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Dirofilaria immitis and *D. repens* are filarioid nematodes of animals and humans, transmitted by the bite of infected mosquitoes. Domestic and wild canids are a major natural host and reservoir for these parasites.

In this study, we designed a duplex real-time PCR protocol targeting the mitochondrial cytochrome c oxidase subunit I (*COI*) gene, detecting both *D. immitis* and *D. repens* using two primer pairs and two *Dirofilaria*-specific hydrolysable probes. The sensitivity and specificity of the primers and probes were tested in both experimental and naturally infected samples. The detection limits of this assay were evaluated using plasmid DNA from *D. immitis* and *D. repens*. No cross-reaction was observed when testing this system against DNA from other filarial nematodes. The detection limit of the real-time PCR system was one copy per reaction mixture containing 5 μl of template DNA. Field application of the new duplex real-time assay was conducted in Corsica. The prevalence rate of *D. immitis* was 21.3% (20/94) in dogs. In a locality where most dogs with *Dirofilaria* spp. infection were found, *D. immitis* and *D. repens* were detected in 5% (20/389) and 1.5% (6/389) of the *Aedes albopictus* population, respectively.

These results suggest that this sensitive assay is a powerful tool for monitoring dirofilariosis in endemic or high risk areas.

1. Introduction

Dirofilarioses are mosquito-borne diseases caused by filarioid nematodes (family Onchocercidae) of the genus *Dirofilaria,* which includes the two main species, *Dirofilaria immitis* and *Dirofilaria repens* (Dantas-Torres and Otranto, 2013). These two species affect canine and feline populations, causing cardiopulmonary and subcutaneous dirofilariosis, respectively (Dantas-Torres and Otranto, 2013; Genchi et al., 2005; McCall et al., 2008). In addition, *D. immitis* and *D. repens* are the causative agents of human dirofilariasis, which can be transmitted to humans by the bite of an infected mosquito, causing pulmonary, subcutaneous and ocular dirofilariosis (Simon et al., 2005; Simon et al., 2009).

Canine, feline and human *Dirofilaria* infections are prevalent in the Mediterranean region (Cancrini et al., 2006). They are endemic in the southern European countries of Italy, Greece, the south of France, Spain, and Portugal (Genchi et al., 2005; Genchi et al., 2009). However, over the last few years, these infections (especially *D. repens*) appear to have spread from the Mediterranean area to Central Europe in countries such as Germany, Austria, the Czech Republic, Slovakia, Hungary, Liechtenstein, Poland, Slovenia and Switzerland (Babal et al., 2008; Fuehrer et al., 2016; Otranto et al., 2013). In Russia, the Ukraine and southern Romania *Dirofilaria* infections appear to be endemic because several autochthonous findings of *Dirofilaria* (especially *D. repens*) in dogs, humans and mosquitoes have been reported (Kartashev et al., 2011; Popescu et al., 2012; Salamatin et al., 2013).

D. immitis and *D. repens* can be transmitted by various mosquito species belonging to different genera, such as *Aedes*, *Anopheles*, *Culex*, *Ochlerotatus, Armigeres, Coquillettidia* and *Mansonia* (Dantas-Torres and Otranto, 2013; Otranto et al., 2013; Pampiglione et al., 1995; Simon et al., 2012). However, the vector competence of several mosquito species remains unclear as it cannot be confirmed

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when entire specimens (including the abdomen) are used for molecular analysis (Fuehrer et al., 2016). The Asian tiger mosquito, *Ae. albopictus*, is a highly invasive mosquito species which continues to spread into European areas where it had not previously been reported (McCall et al., 2008).

The capture and identification of mosquitoes, as well as the search for associated pathogenic nematodes, are indispensable steps for monitoring dirofilariosis in a region. Traditionally, the estimated prevalence of dirofilariosis parasite infections in mosquito vectors was based on the dissection of individual, freshly killed, female mosquitoes. This procedure is labor-intensive, time-consuming and does not resolve the difficulty of parasite species identification (Licitra et al., 2010; Montarsi et al., 2015; Scoles and Kambhampati, 1995). However, in recent years, polymerase chain reaction (PCR)-based techniques have been developed as a tool for detecting DNA from filarioid parasites in biological and environmental samples (Albonico et al., 2014; Latrofa et al., 2012; Licitra et al., 2010). These methods have performed well in terms of sensitivity and specificity towards different filarioid species. For example, when DNA extracted from blood microfilariae was used, the real-time PCR was able to detect $3.2 \times 10–5$ microfilariae/4 μl for *D. immitis*, and $2.4 \times 10–6$ microfilariae/4 μl for *D. repens* (Latrofa et al., 2012) and 4 microfilariae/ml for each single *Dirofilaria* species (Albonico et al., 2014). Nonetheless, these procedures are slow and they have a tendency towards contamination because they involve an additional step such as analysis by agarose gel electrophoresis (Thanchomnang et al., 2010). In 2012, Latrofa et al. (2012) developed a duplex real-time PCR assay for detecting and differentiating between *D. immitis* and *D. repens* in dog blood and mosquitoes using EvaGreen® dye as the source of the fluorescence. However, the SYBR green I dye detects all double-stranded DNA present in the reaction mixture, including non-specific reaction products (Morrison et al., 1998), making it potentially less specific. Indeed, the limited specificity increases false positive results caused by the presence of closely-related species in a test sample (Scoles and Kambhampati, 1995). TaqMan® hydrolysis probes are sequence-specific, dual-labelled, fluorogenic DNA probes and one of the most commonly used chemical probes, offering an alternative approach to the problem of specificity (Mullis, 1990).

We therefore aimed to develop a real-time PCR based on TaqMan® technology, using hydrolysis probes for detection and differentiation of *D. immitis* and *D. repens* in mosquito vectors and blood samples. We applied this tool to a canine dirofilariosis surveillance process as well as to molecular xenomonitoring in an area where both species are endemic, the French Mediterranean island of Corsica.

2. Materials and methods

2.1. Real-time PCR design

The gene encoding the cytochrome oxidase subunit I (*COI*) was chosen as the gene target for the presence of highly-conserved regions with no intra-species variability and for sufficient inter-species variability, allowing discrimination between Filarioidea species. The gene sequences were obtained from GenBank and aligned using version 6.0 of the Molecular Evolutionary Genetics Analysis (MEGA 6) software (Tamura et al., 2013). Reference sequences used for DNA alignments are shown in Table 1. Specific primers and probes for *D. immitis* and *D. repens* were designed from the conserved sequences in the alignment. Once designed, all primers and probes were blasted *in silico* using the NCBI to determine their specificity. In order to distinguish between the signals obtained from the two species, two different fluorescent dyes, FAM and VIC, were used for labeling the probes (Table 2).

Table 1
GenBank accession numbers used for sequence alignment of the filariae (*COI*) DNA sequences.

Reference Sequence	GenBank Accession Numbers
Dirofilaria immitis	AJ537512
Dirofilaria repens	AM749230
Dirofilaria repens	AM749231
Dirofilaria repens	AB973225
Brugia pahangi	AJ271611
Brugia malayi	AJ271610
Onchocerca volvulus	AF015193
Setaria digitata	GU138699
Setaria tundra	KF692106
Loa loa	HQ186250
Wuchereria bancrofti	JN367461

2.2. Real-time PCR specificity and sensitivity testing

To assess the specificity of the oligonucleotide hybridization based on the fluorescent probes developed in this study, DNA extracted from *D. immitis*, *D. repens* (adult worms and microfilariae), *Brugia malayi* and *B. pahangi* (L3) were tested using real-time PCR under the experimental conditions described below. *Mansonella perstans* and *Loa loa* DNA were also used in this assessment. DNA extracted from non-infected laboratory-reared mosquitoes such as *Aedes aegypti* and *Ae. albopictus* and from the blood of uninfected dogs were used as negative controls for the real-time PCR systems. To define the limit of detection (LOD) compared to qPCR, a pUC57 plasmid containing target segments from each *Dirofilaria* species, 444 base pairs long (*D. immitis*) and 430 pb (*D. repens*) (synthesized by Eurogentec, Seraing, Belgium) were used in this assay. Serial dilutions of these constructed plasmids were prepared, equivalent to $10^{8}–10^{-10}$ copies of plasmid per reaction mixture (5 μl of template DNA plasmid). In order to assess intra-assay reproducibility, two replicates from each serial dilution were submitted to the same run.

2.3. Source of samples used to evaluate specificity and sensitivity

D. repens adult filarioids were isolated from subcutaneous nodules of a German Shepherd living in Pau, Pyrénées-Atlantiques (France), and from a woman who was hospitalized at the University Hospital (Dermatology Department, North Hospital, Marseille, France) for subcutaneous nodules on her left thigh (Benzaquen et al., 2015). For *D. immitis*, the adult worms were collected from the heart of a dog that had died from heartworm disease in Cayenne, French Guiana.

D. immitis, *Brugia malayi* and *B. pahangi*-infected and non-infected *Ae. aegypti* mosquitos were provided by the University of Georgia College of Veterinary Medicine, Athens, (USA). In short, infected mosquitoes were fed with microfilaremic blood using an artificial feeding system (Hemotek® feeding system; Discovery Workshops, Lancashire, United Kingdom) (Cosgrove et al., 1994) loaded with 5 ml of infected blood and then incubated for 15 days to allow the parasites to develop. The individual mosquitoes were then placed on a microscope slide, dissected and examined for parasites (L3). Mosquitoes were labelled as infected/uninfected specimens.

D. immitis and *D. repens* microfilariae were obtained from peripheral blood samples collected from a *Dirofilaria*-infected canine. These blood samples (n = 9) came from dogs in France: Pyrénées-Atlantique and Corsica. Dog blood samples were tested by direct microscopy, a rapid Immuno Migration (RIM™) test that detects the presence of female *D. immitis* antigens (Witness® Dirofilaria, Zoetis, Lyon, France) and by conventional PCR using specific primers for *Dirofilaria* spp. as described previously (Rishniw et al., 2006). PCR products obtained after DNA amplification were then

Table 2
Sequence of primers and probe sets designed for molecular detection of *Dirofilaria immitis* and *D. repens*.

Name of primer/probe	Orientation	*Dirofilaria* species	Sequence (5′-3′)	Amplicon size (pb)
COI-DIM-F	Forward	*D. immitis*	TGCTGGTTTGCAAGGTATGC	89
COI-DIM-R	Reverse	*D. immitis*	TCACCGAACCCAACGAAGAA	
COI-DIM-P	/	*D. immitis*	6FAM-CGTAAAATTTTAGATTATCCTGATTGT-TAMRA	
COI-DRE-F	Forward	*D. repens*	TCTTTGGATAGTATAATTTTGGGTTT	231
COI-DRE-R	Reverse	*D. repens*	AAATGCTGATACAACAAAGGA	
COI-DRE-P	/	*D. repens*	6VIC-TTCTTTGTTGTTTTTGTTATTAGATCG-TAMRA	

F: forward, R: reverse, P: probe.

purified and sequenced to identify the species level (Rishniw et al., 2006).

Mansonella perstans and *Loa loa* DNA were extracted from blood samples collected from febrile patients in 2010 from rural Senegalese villages in the Kedougou region (Bassene et al., 2015) and from blood samples from Gabonese children between 2011 and 2014 (Mourembou et al., 2015), respectively. The DNA was then stored in sterile conditions in our laboratory at −20 °C.

2.4. DNA extraction

Total DNA was extracted in a final volume of 100 μl from each mosquito, worm or blood sample using the commercial EZ1® DNA Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Before extraction, samples were digested with 25 μl of proteinase K at +56 °C for 16 h, in line with Dawkins's recommendations regarding the isolation of nucleic acid from nematodes (Dawkins and Spencer, 1989). Genomic DNA was stored at −20 °C for further real-time PCR analysis. DNA samples extracted from reared, uninfected mosquitoes (laboratory colony) and from the blood of uninfected dogs were used as negative controls, using the same extraction protocol. DNA-free water was also included in each reaction to control for possible contamination during the preparation of the mix.

2.5. Real-time PCR assay

The real-time PCR experiment was performed in a total reaction volume of 20 μl containing 10 μl master mix Takyon® (Eurogentec France, Angers, France), 3.5 μl distilled water, 1 μl (20 μM) of each primer, 1 μl (5 μM) probe, and 5 μl DNA template. The amplification program included two initial holds at 50 °C for 2 min and 95 °C for 15 min, followed by 40 cycles consisting of 95 °C for 30 s and 59 °C for one minute. All amplifications in real-time PCR were performed on the thermal cycler CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France).

Once these two primer and probe systems had been individually validated, they were incorporated into a duplex real-time PCR TaqMan® assay (Applied Biosystems, Foster City, CA, USA). Each amplification was performed using the same conditions as in the TaqMan® uniplex reactions, using 0.5 μl of each primer (n = 4), 0.5 μl of each probe (n = 2), and 5 μl DNA (obtained from a mixture of *D. immitis* and *D. repens* DNA). The amplification program was the same as used above.

2.6. Field application of the new real-time PCR assay

Corsica is a French island in the Mediterranean with a total surface area of 8680 km^2. It is located 90 km west of the Italian peninsula, 170 km southeast of the French mainland, and 15 km north of the Italian island of Sardinia. The area is classified as a hot-summer Mediterranean climate. The study focused on the east coast of Corsica.

In September 2014 and June 2015, 94 dogs over the age of 12 months, apparently healthy and housed outdoors, were sampled from 11 localities in Corsica. In the Ventiseri-Solenzara locality (41°55′36″N, 9°24′19″E), military working dogs (n = 23) were taking continuous chemoprophylaxis with preventive medication [Vectra 3D® (Ceva, Libourne, France) and Milbactor® 12.5 mg/125 mg (Ceva, Libourne, France)]. From each animal, 4 ml of blood was drawn from the cephalic vein into EDTA-containing vacutainer tubes and stored at +4 °C until laboratory analysis. The dogs were sampled after obtaining verbal consent from their owners.

Biogents® Sentinel 2 (BGS2) (Biogents AG, Regensburg, Germany) traps were used to collect mosquitoes. According to the manufacturer, the BG-Sentinel mosquito trap offers many improved features concerning quality and functionality. The trap allows for the use of different attractants and can be used with or without CO2. This makes it a very versatile tool for both monitoring and research. In our study, the traps were placed approximatively 1.5 m above ground. They were installed around 17h00 and were recovered the following morning around 10 h 00. The female mosquitoes sampled were frozen at −20 °C prior to further analysis. This sampling was performed in June 2015. In September 2015, traps were installed around 09 h 00 and then recovered two days later, at the same time. After identification, the mosquitoes were stored in silica gel.

2.7. Mosquito identification

Mosquito identification was performed using the Mosquitoes of Europe identification software (Schaffner et al., 2001). Computer-aided identification incorporates morphological identification keys and an ecological approach. All positive mosquitoes were also submitted for molecular identification using previously described primers (Kumar et al., 2007), which amplified DNA sequences of about 600 base-pairs of the *COI* gene. Finally, the sequences obtained were compared with those available in GenBank for species identification.

2.8. DNA sequencing

Of the positive DNA samples in the real-time PCR, only some of them (i.e., 18 for *D. immitis* and six for *D. repens*) were subjected to standard PCR using primers of known sequences of the cytochrome oxidase gene subunit (*COI*) specific for *D. immitis*: DI COI-F1 (5′-AGT GTA GAG GGT CAG CCT GAG TTA-3′) and DI COI-R1 (5′-ACA GGC ACT GAC AAT ACC AAT-3′) and *D. repens* DR COI-F1 (5′-AGT GTT GAT GGT CAA CCT GAA TTA-3′) and DR COI-R1 (5′-GCC AAA ACA GGA ACA GAT AAA ACT-3′) (Rishniw et al., 2006). These primers amplify 203 and 209 base-pair fragments of the *COI* gene, respectively. Conventional PCRs were performed using GeneAmp PCR System 2720 thermal cyclers (Applied Biosystems®, Bedford, MA, USA). All PCR products obtained after the DNA amplification were purified using the PCR filter plate Millipore NucleoFast 96 PCR kit following the manufacturer's recommendations (Macherey Nagel, Düren, Germany). The sequence reaction was carried out using

Table 3
Specificity of the real-time PCR assays.

Real-time PCR assays	Source of DNA						
	Dirofilaria immitis	*Dirofilaria repens*	*Brugia malayi*	*Brugia pahangi*	*Mansonella perstans*	*Loa loa*	Negative controls
COI-DIM	+	–	–	–	–	–	–
COI-DRE	–	+	–	–	–	–	–

(+) amplification, (–) no amplification.

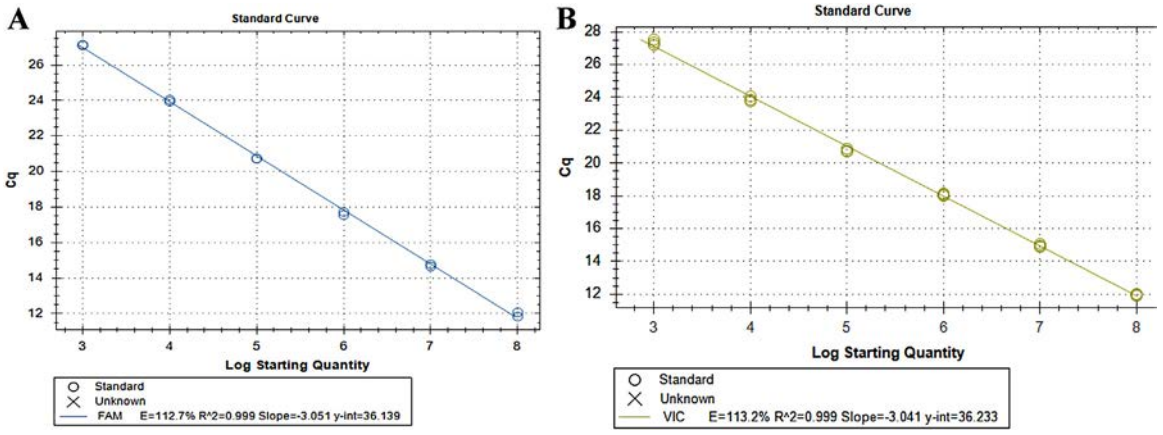

Fig. 1. Standard curves generated from serial dilutions of plasmid DNA from *Dirofilaria immitis* (A) and *Dirofilaria repens* (B).

the BigDye® terminator v3.3 cycle sequencing kit DNA according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). The sequence reaction program contained the following steps: initial denaturation at 96 °C for one min, followed by 25 cycles of denaturation at 96 °C for10 s, annealing at 50 °C for 5 s and extension at 60 °C for 3 min. Sequencing was performed using an ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems®). Finally, all sequences generated were assembled and corrected on ChromasPro 1.7 software (Technelysium Pty Ltd., Tewantin, Australia) and then compared to sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) to confirm species identity.

2.9. Ethics statement

Written informed consent was obtained from the patient (a woman who was hospitalized in the University Hospital) prior to inclusion in the study. Samples from Senegal and Gabon were obtained under ethics numbers N°0000-91/MSP/DS/CNERS and N°00370/MSP/CABMD, respectively. Written informed consent from the parents or legal guardians of each child was provided before inclusion in the study. In terms of the canine samples, blood tests do not fall within the field covered by the regulations on the protection of animals used for scientific purposes, according to Article R.214-88 of the French Rural Code and Maritime Fishing. Moreover, the verbal consent of the dogs' owners was obtained before the dogs were sampled. Finally, when the mosquito traps were placed on private or residential areas, verbal informed consent was obtained from homeowners.

3. Results

3.1. Real-time PCR specificity and sensitivity

In silico evaluation of the specificity of primers and probes showed that the new real-time TaqMan® PCR assay performed well. With 100% query coverage and identification for each sequence, no cross-reactions were observed, with either species of *D. immitis* and *D. repens* or with other filarioid nematodes.

No amplification was observed from genomic DNA from negative controls (uninfected mosquitoes, non-infected dog blood specimens) or any other parasitic nematodes tested. Our system showed species-specific primers and probes (Table 3). When positive control DNA was used as a template, the DNA from two *Dirofilaria* species was amplified in the real-time PCR screening with the primer pair *D. repens* and *D. immitis*. Thus, the combination of the two systems in duplex real-time PCR is suitable for detecting *D. immitis* and *D. repens* DNA in all tested positive samples. The standard curves generated from serial dilutions of DNA plasmid showed both a high correlation coefficient (R^2 of 0.99) and very efficient amplification rates with slope values of −3.21 and −3.04 for *D. immitis* and *D. repens* respectively (Fig. 1). At the end of 35 cycles, the qPCR system was capable of detecting one copy per reaction mixture (5 μl of template DNA) (Fig. 2).

3.2. Field study results for Dirofilaria spp. in mosquitoes and blood specimens from Corsica

A total of 797 female mosquitoes were captured, identified and analyzed using duplex real-time PCR for *D. immitis* and *D. repens*. Morphological identification showed that these mosquitoes belonged to four genera (*Aedes*, *Culex*, *Ochlerotatus* and *Culiseta*). *Ae. albopictus* was the most commonly collected, at 70.89%, followed by *Cx. pipiens* complex, *Oc. caspius*, *Ae. vexans* and *C. annulata* at 21.45%, 4.39%, 2.63% and 0.62%, respectively (Table 4).

Positive mosquito samples for *D. immitis* and *D. repens* were observed in two areas for a single species of mosquitoes: *Ae. albopictus*. In Aléria (42°6′53″N, 9°30′48″E), genomic DNA from *D. immitis* was detected in 18.36% (9/49) of *Ae. albopictus*. Both genomic DNA of *D. immitis* and *D. repens* were detected in 5.14% (20/389) and 1.54% (6/389) of the *Ae. albopictus* population, respectively, in the Solaro (41°54′17″N – 9°19′37″E) locality (Table 4). No mosquitoes were found to be co-infected by both species.

Subsequent sequencing of *COI* gene amplicons from 16 selected positive PCR samples (six for *D. repens* and 10 for *D. immitis*) showed

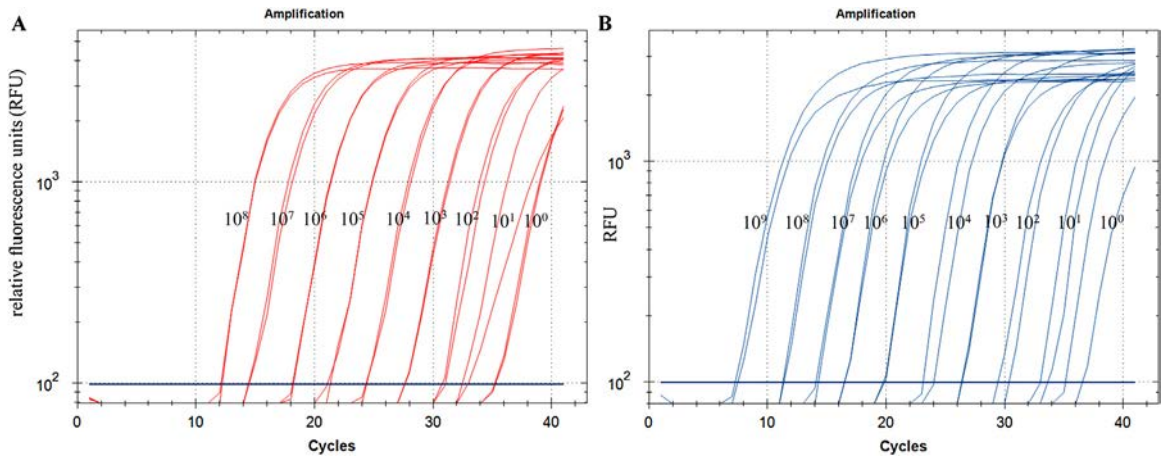

Fig. 2. Determination of the limit of detection (copy per reaction mixture with 5 μl of template DNA plasmid) using serial dilutions of *Dirofilaria immitis* (A) and *Dirofilaria repens* plasmids (B).

Table 4
List of Culicidae captured in Corsica.

Species of mosquitoes (positive/total tested) Capture sites	*Aedes albopictus*	*Aedes vexans*	*Culex pipiens complex*	*Ochlerotatus caspius*	*Culiseta annulata*	Total
Bastia	0/16	0/3	0/17	0/0	0/0	0/36
Ghisonaccia	0/111	0/2	0/123	0/4	0/0	0/240
Aléria	9/49	0/0	0/19	0/0	0/2	9/70
Solaro	26*/389	0/16	0/12	0/31	0/3	26/451
Total	565	21	171	35	5	35/797

The asterisk (*) indicates the mosquito species/capture sites when DNA of *D. immitis* and *D. repens* were simultaneously detected by real-time PCR.

Table 5
Detailed results of selected positive samples sequenced in this study.

Host	Sequence products (N)	Blast analyses (% of similarity)	GenBank accession numbers
Aedes albopictus	6	*Dirofilaria repens* (99.63–100)	AM749234, KF410864
mosquitoes	10	*Dirofilaria immitis* (99.89–100)	LC107816
Dogs	8	*Dirofilaria immitis* (99.85–100)	LC107816

Table 6
Results of molecular identification of 18 mosquitoes.

Sequence products (N)	Blast analyses (% of similarity)	GenBank accession numbers
4	*Aedes albopictus* (99.71–100)	KR068634
2	*Aedes albopictus* (99.42–99.89)	JX679377
7	*Aedes albopictus* (99.72–100)	KC690955 KC690953
5	*Aedes albopictus* (99.85–100)	JQ388786

Table 7
Location, number and positive dogs (duplex real-time PCR) from Corsica.

Location	Number of sampled dogs	Duplex real-time PCR *Dirofilaria*	
		Dirofilaria immitis	*Dirofilaria repens*
Cap Corse	10	0	0
Furiani	1	0	0
Biguglia	1	0	0
Borgo	8	1	0
Castellare	2	0	0
Tallone	1	0	0
Aleria	10	6	0
Ghisonaccia	30	8	0
Ventiseri-Solenzara	23	0	0
Solaro	7	5	0
Lecci	1	0	0
Total	94	20	0

that the closest sequences available in GenBank were those for *D. repens* (accession No. AM749234 and KF410864) and *D. immitis* (accession No. LC107816), which showed 99.63%–100% and 99.89%–100% sequence identity, respectively (Table 5). Moreover, a total of 18 positive *Ae. albopictus* mosquitoes were selected at random and were sequenced to confirm their species identity. The obtained sequences confirmed that the mosquitoes were *Ae. albopictus*, with sequence homology ranging from 99.42% to 100% compared to those available in GenBank (Table 6).

In terms of the blood samples collected from dogs, 20 of 94 specimens (21.27%) were positive for *D. immitis* based on *COI* duplex real-time PCR (Table 7). Identification of *D. immitis* was confirmed by sequencing randomly selected positive samples (n = 8/19) (Table 5). Obtained sequences showed nucleotide similarities of 99.85%–100% with the *D. immitis* sequence from Spain (accession No. LC107816). Finally, no cases due to *D. repens* were detected in the blood samples (Table 7).

4. Discussion

In this study, a duplex real-time PCR assay using TaqMan® was developed for the simultaneous detection of *D. immitis* and *D. repens* in dog blood and mosquito samples. Thanks to the use of hydrolysable probes labelled with two different fluorescent dyes, FAM and VIC, the real-time PCR we developed is able to differentiate between *D. immitis* and *D. repens* DNA. Although the SYBR green I involves relatively inexpensive technology, it does remain,s however, less specific than the TaqMan® chemistry probes (Mullis,

1990). For this reason, we chose this technology to develop a specific real-time PCR system. In the present study, the sensitivity and specificity of primers and probes were confirmed by both *in silico* analyses and experimental laboratory demonstration. The limit of detection of this assay was evaluated using plasmid DNA of *D. immitis* and *D. repens*. Finally, reproducibility was evaluated and the slope (−3.21 for *D. immitis*, and −3.04 for *D. repens*) of standard curves indicated a high PCR efficiency, while the coefficient of determination ($R^2 = 0.99$) suggested a good correlation between threshold cycle values and DNA concentrations. In addition, our real-time detection approach was validated against field samples from dogs and mosquitoes collected in an endemic region (Corsica). Positive cases were identified in both types of samples, suggesting that this assay is suitable for the surveillance of dirofilariosis. However, it should be noted that when the mosquitoes are infected, the system cannot differentiate between immature and infective-stage parasites (L3). Scoles et al. (1993) described the infectivity assay technique to screen mosquitoes for the presence of the L3 infective-stage. Indeed, mosquito dissection is the only available method for identifying vector-stage *Dirofilaria* (Scoles and Kambhampati, 1995). Another advantage of the present molecular method is the possibility of using each system in a simplex assay. This is valid for a laboratory experimental study, when we are interested in knowing the rate of infestation of mosquitoes after an infecting blood meal.

In Corsica, several human cases of dirofilariosis due to *D. repens* have been reported (Basset et al., 2003; Estran et al., 2007; Marty, 1997; Nozais et al., 1994; Pampiglione et al., 1999). However, no formally identified case due to *D. immitis* has been published until now. It is important to note that the anatomical localization and histological criteria are not sufficient to distinguish between *D. immitis* and other filariae. Pulmonary or intra-abdominal localizations have often been associated with *D. immitis*, while subcutaneous and ocular localizations have been attributed to *D. repens* (Pampiglione et al., 1995). This is often not the case, because histopathologic diagnosis may be uncertain if worms are immature or subjected to necropsy, and *D. immitis* may be misidentified as *D. repens* nematodes, or vice versa (Foissac et al., 2013). Recent molecular studies have reported that human subcutaneous and cutaneo-pulmonary dirofilariosis were due to *D. immitis* and *D. repens,* respectively (Benzaquen et al., 2015; Foissac et al., 2013). This indicates that molecular identification can be considered a powerful diagnostic method for screening for dirofilariosis.

In terms of dog dirofilariosis, despite the known endemicity of *Dirofilaria* spp. in the south of France, accurate epidemiological data is limited and outdated. For example, in Corsica, although veterinarians have reported numerous cases of dog *Dirofilaria* infections (B. Davoust, personal communication), there have been no recent published data in this area. In their study, Davoust and Ducos de Lahitte (1989) reported a high prevalence of canine dirofilariosis in Ventiseri-Solenzara (Corsica) with 75% (24/32) and 40% (11/28) in 1983 and 1988, respectively. Our field study showed the presence of canine heartworm disease with a prevalence of 21.27% (20/94). This result is in accordance with previous reports from other endemic European countries in the Mediterranean region, in which the prevalence ranges from 10% to 34% (Greece), 5% to 45% (Italy), 8% to 36% (Spain) and 12% to 17% (Portugal) (Genchi et al., 2005; Montoya et al., 1998; Papazahariadou et al., 1994; Rossi et al., 1996). According to the results obtained in the present investigation, it would appear that dogs in Corsica are more affected by *D. immitis* than by *D. repens* (no cases). Although it is geographically close (<5 km) to a focus of heartworm disease in Solaro, no cases of canine dirofilariosis have been reported in Ventiseri-Solenzara. This could be explained by the fact that the military dogs are well maintained on effective chemoprophylaxis.

Unlike the blood samples from dogs, in mosquito vectors we detected both *D. immitis* and *D. repens* in *Ae. albopictus*. To the best of our knowledge, this is the first report of the presence of *Dirofilaria* spp. DNA in *Ae. albopictus* mosquitoes in France. Previous studies in other endemic regions (e.g., Italy, the USA and Taiwan) have reported that the tiger mosquito was found to be positive for *D. immitis* and *D. repens* (Cancrini et al., 2003a; Cancrini et al., 2003b; Comiskey and Wesson, 1995; Lai et al., 2001; Licitra et al., 2010).

Accordingly, the sequencing of some positive samples confirmed the results obtained with the real-time PCR assay, thus confirming the specificity of the system. All sequences showed nucleotide identitys >99.63% with those of *D. immitis* or *D. repens* available in the GenBank database.

In conclusion, a new, specific and sensitive duplex real-time TaqMan® PCR assay targeting the *COI* gene was developed for detecting and discriminating between *D. immitis* and *D. repens*. This new molecular tool provides a powerful approach for the epidemiological surveillance of dirofilariosis in regions where both parasites are endemic.

Conflict of interest

The authors declare no conflict of interest.

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