Detection of tick-borne pathogens in ticks from dogs and cats in different European countries

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Abstract

Ticks are known to transmit pathogens which globally threat the health and welfare of companion animals and man. In the present study, ticks were collected from dogs and cats presented at their local veterinary practice in Hungary, France, Italy, Belgium (dogs only) and Germany (cats only), and identified based on tick morphology. If more than one tick was collected from an animal, ticks were pooled by tick species for DNA extraction and subsequent examination for the presence of tick-borne pathogens using specific PCR assays. Out of 448 tick samples, 247 (95 from dogs and 152 from cats) were *Ixodes ricinus*, 26 (12 from dogs and 14 from cats) were *I. hexagonus*, 59 (43 from dogs and 16 from cats) were *Dermacentor reticulatus* and 116 (74 from dogs and 42 from cats) were *Rhipicephalus sanguineus* sensu lato (s.l.). In 17.4% of the *I. ricinus* samples, *Anaplasma phagocytophilum* was found. *Borrelia* spp. was mainly identified in *I. ricinus* collected from cats (18.4%) and to a lesser extent in dog-sourced ticks (1.1%), with *Borrelia afzelii* (n=11), *B. garinii* (n=7), *B. valesiana* (n=5), *B. lusitaniae* (n=3) and *B. burgdorferii* sensu strictu (n=3). Only one *I. hexagonus* sample collected from a cat in France was positive for *B. afzelii*. *Babesia canis* was detected in 20.3% of the *D. reticulatus* samples, mainly from Hungary. *Rhipicephalus sanguineus* s.l. was found positive for *Hepatozoon canis* (2.6%), *A. platys* (5.2%) and three *Rickettsia* species (6.9%; *R. massiliae*; *R. raoultii* and *R. rhipicephali*). Furthermore, a total of 66 *R. sanguieus* s.l. ticks were subjected to molecular analysis and were identified as *R. sanguineus* sp. II-temperate lineage, with seven haplotypes recorded. Amongst them, the most prevalent sequence types were haplotype XIII (n=24; 68.6%) and haplotype XIV (n=16; 51.6%) in France and in Italy, respectively, found both in cats and dogs. The results of this study illustrate that tick-borne pathogens are frequently detected in different tick species with differences in infection rate related to both country and host.

Highlights

- Dogs and cats are frequently exposed to tick-borne pathogen infested ticks
- Differences in infection rate depending on country and host
- Haplotypes of *Rhipicephalus sanguineus* belong to the temperate lineage

Keywords: tick, cat, dog, tick-borne pathogens, Europe, *Rhipicephalus sanguineus* sp. II-temperate lineage, haplotypes

Background

The most common ticks infesting dogs and cats in Europe are *Ixodes ricinus*, *I. hexagonus*, *Rhipicephalus sanguineus sensu lato* (s.l.) and *Dermacentor reticulatus*, with differences reported between countries regarding their occurrence. In addition to the potential direct clinical impact, ticks are also important vectors of different pathogens. For example, *Ixodes* spp. ticks are vectors of *Anaplasma phagocytophylum* and *Borrelia burgdorferi* s.l. (respectively the pathogens causing granulocytic anaplasmosis and Lyme disease). *Dermacentor reticulatus* is an important vector of *Babesia canis* which causes significant disease and mortality in dogs and *Rhipicephalus sanguineus* s.l. can transmit a range of pathogens including but not limited to *Anaplasma*, *Rickettsia*, *Babesia*, *Hepatozoon* and *Ehrlichia* spp. (Claerebout et al; 2013; Dantas-Torres, 2008 and 2012; Heyman et al., 2010; Parola et al., 2005; Solano-Gallego et al., 2016; Stich et al., 2008; Stuen et al., 2013).

The ability of ticks to transmit pathogens creates a persistent risk of vector-borne disease infections for human populations and domestic animals. Although tick infestations are commonly associated with rural areas, it has become increasingly clear that ticks are well adapted to urban and suburban environments (Rizzoli et al., 2014; Upsensky, 2014). In addition, the geographical distribution of ticks is expanding due to a number of abiotic and biotic factors, such as climate change, increased travel of dogs and cats with their owners, host expansion as well as changes in habitat and human behavior (Randolph, 2004; Beugnet and Marié, 2009; Dantas-Torres et al., 2012). For *I. ricinus*, a latitudinal and altitudinal spread has been described, as well as increased distribution within endemic areas (Medlock et al., 2013). Similarly *D. reticulatus* is continuing to spread into novel areas (Földvari et al., 2016; Olivieri et al., 2016; Rubel et al., 2016), potentially leading to an increased reporting of canine babesiosis caused by *B. canis* (Phipps et al., 2016). Though cats are exposed to ticks and to the pathogens they transmit, knowledge on the role of cats in the epidemiology or ecology of tick-borne pathogens is limited (Otranto et al., 2017; Pennisi et al., 2015).

The widespread occurrence and the ability of ticks to transmit important pathogens warrant regular screening of cats and dogs for tick infestation. In addition, as companion animals live in close proximity to their owners, the collection of ticks from infected animals combined with a screening for tick-borne pathogens can provide information about the potential infection pressure for the human population as well (Shaw et al., 2001; Baneth, 2014; Otranto et al., 2014). The objective of the present study was to examine the presence of tick-borne pathogens in ticks collected from dogs and cats presented at their local veterinary practice in different European countries.

Methods

Tick collection

Ticks were collected from dogs and cats that were enrolled in two field patient studies to evaluate the efficacy and safety of sarolaner in the field (Becskei et al., 2016; Geurden et al., 2017). Animals were enrolled during the tick season (April to August) prior to the first acaricide treatment. In the dog study, ticks were collected from dogs presented at veterinary practices in Belgium (4 clinics), France (8 clinics), Hungary (5 clinics) and Italy (5 clinics), respectively. In the cat study, ticks were collected in Germany (11 clinics), in France (8 clinics) and from 7 sites in both Hungary and Italy. The veterinary practices participating in the study were selected based on their potential to enroll tick-infested animals. All animals were presented at the veterinary practice for a variety of reasons and not specifically for symptoms related to tick infestation or tick-borne diseases, and only healthy animals infested with 3 or more ticks were selected. Although the development of tick-borne infection or clinical disease was not specifically examined in these studies, no adverse events linked to tick-borne diseases were reported during the 3 month efficacy evaluation period after the collection of the ticks that were examined in this study.

Ticks were identified at species level based on their morphology (Hillyard, 1996; Estrada-Pena et al., 2004). For each individual animal, the ticks were pooled per tick species. If more than one but less than 10 ticks were collected, all of the ticks were pooled in one tick sample. If more than 10 ticks from the same tick species were collected from a dog or a cat, 10 ticks were randomly selected and pooled. All ticks were preserved in 70% ethanol. For *I. ricinus*, the number of ticks found on individual dogs varied from 1 to 64 (mean 2.9) and on cats from 1 to 26 (mean 4.9). For *I. hexagonus*, the number of ticks found on dogs varied from 1 to 516 (mean 3.6) and on cats from 1 to 5 (mean 3.0), to a total of 733 *I. hexagonus* ticks from dogs and 29 from cats. For *D. reticulatus*, the number of ticks found on dogs varied from 1 to 10 (mean 0.9) and in cats from 1 to 4 (mean 2.1), to a total of 152 *D. reticulatus* ticks from dogs and 33 from cats. For *R. sanguineus*, the number of ticks found on dogs varied from 1 to 81 (mean 4.3) and in cats from 1 to 6 (mean 3.7), to a total of 811 ticks found on dogs and 153 on cats.

DNA extraction from pooled tick samples and PCR detection of tick borne pathogens

Genomic DNA was extracted from ticks using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the instruction of the kit's manual for tissue protocol. Prior to the DNA extraction ticks were removed from the 70% ethanol, dried in a Petri dish and washed in water containing washing-up liquid followed by rinsing in distilled water. Depending on the size some ticks were cut in half in a medio-sagittal direction so that the salivary glands remained intact. If fully fed, ticks were cut again into two and only the parts with salivary glands were transferred into a labelled Eppendorf tube containing 100µl PBS. The tick parts were sliced to smaller pieces using sterilized scissors. For each tick sample, a new sterile blade was used to avoid possible contamination between tick samples. After the PCR, the PCR products were run over a 1.5% agarose gel (100V, 40 min), stained with ethidium-bromide and visualized under ultra-violet light. Selected PCR products were purified and sequenced by Biomi Inc. (Gödöllő, Hungary). All of the sequences were compared to the NCBI Nucleotide Database.

A selection of pathogens was examined in the respective tick species, as following:

- *A. phagocytophilum* and *Borrelia* spp in *I. ricinus* and *I. hexagonus*
- *Babesia* spp. in *D. reticulatus*
- *Hepatozoon* spp., *Babesia* spp., *Rickettsia* spp. and *Anaplasma platys* in *R. sanguineus* s.l.

Anaplasma phagocytophilum DNA was detected using a probe-based real-time PCR as described in Courtney et al. (2004) and specific primers targeting the msp2 gene (77bp). *Borrelia* spp. DNA was detected by amplification of the variable 5S-23S intergenic spacer region (IGS), as described by Szekeres et al. (2015). For the detection of *Babesia* spp., a conventional PCR was used to amplify a ~500 bp long fragment of the 18S rRNA gene (Casati et al., 2006). *Hepatozoon* spp. DNA was detected by amplification of a 650 bp fragment of the 18S rRNA gene (Inokuma et al., 2002).

For the detection of *Ehrlichia canis*, a 410 bp long fragment of the *Ehrlichia canis* groEL gene was amplified: 2 µl of extracted DNA were added to 23 µl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5U/µl), 0.5 µl dNTP Mix (10mM), 0.5 µl of each primer (50µM), 2.5 µl of 10x Coral Load PCR buffer (15mM MgCl2 included), and 18.8 µl DW. An initial denaturation step at 95ºC for 10 min was followed by 55 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 15 s and extension at 72°C for 15 s. Final extension was performed at 72°C for 7 min.

For the detection of *Anaplasma platys* and *Rickettsia* spp., a species-specific PCR reaction was used to detect the presence of the 520bp portion of the *Anaplasma platys* p44 gene. The amplification was performed with the primers Apl_p44F3 : 5'-GCT AAG TGG AGC GGT GGC GAT GAC AG-3' forward and Apl_p44R3: 5'- CGA TCT CCG CCG CTT TCG TAT TCT TC – 3' reverse (Arraga-Alvarado et al., 2014), in a 25 µl final volume reaction mixture containing 5 µl DNA template, 1.0 U HotStar Taq Plus

DNA Polymerase (5U/µl) (QIAGEN®, Hilden, Germany), 2.5 µl 10x CoralLoad PCR buffer (15mM MgCl2 incl.), 0.5 µl dNTP mix (10mM), 0.3 µl of each primer (50uM) and 16.2 µl ddH2O. An initial denaturation step at 95°C for 5 min was followed by 40 cycles of denaturation at 95°C for 40 s, annealing at 62°C for 40 s and extension at 72°C for 1 min. Final extension was performed at 72°C for 7 min. DNA of sequenced *A. platys* served as positive control.

For the genus-specific detection of Spotted Fever Group Rickettsiae, a PCR was used (Regnery et al., 1991), to amplify a ~380bp long fragment of the gltA gene with the forward primer RpCS.877p 5'-GGG GGC CTG CTC ACG GCG G-3' and the reverse primer RpCS.1258n 5'-ATT GCA AAA AGT ACA GTG AAC A-3'. 2.5 µl of extracted DNA were added to 22.5 µl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5U/µl) (QIAGEN®, Hilden, Germany), 0.5 µl dNTP Mix (10mM), 0.5 µl of each primer (50µM), 2.5 µl of 10x Coral Load PCR buffer (15mM MgCl2 included), and 18.3 µl DW. An initial denaturation step at 95°C for 5 min was followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 48°C for 30 s and extension at 72°C for 1 min. Final extension was performed at 72°C for 5 min. DNA of *Rickettsia* sp. served as positive control.

Molecular identification of *Rhipicephalus sanguineus* s.l.

To investigate the *R. sanguineus* s.l. tick lineage, 66 tick specimens (35 collected in France and 31 collected in Italy) were selected for further genetic analysis, including at least one tick from each geographical site and host. Partial mitochondrial 16S rRNA (~300 bp) gene sequences were generated using primers and PCR run conditions described elsewhere (Burlini et al., 2010). For phylogenetic analysis, sequences from each haplotype obtained as well as from individual or consensus sequences of the other *Rhipicephalus* spp. from a previous study (Dantas-Torres et al., 2013) were included (i.e., *R. sanguineus* s.l.: KC243835–KC243838; *R. sanguineus* sp. II-temperate lineage: KC243843–KC243847 and KY216135–KY216141; *Rhipicephalus guilhoni*: KC243851–KC243854; *Rhipicephalus pusillus*: KC243855; *Rhipicephalus turanicus*: KC243856–KC243867; *Rhipicephalus bursa*: KC243871). Consensus sequences were generated after alignment with ClustalW program (Larkin et al., 2007) and using the BioEdit software (Hall, 1999). A homologous gene sequence from *I. ricinus* (JF928527) was used as outgroup. Phylogenetic relationship was inferred by Maximum Parsimony analysis (Kimura, 1980) with the general time reversible model in MEGA 6 (Tamura et al., 2013).

Results

Out of 448 tick samples examined, 247 were identified as *I. ricinus*, 26 as *I. hexagonus*, 59 as *D. reticulatus* and 116 as *R. sanguineus* s.l. (Table 1).

The *I. ricinus* tick samples collected from dogs (n=95) and cats (n=152) were examined for the presence of *A. phagocytophilum* and *Borrelia*. *Anaplasma phagocytophilum* was detected in 14.7% (24/95) and in 19.1% (29/152) of the *I. ricinus* tick samples collected from dogs and cats, respectively (Table 2), with the highest frequency of infection in samples collected in Hungary and France. *Anaplasma phagocytophilum* was not detected in Italy or in any of the *I. hexagonus* tick samples. *Borrelia* spp. was detected in a single *I. ricinus* tick sample from a dog in Hungary, whilst the frequency of infection ranged from 14.3% to 33.3% in samples collected from cats in France, Hungary and Germany (Table 2). Out of 29 positive *I. ricinus* tick samples, three each were positive for *B. burgdorferi* s.l. and *B. lusitaniae*, 11 for *B. afzelii*, 7 for *B. garinii* and 5 for *B. valaisiana*. One *I. hexagonus* tick sample collected from a cat in France was positive for *B. afzelii*.

Out of 59 *D. reticulatus* tick samples (43 from dogs and 16 from cats) collected in Hungary, France and Italy, 12 (20.3%) were found to be positive for *B. canis* (Table 3). Out of 116 *R. sanguineus* s.l. tick samples collected from dogs (n=74) and cats (n=42) in France and Italy (Table 4), six (3 samples each from dogs and cats) were positive for *A. platys* was identified, and three samples from dogs in Italy were positive for *H. canis* (2.6%). *Rickettsia* was identified in 5 out of 37 (13.5%) tick samples from dogs in France and in 3 of 26 tick samples (11.5%) from cats in Italy (Table 4). The positive tick samples were identified as *R. massiliae* (n=4 in dogs and n=2 in cats), *R. raoultii* (n=1 in dogs) and *R. rhipicephali* (n=1 in cats).

All 66 *R. sanguineus* s.l. ticks were genetically assigned to the *Rhipicephalus sanguineus* sp. II-temperate lineage, of which 18 (10 in France and 8 in Italy) were collected from cats and 48 (25 in France and 23 in Italy) from dogs (Table 5). Seven haplotypes were identified which shared a high nucleotide identity of 99–100% with those of *R. sanguineus* sp. II-temperate lineage available in GenBank database (Accession numbers KC243844, KY216135, KY216136). Overall, three sequence types were identical to the haplotypes II, VI, VII previously identified in Portugal and in northern Italy (Accession numbers KC243844, KY216135, KY216136), whilst the new representative sequence types were named as haplotypes XIII-XVI. The haplotype XIII was the most prevalent sequence type (n = 24; 36.9%) recorded in two surveyed areas of France country (Gironde and Ariège, Table 5), whilst the haplotype XIV was found in almost all surveyed Italian provinces (Table 5). The phylogenetic analysis confirmed the molecular identification of ticks by clustering all haplotypes identified in the same clade with the consensus sequence of *R. sanguineus* sp. II-temperate lineage, to the exclusion of other *Rhipicephalus* spp. (Fig. 1). All representative new haplotypes obtained are available in the GenBank database under accession numbers MG707293-MG707296.

Discussion

The different tick species in the current study reflect their known geographic distribution in Europe. As expected, the *Ixodes* tick species were found in all selected countries, although to a lesser extent in Italy, whilst *D. reticulatus* was mainly found in Hungary and France (Földvari et al., 2016; Rubel et al., 2016), and *R. sanguineus* s.l. mainly in Italy and in Southern France (Latrofa et al., 2014; René-Martellet et al., 2015). A subset of *R. sanguineus* s.l. ticks was further identified as the *Rhipicephalus* sp. II-temperate lineage (Dantas-Torres et al. 2013), considered as the only representative tick species of the *R. sanguineus* s.l. group in western European countries such as Portugal, Spain and Italy (Dantas-Torres et al., 2013; Latrofa et al., 2014; Dantas-Torres et al., 2017). To the author's knowledge, this is the first report of

Rhipicephalus sp. II-temperate lineage in France. A similar close association between haplotypes of *Rhipicephalus* sp. II-temperate lineage and geographical site of collection has been previously reported in Portugal (Dantas-Torres et al., 2017). The relationship amongst different haplotypes of *Rhipicephalus* sp. II (temperate lineage) and their vector capacities in transmitting pathogens needs to be investigated.All ticks were collected from dogs and cats presented at their veterinary practice within the framework of two field patient studies. Animals were enrolled when adequately infested with ticks, without any restriction in the number of animals enrolled per study site or country (Becskei et al., 2016; Geurden et al., 2017). As the ticks collected from each animal were pooled per tick species for DNA extraction, a positive test result for any tick-borne pathogen confirmed that the specific animal was infested with at least one pathogen-positive tick for that respective tick species (indicating for example that 17.4% of the animals with *I. ricinus* were infested with at least one *A. phagocytophylum* positive tick). As such, the current study was not designed to provide a true prevalence estimate, as in recent studies in the UK (Davies et al., 2017; Abdullah et al., 2017) or Belgium (Claerebout et al., 2013) investigating individual ticks. Also, it is possible that some pathogen infections in these ticks were acquired with the blood meal on the infested animal, rather than being prior infections (Abdullah et al., 2017). The data hence provide an estimate of how many tick-infested animals were at risk of infection with these pathogens through infected ticks.

In the current study, tick-borne pathogens were identified in tick samples collected in all selected countries, although regional differences in infection rate were observed, as previously also reported in the UK (Abdullah et al., 2017; Bettridge et al., 2013; James et al., 2014). Furthermore, differences related to the tick host have been observed in the present study. The *Borrelia* spp. infection rate differed substantially between ticks collected from dogs and cats. Despite being of greater clinical relevance in dogs compared to cats (Krupka and Straubinger, 2010; Little et al., 2010), only one tick collected from a dog was found positive for *Borrelia* DNA. In contrast, the *Borrelia* infection rate was consistently above 10% in the *I. ricinus* ticks collected from cats in France, Germany and Hungary. This difference might be due to the roaming behavior of cats and the more frequent access to habitats of *Borrelia* reservoir hosts, including birds and rodents (Schotthoefer et al., 2015). As different animals have different behavioral patterns, they 'flag' different habitats within the same environment, suggesting that examining ticks from different sentinel animals may provide complementary information on tick-borne pathogens for a specific environment. The results also raise the question if cats potentially act as a carrier for *Borrelia* spp. infected ticks between different habitats in the same environment. Recently, *Borrelia* spp. was reported in *I. ricinus* ticks collected from cats (Davies et al., 2017) and dogs (Abdullah et al., 2017) in the UK, as before in mainland Europe (Claerebout et al., 2013; Pennessi et al., 2015; Rauter and Harting, 2005). The *Borrelia* species identified in the present study (*B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae* and *B. burgdorferi ss*) are consistent with these previous reports (Abdullah et al., 2017; Claerebout et al., 2013; Davies et al., 2017; Krupka and Straubinger, 2010; Stensvold et al., 2015).

In Europe, *A. phagocytophilum* was reported in up to 20.3% of *I. ricinus* ticks (Krol et al., 2016), although lower infection rates have also been reported (Beugnet and Marié, 2009; Mehlhorn et al., 2016; Rizzoli et al., 2014; Smith and Wall, 2013). In this study, 17.4% of the *I. ricinus* tick samples were found to be positive for *A. phagocytophilum.* In Hungary and France, *A. phagocytophilum* infected ticks were consistently found on more than 10% of cats and dogs, and in Germany, where only cats were enrolled, a similar infection rate was found. No *I. ricinus* ticks were found positive for *A. phagocytophilum* in Italy, likely due to the low number of *I. ricinus* tick samples examined. Surprisingly, no *A. phagocytophilum* was detected in any of the *I. ricinus* tick samples collected from dogs in Belgium, which is in contrast to a previous report (Claerebout et al., 2013), although substantial regional differences in *A. phagocytophilum* infection rates were reported in that study as well.

No *I. hexagonus* tick samples were positive for *A. phagocytophilum*, and only one was positive for *B. afzelii*. These findings are somewhat in contrast to previous reports in Europe confirming the importance

of *I. hexagonus* as a vector for tick-borne pathogens (Claerebout et al., 2013; Krol et al., 2016; Nijhof et al., 2007; Schreiber et al., 2014). The limited number of *I. hexagonus* tick samples in the current study might have influenced the observed infection rate.

B. canis was detected in 20.3% of the *D. reticulatus* tick samples, indicating that one out of five animals infected with this tick species were exposed to at least one *B. canis* infected tick. The infected *D. reticulatus* samples were found in Hungary, France and Italy. In Europe, canine babesiosis caused by *B. canis* is known to be highly endemic in these three countries as well as in Switzerland, Serbia, Croatia and northern Spain (Beugnet and Marié, 2009; Földvari et al., 2016). Although *D. reticulatus* and babesiosis is less common in northern European countries, there has been a clear expansion of babesiosis in Belgium, Germany, Poland and The Netherlands, as well as in previously unaffected countries such as the UK (Abdullah et al., 2016). The increasing spread of *D. reticulatus* and the findings of a significant *B. canis* infection rate justify the recommendation for year-round tick control measures in endemic areas (Jongejan et al., 2012).

The current data indicate that dogs and cats are frequently exposed to ticks infected with tick-borne pathogens in different European countries, although both country and host-specific differences were observed. The frequent detection of pathogens emphasizes the need for adequate tick control in dogs and cats.

Competing interests

The study reported here was funded by Zoetis. TG, CB, RHS, SM were current employees of Zoetis. RF, DO and MSL were independent investigators. There were no conflicting interests that could have influenced the conduct and reporting of this study.

Authors' contributions

Authors assisted with the study design, study conduct, interpretation of the data and manuscript writing. All authors read and approved the final version of the manuscript.

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Table 1. The number of tick samples per country and per animal species (dog or cat) as well as total number of tick samples examined

	I. ricinus	*I. hexagonus*	*D. reticulatus*	*R. sanguineus*
		Dog		
Belgium	11	5	0	0
Hungary	50	1	17	0
France	28	5	25	37
Italy	6	1	1	37
Total	95	12	43	74
		Cat		
Germany	63	2	0	0
Hungary	63	0	16	0
France	24	5	0	16
Italy	2	7	0	26
Total	152	14	16	42
Total*	247	26	59	116

* total of cat and dog samples combined

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Table 2. The number of *Ixodes ricinus* tick samples (N) examined per country and animal species, the number (N) and frequency of positive tick samples (%), and sequencing results

	A. phagocytophilum		*Borrelia* spp.	
	N	N (%)	N (%)	Sequencing
			Dog	
Belgium	11	0 (0.0%)	0 (0.0%)	
Hungary	50	10 (20.0%)	1 (2.0%)	*B. afzelii*
France	28	4 (14.3%)	0 (0.0%)	
Italy	6	0 (0.0%)	0 (0.0%)	
Total	95	14 (14.7%)	1 (1.1%)	
			Cat	
Hungary	63	15 (23.8%)	11 (17.5%)	3 B lusitaniae + 5 B afzelii + 1 B garinii + 2 B valaisiana
Germany	63	7 (11.1%)	9 (14.3%)	3 B afzelli + 3 B garinii + 2 B valaisiana + 1 B burgdorferii
France	24	7 (29.2%)	8 (33.3%)	2 B afzelii + 3 B garinii + 1 B valaisiana + 2 B burgdorferii +(1 B afzelii)
Italy	2	0 (0.0%)	0 (0.0%)	
Total	152	29 (19.1%)	28 (18.4%)	
Total*	247	43 (17.4%)	29 (11.7%)	

* total of cat and dog samples combined

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Table 3. The number of *Dermacentor reticulatus* tick samples (N) examined, the number (N) and frequency (%) of *Babesia* positive ticks samples, and identification of *Babesia*.

	N	N *Babesia canis* positive samples (%)
Hungary (dog)	17	3 (17.6%)
Hungary (cat)	16	5 (31.3%)
France (dog)	25	3 (12.0%)
Italy (dog)	1	1 (100%)
Total*	59	12 (20.3%)

* total of cat and dog samples combined

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Table 4. The number of *Rhipicephalus sanguineus* tick samples (N), the number (N) and frequency (%) of positive tick samples for *Anaplasma platys*, *Hepatozoon canis* and *Rickettsia* spp.

		Anaplasma platys	*Hepatozoon canis*	*Rickettsia* spp.
	N	N (%)	N (%)	N (%)
		Dog		
France	37	2 (5.4%)	0 (0.0%)	5 (13.5%)
Italy	37	1 (2.7%)	3 (8.1%)	0 (0.0%)
Total	74	3 (4.1%)	3 (4.1%)	5 (6.8%)
		Cat		
France	16	3 (18.8%)	0 (0.0%)	0 (0.0%)
Italy	26	0 (0.0%)	0 (0.0%)	3 (11.5%)
Total	42	3 (7.1%)	0 (0.0%)	3 (7.1%)
Total*	116	6 (5.2%)	3 (2.6%)	8 (6.9%)

* total of cat and dog samples combined

Table 5. The number (N) of *Rhipicephalus sanguineus* s.l. ticks collected from the different study sites and animal species, the molecular identification, and the country, region and geo-reference.

Country	Region	Geo-reference	N	n/Haplotypes		Accession number
				Dog	Cat	
France	Vaucluse	44°03′N 05°02′E	1	1/II	-	KC243844
	Ariège	43°07′N 01°36′E	8	8/XIII	-	MG707293
	Vaucluse	43°44′N 05°22′E	1	1/II	-	KC243886
	Gironde	45°07′N 0°39′W	6	1/VII; 5/XVI	-	KY216136; MG707296
	Gironde	45°16′N 0°33′W	19	9/XIII	1/XVI; 2/II; 7/XIII	KC243844; MG707293; MG707296
Italy	Pavia	45°15′N 08°52′E	3	1/VI	1/XIV; 1/XV	KY216135; MG707294; MG707295
	Brescia	45°24′N 09°55′E	7	1/II; 6/XIV	-	KC243844; MG707294
	Milano	45°28′N 09°11′E	1	-	1/XIV	MG707294
	Pavia	45°01′N 09°08′E	2	1/II; 1/XIV	-	KC243844; MG707294
	Pavia	45°08′N 09°06′E	1	-	1/II	KC243844
	Milano	45°32′N 09°14′E	6	3/XIV; 3/VI	-	KY216135;MG707294
	Pavia	45°28′N 09°11′E	4	3/II; 1/XIV	-	KC243844; MG707294
	Pavia	45°01′N 09°08′E	2	-	1/II; 1/VI	KY216135; KC243844
	Pavia	45°08′N 09°06′E	3	1/VI; 2/XIV	-	KY216135; MG707294
	Brescia	45°24′N 09°55′E	1	-	1/II	KC243844
	Pavia	45°19′N 08°52′E	1	-	1/XIV	MG707294