealed, for the first time in Spain, the occurrence of likely mature infection of *D. immitis* in *Cx. pipiens,* as well as with other yet uncharacterized nematodes, supporting its role as a potential vector of these filarids.
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1. Introduction

Mosquitoes are vectors of agents of infectious and parasitic diseases to humans and animals, such as malaria, arbovirosis and filariosis. Filarioid nematode parasites affect millions of people and animals worldwide, and represent a major health hazard with significant economic implications (Laaksonen et al., 2010). Canine dirofilariosis is a major veterinary health problem in tropical, subtropical and temperate regions of the world (Simón et al., 2012), with at least 70 species of mosquitoes being considered as potential vectors (Cancrini et al., 2006).

Dirofilariosis is endemic in the Mediterranean region, affecting southern European countries including Portugal, Spain, France and Italy (Genchi et al., 2005; Morchón et al., 2012). In recent years, climate change, increased transport of animals (including dogs), and the dispersal of invertebrate vector species of filarioid nematodes, have led to the geographical expansion of dirofilariosis (Genchi et al., 2014), which is usually associated with *Dirofilaria repens* (Tappe et al., 2014). Humans are only accidental hosts as the parasite cannot develop to the adult stage (dead-end host), but the observed increase in the number of cases of human dirofilariosis in recent decades has led to its classification as an emerging zoonosis (Pampiglione and Rivasi, 2001).

D. immitis is present in Spain, with prevalences ranging from 0.8% to 36.7% in dogs along the Iberian Peninsula, and 19%-39% in the Balearic and Canaries islands (reviewed by Diosdado et al.,

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2016). In other vertebrate hosts, the detection of *D. immitis* ranged between 1.7% and 32% in the red fox (*Vulpes vulpes*) (Gortázar et al., 1998), and 2.1% in the wolf (*Canis lupus*) and Eurasian otters (*Lutra lutra*) (Morchón et al., 2012). In cats, seroprevalence values of 11.4% and 18.1% were detected in Barcelona and the Canary Islands, respectively (Montoya-Alonso et al., 2014, 2015). In neighbouring Portugal, canine dirofilariosis is endemic with an overall national seroprevalence of 2.1%- 15.1% (Alho et al., 2014; Vieira et al., 2015).

The incrimination of vectors of *Dirofilaria* in Spain has led so far to the detection of *D. immitis* DNA in the abdomen of two *Culex pipiens* (haplotype H1) in Salamanca (western Spain) and in *Cx. theileri* in the Canary Islands, and no mature infections have been found, as no DNA was detected in the head-thorax of the analysed specimens (Morchón et al., 2007, 2011).

Studies that will turn preliminary results into definite evidence for irrefutable implication of *Cx. pipiens* as a potential vector for canine heartworm disease transmission are of great relevance (Cancrini et al., 2006; Morchón et al., 2007; Yildirim et al., 2011; Latrofa et al., 2012), especially considering its abundance and wide geographic distribution, its genetic heterogeneity, having two bioforms with different behaviours (Gomes et al., 2015). The so-called pipiens form is characterized as being anautogenous, eurygamous, and preferably ornithophilic, while the *molestus* form is autogenous, stenogamous and preferably mamophilic. Therefore, disclosure of which form(s) of the vector may be implicated in *Dirofilaria* spp. transmission is undoubtedly relevant. Although Spain is an endemic country for canine heartworm disease (Simón et al., 2012), the implementation of appropriate control measures call for a more profound understanding of the epidemiology of this disease, of which the identification of the vector species involved, is of paramount importance to understand the dynamics of filarioid transmission to both animals and humans. Despite the high level of risk for dirofilariosis in the Extremadura region of Spain (Simón et al., 2014), there is no information regarding the distribution and prevalence of filarioid nematodes in mosquito vectors.

The objectives of this study were (i) to bring new insights on the distribution of these filarioid nematodes in field-collected mosquitoes from Extremadura (southwest Spain), (ii) to identify which species of mosquitoes could be involved in their transmission and (iii) assess the vector genetic variation.

2. Material and methods

2.1. Study region, mosquito collection and identification

The study was conducted in the Extremadura region in Southwest Spain (39°12′N 6°09′W). This region is characterized by a Mediterranean climate (Kottek et al., 2006), except to the north, where it is continental, and in the west, where due to the Atlantic Climate influence, displays milder characteristics. Most of the territory has an altitude between 200 and 600 m above sea level. The median annual rainfall is 605 mm (400–1.500 mm), with mean annual temperature ranging from 13 °C (in the North) to 18 °C (in the South), winter average temperatures slightly below 7.5 °C and summer average temperatures between 22 °C and 26 °C.

Mosquitoes were collected monthly from January of 2012 to December of 2013. CDC miniature light-traps (Model 512; John W. Hock Company, Gainesville, FL, USA) were placed outdoors in 21 stations distributed along 18 municipalities. Traps were placed at approximately 1 m from the ground and run for 24 h, without any baiting. Captured adult specimens were initially stored at −80 °C and subsequently morphologically identified to species and/or species complex under a stereomicroscope, following Becker et al. (2010). Each mosquito was transferred to individual, sterilized 1.5 ml vials and stored at −20 °C before being processed for DNA extraction.

2.2. DNA extraction and molecular identification

Female mosquitoes were dissected into head-thorax and abdomen, under the stereomicroscope using sterile needles, to discriminate between *Dirofilaria* spp. infective/infected status, respectively, in case a positive amplification result for *Dirofilaria* DNA detection was obtained (Ferreira et al., 2015). Genomic DNA was extracted using the method described by Collins et al. (1988). After DNA precipitation and washing (70% ethanol), the sediment was resuspended in 100 μl of TE buffer (pH 7.0) and stored at −20 °C. Negative controls (no DNA template) were performed for each extraction procedure.

To detect and identify *Dirofilaria* at the species level, PCR amplification of the internal transcribed spacer-2 (ITS-2) of *Dirofilaria* rDNA was performed using primers DIDR-F1 (5′-AGTGCGAATTGCAGACGCATTGAG-3′) and DIDR-R1 (5′-AGCGGGTAATCACGACTGAGTTGA-3′), as described by Rishniw et al. (2006). Optimal conditions for PCR amplification were as follows: initial denaturation at 94 °C for 2 min, 32 cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min.

As a confirmation protocol (for *Dirofilaria* detection/identification), amplification and sequencing of the filarial mitochondrial DNA cytochrome oxidase subunit I (COI) gene was also performed in a subsample of mosquitoes that tested *Dirofilaria* DNA positive by ITS-2 PCR. This was carried out following conditions described by Casiraghi et al. (2001), using primers COIintF (5′-TGATTGGTGGTTTTGGTAA-3′) and COIintR (5′-ATAAGTACGAGTATCAATATC-3′), and the cycling profile we used was an initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 45 s, extension at 72 °C for 90 s, with a final extension of 74 °C for 7 min.

In order to identify the members of the *Cx. pipiens* complex a PCR assay targeting species-specific polymorphisms in the intron-2 of the acetylcholinesterase-2 (ACE-2) gene was performed (Smith and Fonseca, 2004). Specific primers ACEpip (5′-GGAAACAAC GACGTATGTACT-3′), ACEquin (5′-CCTTCTTGAATG GCTGTGGCA-3′), ACEtorr (5′-TGCCTGTGCTACCAGTGATGTT-3′) and the universal primer B1246s (5′-TGGAGCTCCTCT TCACGG-3′) were used to identify *Cx. pipiens*, *Cx. quinquefasciatus* and *Cx. torrentium*, respectively. Genomic DNA from homozygous *Cx. pipiens* and *Cx. quinquefasciatus* were used as positive controls. To differentiate between *molestus* and *pipiens* biological forms of *Cx. pipiens* PCR amplification of the flanking region of microsatellite CQ11 was performed according to Bahnck and Fonseca (2006) using primers pipCQ11R (5′-CATGTT GAGCTTCGGTGAA-3′), specific for the *pipiens* form (200 bp amplicon), and molCQ11R (5′-CCCTCCAGTAAGGTATCAAC-3′), specific for the *molestus* form (250 bp amplicon) along with the universal primer CQ11F2 (5′-GATCCTAGC AAGCGAGAAC-3′). This analysis was carried out in *Cx. pipiens* specimens for which a *Dirofilaria* spp. DNA amplification had been obtained and with a subsample of negative mosquitoes. Positive controls (i.e. DNA from *molestus* and *pipiens* forms) were included in the PCR assays.

Finally, the haplotype of *Cx. pipiens* ITS-2 polymorphisms was assessed on the dirofilarial positive mosquitoes, by amplifying rDNA with primers 5.8S (5′-TGTGAACTGCAGGACACATG-3′) and 28S (5′-ATGCTTAAATTTAGGGGGTA-3′) (Bargues et al., 2006). The thermal cycler was set at 94 °C for 5 min, followed by 30 cycles with a denaturation step for 30 s at 94 °C, annealing for 30 s at 50 °C, and extension for 1 min at 72 °C, and a final extension for 7 min at 72 °C.

All the obtained amplicons by the different methods were analyzed by electrophoresis in 1.5% agarose gels stained with

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