# Detection of heartworm infection in dogs via PCR amplification and electrospray ionization mass spectrometry of nucleic acid extracts from whole blood samples

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**Objective**—To develop and evaluate a rapid and accurate assay involving PCR amplification and electrospray ionization mass spectrometry of nucleic acid extracts from whole blood samples for the detection of *Dirofilaria immitis* infection in dogs.

**Sample**—Whole blood nucleic acid extracts from 29 dogs experimentally infected with *D immitis* (and in which circulating *D immitis* antigen was detected) and 10 uninfected dogs.

**Procedures**—16 of the 29 whole blood samples from infected dogs were examined at the time of collection for circulating microfilaria. Nucleic acids were extracted from all whole blood specimens and underwent PCR amplification with 12 PCR primer pairs designed to detect a wide range of pathogens (including the *Wolbachia* endosymbiont of *D immitis*) and electrospray ionization mass spectrometry.

**Results**—On the basis of assay results, heartworm infection was detected in 13 of 13 antigen-positive dogs of unknown microfilaria status, 11 of 11 antigen-positive dogs with circulating microfilaria, 0 of 3 antigen-positive dogs tested at 3 months after larval infection, 0 of 2 antigen-positive dogs with occult infections, and 0 of 10 uninfected dogs.

**Conclusions and Clinical Relevance**—With the assay under investigation, it was possible to identify *D immitis* infection in dogs with circulating microfilaria via detection of the obligate *Wolbachia* endosymbiont of *D immitis*. It was not possible to identify dogs with occult infections, which suggested that circulating microfilaria must be present to detect infection with this assay, although further studies would be required to verify that finding. (*Am J Vet Res* 2012;73:854–859)

Parasitic filarial nematodes cause diseases in humans and other animals. In humans, various types of filarial nematodes, such as *Onchocerca vulvulus* and *Wuchereria bancrofti*, are estimated to infect 120 million people worldwide annually; 40 million people develop infection-associated clinical symptoms.[1] *Dirofilaria immitis* is a mosquito-borne nematode that infects both dogs and cats. In dogs, *D immitis* infections are typically subclinical initially but, if untreated, can progress to development of chronic respiratory tract problems or cardiac abnormalities and can eventually result in death. Incidence of *D immitis* infections in dogs can be > 50% in some regions, and this rate is increasing in Europe.[2,3]

**Abbreviations**

| | |
|---|---|
| ESI | Electrospray ionization |
| MS | Mass spectrometry |
| RT | Reverse transcription |

Early detection and treatment of heartworm infection in dogs when the parasite burden is low can lessen the effects of the parasitic infection as well as the possible adverse effects from treatment. Additionally, the ability to measure the parasite burden during treatment can be used to monitor the efficacy of the treatment regimen as well as to confirm when the parasite infection has been eliminated.

*Dirofilaria immitis*, similar to other filarial nematodes, harbor a distinctive bacterial endosymbiont from the *Wolbachia* group.[4] Research has shown that this *Wolbachia* endosymbiont is necessary for the proper development of adult *D immitis*.[5] Administration of tetracycline to kill the *Wolbachia* endosymbionts in some filarial nematodes prevents development of larval stages and inhibits embryogenesis in adult heartworms.[6]

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During infection with *D immitis*, these endosymbiotic bacteria can elicit an immunologic response from the host.[7] Although there is evidence of such an immune response caused by the endosymbionts, the exact role of the *Wolbachia* organisms in the pathological changes associated with heartworm infection is unknown.[8,9]

In the study reported here, a broad-range PCR–ESI-MS assay that was designed to detect a wide range of vector-borne pathogens was used to indirectly detect *D immitis* infection in dogs. In previous studies,[10–13] several of the assay primers were successfully used to detect flaviviruses and *Ehrlichia*, *Rickettsia*, and *Borrelia* spp. The strength of this assay is that the primers broadly amplify nucleic acids of a group of pathogens; the resulting base count signatures can be used to detect and identify pathogens to the species level, as demonstrated by the detection and species identification of several *Ehrlichia* organisms (eg, *Ehrlichia chaffensis and Ehrlichia ewingii*) from clinical specimens.[11] The assay used also involves primers for the broad-range detection of *Babesia* spp and Alphaproteobacteria, including the *Wolbachia* endosymbionts. Thus, the purpose of the study reported here was to evaluate whether an assay involving PCR amplification and ESI-MS of nucleic acid extracts from whole blood samples could be used for the detection of *Dirofilaria immitis* infection in dogs through identification of the obligate *Wolbachia* endosymbiont.

## Materials and Methods

**Infection and blood sample collection**—Blood samples were obtained from a company[a] that maintains dogs infected with *D immitis*. Whole blood samples (10 mL of blood/dog, of which 1.25 mL was used for extraction) were collected from 29 *D immitis*–infected dogs and 10 uninfected dogs in the company's facility at the time of the study. Infected dogs were Beagles that had been previously inoculated with adult or larval *D immitis* under the guidance of an animal ethics committee. Adult heartworms were inoculated intraventricularly, and larvae were inoculated SC. Prior to blood sample collection, infection in these dogs was confirmed on the basis of results of screening whole blood samples for *D immitis* antigen. Uninfected dogs were also screened to confirm the absence of circulating *D immitis* antigen prior to collection of whole blood samples for use in the present study. For 16 of the 29 infected dogs, blood samples were examined for circulating microfilaria at the time of sample collection, and the number of parasites/20 µL was recorded for each dog. Each blood sample was collected in a 7-mL tube containing EDTA, immediately frozen, and transported for analysis.[b]

**Extraction of DNA from blood samples**—A combination of bead-beating cell lysis and magnetic bead isolation was used to extract nucleic acids from whole blood samples obtained from infected and uninfected dogs. For homogenization, 1.25 mL of each whole blood sample was placed in a single 2.0-mL screw-cap tube[c] filled with 1.35 g of 0.1-mm yttrium-stabilized zirconium oxide beads.[d] To each tube containing 1.25 mL of blood, proteinase K solution[e] (25 µL), 20% SDS solution[f] (142 µL), extraction control[g] (1 µL), and antifoam A[h] (10 µL) were added. The mixture was then homogenized in a tissue homogenizer[i] at 6,200 revolutions/min for 90 seconds 3 times (5-second interval between events); total homogenization time was 270 seconds. Each homogenized lysate was incubated at 56°C for 15 minutes and then centrifuged for 3 minutes at 16,000 × *g* in a benchtop microcentrifuge. Subsequently, nucleic acids were isolated with a magnetic particle processor.[j] One milliliter of lysate was transferred to a 24-well deep-well plate[j] along with lysis buffer[g] (1.1 mL) and magnetic particles[g] (160 µL). Each lysate mixture was incubated for 16.5 minutes in the lysis buffer at 56°C. Specimens were then washed once in wash buffer[k] and 3 times in another wash buffer[l] (1-minute incubation for each wash step). The magnetic beads were then dried for 3 minutes at 65°C, and nucleic acids were eluted into 250 µL of elution buffer[g] by incubating the magnetic particles at 65°C for 3 minutes.

**PCR and RT-PCR assay procedures**—Detection of heartworm infection was performed with an assay involving PCR primers that were designed to detect a wide range of vector-borne pathogens by targeting conserved regions of DNA that border variable regions (**Appendix 1**). Primer pairs BCT3511, BCT3517, BCT2328, and INV4855 were run as a multiplex PCR procedure. A 1-step RT-PCR procedure was performed for the reactions containing primer pairs VIR2217 (single-plex reaction) and VIR2230 with BCT3570 (multiplex reaction). The remaining primer pairs were run in single-plex PCR procedures. For one of the primer pairs in the reactions, an internal positive control made from cloned synthetic DNA[m] (20 copies/reaction) was included. Each internal control was designed to be identical to the expected amplicon, with the exception of a 5-bp deletion to enable the control to be distinguished from the target-derived amplicon.

The PCR procedure was performed in a 50-µL reaction volume containing 10 µL of nucleic acid extract in a reaction mix that combined a 750nM concentration of each primer as previously described.[10] The 1-step RT-PCR procedure was performed in a 50-µL reaction volume containing 10 µL of nucleic acid extract in a reaction mix that combined a 750nM concentration of each primer as previously described.[14] Because all reactions for a sample were run in the same 96-well RT-PCR plate, cycling conditions were used for both the RT-PCR and PCR procedures as previously described.[14]

**16S DNA sequence analysis**—The presence of the *Wolbachia* endosymbiont in *D immitis*–infected dogs was confirmed via 16S DNA sequencing of a *Wolbachia*-positive specimen with primers 4F and 801R (**Appendix 2**) to amplify an 800-bp region of the 16S gene. The primers have an M13 tag sequence, which was used for the sequencing reactions. Nucleic acid extract from a blood sample of an infected dog included in the study was used as a representative extract of all *D immitis*–infected study dogs. Each 16S PCR assay was performed in a 40-µL reaction volume containing 1 µL of nucleic acid extract in a reaction mix composed of 1 U of immolase *Taq* polymerase, 20mM Tris (pH, 8.3), 75mM KCl, 1.5mM $MgCl_2$, 0.4M betaine, 200µM deoxyadenosine triphosphate, 200µM deoxycytidine triphosphate, 200µM deoxythymidine triphosphate, 200µM deoxyguanosine triphosphate, 20mM sorbitol,

Table 1—Results of a PCR–ESI-MS assay performed with each of 2 primer pairs (BCT3575 and BCT3570) to detect the *Wolbachia* endosymbiont of *Dirofilaria immitis* in whole blood samples collected from 29 dogs in which adult or larval *D immitis* had been previously inoculated.

| Microfilarial status at time of blood sample collection | *D immitis* inoculation | Interval after *D immitis* inoculation (mo) | *Wolbachia* endosymbiont detected with BCT3575 | | *Wolbachia* endosymbiont detected with BCT3570 | |
|---|---|---|---|---|---|---|
| | | | Yes | No | Yes | No |
| Not determined (n = 13) | 6 F and 6 M adults (5) | 5 (5) | 5 | — | 4 | 1 |
| | 10 F and 10 M adults (1) | 35 (1) | 1 | — | — | 1 |
| | 50 larva (3) | 22 (2) | 2 | — | — | 2 |
| | | 33 (1) | 1 | — | 1 | — |
| | 250 larva (2) | 7 (2) | 2 | — | 2 | — |
| | 300 larva (1) | 7 (1) | 1 | — | 1 | — |
| | 400 larva (1) | 7 (1) | 1 | — | 1 | — |
| Negative (0 microfilaria/20 µL of blood [5]) | 10 F and 10 M adults (1*) | 7 (1) | — | 1 | — | 1 |
| | 13 F and 13 M adults (1*) | 19 (1) | — | 1 | — | 1 |
| | 400 larva (3) | 3 (3) | — | 3 | — | 3 |
| Positive (2 to 460 microfilaria/20 µL of blood [11]) | 10 F and 10 M adults (3) | 19 (1) | 1 | — | 1 | — |
| | | 55 (2) | 2 | — | 2 | — |
| | 13 F and 13 M adults (1) | 19 (1) | 1 | — | 1 | — |
| | 21 F and 20 M adults (1) | 24 (1) | 1 | — | 1 | — |
| | 50 larva (2) | 10 (2) | 2 | — | 2 | — |
| | 300 larva (4) | 11 (3) | 3 | — | 2 | 1 |
| | | 19 (1) | 1 | — | 1 | — |

Values in parentheses represent the number of dogs (1 sample/dog).
*Dog with occult infection.
— = Not applicable. F = Female. M = Male.

2 µg of sonicated poly A RNA/mL, 500 µg of ultrapure bovine serum albumin/mL, and a 750nM concentration of each primer. The following PCR cycling conditions were used to generate the 16S amplicons on an 96-well thermocycler[n]: 95°C for 10 minutes, followed by 8 cycles of 95°C for 15 seconds, 50°C for 45 seconds, and 72°C for 90 seconds, with the 50°C annealing temperature increasing 0.6°C for each cycle. The PCR assay then continued for 37 additional cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 60 seconds. The PCR cycle ended with a final extension of 4 minutes at 72°C, followed by a 4°C hold. The PCR amplicons were sequenced[o] with a DNA sequencer.[p]

**MS and analysis**—Mass spectrometry was performed with a biosensor.[q] After PCR amplification, 30-µL aliquots of each PCR product were desalted and analyzed via MS as previously described.[10,11,15,16] Dogs were considered infected with *D immitis* when results yielded the appropriate base count for either the BCT3575 or BCT3570 primer pair.

## Results

**Infection status of dogs**—For all 29 dogs that had been inoculated with *D immitis* (adult or larval forms), circulating *D immitis* antigen was detected in the blood samples at the time of collection. Samples were collected from the infected dogs at 3 to 55 months after inoculation (**Table 1**). Blood samples from all uninfected dogs yielded negative results for circulating *D immitis* antigen.

**Detection of *D immitis* infections in canine blood samples via the PCR–ESI-MS assay**—Whole blood samples from 29 dogs infected with *D immitis* and 10 uninfected dogs were used to estimate the ability of the assay under investigation (which was designed to detect a wide range of clinically relevant vector-borne pathogens) to identify *D immitis* infection in dogs via detection of the obligate *Wolbachia* endosymbiont of *D immitis*. Two of the assay primer pairs, BCT3575 and BCT3570, target Alphaproteobacteria and result in specific amplicons in the blood samples from dogs infected with *D immitis* (**Figure 1**). These signatures were not observed in blood samples collected from uninfected dogs. These base count signatures were also unique, compared with those of other Alphaproteobacteria that have been screened with these primers (data not shown).

Blood samples collected from 16 of the 29 infected dogs were examined for circulating microfilaria at the time of sample collection; microfilarial status was not determined for the other 13 infected dogs. In the 13 *D immitis* antigen–positive blood samples for which the microfilarial status was unknown, the *Wolbachia* endosymbiont of *D immitis* was detected by BCT3575 and BCT3570 in all samples and in 9 samples, respectively (Table 1). In 11 blood samples in which microfilaria concentrations ranged from 2 to 460 microfilaria/20 µL of blood, the *Wolbachia* endosymbiont of *D immitis* was detected by BCT3575 and BCT3570 in all samples and in 10 samples, respectively. Two dogs had occult infections; the blood samples collected from those dogs were *D immitis*–antigen positive but microfilaria negative. The endosymbiont was not detected in the blood samples collected from dogs with occult heartworm infections. Additionally, the endosymbiont was not detected with either primer pair in blood samples collected from 3 dogs that had been inoculated with *D immitis* larvae 3 months earlier. The *Wolbachia* endosymbiont of *D immitis* was not detected by BCT3575 or BCT3570 in any blood sample collected from the 10 uninfected dogs. For all blood samples (from infected and uninfected dogs), no other pathogens were detected via the PCR–ESI-MS assay.

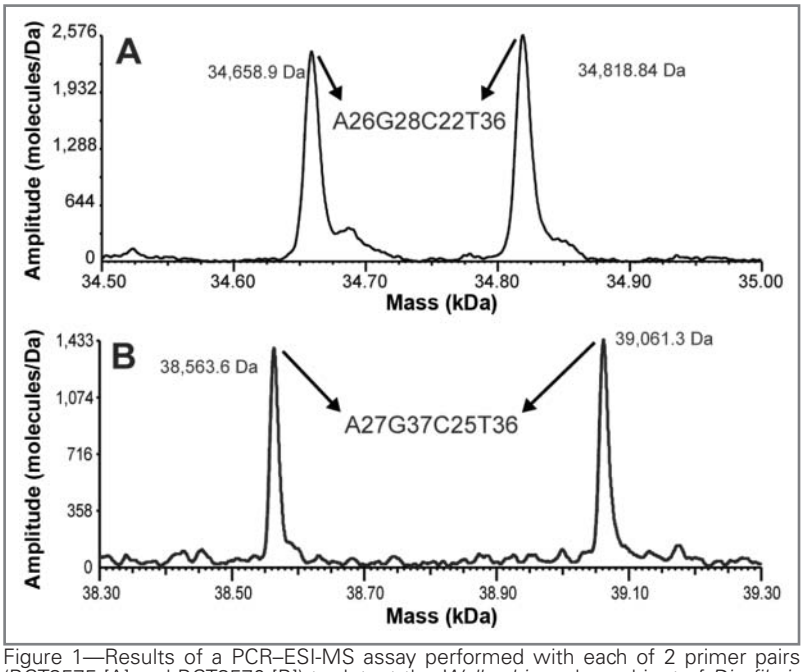


Figure 1—Results of a PCR–ESI-MS assay performed with each of 2 primer pairs (BCT3575 [A] and BCT3570 [B]) to detect the *Wolbachia* endosymbiont of *Dirofilaria immitis* in a whole blood sample collected from a dog in which heartworm infection had been experimentally induced. For *Wolbachia* endosymbiont amplicon detection, primer pair BCT3575 targets *rpoB* and primer pair BCT3570 targets *gltA*; targets are conserved regions of endosymbiont DNA that border variable regions. The mass for both the forward and reverse strands of the amplicons are given. Between the major peaks in each panel, the numbers after the letters A, G, C, and T represent the amplicon base composition (ie, the number of adenine [A], guanine [G], cytosine [C], and thymine [T] bases).

**Identification of the *Wolbachia* endosymbiont of *D immitis* via 16S DNA sequencing**—The presence of the *Wolbachia* endosymbiont of *D immitis* in the blood samples collected from infected dogs was confirmed via sequencing of the 16S amplicon from a representative sample of 1 infected dog. The sequencing results obtained matched that of the previously sequenced *Wolbachia* endosymbiont of *D immitis* (719/720 nucleotides; GenBank accession No. AF088187). No other 16S sequence was detected in the blood sample collected from that *D immitis*–infected dog.

## Discussion

Companion and domestic animals can become infected with a wide range of vector-borne pathogens (bacteria, viruses, protozoa, and nematodes), which can be both life-threatening and difficult to diagnose. These animals also can serve as sentinel species for the spread of vector-borne pathogens to humans.[17,18] To this end, we have developed a single broad-range PCR–ESI-MS assay to detect and identify a wide range of vector-borne pathogens important to animal health, including Spirochaetes (eg, *Borrelia* and *Leptospira* spp), Gammaproteobacteria (eg, *Francisella* spp), Alphaproteobacteria (eg, *Ehrlichia*, *Anaplasma*, *Rickettsia*, *Bartonella*, and *Wolbachia* spp), flaviviruses (eg, Powassan virus and tick-borne encephalitis virus), and protozoa (eg, *Babesia* spp). In the present study, we demonstrated the ability of this assay to identify *D immitis* infection in dogs via detection of the heartworm *Wolbachia* endosymbiont in whole blood samples. The entire assay process, from specimen preparation to result reporting, can be completed within 6 hours for 1 sample; moreover, the biosensor system[q] used in the present study has a potential throughput of > 150 samples/24 h. Assay results provide veterinarians with useful information about a wide range of infectious organisms, including coinfections. To our knowledge, this is the first report of heartworm infection in dogs that was directly detected via assessment of blood samples by use of the PCR–ESI-MS assay.

Identification of *D immitis* infection has been routinely performed via microscopic detection of the circulating microfilaria or detection of circulating *D immitis* antigen via an ELISA or other immunochromatographic tests. Visual detection of circulating microfilaria requires examination of ≤ 1 mL of blood by a skilled technician, and a positive result can be overlooked. It has been previously reported that even in areas with a high prevalence of heartworm infection, approximately 20% of the infected dogs may not have circulating microfilaria, which creates challenges for visual detection methods.[19] In the present study, the *Wolbachia* endosymbiont was detected in all blood samples that contained microfilaria, but it was not detected in blood samples from 2 dogs with occult infections, suggesting that circulating microfilaria might be required to detect the heartworm endosymbiont in canine blood samples. However, further controlled investigations of blood samples collected from dogs with occult infections would be needed to test this hypothesis. Nevertheless, results of the present study have suggested that the PCR–ESI-MS assay could be used in place of microscopy to detect *D immitis* infections in dogs with circulating microfilaria.

Similar to *D immitis*, *Wolbachia* bacteria are harbored by many other filarial nematodes, including pathogens of other domesticated animals (eg, *Parafilaria bovicola* in horses and *Onchocerca* spp in cattle) and humans (eg, *W bancrofti*, *Onchocerca volvulus*, and *Brugia malayi*).[6] Due to the broad-range design of the assay, it may be possible to detect these and other filarial nematode infections in humans and other animals via detection of their respective obligate endosymbionts, although further studies would be required.

Heartworm is one of many vector-borne diseases that infect dogs. In the study reported here, a broad-range PCR–ESI-MS assay designed to detect vector-borne pathogens was used to identify *D immitis* infection in dogs through detection of the heartworm *Wolbachia* endosymbiont in whole blood samples. By use of the assay, whole blood samples collected from experimentally infected dogs with circulating microfilaria were analyzed and results confirmed *D immitis* infection. This assay can also detect a wide range of vector-borne bacterial, viral,

and protozoal pathogens (including co-infections) in a single blood sample. Further controlled studies to evaluate the use of this PCR–ESI-MS assay for the diagnosis of *D immitis* infection in dogs in clinical settings are warranted.

a. TRS Labs Inc, Athens, Ga.
b. Ibis Biosciences Inc, Carlsbad, Calif.
c. Sarstedt AG & Co, Newton, NC.
d. Glen Mills Inc, Clifton, NJ.
e. Qiagen Inc, Valencia, Calif.
f. Ambion Inc, Austin, Tex.
g. Abbott Molecular Inc, Des Plaines, Ill.
h. Sigma Chemical Co, St Louis, Mo.
i. Precellys 24, Bioamerica Inc, Miami, Fla.
j. Kingfisher Flex, Thermo Scientific, Waltham, Mass.
k. Wash buffer I, Abbott Molecular Inc, Des Plaines, Ill.
l. Wash buffer II, Abbott Molecular Inc, Des Plaines, Ill.
m. Blue Heron Biotechnology Inc, Bothell, Wash.
n. MJ Dyad, MJ Research Inc, Waltham, Mass.
o. SeqWright Inc, Houston, Tex.
p. ABI Prism 3730xl DNA sequencer, Applied Biosystems Inc, Foster City, Calif.
q. PLEX-ID biosensor, Abbott Molecular Inc, Des Plaines, Ill.

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## Appendix 1

Primers, gene targets, and bacterial targets used in a PCR–ESI-MS assay to identify *Dirofilaria immitis* infection in dogs through detection of the heartworm *Wolbachia* endosymbiont in whole blood samples.

| Primer pair | Primer identifier | Primer sequence (5′ to 3′) | Gene target | Bacterial target clade or genus |
|---|---|---|---|---|
| BCT3511 | BCT8229F | TGCATTTGAAAGCTTGGCATTGCC | gyrB | All Spirochaetes |
| | BCT8230R | TCATTTTAGCACTTCCTCCAGCAGAATC | | |
| BCT3514 | BCT8235F | TTTGGTACCACAAAGGAATGGGA | rpoC | All Spirochaetes |
| | BCT8236R | TGCGAGCTCTATATGCCCCAT | | |
| BCT3517 | BCT8241F | TGCTGAAGAGCTTGGAATGCA | flagellin | All *Borrelia* spp |
| | BCT8242R | TACAGCAATTGCTTCATCTTGATTTGC | | |
| BCT2328 | BCT5602F | TGAGGGTTTTATGCTTAAAGTTGGTTTTATTGGTT | asd | *Francisella tularensis* |
| | BCT5603R | TGATTCGATCATACGAGACATTAAAACTGAG | | |
| BCT1083 | BCT2764F | TAAGAGCGCACCGGTAAGTTGG | RNaseP | All *Rickettsia* spp |
| | BCT2763R | TCAAGCGATCTACCCGCATTACAA | | |
| BCT3570 | BCT8336F | TGCATGCAGATCATGAACAGAATGC | gltA | Alphaproteobacteria |
| | BCT8337R | TCCACCATGAGCTGGTCCCCA | | |
| BCT3575 | BCT8346F | TGCATCACTTGGTTGATGATAAGATACATGC | rpoB | Alphaproteobacteria |
| | BCT8347R | TCACCAAAACGCTGACCACCAAA | | |
| INV4443 | INV10034F | TGCGCAAATTACCCAATCCTGACAC | 18S rRNA | All *Babesia* spp |
| | INV10035R | TCCAGACTTGCCCTCCAATTGGTA | | |
| INV4855 | INV10812F | TGAGAGAAATCGTACACATTCAAGCGGG | β-tubulin | All *Babesia* spp |
| | INV10813R | TCCATGTTCGTCGGAGATGACTTCCCA | | |
| VIR2217 | VIR5397F | TGTGTCTACAACATGATGGGAAAGAGAGA | RdRp | Flaviviruses |
| | VIR5398R | TGCTCCCAGCCACATGTACCA | | |
| VIR2230 | VIR5420F | TCACACCGTGGCTGGCATGGCA | RdRp | Flaviviruses |
| | VIR5421R | TCCTCTGGGCCTTCCCATGTCCA | | |
| PLN4437 | PLN10022F | TGACGAGTTCATGAGGGCAGGC | | Extraction control |
| | PLN10023R | TCTGGCCTTTCAGCAAGTTTCCAAC | | |

*Appendix 2 appears on the next page*

## Appendix 2

Primers used for amplification and sequencing of 16S DNA of the heartworm *Wolbachia* endosymbiont in a whole blood sample collected from a dog with experimentally induced *D immitis* infection.

| Primer | Primer sequence (5′ to 3′) |
|---|---|
| 4F | M13F/TTGGAGAGTTTGATCCTGGCTC |
| 801R | M13R/GGCGTGGACTTCCAGGGTATCT |
| M13F | CCCAGTCACGACGTTGTAAAACG |
| M13R | AGCGGATAACAATTTCACACAGG |