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Short Communication

Detection of *Wolbachia* in *Dirofilaria* infected dogs in Portugal

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ABSTRACT

Wolbachia pipiens, an intracellular endosymbiont bacteria of filarial nematodes, has been implicated in the pathogenesis of filarial diseases, in particular in heavy *Dirofilaria* spp. infections. Antibiotic therapy (doxycycline) against *Wolbachia* has been proven to be suitable adjunct therapy, prior to adulticide treatment of canine dirofilariosis. Despite its importance, investigation on the *Wolbachia*/*Dirofilaria* complex in Portugal had not been undertaken so far. This study reports the first detection of *Wolbachia* in *Dirofilaria* spp. infected dogs in the context of an ongoing epidemiological survey in central-south regions in the country. *Wolbachia* DNA was detected by PCR in 52.6% (20/38) of canine blood samples positive for *Dirofilaria immitis* based on parasitological (Knott's and Acid Phosphatase) and serological (*Witness®Dirofilaria*) methods. No *Wolbachia* DNA could be detected in samples from dogs with occult infections (parasite negative but antigen positive).

The lack of *Wolbachia* detection in some microfilaremic dogs was somewhat unexpected and needs to be elucidated in further studies, as the presence or absence of these bacteria in association with microfilaria is of importance for veterinarians in the management and control of canine dirofilariosis.

1. Introduction

Many studies have recently been conducted on *Wolbachia pipientis*, an intracellular bacterium of the Anaplasmataceae family and, so far, the only species of the genus (Merçot and Poinsot, 2009). First discovered in arthropods, where they are widespread, it can also be found in some nematodes, in particular in agents of human and animal Onchocercidae, such as *Onchocerca volvulus*, *Wuchereria bancrofti* and *D. immitis* (Slatko et al., 2010; Martin and Gavotte, 2010; Ferri et al., 2011).

Although little is known about the interaction between *W. pipientis* and their nematode hosts, available evidence suggests that it plays an essential role in the biology of Dirofilariae (Genchi et al., 2011). In particular, filarial worms seem to need the bacteria to complete their life cycle or for embryogenesis. Therefore, the use of antibiotics (doxycycline) as an adjunct therapy targeting *W. pipientis* prior the administration of antiparasitic treatment, has been proven to contribute to the significant reduction in pathological side effects often induced by filaricidal drugs, particularly in heavy canine *Dirofilaria* infections (Grandi et al., 2010).

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As a result, antibiotic therapy became a useful approach for treatment and control of filariosis (Genchi et al., 2011; McHaffie, 2012).

Whilst *W. pipientis* has been found in all studied adult *Dirofilaria* spp. worms, two recent studies on canine dirofilariosis produced contradictory results. Rossi et al. (2010) detected *W. pipientis* DNA in all microfilaremic dogs infected with *D. immitis* in Brazil, but Tabar et al. (2013) found *W. pipientis* DNA in only 30.6% of microfilaremic dogs in the Mediterranean.

The present study aims to elucidate the relationship between *D. immitis* and *W. pipientis* in canine populations in Portugal in order to improve approaches for the control of animal dirofilariosis.

2. Materials and methods

Blood samples were collected from 308 dogs from kennels in three districts of central Portugal (Setúbal, Santarém and Coimbra), from October to November 2011. The sample included 183 females and 125 males, ranging from 6 months to 16 years (median 4.9 years ± 3.3 SD) most of them mixed breed.

The presence of *D. immitis* circulating adult female antigen was tested using a commercial kit (*Witness® Dirofilaria*, Synbiotics). Blood microfilariae were detected and identified through Knott's modified and Acid Phosphatase techniques (Genchi et al., 2007).

DNA was extracted from blood pellets (from Knott's technique) using the CTAB (Cetyltrimethylammonium bromide) method, adapted from (Stothard et al., 1996). Briefly, 50 μl whole blood was incubated with CTAB buffer and 0.2 mg Proteinase K (Bioline) at 56 °C for 2 h, with agitation. DNA precipitation was done with 0.6 ml absolute ethanol and the pellet hydrated in 50 μl TE buffer (pH 7.0).

Wolbachia pipientis DNA was detected by PCR primers wolbF and wolbR, which amplify a fragment of 1018 bp of the 16S rRNA gene (Foster et al., 2008). PCR reactions were carried out with Illustra™ puReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK), using a final volume of 25 μl and a primer concentration of 1 μM and 2 μl DNA per reaction. PCR conditions were as in Foster et al. (2008) but with an annealing temperature of 56 °C. PCR sensitivity was tested with serial dilutions (by a factor of 10) of DNA from a female adult worm of *D. immitis* and from canine blood samples with known concentrations of <5 ($n = 2$) or >20 ($n = 2$) microfilariae per 20 μl of blood. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. PCR products were purified with QIAquick Gel Extraction Kit (Qiagen) and sequenced on both strands by Stab Vida (Portugal). Sequences were edited and aligned using BioEdit (Hall, 1999) and compared to other similar sequences available in Genbank, as identified through BLAST.

Positive controls included DNA from a female *D. immitis* adult worm and DNA from *Drosophila melanogaster* known to harbour *W. pipientis* (by PCR).

Table 1
Correlation of *D. immitis* detection by Knott's and Witness® *Dirofilaria* tests.

Techniques	Witness® *Dirofilaria*		
	Ag+	Ag−	Total
Knott			
Mf+	24	14	38
Mf−	9	261	270
Total	33	275	308

Mf, blood microfilariae; Ag, *D. immitis* antigen.

3. Ethical considerations

The study was approved by the Commission on Ethic and Animal Welfare of the Faculty of Veterinary Medicine, Universidade Técnica de Lisboa, and all procedures were performed according to national and European legislations.

4. Results

Out of the 308 canine blood samples analysed, 47 samples (15.3%) were positive for *D. immitis* by at least one of the three diagnostic tests used (Knott's, Acid Phosphatase and Witness® *Dirofilaria*). *Dirofilaria immitis* antigen was detected in 33 samples (10.7%), while microfilariae were detected in 38 (12.3%) samples. Twenty four samples (7.8%) where positive for both parasitological and serological tests whereas 14 (5.1%) cases were only positive for blood microfilariae and *D. immitis* antigen was exclusively detected in nine (3.3%) samples (Table 1).

Microfilariae were identified as *D. immitis* in 37 of the 38 microfilaraemic dogs by Acid Phosphatase (AP) technique; one positive blood sample (Knott's) failed to stain hampering species identification.

For *W. pipientis* DNA detection, a PCR with a sensitivity to detected down to 14 pg of DNA from adult female *D. immitis*, and 0.16 ng or 3.7 ng of DNA from canine blood with >20 and <5 microfilariae per 20 μl of blood, respectively, was used. The estimated detection limit was approximately 0.01–0.05 microfilariae per reaction mixture (Fig. 1).

In the 47 *Dirofilaria* positive samples, *Wolbachia* DNA was detected by PCR in 20 samples (42.6%), all of which from microfilaremic dogs (Fig. 1). Negative PCR reactions were confirmed by using higher and lower DNA dilutions than in the initial reaction.

The 16S PCR product obtained from two blood samples was sequenced (accession numbers HG328332 and HG328333) and found to have 100% identity (99% query coverage) to sequences attributed to *Wolbachia* sp. symbionts of nematodes (GenBank AF304445.1, AY652762.1 and Z49261.1).

5. Discussion

This survey of dogs in kennels in central Portugal found a global prevalence of *D. immitis* infection of 15.3%, based on both parasitological and antigen detection methods.

Data analysis showed only a moderate level of agreement between the tests (Landis and Koch, 1977). Among