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MOLECULAR INVESTIGATION OF VECTOR-BORNE PATHOGENS IN RED FOXES (*VULPES VULPES*) FROM SOUTHERN FRANCE

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ABSTRACT: Because of their free-ranging nature, the probability of wild animals being exposed to vector-borne pathogens is likely higher than that of humans and pets. Recently, the red fox (*Vulpes vulpes*) has been suspected as being a reservoir or host of several pathogens of veterinary and public health importance. We conducted a molecular survey on 93 red foxes hunted in 2008–18, in the departments of Bouches-du-Rhône and Var, in southeastern France, for pathogens including *Leishmania infantum*, Piroplasmida, *Hepatozoon* spp., nematodes, *Coxiella burnetii*, *Borrelia* spp., *Rickettsia* spp., and *Anaplasmataceae*. Spleen samples were screened for the presence of vector-borne pathogens by PCR followed by sequencing. Pathogens were detected in 94% (87/93) of red foxes, and coinfections were identified in 24% (22/93) of foxes. We identified DNA from *Hepatozoon canis*, *L. infantum*, and *Babesia vogeli* in 92% (86/93), 15% (14/93), and 3% (3/93) of red foxes, respectively. We also found DNA of nematodes in 3% (3/93) of foxes; *Spirocerca vulpis* was identified in one fox and *Dirofilaria immitis* in the two others. Interestingly, *C. burnetii* genotype 3, previously described in humans from the same region, was identified in 3% (3/93) of foxes and *Anaplasma platys* in 2% (2/93) of foxes. We did not detect DNA of *Borrelia* spp., *Bartonella* spp., or *Rickettsia* spp. In our study, the prevalence of pathogens did not vary by fox origin, sex, or tick carriage. Molecular evidence of *B. vogeli*, *H. canis*, *S. vulpis*, *D. immitis*, *C. burnetii*, and *A. platys* in red foxes has not previously, to our knowledge, been reported from southern France. We propose that red foxes are potential reservoirs for several pathogens, including major zoonotic agents such as *L. infantum*. They could be incidental hosts for pathogens, such *C. burnetii*. The high prevalence for *H. canis* suggests an important role of foxes in domestic dog (*Canis lupus familiaris*) infection. These animals may pose a threat to human and animal health.

Key words: *Coxiella burnetii*, *Dirofilaria immitis*, France, *Hepatozoon canis*, *Leishmania infantum*, red fox, *Spirocerca vulpis*, vector-borne pathogens.

INTRODUCTION

Vector-borne diseases (VBD) are transmitted to animals and humans by blood-sucking arthropods (Otranto and Dantas-Torres 2010). Their transmission requires an introduced and/or established vector population, a pathogen, and suitable environmental and climatic conditions across the full cycle of VBD transmission in hosts (Randolph and Rogers 2010). The latter affects everything from vector survival and abundance; pathogen growth and survival in hosts, reservoir organisms, and vector organisms; vector activity and biting rates; and host exposures to disease vectors (Aguirre 2009; Semenza and Suk 2018). Wild and domestic carnivores are considered the most important source of human infections with zoonotic agents (Cleaveland et al. 2001). Recently, intensive clinical and epidemiologic studies have been carried out, especially in domestic canids, but data on VBDs in wild canids are still rare in many countries (Deždek et al. 2010; Zanet et al. 2014).

In Europe, foxes are the most widespread and abundant of wild carnivores (Dumitrache et al. 2015; Otranto et al. 2015a, b). The red fox (*Vulpes vulpes*) is widely distributed in the northern hemisphere, including in metropolitan France. This animal is not highly specialized and lives in various habitats (including periurban areas), mainly because of the availability of food, absence of preda-

tors, and human tolerance (Criado-Fornelio et al. 2018). During their migrations into suburban and urban environments, they can create problems, such as predation (chickens and rabbits), scavenging trash cans, and damaging gardens (Torina et al. 2013). The red fox establishes a link between wild and urban environments, and its huge population size and widespread abundance make it an important reservoir of pathogens for both pets and humans (André 2018).

Foxes have long been known to be the main reservoir for sylvatic rabies in western and central Europe, which was very common and a threat to human and animal health before the oral fox vaccination campaign (Duscher et al. 2015). Recently, they have been recognized as a potential reservoir for vector-borne pathogens, such as *Leishmania infantum* (Verin et al. 2010; Davoust et al. 2014; Karayiannis et al. 2015), *Babesia* spp. (Cardoso et al. 2013; Najm et al. 2014a; Hodžić et al. 2015), *Hepatozoon canis* (Cardoso et al. 2014), *Anaplasmataceae* bacteria family such as *Anaplasma phagocytophilum* and *Ehrlichia canis* (Härtwig et al. 2014; André 2018), *Rickettsia* spp. (Ortuño et al. 2018), *Bartonella* spp. (Kaewmongkol et al. 2011; Gerrikagoitia et al. 2012), and filarial nematodes (Tolnai et al. 2014; Otranto et al. 2015a; Hodžić et al. 2016). In addition, they could be an excellent sentinel for pathogens circulating in the environment as well as a possible source of other pet and human VBDs, mainly because of their proximity to urban or agricultural areas and frequent exposure to different arthropod vectors (Aguirre 2009; Torina et al. 2013; Härtwig et al. 2014; Hodžić et al. 2014).

The role of red foxes in VBD epidemiology is poorly investigated in France. Our study aimed to assess the prevalence and diversity of pathogens belonging to Piroplasmida, *Anaplasmataceae* bacteria, *Filarioidea*, *Hepatozoon* spp., *Borrelia* spp., *Bartonella* spp., and *Rickettsia* spp., *L. infantum*, and *Coxiella burnetii*, in spleen samples from free-ranging red foxes from southern France, a region known to be endemic for numerous VBDs.

MATERIALS AND METHODS

Study area and sample collection

The study was conducted in the departments of Bouches-du-Rhône and Var in southeastern France (Fig. 1). Wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), and red fox are the main wild large mammalian species in the area. A military hunting society is authorized to regulate the population size of these three species. During the past decade (2008–18), spleen samples were collected from 93 hunted red foxes, including 57 (61%) from the military camp of Carpiagne (43°14′54″N, 5°30′43″E), 33 (35%) from the camp of Canjuers (43°38′49″N, 6°27′56″E), and three (3%) from the city of Hyères. After hunting, the necropsies were performed aseptically in the laboratory by veterinarians, and spleen samples were kept at –20 C until DNA extraction. Gender and tick carriage were registered for each animal.

Sample treatment and DNA extraction

About 25 mg of each spleen sample was crushed and resuspended in 200 µL of G2 lysis buffer. Mechanical lysis used glass powder, for 30 s, on a Fastprep-24 device (MP Biomedicals, Chicago, Illinois, USA). After that, and for maximum yield, we performed an overnight digestion at 56 C with 15 µL of proteinase K. We extracted DNA from 200 µL of mixture employing a commercial DNA extraction kit QIAamp DNA Mini Kit® (Qiagen, Courtaboeuf, France) through the BIOROBOT EZ1 (Qiagen) per the manufacturer's instructions. We concentrated DNA in 200 µL of distilled water and kept the mixture at –20 C until analysis.

PCR protocols and sequencing

Quantitative real-time PCR (qPCR) systems were applied for the detection of *L. infantum*, Piroplasmida, and *Anaplasmataceae* bacteria and *Borrelia*, *Bartonella*, and *Rickettsia* spp. of the spotted fever group, targeting, respectively, minicircle kinetoplast DNA, 5.8S ribosomal RNA (rRNA), 23S rRNA, 16S rRNA, internal transcribed spacer (ITS), and *gltA* genes. After that initial screening, quantitative PCR (qPCR)-positive samples were confirmed by conventional PCR and sequencing. Primers, probes, thermal conditions, and references are detailed in Table 1.

For the initial detection of filarial infections, we used a qPCR targeting the 28S rRNA gene (Laidoudi et al. 2019). After that, a triplex qPCR was used to detect Cox-1 DNA from *Dirofilaria immitis*, *Dirofilaria repens*, and *Acanthocheilonema reconditum*. To detect the occult infections

FIGURE 1. Map of southeast France, showing the study areas where the red foxes (*Vulpes vulpes*) were sampled. Samples were screened for vector-borne pathogens. The area is known to be endemic for several vector-borne pathogens. Arrows indicate the sampling sites.

from *D. immitis*, we developed a specific qPCR for the detection of *Wolbachia* endosymbiont of *D. immitis* targeting the *ftsZ* gene, and we screened samples. Simultaneously, all samples were submitted to a PCR with primers able to amplify a 1,230-base pair fragment length of the 18S rRNA gene of Nematoda species, followed by sequencing (Laidoudi et al. 2019).

Screening for *C. burnetii* was performed by two independent qPCR tests (*IS1111* and *IS30A*; Table 1). Samples found positive by the two qPCRs were submitted to the multispacer typing (MST) using primer pairs for Cox2, Cox5, and Cox 37 (Glazunova et al. 2005). The amplicons were sequenced; the sequences obtained were compared with those reported in the *C. burnetii* reference database (IHU Méditerranée Infection 2019). For *Hepatozoon* spp. detection and for species identification, a primer pair able to amplify a 620-base pair fragment length of the 18S rRNA gene was employed (Table 1).

The qPCR reactions were prepared in a final volume of 20 µL containing 10 µL of Master Mix Roche (Eurogentec, Liège, Belgium), 0.5 µL of each forward and reverse primer and Uracil-DNA glycosylase (Eurogentec), 0.5 µL of the labeled probes (Table 1), 3 µL of DNase/RNase-free distilled water, and 5 µL of the DNA sample. We added DNA of the target pathogens as positive controls and master mixtures as negative controls for each test. We performed qPCR amplification under the following thermal conditions: incubation at 50 C for 2 min and an initial denaturation step at 95 C for 5 min, followed by 40 cycles of denaturation at 95 C for 5 s and annealing-extension at 60 C for 30 s, in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, California, USA). We considered a result positive when the cycle threshold was lower than 35 cycle threshold for all qPCR assays.

The PCR reactions were performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA) and prepared in a 50 µL volume, including 25-µL AmpliTaq Gold Master mix, 1 µL of each primer, 18 µL distilled water, and 5 µL of the DNA sample. The DNA for the target pathogen and master mixture were added as positive and negative controls, respectively, in each assay. Cycler conditions included an initial denaturation step at 95 C for 15 min, followed by 40 cycles of 1 min at 95 C, 30 s annealing at the hybridization temperature for each PCR assays (Table 1), followed by 1 min at 72 C, and a final extension step for 5 min at 72 C. Amplicons were separated and visualized by electrophoresis in 1.5% agarose. All positive samples were purified and directly sequenced using the commercial Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, California, USA) with an ABI automated sequencer (Perkin Elmer). Obtained sequences were edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) and compared using BLAST (National Center for Biotechnology Information 2019) for similarity to sequences available in GenBank. Molecular phylogenetic and evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Statistical analyses

Results were treated and described in XLSTAT Software (version 2018.7, Addinsoft, New York, New York, USA). Prevalence of pathogens was calculated and the chi-squared or Fisher's exact tests compared proportions of positivity according to the region, sex, or tick carriage. A $P<0.05$ was considered as statistically significant.

TABLE 1. Primers and probes used to screen samples from red foxes (*Vulpes vulpes*) from southeast France for vector-borne pathogens.

Targeted microorganisms	Target gene	Name[a]	Primers (5′-3′) and probe	Tm[b] (C)	Reference
Leishmania infantum	kDNA	RV1	CTTTTCTGGTCCTCCGGGTAGG	—	Mary et al. 2004
		RV2	CCACCCGGCCCTATTTTACACCAA		
		Probe. Leish	FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA		
Leishmania spp.	ITS I	rDNA-10 F	CAATACAGGTGATCGGACAGG	55	Ogado Ceasar Odiwuor et al. 2011
		rDNA-14R	CACGGGGATGACACAATAGAG		
Piroplasmida	5.8S	5.8S-F5	AYYKTYAGCGRTGGATGTC	—	Dahmana et al. 2019
		5.8S-R	TCGCAGRAGTCTKCAAGTC		
		5.8S-S	FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB		
	18S	piro18S-F1	GCGAATGGCTCATTAIAACA	58	
		piro18S-R4	TTTCAGMCTTGCGACCATACT		
Nematoda	28S	Fil-28S-F	TTGTTTGAGATTGCAGCCCA	—	Laidoudi et al. 2019
		Fil-28S-R	GTTTCCATCTCAGCGGTTTC		
		Fil-28S-P	FAM-CAAGTACCGTGAGGGAAAGT-TAMRA		
Dirofilaria immitis, *Dirofilaria repens*, *Acanthocheilonema reconditum*	COI	Fil.COI.749F	CATCCTGAGGTTTATGTTATTATTTT	—	
		Fil.COI.914R	RCWGTATACATATGATGRCCYCA		
		D.imm.COI.777P	FAM-CGGTGTTTGGGATTGTTAGTG-TAMRA		
		D.rep.COI.871P	VIC-TGCTGTTTTAGGTACTTCTGTTTGAG-TAMRA		
		A.rec.COI.866P	Cy5-TGAATTGCTGTACTGGGAACT-BHQ-3		
Nematoda		F.18S.631	TCGTCATTGCTGCGGTTAAA	54	
		R.18S.1825	GGTTCAAGCCACTGCGATTAA		
Wolbachia endosymbiont of *Dirofilaria immitis*	ftsZ	W.imm.ftsZ.784f	GGAGGGGAAAATAGGGCAAT	—	This study
		W.imm.ftsZ.1006r	TACCTTCCATCGCTTGATCG		
		W.imm.ftsZ.886p	FAM-ACGGGTAGTGGGGACATGA-TAMRA		
Hepatozoon spp.	18S	H14HepaF	GAAATAACAATACAAGGCAGTTAAAATGCT	58	Hodžić et al. 2015

Table 1. Continued.

Targeted microorganisms	Target gene	Name[a]	Primers (5′-3′) and probe	Tm[b] (C)	Reference
H14HepaR			GTGCTGAAGGAGTCGTTTATAAAGA		
Coxiella burnetii	IS1111	F	CAAGAAACGTATCGCTGTGGC	—	Mediannikov et al. 2010
		R	CACAGAGCCACCGTATGAATC		
		S	FAM-CCGAGTTCGAAACAATGAGGGCTG-TAMRA		
	IS30A	F	CGCTGACCTACAGAAATATGTCC	—	
		R	GGGGTAAGTAAATAATACCTTCTGG		
		S	FAM-CATGAAGCGATTTATCAATACGTGTATGC-TAMRA		
	Cox 2	F	CAACCCTGAATACCCAAGGA	59	de Santi et al. 2018
		R	GAAGCTTCTGATAGGCGGGA		
	Cox 5	F	CAGGAGCAAGCTTGAATGCG	59	
		R	TGGTATGACAACCCGTCATG		
	Cox 37	F	GGCTTGTCTGGTGTAACTGT	59	
		R	ATTCCGGGACCTTCGTTAAC		
Anaplasmataceae	23S	TtAna-F	TGACAGCGTACCTTTTGCAT	—	Dahmani et al. 2015
		TtAna-R	GTAACAGGTTCGGTCCTCCA		
		TtAna-S	FAM-CTTGGTTTCGGGTCTAATCC-TAMRA		
		Ana23S-212f	GTTGAAAARACTGATGGTATGCA	55	
		Ana23S-753	TGCAAAAGGTACGCTGTCAC		
Borrelia spp.	16S	Bor-16S-3F	AGCCTTTAAAGCTTCGCTTGTAG	—	Parola et al. 2011
		Bor-16S-3R	GCCTCCCGTAGGAGTCTGG		
		Bor-16S-3P	FAM-CCGGCCTGAGAGGGTGAACGG-TAMRA		
Bartonella spp.	16S–23S internal transcribed spacer	TTB23s F	GGGGCCGTAGCTCAGCTG	—	Raoult et al. 2006
		TTB23s R	TGAATATATCTTCTCTTCACAATTTC		
		TTB23s P	6-FAM-CGATCCCGTCCGGCTCCACCA-TAMRA		
Rickettsia spp.	citrate synthase (gltA)	RKNDO3-F	GTGAATGAAAGATTACACTATTTAT	—	Rolain et al. 2002
		RKNDO3-R	GTATCTTAGCAATCATTCTAATAGC		
		RKNDO3-S	FAM-CTATTATGCTTGCGGCTGTCGGTTC-TAMRA		

[a] F = forward; R = reverse; S = probe.

[b] — = 60 C (annealing temperature, for quantitative PCR).

RESULTS

This study involved 93 red foxes, including 57 from Carpiagne (61%), 33 from Canjuers (35%), and three (3%) from Hyères. Overall, there were 56% (52/93) male and 44% (41/93) female. Among foxes, 49% (46/93) carried ticks identified as *Rhipicephalus turanicus* by keys (Estrada-Pena et al. 2004), including 74% of foxes (n=42) from Carpiagne, 6% (n=2) from Canjuers, and 67% (n=2) from Hyères. Molecular analyses showed that 94% (87/93) of the foxes were infected by at least one pathogen. Remarkably, a high infection rate of 92% (86/93) for *H. canis* was observed (Table 2). Prevalence was 15% (14/93) for *L. infantum* and 3% (3/93) for *Babesia vogeli* and *C. burnetii*. Two foxes (2%) were infected by *Anaplasma platys*. In addition, 3% (3/93) of foxes had nematode infections, including two cases of *D. immitis*, detected by the triplex qPCR and sequencing, and one infection by *Spirocerca vulpis*, confirmed after sequencing the 18S gene. All foxes tested negative for the DNA of the other filarial pathogens (*D. repens*, *A. reconditum*), the *Wolbachia* endosymbiont of *D. immitis*, and *Borrelia*, *Bartonella*, and *Rickettsia* spp. No statistically significant difference was observed between the prevalence of infections and the foxes' origin, sex, or tick carriage (Table 2).

Monoinfections were detected in 70% (65/93) of the foxes, including 64 cases of *H. canis* and one case of *L. infantum* infection. Coinfections were present in 24% (22/93) of the foxes, all of which were coinfected by *H. canis*. One fox was infected with three pathogens (*L. infantum*, *B. vogeli*, and *H. canis*; Table 3).

The ITS1 sequences obtained with *Leishmania*-specific primers were similar to each other and also showed 99–100% identity with *L. infantum* sequences available in GenBank database (accession nos. KX664454, KX664454, and KX664449) and 96–99% identity with *L. donovani* (Genbank nos. FN677363 and CP029526; Fig. 2). An obtained sequence for the 18S rRNA of piroplasms showed 98–100% identity with *B. vogeli* sequence available in GenBank (Fig. 3).

TABLE 2. Molecular results and distribution of vector-borne pathogens surveyed in samples from red foxes (*Vulpes vulpes*) from southeast France and shows the prevalence of pathogens and their distribution according to fox origin, gender, and tick carriage.

Pathogen	No. positive (% positive) by risk factor[a]											
	Prevalence			Region				Gender			Tick carriage		
	Negative	Positive	P	Carpiagne	Canjuers	Hyères	P	Male	Female	P	Yes	No	P
Leishmania infantum	79 (85)	14 (15)	<0.0001	5 (9)	8 (24)	1 (33)	0.100	9 (17)	5 (12)	0.689	6 (13)	8 (17)	0.805
Babesia spp.	90 (97)	3 (3)	<0.0001	3 (5)	0 (0)	0 (0)	0.381	1 (2)	2 (5)	0.841	2 (4)	1 (2)	0.985
Hepatozoon canis	7 (7)	86 (93)	<0.0001	53 (93)	31 (94)	2 (67)	0.223	48 (92)	38 (93)	1.000	45 (98)	41 (87)	0.113
Nematoda	90 (97)	3 (3)	<0.0001	1 (2)	2 (6)	0 (0)	0.509	1 (2)	2 (5)	0.841	1 (2)	2 (4)	1.000
Coxiella burnetii	90 (97)	3 (3)	<0.0001	3 (5)	0 (0)	0 (0)	0.379	1 (2)	2 (5)	0.841	3 (7)	0 (0)	0.230
Anaplasma platys	91 (98)	2 (2)	<0.0001	2 (3)	0 (0)	0 (0)	0.541	1 (2)	1 (2)	1.000	1 (2)	1 (2)	1.000
Borrelia spp.	93 (100)	0 (0)	<0.0001	0 (0)	0 (0)	0 (0)	—	0 (0)	0 (0)	—	0 (0)	0 (0)	—
Rickettsia spp.	93 (100)	0 (0)	<0.0001	0 (0)	0 (0)	0 (0)	—	0 (0)	0 (0)	—	0 (0)	0 (0)	—

[a] — = not applicable.

TABLE 3. Mono and mixed infections detected surveyed in samples from red foxes (*Vulpes vulpes*) from southeast France (N=93).

Status	Infections	No. infected	% Infected
Monoinfection	*Leishmania infantum*	1	1
	Hepatozoon canis	64	69
	Leishmania infantum + *Hepatozoon canis*	12	13
Coinfection	*Babesia vogeli* + *Hepatozoon canis*	2	2
	Dirofilaria immitis + *Hepatozoon canis*	1	1
	Spirocerca vulpis + *Hepatozoon canis*	1	1
	Coxiella burnetii + *Hepatozoon canis*	3	3
	Anaplasma platys + *Hepatozoon canis*	2	2
	Leishmania infantum + *Babesia vogeli* + *Hepatozoon canis*	1	1
Totals		87	94

Forty sequences of 550- to 600-base pairs for the 18S rRNA gene of *Hepatozoon* spp. were obtained, and they showed 99–100% identity with *H. canis* sequences available in GenBank database (Fig. 4). Overall, 36 sequences showed high identity with those previously obtained in wild canids, including nine sequences with close (>99%) identity to those identified in red foxes from Spain (no. AY150067.2), golden jackal (*Canis aureus*) from Austria (no. KX712123), and pampas fox (*Lycalopex gymnocercus*) from Brazil (no. KX816958); 27 sequences were closer (99% similarity) to those found in red foxes from the Czech Republic (nos. KU893123 and KU893122) and in golden jackal from Romania (no. KX712126). In addition, four sequences were identical to each other and also showed >99% identity with *H. canis* (no. LC331054) detected in a dog from Zambia.

The two obtained sequences, of about 1,010 base pairs, from *D. immitis*–positive samples (RC37 from Carpiagne and RC69 from Canjuers) were identical to each other and showed >99% identity with *D. immitis* strains (nos. AB973231 and AB973230). Another sequence obtained from another fox was 100% identical with *S. vulpis* isolate 2017 (no. MG957120; Fig. 5).

Genotyping of the three *C. burnetii*–positive samples showed sequences for two of them with a perfect match (100%) with Cox2.5, Cox5.6, and Cox37.7. These foxes were originally from Carpiagne. This combination corresponds to the MST3 genotype currently recognized in the *Coxiella* MST database isolated previously from a human heart valve from Marseille, France (Glazunova et al. 2005).

Two sequences of 440 and 417 base pairs for the partial 23S rRNA gene of *Anaplasmataceae* were obtained from two foxes from Carpiagne (RCP2 and RCP50). They were similar when compared with each other and showed 98% identity with *A. platys* strain ChieCal05 (no. KM021425), strain ChieGuy88 (no. KM021414), and strain Gard1 (no. KM021412; Fig. 6).

DISCUSSION

We report the presence of *L. infantum*, *B. vogeli*, *H. canis*, *D. immitis*, *S. vulpis*, *C. burnetii*, and *A. platys* in red foxes from southern France. A high infection rate was observed. These results supported red foxes as potential reservoirs for pathogens and represent an important sanitary risk for local human and animal (domestic and wild) populations.

Leishmania infantum is the main *Leishmania* species identified in southern France. It has been described since 1968 in red foxes from Cevennes, France, by culturing the parasite (Rioux et al. 1968). Our results confirmed the previous studies, which reported a prevalence of 9% (8/92) among red foxes from the Var area, southeastern France, including 7% (6/90) from the camp of

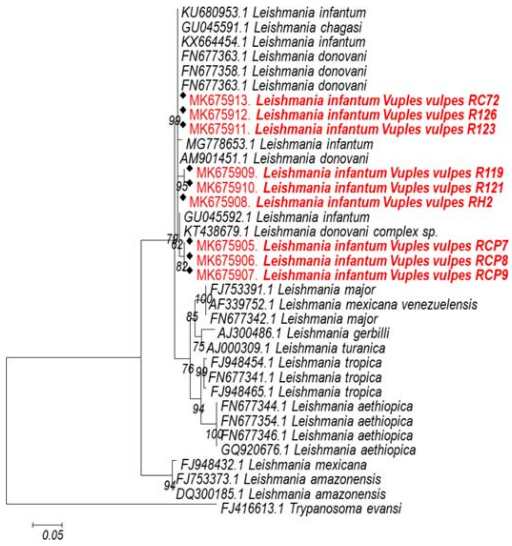

FIGURE 2. Molecular phylogenetic analysis, based on ITS1 partial gene, showed the position of *Leishmania infantum* strains detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–1095.40) is shown. Initial trees for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood approach and then selecting the topology with superior log-likelihood value. The analysis involved 35 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 266 positions in the final dataset.

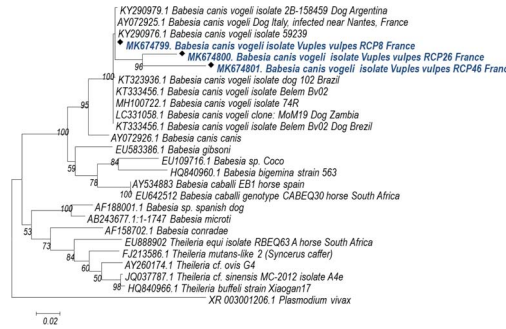

FIGURE 3. Molecular phylogenetic analysis, based on the 18S rRNA partial gene, showed the position of *Babesia vogeli* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–3069.06) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood approach and then selecting the topology with superior log-likelihood value. The analysis involved 26 nucleotide sequences. There were 556 positions in the final dataset.

Canjuers and two from Hyères (Davoust et al. 2014). An almost similar prevalence of 14% (23/162) was observed in Spain (Sobrino et al. 2008). Molecular studies in central and southern Italy showed prevalences of 52% (48/92) and 40% (20/50), respectively (Dipineto et al. 2007; Verin et al. 2010). Other studies showed an infection rate of 75% (50/67) in red foxes living in central Spain (Criado-Fornelio et al. 2000), and 29% (14/48) in foxes from *L. infantum* periendemic area in the north (del Río et al. 2014). *Leishmania infantum* isolates here were highly identical to isolates 119 and 11PSAZ, isolated from a human and *Phlebotomus sergenti* from Azilal, Morocco (Zouirech et al. 2018). It will be very important to isolate *L. infantum* strains from red foxes and compare them to reference strains that are pathogenic to humans and pets. Domestic dogs were always considered the main reservoir host for *L. infantum*, despite the extension of vaccination in some countries (Otranto et al. 2015b). The presence of *L. infantum*–infected red foxes represents a serious risk to humans, pets, and sylvatic fauna susceptible to leishmaniosis. Further, this risk is insidious, misunderstood, and all the more relevant because domestic dogs, considered the traditional reservoir for leishmaniosis, are more frequently vaccinated or benefit from chemoprophylaxis (e.g., insecticide collars, spot-on; Davoust et al. 2014). For better comprehension of the role of the red fox in the *L. infantum* transmission cycle, other studies are needed, as demonstrated in Brazil for the crab-eating foxes (*Cerdocyon thous*). Gomes et al. (2007) showed that one of the three healthy *Leishmania chagasi* PCR-positive crab-eating foxes was able to infect 1/12 sandflies, which fed on it, suggesting a sylvatic

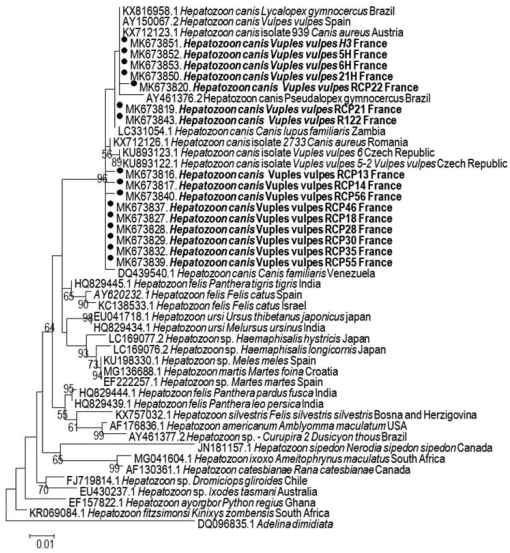

FIGURE 4. Molecular phylogenetic analysis showed the position of *Hepatozoon canis* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history based on the *Hepatozoon* spp. 18S rRNA genes was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–1887.73) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood approach and then selecting the topology with superior log-likelihood value. The analysis involved 48 nucleotide sequences. There were 518 positions in the final dataset.

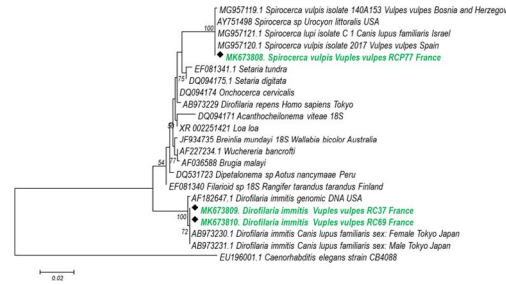

FIGURE 5. Molecular phylogenetic analysis, based on the 18S rRNA partial gene, showed the position of *Spirocerca vulpis* and *Dirofilaria immitis* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–2408.13) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood approach and then selecting the topology with superior log-likelihood value. The analysis involved 22 nucleotide sequences. There were 935 positions in the final dataset.

cycle of *L. chagasi* among crab-eating foxes in Brazil.

We report the presence of Apicomplexa parasites (*B. vogeli* and *H. canis*) in French red foxes. The *B. vogeli*–positive foxes were from Carpiagne, and all of them carried ticks. *Babesia vogeli* is a globally distributed, tick-borne pathogen (Otranto et al. 2009). This parasite has been reported and characterized in 33% dogs and 22% brown dog ticks (*Rhipicephalus sanguineus*) from southern France (René et al. 2012). Several studies in Europe showed the presence of *Babesia* spp. DNA in foxes or their ticks, suggesting a potential role of the red fox in natural endemic cycles of these protozoa (Najm et al. 2014a; Criado-Fornelio et al. 2018). Therefore, the red fox can be regarded as the natural host for these piroplasms. Here, *B. vogeli* isolates detected on foxes were similar to canine isolates, and two new variants showed similar identity with an Italian dog infected near Nantes, France (no. aAY072925).

The highest *H. canis*–infection rate in red foxes was registered in the present study. High prevalence, although less than in our study, was reported in Portugal (75%; Cardoso et al. 2014), central Italy (49%; Ebani et al. 2017), Germany (45%; Najm et al. 2014b), and in northern Spain (28%; Gimenez et al. 2009). All these findings suggest that red foxes represent an important reservoir of *H. canis* infection for domestic dogs. The *R. sanguineus* s.l. tick, which benefits from a worldwide distribution, including in the Mediterranean, is the main vector of this parasite (Otranto et al. 2015b). The transmission of *H. canis* in ticks may occur transstadially from larvae to nymphs (Giannelli et al. 2013) and from these to the adult stages (Baneth et al. 2001). In our study, the prevalence varied insignificantly (P=0.113) between animals carrying ticks and animals without ticks. Other transmission routes could be possible and might contribute

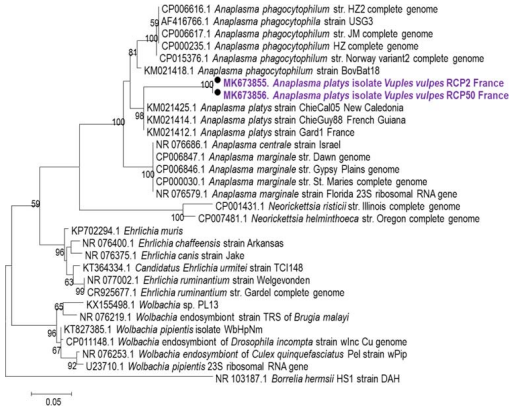

FIGURE 6. Molecular phylogenetic analysis showed the position of *Anaplasma platys* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history based on the 23S rRNA partial gene of the *Anaplasmataceae* bacteria was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–2055.15) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood approach and then selecting the topology with superior log-likelihood value. The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 370 positions in the final dataset.

to the spread of this protozoan and explain this high prevalence in foxes, for example, by vertical transmission or predation of canid hosts on another intermediate hosts, as shown for *Hepatozoon americanum* (Johnson et al. 2009). The *H. canis* isolates that we detected were highly identical to those detected on wild canids and dogs (Hodžić et al. 2015). Therefore, the epizootiologic importance of possible fox *H. canis* strains needs to be studied further before definitive conclusions can be drawn (Cardoso et al. 2014).

Dirofilaria immitis was identified in two foxes and *S. vulpis* in a third. *Dirofilaria immitis* and *D. repens* are the most important filarial pathogens, mainly affecting domestic carnivores, such as dogs and cats, but also humans and wild carnivores (McCall et al. 2008). They have a large geographic distribution, mainly in southern Europe (Spain, Portugal, Italy, and France), and especially in the Mediterranean (Tahir et al. 2019). In France, *D. immitis* is more frequently found in the south, along the Mediterranean coast: Bouches-du-Rhône (our study area), Vaucluse, and Corsica Island (5–15%), and to a lesser extent, Dordogne and Haute-Garonne (Doby et al. 1986). Low prevalence, <1.5%, of canine dirofilariasis has been reported in France between 1986–89 (Morchón et al. 2012). No data on the infection in foxes are available in France. In Italy, 7% of 132 foxes living in a canine *Dirofilaria*-endemic area were infected (Magi et al. 2008). Studies in Spain showed that prevalence of *D. immitis* infections is higher in foxes from irrigated areas (32%) compared with foxes from semi-arid regions (2%); heartworm was absent in foxes from the mountain (Gortazar et al. 1994). The role of red foxes in *D. immitis* transmission to other canids and/or humans is uncertain and needs further investigation. Strains detected in this study are similar to other *D. immitis* strains from dogs (nos. AB973231 and AB973230). Interestingly, we detected *S. vulpis* on a red fox. This nematode is a new species, recently described from red foxes in Europe (Bosnia and Herzegovina, Italy, and Spain), suggesting a high genetic diversity of the *Spirocerca* spp. infecting canids (Rojas et al. 2018). *Spirocerca vulpis* isolated in this study was similar to isolate 2017 (no. MG957120) described in red foxes from Spain. Knowledge on this species is limited, and additional studies are needed to clarify its importance. No experimental model is available to study pathogenesis of this nematode, and its presence in the spleen remains unexplained.

Coxiella burnetii is the etiologic agent of Q fever, with a worldwide distribution (Eldin et al. 2017). We found *C. burnetii* DNA in three foxes from Carpiagne; all of which carried ticks. In recent studies, 2/12 foxes (17%) in Spain (Millán et al. 2016) and 3/153 (2%) in central Italy were PCR positive (Ebani et al. 2017), whereas no foxes (n=105) tested positive in southern Italy (Santoro et al. 2016). Other studies described *C. burnetii* in ticks and fleas collected on red foxes (Psaroulaki et al. 2014a, b). *Coxiella burnetii* can infect a broad range of vertebrate and

invertebrate hosts (Meredith et al. 2015). Even if the prevalence of infection is low, our study suggested a possible spillover in the fox population. In our study, the identified genotype, MST3, has been previously isolated on a human heart valve from Marseille (Glazunova et al. 2005). This suggests the circulation of a specific *C. burnetii* genotype in southern France.

Anaplasma platys strains had the most similarity to strains detected in domestic dogs from France (no. KM021412), French Guiana (no. KM021414), and New Caledonia (no. KM021425). *Anaplasma platys* has been identified in 14.5% (10/69) of foxes from Portugal, which suggested a possible role of foxes as reservoir of this dog's disease (Cardoso et al. 2015). Red foxes are also suspected to be reservoir for other *Anaplasmataceae* sp., especially *A. phagocytophilum* and *E. canis* in Europe (Ebani et al. 2011; Torina et al. 2013; Hodžić et al. 2015; Millán et al. 2016). All foxes tested negative for *Borrelia* spp., *Bartonella* spp., and *Rickettsia* spp. DNA. In a study conducted on ticks and fleas sampled from foxes from the same region (southern France), 45% (33/73) of ticks (*R. turanicus*) and 10.5% (2/19) of fleas (*Archaeopsylla erinacei*) were found to be infected with *Rickettsia massiliae* and *Rickettsia felis*, respectively (Marié et al. 2012). *Bartonella* DNA was not found in any of the ticks or fleas in that study.

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