DETECTION OF WOLBACHIA DNA IN BLOOD FOR DIAGNOSING FILARIAL-ASSOCIATED SYNDROMES IN CATS

Running title: Detection of Wolbachia DNA in Feline Blood

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Abstract

A fundamental role for the endosymbiotic bacteria *Wolbachia pipientis* in the pathogenesis of *Dirofilaria immitis* infections has emerged in recent years. Diagnostic opportunities arising from this breakthrough have not yet been fully exploited.

This study was aimed at developing conventional and real-time PCR assays to carry out a molecular survey in a convenience sample of cats living in a *D. immitis* endemic area, and to evaluate the detection of bacterial DNA in blood as a surrogate assay for diagnosing filarial-associated syndromes in cats.

*COI* and *FtsZ* loci were used as targets for *D. immitis* and *Wolbachia* PCR assays, respectively, and Real-Time TaqMan® PCR assays were used only for *Wolbachia*. A convenience sample of 307 disease-affected or healthy cats examined at a University facility were PCR tested and their medical records investigated.

Conventional nested PCR for *Wolbachia* amplified the endosymbionts of both *D. immitis* and *D. repens* while real-time PCR was highly specific only for the former. Observed prevalences of 0.3% and 10.4% were found using conventional nested PCR assays for *D. immitis* and real-time PCR for *Wolbachia*, respectively. Similar prevalences were established using the Wolbachia nested PCR (98% concordance with real-time PCR). The group of *Wolbachia*-positive samples had a significantly higher proportion of subjects with respiratory signs (29.0% vs. 9.7%; \( p = 0.002 \)). The findings of this study indicate that a highly sensitive PCR assay can be used to detect the *Wolbachia* organism in the peripheral blood of cats with respiratory signs.

Keywords

*Wolbachia, Dirofilaria immitis, PCR, cats, diagnosis*
Introduction

Heartworm disease due to the nematode *Dirofilaria immitis* is globally widespread and is a leading cause of morbidity and mortality in dogs and cats in endemic areas. The prevalence in cats is consistent with the prevalence in dogs in a given area, even if in a lower proportion (5-20%) (10,21). Nevertheless, feline prevalence data might be biased by a very low testing frequency estimated as 0.06% versus a meaningful 33% in dogs (22). These testing frequencies clearly demonstrate that feline filariosis has not yet been perceived as an actual clinical problem by practitioners who erroneously believe that low infection rates occur in cats (9,22). Certainly, until now, a combination of physical examination and ancillary methods have been recommended for establishing the likelihood of feline filariosis *intra vitam* (2,7).

Filarial nematodes harbour *Wolbachia* endosymbionts. *Wolbachia* is an intracellular Gram-negative bacterium, belonging to the order Rickettsiales and found in 20-80% of the arthropod species and in the nematodes of the Onchocercidae family (4,14,36). In addition to the demonstrated symbiotic relationships regarding fecundity and long-term survival, *Wolbachia* has an important role in the pathogenesis of filarial infections of mammalians (19,31). In cats, the peculiar pathobiology of filariosis characterised by the death of the majority of immature adults (L5 larvae) arriving in the lung vasculature has been advocated as the cause of the recently described Heartworm-Associated Respiratory Disease (HARD) (3). The reaction was partially ascribed to the release of *Wolbachia* antigens after the disintegration of the worms (24). *Wolbachia* surface proteins (WSPs) are highly immunogenic and elicit a strong inflammatory response (23). Some authors have speculated that a diagnosis of filariosis could be attained serologically by detecting anti-*Wolbachia* antibodies. Indeed, in cats, antibodies against WSPs are...
promptly produced as early as 2 months after infection and last for more than 8 months (26).

Unfortunately, the increase in IgG persists months beyond the disappearance of adult nematodes and beyond the duration of *D. immitis* specific antibodies (17,18). Thus, their diagnostic suitability is limited. Conversely, PCRs targeting *Wolbachia* were developed for detecting the bacteria either in tissues, nematodes (8,20,25,32) or the blood of filarial-infected dogs (30). However, those studies reporting *Wolbachia* PCRs were not aimed at establishing diagnostic reliability but rather served as an insight into filarial pathogenesis. Thus, the authors hypothesised that the diagnostic expediency of PCR testing and, in particular of *Wolbachia* PCRs, were not fully exploited.

This study was aimed at developing conventional and real-time PCR assays to be employed in carrying out a molecular survey in a convenience sample of cats from a *D. immitis* endemic area, and for evaluating and comparing the detection of filarial and bacterial DNA in feline blood as surrogate assays for diagnosing feline filarial-associated syndromes in cats.

**Materials and Methods**

**Case material**

The present study utilised feline blood samples (ethylenediaminetetraacetic acid [EDTA]-anticoagulated) from a convenience-sampled population which had already been used in a different study (12). They had been obtained from cats seen at the Veterinary Teaching Hospital, University of Bologna, Italy, and underwent routine blood testing at the Veterinary Clinical Pathology Service between 1 January and 31 December 2006. The samples were frozen daily at -20 °C and stored until further analysis. Most of the samples had been collected from sick cats requiring haematological evaluation as part of a diagnostic profile whereas a minority of samples
were collected from apparently healthy cats which were at the hospital for pre-surgical testing or a pre-anaesthetic check. The samples collected from cats during repeated presentation were discarded (n=192). Only those samples with a minimum blood volume of 100 μl after routine haematological testing were included. The medical records of the subjects included in the study were retrieved, and the examiner was blinded as to the previous molecular findings. Due to its retrospective nature which examined a heterogeneous sample, the medical records were largely incomplete, and a definitive diagnosis had been obtained and/or reported in only a minority of cases. Nevertheless, in those cases without a definitive diagnosis, to complete the medical record, the clinician was forced to indicate the affected organ system on a definite field of the electronic medical records. This tool was used to categorise most of the subjects included in the study.

DNA extraction
The DNA was extracted from whole blood samples after thawing at room temperature; the extraction was accomplished by using the QIAamp® DNA blood mini kit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. The DNA was eluted into 200 μl Buffer AE and stored at -20° C until use.

A subset of 25 samples was repurified from frozen blood using the NucleoSpin ® kit (Macherey and Nagel, Milan, Italy) following the manufacturer’s instructions.

Conventional PCR and PCR sensitivity
Two different nested PCRs were set up for *D. immitis* and *Wolbachia* gDNA detection. The genomic DNAs of both *D. immitis* and *Wolbachia* were obtained from 3 dogs diagnosed with filariosis by means of the SNAP® Filaria RT Antigene (IDEXX Laboratories, Milan, Italy) and microfilarial detection on blood smear observations. Further molecular characterisation of the
gDNA was accomplished with primer pairs DIDR_F1 and DIDR_R1, as described by Rishniw et al. (28).

The primer pairs were designed on the Cytochrome oxidase subunit I (COI) gene of *D. immitis* (Accession number GI:40255343) as well as on cell division protein FtsZ of *Wolbachia* (GI:32562974). The prefixes Fil_COIclon and COIfel were assigned to the outer and inner primers for *D. immitis*, respectively. The prefixes Wol1 and Wol7 were assigned to the *Wolbachia* outer and inner primers, respectively. All the primers used in this study are reported in Table 1.

The first round PCR produced the expected size bands of 634 and 267 bp for *D. immitis* and *Wolbachia*, respectively. Therefore, the PCR products were purified using commercial kits (Perfectprep purification, Eppendorf, Milan, Italy). The purified amplicons were sequenced using a Big Dye Terminator v1.1 kit (Applied Biosystems, Milan, Italy), purified with Centri-Sep columns (Applied Biosystems, Milan, Italy) and electrophoresed on an ABI Prism 310 sequencer after denaturation with HiDi Formamide (Applied Biosystems, Milan, Italy) at 95°C for 5 min. Sequences covering almost 90% of the amplicons were 100% homologous with *D. immitis* and its *Wolbachia* endosymbiont. Consequently, the first round PCRs were cloned into the pCR-4 TOPO (Invitrogen, Milan, Italy) plasmid vectors. The plasmids were used to transform TOP10 chemically competent *E.coli* (Invitrogen, Milan, Italy) and were purified using the Plasmid purification kit (Sigma-Aldrich, Milan, Italy).

The plasmid copy number was determined by spectrophotometry. The plasmids were then linearised with *Sphl* endonuclease (New England Biolabs, Euroclone, Milan, Italy), and serial 10-fold dilutions were prepared for a PCR sensitivity assay. The precise serial dilutions of the linearised plasmid were also used to calibrate the real-time PCR assay for *Wolbachia* detection.
For the assessment of the specificity, in addition to the sequencing of 3 Wolbachia-positive samples, PCR assays were used to assess canine and feline genomic templates. In particular, Fil_COIclon and COIfel primers were assayed against canine gDNA containing Acanthocheilonema (Dipetalonema) reconditum and Dirofilaria repens canine gDNA while conventional and real-time Wolbachia PCRs were checked in PCR assays using Ehrlichia canis, Anaplasma platys (34), Bartonella henselae, and D. repens canine and feline gDNA which were previously positive in routine testing as templates.

To reduce the risk of contamination, the two nested PCR assays were set up with an internal control known as a mimic. The mimics were obtained as described by Ballagi-Pordány and Belák (1) with only slight modifications. Briefly, mimics are PCR amplicons which include the sequences recognised by the primers at each end of a different target (off-target) so that the sequence length between each target primer site is different from that of the target sequence. Mimics are easily and cheaply obtained by amplifying the off-target with a primer pair composed of a 3’ end specific for the off-target and a 5’ oligonucleotidic tail made up of sequences of the target. Unlike the original method, in this study, the 5’ oligonucleotidic tail was 2-3 bp shorter than the target primers in order to facilitate the recognition of the target sequence of the gDNA with respect to the mimics (Fig. 1). The PCR products were diluted 10^{-10} in molecular biology grade water and used in the PCR mixtures.

The PCR mixture for all PCR assays included 2.5 μl 10X PCR buffer (Invitrogen, Milan, Italy), 1.5 mM magnesium chloride, 300 nM each of forward and reverse primers, 250 nM dNTPs (10 mM dNTP Mix, PCR Grade®, Invitrogen), 1U recombinant Taq Polymerase (Invitrogen, Milan, Italy) and 2 μl of template brought up to 25 μl with mimics diluted 10^{-10} in molecular biology grade water (Eppendorf, Milan, Italy). Each PCR run included negative controls represented by
molecular biology grade water. The PCRs were carried out using an EP-gradient S thermalcycler (Eppendorf, Milan, Italy). The first *D. immitis* PCR round included an initial denaturation at 95°C for 4 minutes followed by 40 cycles at 94°C for 30 seconds, at 56.5°C for 30 seconds and at 72°C for 45 seconds, and a final extension step at 72°C for 5 minutes. The nested *D. immitis* PCR round differed only with respect to an annealing temperature of 54.5°C and an extension step of 30 seconds. The *Wolbachia* first round PCR consisted of an initial denaturation at 95°C for 4 minutes, followed by 40 cycles at 94°C for 30 seconds, at 51°C for 30 seconds and at 72°C for 30 seconds, and, finally, at 72°C for 5 minutes. The *Wolbachia* second round differed only with respect to an annealing temperature of 57°C. In both cases, the second round template was represented by a 1:10 dilution of the first round PCR mixture. The PCR products were evaluated after electrophoresis on 1.5% agarose gel and gel staining with ethidium bromide.

All conventional PCR reactions were carried out in duplicate. The last dilution yielding a positive result was assumed to be the limit of detection.

**Real-time Taqman™ PCR**

A Taqman™ assay was designed using Primer Express v3 software (Applied Biosystems, Milan, Italy) within the *Wolbachia* cloned sequences (Table 1), purchased (Proligo, Sigma-Aldrich, Milan, Italy) and used to re-assay all the samples. The real-time PCRs were carried out with a mixture composed of 10 μl of PCR mix 2x (Maxima probe master mix, Fermentas, Milan, Italy), 900 nM each of forward and reverse primers, 300 nM of Taqman™ probe, 2 μl of template and molecular biology grade water to reach a final volume of 20 μl. The real time PCRs were carried out with a 4 step protocol: initial denaturation at 95°C for 10 min. followed by 45 cycles at 92.5°C for 15 seconds, at 54°C for 15 seconds, at 54°C for 10 seconds with signal acquisition and at 72°C...
for 25 seconds in a StepOne ™ thermal Cycler (Applied Biosystems, Milan, Italy). The calibration was carried out by assessing each sample in triplicate.

The findings of both the conventional and the real-time PCR assays were considered positive when at least one out of the two replicates yielded a specific amplicon or a fluorescent signal.

Statistical analysis

The chi-square test was used to evaluate the differences in presenting clinical signs, and the odds ratio with a 95% confidence interval was calculated. A $P$ value $< 0.05$ was considered statistically significant.

Results

Sequencing

All positive controls, 3 randomly chosen samples positive for *Wolbachia* and the sole sample positive for *D. immitis* were sequenced. All confirmed 100% homology with the reference sequences.

Nested PCR assay sensitivity and specificity

Both nested PCR assays showed similar sensitivity performances. An assay sensitivity of about 900 copies/reaction for the first round and of 9 copies/reaction for the second round were obtained in both conventional PCR assays (Fig. 2). As expected, the internal standard did not show interference since similar assay sensitivity was demonstrated with or without the inclusion of mimics in the PCR mixtures (Fig. 2 and 3). The *D. immitis* nested PCR did not yield PCR products when canine gDNA positive for *Acanthocheilonema (Dipetalonema) reconditum* (Fig. 4) and *D. repens* were amplified. The *Wolbachia* nested PCR did not yield products when canine genomic DNA PCR positive for *E. canis, A. platys*, or when feline genomic DNA positive for *B.*
henselae by PCR were used as templates. Conversely, nested PCR amplified *Wolbachia* from *D. repens* positive gDNA samples.

**Retrospective survey using conventional nested PCR**

One out of 307 samples was positive when using the *D. immitis* nested PCR. The sample was already positive after the first round of PCR. Thirty-four out of the 307 samples were positive when using the *Wolbachia* nested PCR. All except one sample were negative after the first round and positive only after the second round. The only sample which was positive when using the first round was referred to as the *D. immitis* positive sample. Thus, approximately less than 900 and more than 9 copies/reaction of *Wolbachia* could be estimated in all cases without circulating *D. immitis*. The observed prevalences using conventional nested PCR were 0.3 % (0.0 % - 1.8 %; CI 95%) and 11.1 % (7.8 % - 15.1 %; CI 95%) for *D. immitis* and *Wolbachia*, respectively.

**Retrospective survey using real-time PCR and concordance with conventional PCR**

Real-time PCR was linear over 8 orders of magnitude from 9x10^6 to 9 target/reaction with a coefficient $R^2$ of 0.998. The mean coefficient of variation of each triplicate calibration point was 0.42% (range 0.22%-0.92%) and there was PCR efficiency of 97.521 (Fig. 5). The limit of detection was 9 targets/reaction although 1 out of 3 replicates at 0.9 copies/reaction yielded a signal. If the 0.9 target calibration point was included, the calculated efficiency was 99.212 with an $R^2$ of 0.999. Real-time Taqman™ PCR did not originate a signal when canine genomic DNA previously positive for *E. canis, A. platys* and *D. repens* by PCR or when a feline genomic DNA previously positive for *B. henselae* by PCR were used as templates. Using a real-time PCR assay, 32/307 samples were positive and the observed prevalence was 10.4% (7.2 % - 14.4 %; CI 95%). Concordant results were found in 301/307 (98.0%) cases (271 negatives and 30 positives). Four samples which were positive when using nested PCR were negative when using real-time PCR.
and, vice versa, two samples which were negative when using nested PCR were positive when using real-time PCR.

**Features of the sample**

The analysis of medical records allowed the classification of the clinical problem in 258/307 cases (84.0%). The sample was divided into 2 subgroups based upon the Wolbachia status obtained using real-time PCR (227 Wolbachia PCR negative; 31 Wolbachia PCR positive); the involvement of the respiratory organ system occurred more frequently in the Wolbachia positive group (Odds ratio = 4.1 (1.7 % - 10.0 %; p = 0.002)). The complete findings of the analysis of the medical records are reported in Table 2.

**Discussion**

In this study, the set-up of both the conventional nested PCR for *D. Immitis* and Wolbachia and the real-time quantitative PCR for Wolbachia only were carried out. Both the conventional and the real-time assays gave satisfactory results in terms of sensitivity and specificity even though the nested PCR could not differentiate between the endosymbiont of *D. immitis* and *D. repens*.

Whenever real-time thermal cyclers are available, the real-time technique is preferred due to the high sensitivity and even higher specificity achieved through the use of TaqMan™ hydrolysis probes. Furthermore, the reaction occurs in closed reaction tubes circumventing the risk of carry-over of the amplicons. Conversely, in nested PCR, the risk of contamination due to PCR products is elevated due to the opening of the first round tube. To limit this drawback of end-point nested PCR, an additional improvement was represented by the introduction of internal controls known as mimics (1). The technique was intended to limit the possibility of carry-over and false positive results in nested reactions. In this study, a slightly modified mimic technique was set up. The
modified mimics performed adequately without any detrimental effect on the sensitivity of the assay. Thereafter, although the real-time technique is very fast and effective, both end-point and real-time PCRs were shown to readily and effectively detect their target cloned in plasmids. The PCR assays were employed for retrospectively assessing the presence of both filarial and Wolbachia DNA in feline gDNA purified from a convenience sample of cats examined at the Veterinary Teaching Hospital of the University of Bologna. Although the limit of detection of the PCR assays was almost identical, the calculated percentage of the positive samples varied markedly. Indeed, only 1 positive case of D. immitis was present as opposed to 34 and 32 positive cases for Wolbachia using conventional nested or real time PCRs, respectively.

In the endemic area of Northern Italy, the prevalence of feline filariosis estimated by the presence of antibodies against D. immitis was approximately 24% while it was about 50-84% in untreated dogs (10). In the same endemic area, it was shown that 6.7% of cats harboured filarial nematodes (11). In this regard, the negligible prevalence (0.3 % (0.0 % - 1.8 %; CI 95%)) assessed by nested PCR unquestionably represents an underestimated value. Likely explanations are related to feline filarial pathobiology; cats harbour fewer (fewer than 6) parasites with a shorter lifespan than dogs. Cats are frequently parasited by one or only a few adult male nematodes; there are also very few microfilariae and their presence is transient due to the strong immune response of cats (21,27).

Conversely, the remarkably higher observed prevalence of Wolbachia (11.1% (7.8% - 15.1%; CI 95%)) using nested PCR or 10.4% (7.2% - 14.4%; CI 95%) using real-time PCR) is more consistent with the actual prevalence of filariosis.

The findings of the study may have different explanations which, unfortunately, cannot be addressed herein due to the inherent weakness of retrospective studies. Indeed, the discrepancy of the observed prevalence between D. immitis and Wolbachia could be: 1) both D. immitis and
Wolbachia were present, although only endosymbiotic bacteria, released either by living adult worms lying in vessels or microfilariae, could readily be detected by PCR because they were more abundant; 2) a massive release of Wolbachia in cats occurs within 90 days from infection during the migration of L5 immature adults before their arrival in the pulmonary vessels. It was shown that, unlike what happens in dogs, the majority of nematodes die prior to becoming adults due to the strong immune response of cats; 3) Wolbachia bacteria entered the host as endosymbionts of the nematodes but remained, infecting host cells after the disappearance of the adult nematodes; 4) sources other than D. immitis could have released Wolbachia.

Verifying the hypothesis that there was a greater likelihood of detecting Wolbachia DNA in infected animals than detecting D. immitis DNA was within the aims of this study. Wolbachia could be found in all the developmental stages of D. immitis; in adults, Wolbachia was present in the hypodermal cells of the lateral chords and in the reproductive organs of females (15,16).

Overall, 1 nematode harbours thousands of bacteria and the bacteria are released en masse when the parasite is killed by adulticidal therapy or dies spontaneously (6). As a result of the above-mentioned evidence, a PCR targeting Wolbachia DNA would be more suitable than a PCR targeting D. immitis DNA, and the positive results of a Wolbachia PCR could be considered the surrogate of a positive filarial test and diagnosis thereof.

Nonetheless, indirect evidence supports alternative explanations. In this study, the group of Wolbachia-positive samples showed a significantly higher prevalence of respiratory disorders, although positive cases showed a wide range of clinical syndromes. Typically, feline filariosis is characterised by physical signs which may vary greatly from asymptomatic to fatal cases. Many cats never present any signs during their lifetime; in other cases, signs and symptoms may be acute or chronic, and mild or severe (7,11,35). Typically, respiratory signs characterise the HARD
syndromes. Even though arteritis and thromboembolic disease secondary to the presence of adult

*D. immitis* in the pulmonary arteries could explain a few of the manifestations of feline filarial
disease at post mortem examination, it was nevertheless demonstrated that severe pulmonary
lesions occur in the absence of adult worms (5,8). Macrophages containing *Wolbachia* have been
found in the lungs, kidneys and liver of dogs infected with *D. immitis* (18). Evidence suggests
that, in cats, even transient filarial infections cause enduring and even worsening lung lesions (4).

Lung lesions are typically characterised by both parenchymal and vascular inflammation evolving
into arteriolar occlusive hypertrophy. Typically, the WSP antigen was demonstrated in lung
lesions (8,17,18). The WSP antigen of *Wolbachia* elicits a strong inflammatory response itself in
the host so a pivotal role in the HARD syndromes was hypothesised (24). However, a recent study
failed to demonstrate a clear association between the presence of either *Wolbachia* DNA or WSPs
in lung lesions and the severity of pulmonary lesions (8).

The possibility of other sources of *Wolbachia* organisms merits further investigation. The
occurrence of cutaneous dirofilariosis sustained by *D. repens* is reported in limited case series and
the presence of *D. repens* in cats was demonstrated in a central region of Italy (33). Both
conventional and real-time PCR assays were designed on the nucleotidic sequences of the
*Wolbachia* endosymbiont of *D. immitis* since only partial *FtsZ* nucleotidic sequences are available
in public databases to date. Indeed, nested PCR, but not real-time PCR, could amplify the
*Wolbachia* endosymbiont of *D. repens*. Though the possibility that the few discordant cases, in
particular the nested PCR positives which were negative using the real-time PCR, could be
ascribed to the presence of the *D. repens* endosymbiont; overall, the observed prevalences
obtained using the two PCR methods overlap.
In addition to filaroid parasites, other sources, represented by arthropods, are possible and could not be ruled out. Indeed if, on the one hand, common nematodes of cats were not reported to harbour *Wolbachia* (4), on the other hand, ticks and fleas harbour *Wolbachia* (29) and no studies have investigated the possibility that *Wolbachia* are transmitted during the feeding of hematophagous arthropods. In addition, the absence of *FtsZ* sequences of the *Wolbachia* endosymbiont in cat fleas and ticks impedes ruling out cross-amplification using *in silico* methods.

Having stated that those uncertainties were not fully addressed herein, a prospective study investigating a combination of filarial or even *Wolbachia* (13) antibody assays and PCR assays is warranted in order to clarify the issues related to the universality of *Wolbachia* primers and probes, and the role of *D. repens* as well as the exact significance of PCR positivity. Such a study could also establish the accuracy of a combination of a *D.immitis* antibody assay and a *Wolbachia* PCR assay as well as argue, for or against, the use of antibiotic therapy targeting *Wolbachia*.

In this complex scenario, the findings of this study add a small piece to the puzzle and further support the role of *Wolbachia* in the pathogenesis of filarial-associated syndromes and the HARD syndrome, in particular.

In conclusion, the findings of this study indicate that testing *Wolbachia* by means of PCR could be suitable for reaching a diagnosis of filarial-associated syndrome in cats and could therefore be convincingly introduced into the clinical setting.

References


Captions

Figure 1.
Schematic representation of the mimic technique. In A, the method of obtaining the mimic amplicons is described. In B, the use of the mimic amplicons in the first *D. immitis* PCR round is shown.

Figure 2
Sensitivity of the *Dirofilaria Immitis* nested PCR (A) and *Wolbachia* nested PCR (B) assays. Precise serial dilutions of the linearised PCR 4 TOPO® vector containing the *D. Immitis* COI and *Wolbachia* FtsZ sequences were used as a PCR template. The amounts of target are indicated above each lane. (A) Lanes 2-9: 1st PCR round. Upper bands of expected 634 bp size represent the *Fil_COIclon* amplicons whereas the lower bands of 280bp represent the mimic internal standard. Lanes 11-15: 2nd PCR round. Upper bands of expected 406 bp size represent the COIfel amplicons whereas the lower bands of 237bp represent the mimic internal standard. (B) Lanes 2-9: 1st PCR round. Upper bands of expected 267 bp size represent the Wol1 amplicons whereas the lower bands of 220bp represent the mimic internal standard. Lanes 11-15: 2nd PCR round. Lower bands of expected 147bp size represent the Wol7 amplicons whereas the upper bands of 309bp represent the mimic internal standard. MWM= molecular weight marker, 100 bp ladder. The 500 bp band is indicated by an arrowhead.
Figure 3.
Sensitivity of the 1st round *Wolbachia* nested PCR without mimic internal standard. This figure should be compared to Figure 2, lanes 2-9. An almost identical pattern to Fig. 2 was seen indicating the same limit of detection of 900 copies/reaction.

Figure 4.
Molecular identification of *Dirofilaria immitis*. Amplicons obtained using different filarial gDNA templates. Lanes 1-4: PCRs using primer of *D. immitis* COI-fel; Lanes 6-9: PCRs using a primer of *D. immitis Fil COI-clon* and lanes 11-14: PCRs using primers DIDR_F and DIDR_R. Ar: *Acanthocheilonema (Dipetalonema) reconditum* templates; Di: *D. immitis* templates; CTR -: no DNA control. The lowest bands of lanes 1-4 and 6-9 represent the mimic internal standard PCR product. MWM= molecular weight marker, 100 bp ladder. The 500 bp band is indicated by an arrowhead.

Figure 5
Calibration curve (upper) and the exponential PCR curves (lower) of the real-time PCR *Wolbachia* assay.

Table 1
Primers and probe sequences used in the study
Table 2

Organ system involved at presentation according to the medical records of the convenience sample.
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<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>R_Fil_COIclon_is</td>
<td>agtcccaataccacatctctctctctctctctctctctctct</td>
<td><em>D.immitis</em> – 280 bp</td>
</tr>
<tr>
<td>Fwd_COIfel_is</td>
<td>tcctgggagtagttgaataacatgtctgtctctctctctct</td>
<td>Mimic for 2° round PCR for</td>
</tr>
<tr>
<td>R_Fil_COIfel_is</td>
<td>ctaatactccaaacacccggtacacaaaaaggttacatggaagc</td>
<td><em>D.immitis</em> – 238 bp</td>
</tr>
<tr>
<td>System</td>
<td>Overall sample (n°=258)</td>
<td>PCR <em>Wolbachia</em> negative group (n°=227)</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td></td>
<td>n° (%)</td>
<td>n° (%)</td>
</tr>
<tr>
<td>Respiratory</td>
<td>31 (12.0)</td>
<td>22 (9.7)</td>
</tr>
<tr>
<td>Digestive</td>
<td>83 (32.2)</td>
<td>73 (32.2)</td>
</tr>
<tr>
<td>Urogenital</td>
<td>40 (15.5)</td>
<td>37 (16.3)</td>
</tr>
<tr>
<td>Nervous</td>
<td>28 (10.9)</td>
<td>24 (10.6)</td>
</tr>
<tr>
<td>Others</td>
<td>76 (29.5)</td>
<td>71 (31.3)</td>
</tr>
</tbody>
</table>