Detection of Wolbachia DNA in blood from dogs infected with Dirofilaria immitis

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A B S T R A C T

Dirofilaria immitis is the causative agent of heartworm disease in canines and felines, and pulmonary dirofilariosis in man. It harbors a symbiotic intracellular bacterium from the genus Wolbachia that plays an important role in its biology and contributes to the inflammatory pathology of the heartworm. This endosymbiont is sensitive to the tetracycline family of antibiotics prompting its use in the treatment of filariasis. To track Wolbachia during treatment, primers were designed based on the FtsZ gene from Wolbachia. These primers amplify a single PCR product with the expected size from DNA samples derived from various species of worms that harbor Wolbachia (D. immitis, Brugia malayi and Brugia pahangi). The detection limit of Wolbachia DNA in the assay was 80 pg of D. immitis DNA. Furthermore, the primer set successfully amplified the expected PCR product using blood samples from dogs harboring the heartworm and circulating microfilariae.

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1. Introduction

Wolbachia are intracellular alphaproteobacteria endosymbionts sheltered in a broad range of insects and nematodes (Pfarr and Hoerauf, 2007). According to surveys based on DNA amplification, 20% of the arthropod species are infected with Wolbachia, rendering this bacterium the most ubiquitous intracellular symbiont yet described (Bourtzis, 2008).

Several species of filariae, including the important parasites of humans and animals (e.g., Onchocerca volvulus, Wuchereria bancrofti and Dirofilaria immitis), harbor this bacterium concentrated in intracytoplasmic vacuoles within the hypodermal lateral cords of male and female worms and female reproductive organs (Taylor and Hoerauf, 1999). Wolbachia is important for the biology of the filarial host, being involved in the immunophatogenesis of filarial infections of mammals and in the side-effects of adulticidal therapy (Simoncini et al., 2001).

D. immitis is a filarial nematode that causes canine heartworm disease in many countries worldwide (Genchi et al., 2005). In Brazil, D. immitis is prevalent in all regions, but dogs living in coastal regions are more susceptible to infection (Labarthe and Guerrero, 2005).

The death of adult filarial worms, either due to treatment or natural causes, results in an exacerbated inflammatory lung response in dogs infected with D. immitis. This response is partially attributed to the release of Wolbachia antigens after disintegration of the worms (McCall et al., 2008). Therefore, Wolbachia is being targeted during therapy (Hoerauf et al., 2000, 2002). Specific antibiotic treatments aimed at reducing bacteria levels in worms have a beneficial effect by reducing filarial induced pathology (Debrah et al., 2006). The determination of the specific moment that Wolbachia infection is reduced prior to adulticidal treatment can play a pivotal role in controlling the severity of adverse effects of anti-parasitic therapy. The development of diagnostic tools to detect the endosymbiont is mandatory to allow the establishment of more effective treatment protocols and control cure criteria.

Analysis of Wolbachia phylogeny has only been based on a few genes (protein wsp, protein ftsZ, citrate synthase gktA, groEL chaperone, and small subunit ribosomal RNA [16S]) (Bandi et al., 1998; Casiraghi et al., 2001, 2005; Werren et al., 1995). Two genes (ftsZ and 16S rDNA) cluster of Wolbachia into eight supergroups: A, B, E, F, G and H encompassing arthropod Wolbachia and supergroups C and D consisting of nematode Wolbachia (Baldo et al., 2006; Casiraghi et al., 2003).

The product of the ftsZ gene controls the bacterial cell cycle being involved in the regulation of the cell division (Lutkenhaus, 1990). It was sequenced, showing a conserved and a highly divergent region allowing the discrimination of the bacterial genus.

The purpose of this study was to develop a PCR-based method, amplifying the Wolbachia ftsZ gene in order to detect bacterial DNA in peripheral blood of dogs infected with D. immitis.
2. Materials and methods

2.1. Selection of dogs and blood collection

After the owners' consent and agreement, the blood samples from 18 dogs from Arraial do Cabo, Rio de Janeiro, Brazil (22.92417°S; 42.22431°W) were collect and kept at 4 °C until processing.

2.2. Microfilariae blood count

Microfilariae detections were performed using a modified Knott's test. Smears of 20 µL (25 × 15 mm) of homogenized blood were fixed and stained with Giemsa for microfilariae counts. Two slides of each sample were prepared and examined independently by two veterinarians at 40× magnification that made two counts each. The same two veterinarians examined all samples. The final microfilariae count represents the mean of all the counts and extrapolated to 1 mL (Bendas et al., 2008).

2.3. DNA extraction

2.3.1. Adult nematodes

A D. immitis positive control was prepared from adult worms obtained at necropsy of naturally infected dogs from Rio de Janeiro state, Brazil. The worms were extensively washed and stored frozen at -20 °C. Brugia pahangi and Brugia malayi worms were kindly donated by Dr. John McCall.

DNA extractions were carried out from 0.5 cm sections of worms. After mechanical homogenization, DNA was extracted from cells using illustra™ GenomicPrep Cells and Tissue DNA Isolation Kit (GE Healthcare), according to the manufacturer's suggested protocol.

2.3.2. Dog blood samples

Blood samples from 13 dogs that were positive for (i) the presence of D. immitis (presence of peripheral microfilaria (KNOTT test) and (ii) search of D. immitis antigens (SNAP 3DX, IDEXX Laboratories Inc.) were used as positive controls. Five samples from healthy uninfected dogs (free of microfilariae and antigens) were used as negative controls. Samples were always used following blind procedures. DNA was extracted from 200 µL of whole blood using the illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare, Chalfont, St. Giles, UK), according to the manufacturer's instructions.

2.4. PCR amplification and sequencing

Forward (5'-ATA ACA GCA GGA ATG GGT GGT-3') and reverse (5'-TCA GGC ACT CTA TTT GCT GCA-3') oligonucleotides were designed for species-specific amplification based on conserved sequences of Wolbachia ftsZ gene (GenBank accession numbers AJ010272, AJ010273, AJ495000, A523519 and AJ131709) previously aligned by MEGA 4 software. The in silico specificity of the primers was tested by BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

PCR mixtures were composed of 1× buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3 and 1.5 mM MgCl2), 200 µM each of dNTPs (Invitrogen, Carlsbad, CA, USA), 7.2 pmol of both primers, 0.75 unit of AmpliTaq Gold™ DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and 30 ng of genomic DNA was used for each PCR reaction. The reactions were performed on a PXe thermal cycler (Thermo Electron Corporation, Waltham, MA, USA) under the following conditions: 94 °C for 2 min; 40 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s with a final extension at 72 °C for 10 min. PCR products were analyzed on 1.5% agarose gels stained by ethidium bromide and visualized under ultraviolet light.

2.5. Analytical sensitivity of the proposed PCR for detecting Wolbachia DNA

To test the sensitivity of the proposed PCR for detecting Wolbachia DNA, a dilution series (5 ng–4.8 pg) of Wolbachia DNA was generated using distilled water or 40 ng of DNA from healthy, uninfected dogs (negative for microfilariae by KNOTT test and D. immitis antigens). This set of experiments was carried out in order to define the analytical sensitivity of the assay in the presence of DNA from dogs that corresponds to the real scenario where the test will be applied.

2.6. DNA sequencing

Amplification products were purified using Wizard SV gel and PCR clean-up system Kit (Promega, Madison, WI, USA). Amplicons were sequenced (forward and reverse) with BigDye Terminator V3.1 Cycle Sequencing Ready Kit’ (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA) followed by analyses on a 48-capillary 3730 DNA Analyzer (Applied Biosystems). The reactions were carried out according to manufacturer's instructions.

2.7. Other bacterial DNA

The DNAs from Rickettsia typhi, Borrelia burgdorferi, Coxiella burnetii, Bartonella spp., Rickettsia rickettsii were provided by the Laboratory of Hantavirosis and Rickettsiosis, Fiocruz, Rio de Janeiro, Brazil. These DNAs were used as a control to test the specificity of the reaction of both Wolb Ftsz and Wolb Rtsz primers during the standardization of PCR experiments.

3. Results and discussion

A single amplification product of the expected fragment size was detected by the proposed PCR in a variety of samples (Fig. 1). The initial DNA tested came from D. immitis harboring Wolbachia. Considering the possibility that the worm harboring Wolbachia could influence the results, samples from B. malayi and B. pahangi also were tested. Each gave the expected band, suggesting that the PCR reaction can detect Wolbachia independent of the host. The sequences of the amplified PCR products confirmed that the amplified DNA represents DNA from Wolbachia bacteria (data not shown).

Next, we tested blood samples from dogs positive for infection by D. immitis as previously determined by traditional methods, KNOTT test and D. immitis antigens. A PCR product was detected in all D. immitis positive samples (Fig. 1, Lanes 6–8). PCR reactions

![Fig. 1. Detection for the presence of Wolbachia by PCR using primers against Wolb Ftsz and Wolb Rtsz. Amplicons of 550 bp was obtained in all samples from worms (Lanes 1–4) and blood of microfilaricaridicogs (Lanes 6–8). Lane L, 100 bp ladder; Lane 1, D. immitis; Lane 2, B. malayi (male sample); Lane 3, B. malayi (female sample); Lane 4, B. pahangi (female sample); Lane 5, no DNA; Lanes 6–8, blood samples from D. immitis infected dogs; Lanes 9–11, blood samples from dogs negative for infection; Lane 12, R. typhi DNA; Lane 13, B. burgdorferi DNA; Lane 14, Bartonella spp. DNA; Lane 15, C. burnetii DNA; Lane 16, R. rickettsi DNA; Lane 17, D. immitis DNA.](image-url)
performed on blood samples from non-infected dogs did not generate a PCR product confirming the reliability of the results regarding the finding of false positive results (Fig. 1, Lanes 9–11).

To confirm the specificity of the primer sets, PCR reactions were performed in the presence of DNA from other Alphaproteobacteria (*Rickettsia, Ehrlichia* and *Anaplasma*), closely related to *Wolbachia*. The results consistently showed the absence of amplified products (Fig. 1, Lanes 12–16). The distinction of *Wolbachia* from other alphaproteobacteria is important due to the possibility of co-infection with *Rickettsia* or *Anaplasma*, pathogens also capable of causing major diseases in humans and domestic animals (Blanco et al., 2008).

The analytical sensitivity of the PCR reaction was determined by assaying the detection limit (Fig. 2). *D. immitis* DNA, representing the *Wolbachia* DNA, was serially diluted into distilled water or into DNA purified from a healthy, uninfected dog. In both series, the reactions in the presence of dog DNA also demonstrate the specificity of the primer set for *Wolbachia* DNA.

Doxycycline has been used as part of new protocols to treat heartworm infected dogs, usually associated with *Rickettsia* or *Anaplasma*. In general, authors suggest that the use of the antibiotic reduces the potential risk for thromboembolism (Bazzocchi et al., 2008; McCall et al., 2008).

The future development of a specific method based on sensitive DNA amplification capable of detecting the reduction of bacterial load using clinical material from dogs will better address the therapy control. The description of a qualitative PCR is the first step to enable these future studies.

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Table 1 Detection of *Wolbachia* DNA by PCR in blood of microfilaremic dogs. Mf/mL corresponds to the number of microfilariae per milliliter of blood.

<table>
<thead>
<tr>
<th>Mf/mL</th>
<th>No. of dogs</th>
<th>PCR +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>5</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>150–300</td>
<td>4</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>300–10,000</td>
<td>4</td>
<td>4 (100%)</td>
</tr>
<tr>
<td>&gt;10,000</td>
<td>5</td>
<td>5 (100%)</td>
</tr>
</tbody>
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Fig. 2. Analytical sensitivity of the proposed PCR for detecting *Wolbachia* DNA. Successive dilutions were made in decreasing concentrations of DNA extracted from adult *D. immitis* in purified water (Lanes 11–19). The initial concentration of the DNA was 5 ng and dilutions of 1/2 to 1/1024. The same dilution of *Wolbachia* DNA was carried out in the presence of 40 ng of DNA from a healthy dog (free of microfilariae and antigens for *D. immitis*) (Lanes 1–9). Lane L, 100 bp ladder; Lane 10 and 20, negative control (no DNA).

References


