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Occurrence of *Dirofilaria immitis* and Tick-Borne Infections Caused by *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Ehrlichia canis* in Domestic Dogs in France: Results of a Countrywide Serologic Survey

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Abstract

The occurrence of *Dirofilaria immitis* antigen and antibodies against tick-borne pathogens in French dogs has been analysed based on 1,050 blood samples. Serum samples of 919 dogs (group A) were sent for a variety of diagnostic investigations, further 131 dogs (group B) were tested for a tentative diagnosis of heartworm disease. All samples were tested for *D. immitis* antigen. Samples in group A were also tested for specific antibodies against three tick-borne pathogens (*Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Ehrlichia canis*). Results were plotted in geographical maps. Occurrence of *D. immitis* antigen in group A (0.22%; 95% CI: 0.03–0.78%) was significantly lower ($p < 0.0001$) than in group B (6.87%; 95% CI: 3.19–12.64%). Heartworm infections in both groups were regionally restricted to the areas of Bouches-du-Rhône in the South of France and Corsica. In group A, the calculated seroprevalence was 2.72% (95% CI: 1.77–3.99%) for *A. phagocytophilum*, 1.09% (95% CI: 0.52–1.99%) for *B. burgdorferi* and 0.33% (95% CI: 0.07–0.95%) for *E. canis* with a distribution of the positive cases throughout the country. This study represents the first data of *A. phagocytophilum* seroprevalence in the French dog population.

Introduction

Concern over vector-borne diseases in domestic dogs is evidenced by the common use of preventative measures against ticks, mosquitoes and heartworm in small animal practice. While infection with these agents may be prevented to some extent through vector avoidance or other control measures, morbidity and mortality due to these diseases continue to occur in domestic dogs. The role that these agents play in animal and human health has become evident over the last few decades and the need for further data on the distribution and prevalence of these infections has become apparent.

Heartworm (*Dirofilaria immitis*) is transmitted by blood-sucking female mosquitoes (primarily *Culex, Aedes* and *Anopheles*) and is classified as a zoonotic agent. Heartworm infection in dogs and cats has been detected mainly in southern European countries (Spain, Portugal, southern France and Greece) with isolated reports from Turkey and eastern European countries (Trotz-Williams and Trees 2003; Genchi et al. 2005). However, Europe's largest endemic area is located along the Po valley in northern Italy. In France, *D. immitis* occurs mainly in the South including Corsica, but infections were also seen in the department of Dordogne in the Southwest, as well as Brittany and Normandy (Doby et al. 1986a,b; Ducos de Lahitte 1990).

The tick *Ixodes ricinus* is widely prevalent in Europe and acts as an important vector for spirochaetes from the *Borrelia burgdorferi* sensu lato complex (Gray et al. 2002). Today, Lyme disease (Steere et al. 1983) caused by *B. burgdorferi* (Burgdorfer et al. 1982; Johnson et al. 1984) is the most frequent vector-transmitted infectious disease in humans in the northern hemisphere (Piesmann and Gern 2004). Alongside *Borrelia*, *Ixodes* ticks can be infected with the rickettsial bacteria *Anaplasma phagocytophilum* (previously *Ehrlichia equi* or *Ehrlichia phagocytophila* (Dumler et al. 2001)), which establishes intracellular infection in the host. Transmitted to dogs, *A. phagocytophilum* can cause the clinical picture of canine granulocytic ehrlichiosis (CGE) (Greig et al. 1996; Pusterla et al. 1997). So far, the role of this infection in the French dog population is unknown.

Just like *A. phagocytophilum*, the bacterium *Ehrlichia canis* belongs to the family of Anaplasmataceae (Dumler et al. 2001) and is also an obligately intracellular (monocytic cells) organism. However, *E. canis* is transmitted by the tick *Rhipicephalus sanguineus* (Groves et al. 1975; Lewis, Jr. et al. 1977), which in Europe occurs in Mediterranean climates. *E. canis* was first described in France in 1937 (Donatien and Lestoquard 1937), with sporadic autochthonous cases occurring thereafter (Cabassu et al. 1980). Studies on military dogs have been carried out in France in the past, identifying *E. canis* prevalence ranging from 0% in some kennels to 87.5% in a kennel in Bastia, Corsica (reviewed by Trotz-Williams and Trees 2003).

The current discussions on climate change (global warming) raise renewed interest in the origin and endemic behaviour of arthropod-borne diseases. Factors determining the establishment of arthropod-borne diseases are habitat changes that lead to increased numbers of mosquitoes and ticks, our lifestyle with increasing travel of pets throughout the European Union, and the availability of suitable wild animal reservoirs (Genchi et al. 2001; Trotz-Williams and Trees 2003; Bourdeau 2008).

France borders several climatic and ecological influences: Oceanic to the West, Continental to the East, and Mediterranean to the South. It is therefore a country that is directly exposed to the spreading of vector-borne diseases which initially established in only one of these habitats but expanded into adjacent, different types of habitat.

The first aim of the study was therefore to collect current data on the occurrence and distribution of heartworm infection in two differently preselected canine populations in France. It was also aimed to clarify whether this mosquito-transmitted infection is spreading into the North of the country. Because this was a first opportunity to test a large number of canine blood samples from France, almost nationwide and serologically standardised, anoth-

er aim was to capture the respective specific seroprevalences for *A. phagocytophilum, B. burgdorferi* sensu lato and *E. canis* with the same test system, and to analyse and describe the data geographically. Furthermore, the occurrence of antibody reactions against the largely unknown bacterium *A. phagocytophilum* in dogs in France in conjunction with the significance of mixed infections with *B. burgdorferi* was to be studied.

Material and methods

General

Serum samples from dogs in France were used for this study. The samples were taken by local veterinarians and submitted to a diagnostic laboratory for various analyses. The origin of the dogs was determined using the postcode supplied with the submission.

Group A

The blood samples were submitted to Vet Med Labor GmbH (Division of IDEXX Laboratories, Ludwigsburg, Germany) by veterinarians from all over France. The data for group A (n = 919) were collected between 2nd August and 9th December 2006. The samples had been submitted for endocrinology, biochemistry and allergy testing, allowing no preselection on history or typical symptoms for one of the infectious diseases in the dog population.

Serological testing in group A was performed using a rapid assay test system (IDEXX SNAP® 4Dx®) following the manufacturer's directions for use (Fig. 1). SNAP® 4Dx® is an enzyme immunoassay. A test unit consists of a coated membrane matrix with five spots in the reaction area (result window). Three spots are impregnated with specific peptide antigen of *A. phagocytophilum* (synthetic peptide from the major surface protein (p44/MSP2)), *B. burgdorferi* sensu lato (C_6 peptide) and *E. canis* (peptides from p30 and p30-1 outer membrane proteins), respectively. The *D. immitis* analyte for both the SNAP® assays is derived from antibodies specific to the heartworm antigen. The fifth spot serves as a positive control. A two-chamber system contains wash solution and substrate solution, which flow across the coated membrane upon activation of the test.

Group B

Group B included blood samples of 131 dogs from France with a tentative diagnosis of heartworm infection based on clinical signs, submitted by veterinarians to a private veterinary diagnostic laboratory (Vet Med Labor GmbH, Division of IDEXX Laboratories, Ludwigsburg, Germany) between 1st October 2005 and 28th February 2007. For the detection of *D. immitis* antigen, an enzyme immunoassay was used detecting soluble heartworm antigen in the blood, just like the SNAP® 4Dx® test, except that it also allows semiquantification. The

Fig. 1 SNAP® canine heartworm (left) and canine SNAP® 4Dx® (right) test devices demonstrated with a canine serum sample positive for *Dirofilaria immitis* antigen

Table 1 Occurrence of *Dirofilaria immitis* antigen and seroprevalences of tick-borne infections in dogs from France (group A, n = 919)

Causative organism	Antigen (Di) or antibodies, positive/all tested dogs	Rate	95 % Confidence interval
Dirofilaria immitis	2/919	0.22 %	[0.03–0.78 %]
Anaplasma phagocytophilum	25/919	2.72 %	[1.77–3.99 %]
Borrelia burgdorferi	10/919	1.09 %	[0.52–1.99 %]
Ehrlichia canis	3/919	0.33 %	[0.07–0.95 %]

Table 2 Proportion of single and double infection in positive samples (n = 37) in group A
Ap: Anaplasma phagocytophilum, Bb: Borrelia burgdorferi sensu lato*, Di: Dirofilaria immitis, Ec: Ehrlichia canis*

	Ap (alone)	*Bb (alone)*	*Di*	*Ec (alone)*	*Ap + Bb*	*Ap + Ec*
Positive samples	22	8	2	2	2	1
Positive tests (total)	37	37	37	37	37	37
Percentage	59.5 %	21.6 %	5.4 %	5.4 %	5.4 %	2.7 %

test, IDEXX SNAP® canine heartworm, was performed following the manufacturer's directions for use (Fig 1).

Statistical analyses

Differences between both groups were analysed for significance using the Fisher's exact test with the validated statistic software Testimate 6 from IDV Gauting. Differences were regarded as significant at a level of $p < 0.05$ two-sided.

Data for groups A and B in conjunction with their corresponding postcode were analysed and the geographical distribution was reported on administrative maps.

Results

Group A

The origin of the samples tested in group A (n = 919) is shown in Fig. 2. The samples were submitted from all over France with high regional activity (> 31 submissions) in Bouches-du-Rhône and Alpes-Maritimes in the South of France as well as Val-d'Oise, Essonne, Hauts-de-Seine and Val-de-Marne near Paris. Only seven regions were not represented with samples. Results obtained for group A using the rapid assay test are shown in Table 1, 2.

D. immitis antigen was found in the blood samples of two dogs (0.22 %; 95 % CI: 0.03–0.78 %) from Arles (Bouches-de-Rhône) and Prunelli di Fiumorbo (Corsica), respectively (Fig. 3).

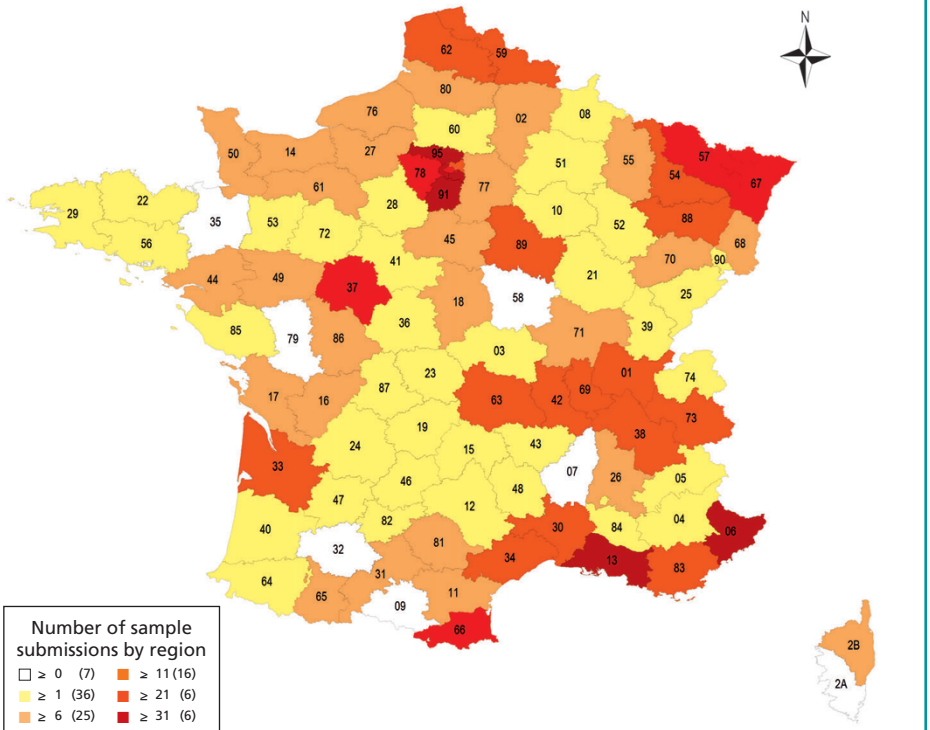

Fig. 2 Origin of 919 canine serum samples (group A) tested for *Dirofilaria immitis* antigen and antibodies against *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Ehrlichia canis*

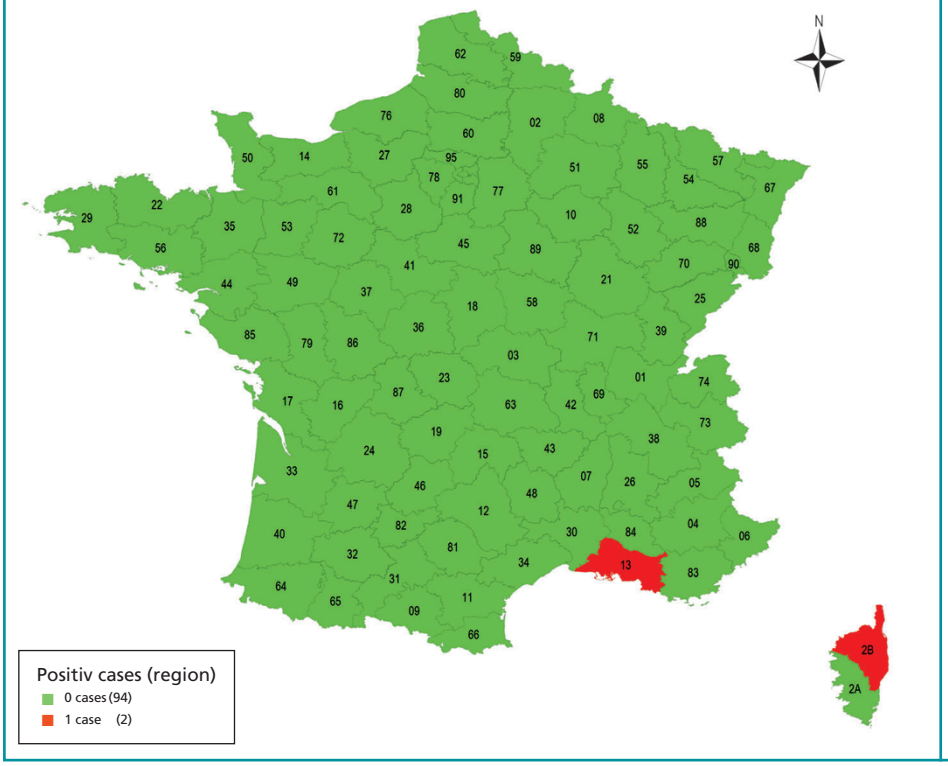

Fig. 3 Origin of canine serum samples from group A tested positive for *Dirofilaria immitis* antigen

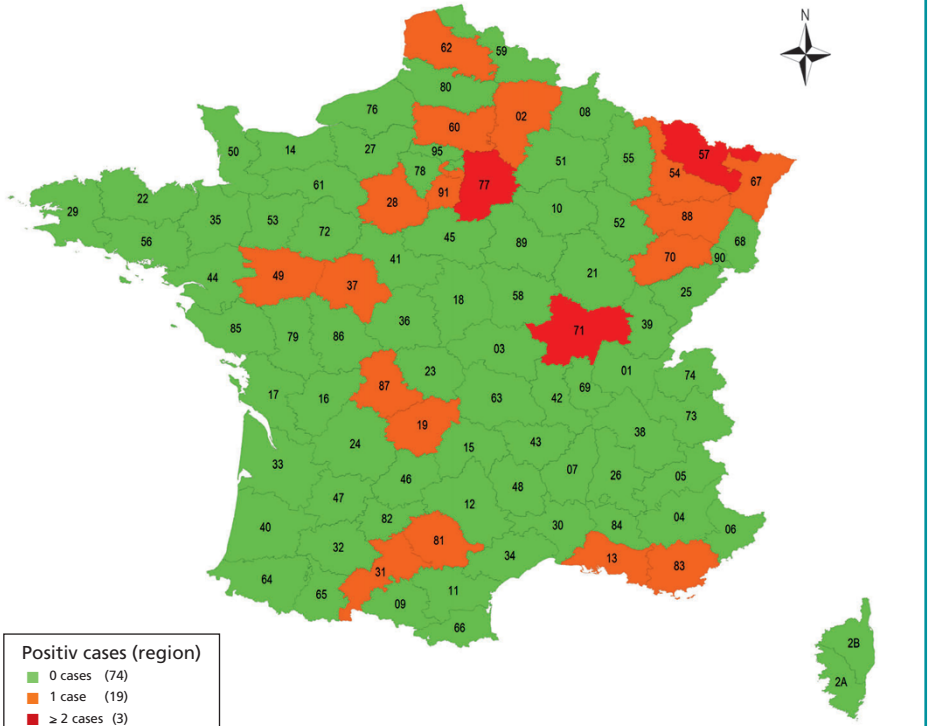

Fig. 4 Origin of canine serum samples from group A tested positive for specific antibodies against *Anaplasma phagocytophilum*

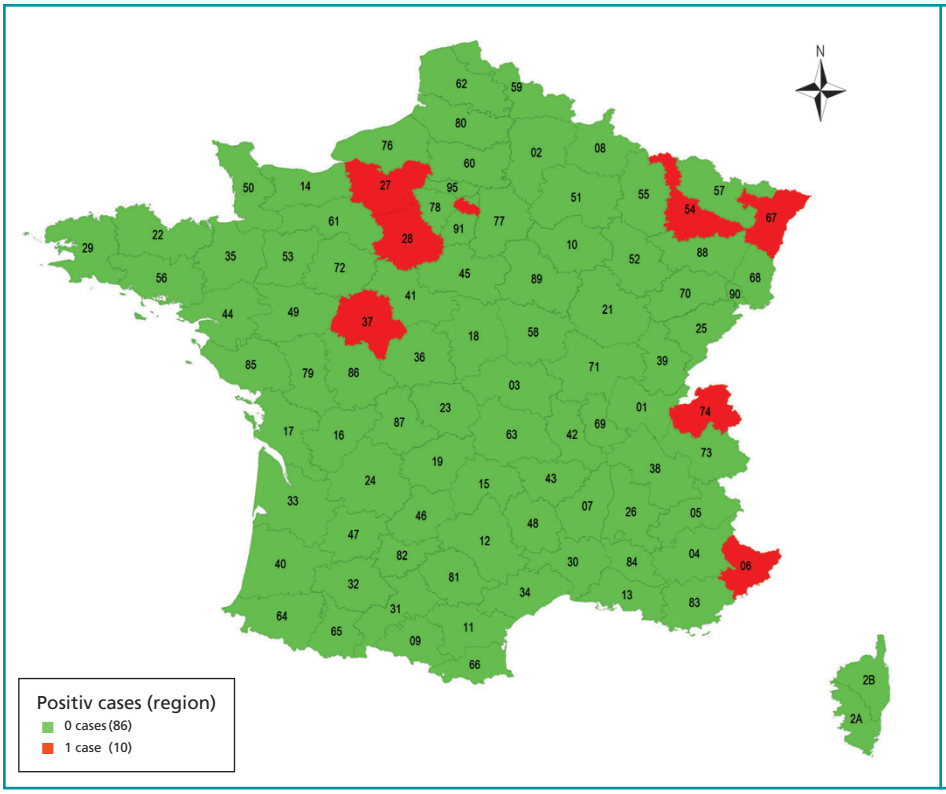

Fig. 5 Origin of canine serum samples from group A tested positive for specific antibodies against *Borrelia burgdorferi* sensu lato

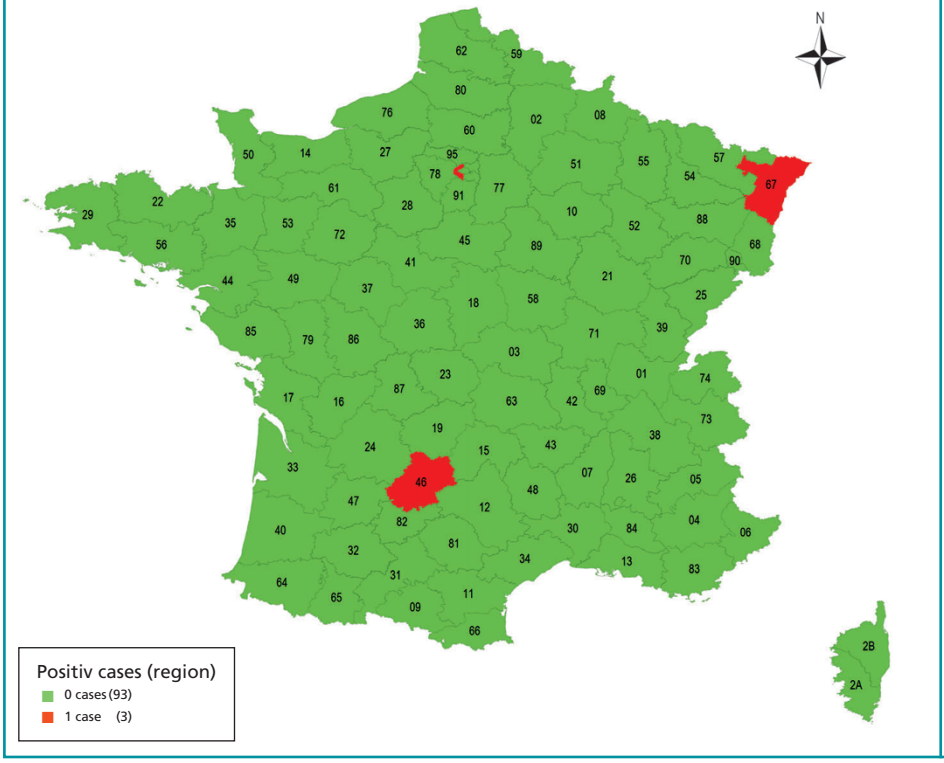

Fig. 6 Origin of canine serum samples from group A tested positive for specific antibodies against *Ehrlichia canis*

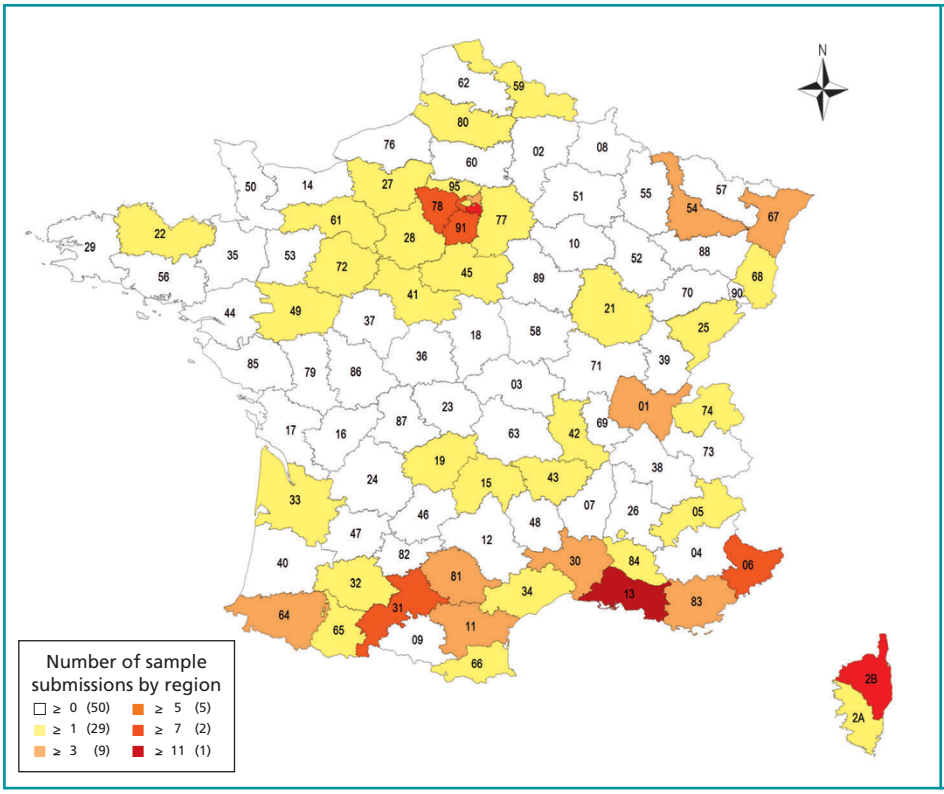

Fig. 7 Origin of 131 canine serum samples (group B) tested for *Dirofilaria immitis* antigen with a tentative diagnosis of heartworm infection based on clinical signs

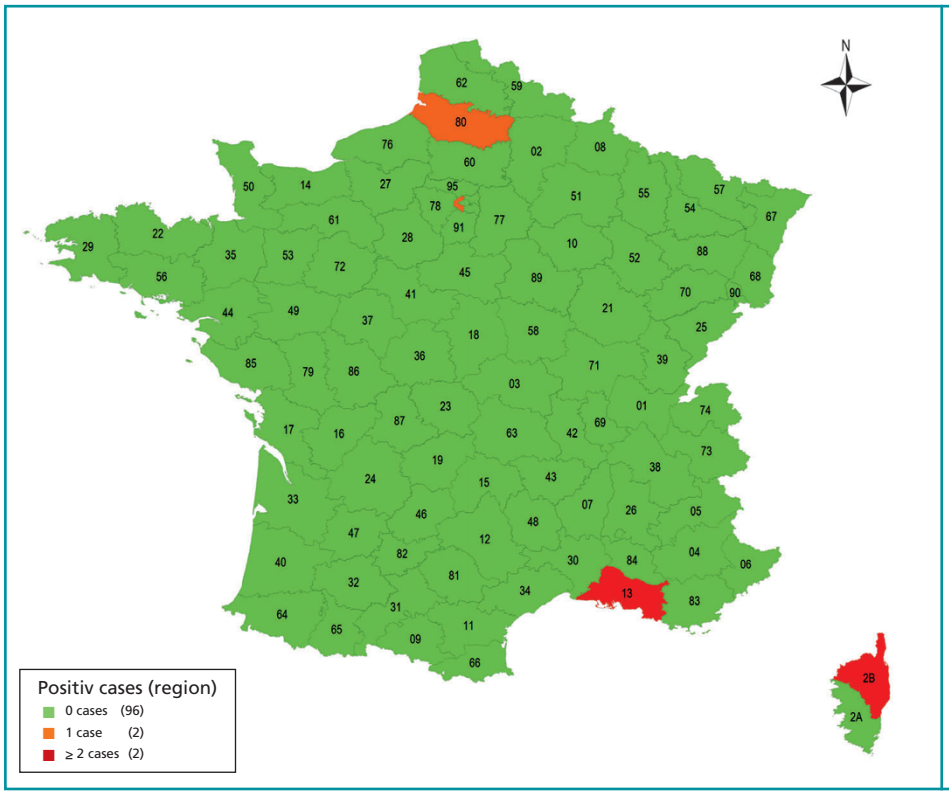

Fig. 8 Origin of canine serum samples from group B tested positive for *Dirofilaria immitis* antigen

In group A, the calculated seroprevalences for the tick-borne pathogens was 2.72% (95% CI: 1.77–3.99%) for *A. phagocytophilum*, 1.09% (95% CI: 0.52–1.99%) for *B. burgdorferi* and 0.33% (95% CI: 0.07–0.95%) for *E. canis* with distribution of the positive cases throughout the country (Figs. 4–6). Furthermore, concurrent infections of *Anaplasma* with either *B. burgdorferi* (n = 2; 0.22%) or *E. canis* (n = 1; 0.11%) were determined.

Group B

The origin of the samples tested in group B (n = 131) is shown in Fig. 7. High regional activity in sample submissions (> 11 submissions) occurred in the South of France (Bouches-du-Rhône).

D. immitis antigen was found in the blood samples of nine dogs (6.87%; 95% CI: 3.19–12.64%) (Fig. 8). Of these nine positive dogs, seven were from the South of France: n = 3 from Bastia (Corsica) and n = 4 from Bouches-du-Rhône (Raphele-les-Arles, Marseille, Lambesc and Simiane Collongue). Two of the nine positive dogs were from the northern part of the country: n = 1 from Gennevilliers in the region of Hauts-de-Seine around Paris (this dog had arrived from Martinique, French West Indies, in France in October 2005) and n = 1 from Amiens in the Somme region (this dog had arrived in France in July 2005 after living in French Guyana for 4 years).

Differences between the two groups regarding the rate of *D. immitis* infection

There is proof of a significant difference between the two groups ($p < 0.0001$).

Differences between the two groups regarding the geographic distribution of *D. immitis* infection

When the occurrence for the separate regions was calculated, it became evident that the distribution of heartworm infection in both groups was statistically not different ($p = 1.0$) and regionally restrict-

ed to the departments of Bouches-du-Rhône and Corsica in the South of France. The two cases from the northern part of the country were excluded due to their known travel history.

Discussion

The distribution areas of *D. immitis* in France primarily reflect the regions of origin of filaria-positive dogs, identified in a study with veterinarians participating from 62 departments (Ducos de Lahitte 1990; Chauve 1997). In this study, 5,503 dogs – mainly from kennels – were blood-tested. 106 (= 1.9%) dogs were positive for microfilaria. Species differentiation using activity pattern of acid phosphatase staining in unsheathed microfilaria determined that 40 (= 0.7%) dogs were infected with *D. immitis*, 75 (= 1.4%) were infected with *D. repens* and 3 (= 0.05%) were infected with *Acanthocheilonema reconditum*. The region of origin of the positive *D. immitis* dogs was confined to Corsica, the departments Vaucluse, Bouches-du-Rhône and Haute-Garonne in the South of France, and the Dordogne to the East of Bordeaux.

However, *D. immitis* infections have been reported beyond the Mediterranean, namely in field studies with positive findings in Normandy and Brittany in the Northwest of France. Blood tests in 215 hunting dogs and 85 military dogs from eight local departments (Finistère, Morbihan, Ille-et-Vilaine, Manche, Calvados, Orne, Sarthe, Vienne) determined 15 (= 5%) infected hunting dogs, whereby 11 (= 3.7%) of these dogs, originating from Cherbourg (Normandie/Manche) and Monterfil (Bretagne/Ille-et-Villaine), were positive for *D. immitis* (Doby et al. 1986a). A further study points to Brittany as an endemic region for *D. immitis*, whereby 30 hunting dogs from West of Rennes were tested and 3 (= 10%) of the dogs showed microfilaraemia (Doby et al. 1986b). However, these results appear questionable as the microfilariae, detected using a filtration method, were related to *D. immitis* only on the basis of morphological criteria. This approach is not well suited and relies on specialist training to accurately differentiate the filariae. Current research suggests that more reliable larvae differentiation can be achieved using molecular diagnostic techniques such as PCR. In a recent study, three out of five morphological identifications had to be corrected after using PCR, demonstrating the inaccuracy of morphological diagnosis (Rishniw et al. 2006).

The prevalence of *D. immitis* infection in Europe is reported between 0 and over 60% (Trotz-Williams and Trees 2003), whereas the detection method plays a decisive role in diagnosing this infection. Many studies may underestimate the prevalence if they are only testing for microfilariae and not also for heartworm antigen to include occult infection. A study by Courtney and Zeng (2001) suggests that specificity and sensitivity of different ELISA tests for the detection of specific antigen are between 94–98% and 30–94%, respectively. Sensitivity is crucially dependent on the number of adult female worms (0, 1–2 or >2). The rapid assay test deployed in the study presented here showed an average sensitivity of 67% (95% CI: 58–75%), with 35% (95% CI: 16–60%) at 0, 65% (95% CI: 53–75%) at 1–2, and 94% (95% CI: 76–98%) at more than two adult female worms, and showed a specificity of 98% (95% CI: 92–100%) (Courtney and Zeng 2001).

The occurrence of *D. immitis* antigen in group A of the study presented here was significantly lower than in group B. This was not surprising as group B comprised dogs with a tentative diagnosis of heartworm infection based on clinical signs, while the blood samples of group A were submitted to a diagnostic laboratory for reasons not preselecting for any infectious disease. Therefore these can be regarded as random samples. This conclusion is supported by the fact that samples from group A came from all over France, while those from group B came mainly from the South of France with its known *D. immitis* endemic areas. When the occurrence was calculated for separate regions, it became evident that heartworm infection in both groups (A and B) was regionally restricted to the departments Bouches-du-Rhône and Corsica in the South of France. This cor-

responds with the findings from Genchi et al. (2005), who predict the most favourable developmental conditions for *D. immitis* for these regions, using a geographical information system (5–10 yearly average predicted number of heartworm generations).

Our data indicates that an expansion of the southern endemic areas of *D. immitis* into the North has not occurred. It also indicates that dogs in the southern areas of the country are at a higher risk of heartworm infection. The only two positive cases from the northern part of the country likely came into France from abroad: one dog was brought in from Martinique (French West Indies) and one from French Guyana.

During the past twenty years, *D. immitis* has slowly spread in the northeastern areas of the USA through to Canada. It is considered that one of the reasons is the growing population of coyotes (Bowman 2007). The coyote *Canis latrans* is a canine indigenous to America which allows, unlike fox (Magi et al. 2007) and raccoon dog (Nakagaki et al. 2007), efficient growth and development of *D. immitis*, with prevalences of 24–57 % in 2000 to 2002 (Bowman 2007). In contrast to the USA, one of the reasons why *D. immitis* is not spreading further North in Europe and particularly in France, despite favourable climatic conditions and the existence of suitable vectors (Genchi et al. 2005), may be the lack of a suitable wild animal reservoir such as the coyote.

In the study presented here, positive cases for tick-borne pathogens in the dogs of group A are distributed throughout the country, unlike heartworm infection. The distribution of positive cases for *B. burgdorferi* and *E. canis* is consistent with the results from Bourdeau (2008), which are based on data obtained from veterinary clinics in national surveys.

The number of positive samples for *E. canis* (3/919) corresponds with results recently published (Bourdeau 2008), which estimate the global annual prevalence of canine ehrlichiosis in France within an interval of 0.9 to 3 cases per thousand dogs with a proposed annual incidence of 2.1 per thousand (based on the evaluation of 20,000 annual clinical cases). The sensitivity of the *E. canis* test performed in this study was 95.7 % (134/140) in comparison to IFA (immunofluorescence assay) and/or IB (immunoblotting) (O'Connor et al. 2002). Test specificity was 100 % as compared to IFA and IB in two separate surveys (O'Connor et al. 2004, 2006). It should be noted that some strains of *E. chaffeensis* (the causative agent of human monocytotropic ehrlichiosis) express proteins homologous to those of *E. canis*. As a result, some *E. chaffeensis* infections will induce cross-reacting antibodies on the SNAP® *E. canis* peptide (O'Connor et al. 2004). Although this agent is known to be prevalent throughout the United States and probably southern China and South America (Neer and Harrus 2006), its occurrence in France would be rather unusual.

Furthermore, a recent survey suggests that the overall annual prevalence of clinical canine *B. burgdorferi* sensu lato infection in France (based on information given by veterinary clinics) should be 0.03 to 0.06 cases per thousand dogs with a proposed average annual incidence of 0.05 per thousand (500 cases per year) (Bourdeau 2008). This is much lower (200-fold) than the findings in our study (10 positive cases in 919 dogs tested) and indicates that this infection is largely unknown to French veterinarians, or that its significance is underestimated. The difference may also be explained to some extend by the fact that our data captures active bacterial infection, whereby only 5 % of infected dogs actually develop disease (Levy and Magnarelli 1992).

The highly specific peptide antigen C_6 deployed in the rapid assay test used in the presented study is expressed on the surface of metabolically active *Borrelia* in the invariable region 6 (IR_6) within the VlsE protein (VMP-like sequence, expressed) (Liang and Philipp 1999; Liang et al. 1999). The result of the rapid assay test can therefore provide information on the potential activation state of the spirochaetes, the infection, as well as its worthiness for treatment (Krupka et al. 2007; Straubinger et al. 2008; Levy et al. 2008). Test sensitivity was

94.4% (238/252) when compared to a combination of IFA and IB tests (O'Connor et al. 2004). The C_6 analyte has been shown, in both humans and canines, not to react to antibodies elicited following *B. burgdorferi* vaccination (O'Connor et al. 2004; Marques et al. 2002). SNAP® specificity was 99.5% when used on field samples from dogs in North Carolina (Duncan et al. 2003).

To our knowledge, this study represents the first data on seroprevalence of *A. phagocytophilum* in the French dog population. The fact that this was the tick-borne pathogen with the highest prevalence in this study should prompt veterinarians to consider this infection seriously in their diagnostic routine. In a selected group of samples, SNAP® 4Dx® sensitivity and specificity were 99.1% and 100%, respectively, relative to the IFA (Chandrashekar et al. 2007). There appears to be some cross-reactivity between *Anaplasma platys* and *A. phagocytophilum* with the rapid assay performed in this study (Alleman and Wamsley 2008), but so far there is only one genetically proven report of *A. platys* infection in a French dog (Beaufils et al. 2002).

Moreover, co-infection of *A. phagocytophilum* with *B. burgdorferi* from *Ixodes* exposure or *A. platys* with *E. canis* from *Rhipicephalus* exposure was determined. The possibly concurrent incidence of an intracellular (*A. phagocytophilum*) and extracellular (*B. burgdorferi*) infection suggests the possibility of a two-way immunological influence for anaplasmosis and canine lyme disease (Krupka et al. 2007; Straubinger et al. 2008). For example, an additional infection with *A. phagocytophilum* could induce immune modulation in a dog that is already infected with *B. burdorferi*. It is now clear that dogs co-infected with *B. burgdorferi* and *A. phagocytophilum* are almost twice as likely to develop clinical disease as dogs infected with either agent alone (Beall et al. 2008).

There are some limitations to this countrywide serologic survey with regard to tick-borne pathogens. A positive antibody test is not equivalent to the existence of an agent in a particular geographic region; it is evidence only of prior exposure at some point and some location in the dog's history. Areas experiencing immigration are likely to also exhibit an import of dogs from other regions of the country. Pets testing positive in these areas may therefore well have been exposed elsewhere.

Conclusion

This data will provide veterinarians with an increased awareness of the vector-borne disease agents common in their practice areas, and elevate their consideration of these infections when taking a travel history, evaluating animals presenting with clinical signs of these diseases and choosing appropriate diagnostic or prophylactic procedures. *D. immitis*, *B. burgdorferi* and *A. phagocytophilum* can also cause disease in humans. The public health implications of the study are therefore significant. Dogs may serve as indicators to identify the presence of vector-borne disease agents of both veterinary and public health importance.

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