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Clinical signs, seasonal occurrence and causative agents of canine babesiosis in France: Results of a multiregional study

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ABSTRACT

Canine babesiosis (or piroplasmosis) is an emerging tick-borne disease caused by intraerythrocytic protozoa of the genera *Babesia* and *Theileria*. In dogs, infection by these parasites usually induces a haemolytic syndrome that can be fatal when complicated. Canine babesiosis prevalence is high in France, with *Babesia canis* thought to be the main etiological agent of the disease. This article presents the results of a multiregional prospective longitudinal survey on canine babesiosis conducted in France from October 2006 to December 2007. A total of 836 cases were reported by veterinarians using a multiple choice questionnaire and blood samples from 70 dogs were analyzed using PCR-RFLP to identify species responsible for canine babesiosis cases across the country. The main clinical signs reported were lethargy (98%), anorexia (98%) and hyperthermia $\geq 39\,^{\circ}C$ (80%) followed by pale mucous membranes (54%), modification of urine aspect (45%) and splenomegaly (33%). The dog population at risk was mainly represented by young dogs living in rural areas. Twenty-five out of the 70 blood samples (36%) tested by diagnostic PCR were found to contain *Babesia*/*Theileria* genus-specific DNA and all had profiles similar to that of *Babesia canis* genomic DNA after restriction fragment length polymorphism analyses. The survey results provide a reference for further molecular studies to assess the species and vectors involved in the transmission of the disease in France and across the Mediterranean basin.

1. Introduction

Canine babesiosis (or piroplasmosis) is an emerging tick-borne disease caused by intraerythrocytic protozoa of the genera *Babesia* and *Theileria* also called "piroplasms" (Irwin, 2009). In dogs, infection by these parasites usually induces a syndrome characterized by fever, lethargy, anorexia, anaemia and thrombocytopenia that can be fatal when complicated (Irwin, 2009; Solano-Gallego and Baneth, 2011). However, the severity of the disease depends on various factors such as the *Babesia* species involved, the age and immune status of the host and the presence of other infections or diseases (Irwin, 2009). Four *Babesia* species known to infect dogs have been identified to date in Europe: *Babesia canis, Babesia vogeli, Babesia gibsoni* and *Babesia microti*-like, also known as *Theileria annae* or *Babesia* sp. "Spanish dog" (Solano-Gallego and Baneth, 2011; Matijatko et al., 2012). In Europe, *B. canis* is the most widely distributed of these species, coinciding with the distribution of its known vector *Dermacentor reticulatus*. *B. vogeli* is more often found around the Mediterranean basin where *Rhipicephalus sanguineus* is the predominant tick species. The *Babesia microti*-like species seems to be centred in the northwest of Spain whereas the occurrence of *B. gibsoni* is reported more sporadically (Irwin, 2009; Solano-Gallego and Baneth, 2011).

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Canine babesiosis has a high prevalence in France (Bourdoiseau, 2006) and *B. canis* is thought to be the main etiological agent of the disease (Bourdoiseau, 2006; Beugnet and Marié, 2009). *B. vogeli* has recently been confirmed to be responsible for dog infections in southern France but the prevalence of infection is not known (René et al., 2012). Climate change, vector migration and the increase in pet travel may all affect the future distribution of *Babesia* or *Theileria* including those that have yet to be detected by current molecular methods.

To provide a point of reference to study the epidemiology and clinical expression of canine babesiosis in France, we present here the results of a multiregional prospective survey conducted from October 2006 to December 2007. A total of 836 cases of canine babesiosis were reported by 41 veterinary clinics throughout mainland France and the main clinical and epidemiological data were collected from questionnaires answered by veterinarians. Seventy dog blood samples were analyzed using restriction fragment length polymorphisms of *Babesia*-specific PCR products (PCR-RFLP) to identify the species responsible for canine babesiosis in France.

2. Material and methods

2.1. Questionnaire records

Mainland France is divided into 96 administrative *départements* (for simplicity translated here as "departments"), which provides a convenient 'grid' for sampling clinics across the country. Veterinary practices were selected taking into account their size (associations of veterinarians were preferred) as well as the experience and the interest of veterinarians in the study. Forty-one veterinary clinics, one per department (Fig. 1), agreed to participate in the study which corresponded to 2% of all veterinary clinics practising in France in 2007 according to data provided by a French veterinary institution (Conseil National de l'Ordre des vétérinaires, Paris). For all suspected cases of canine babesiosis (according to clinical criteria and/or blood smear results), veterinarians had to complete multiple choice questionnaires where the following information was requested (Tables 1 and 2): examination date; age, sex, breed and lifestyle of dog; major clinical signs and results of blood smear examination (observation or not of typical forms of piroplasms).

2.2. Blood sample collection and analysis

2.2.1. Blood sample collection and DNA extraction

Fresh blood was taken from ear punctures of all dogs suspected to have contracted canine babesiosis. Blood smears were prepared, air dried, stained using the May Grünwald Giemsa method and stored at room temperature before observation by light microscopy. If, based on clinical evidence and/or the blood smear observation, a suspected case required confirmation, veterinarians were asked to send blood smears and/or venous blood samples for microscopic confirmation or PCR analysis using franked parcels provided for this purpose. Venous blood samples were taken from the cephalic or jugular veins of dogs using blood collection tubes containing anticoagulant EDTA and stored at 5 °C ± 1 °C until analysis. Subsequent DNA extraction and PCR analysis were performed in the Laboratory of Parasitology and Parasitic Diseases of VetAgro Sup (Marcy l'Etoile, France). Total DNA was extracted from blood/EDTA samples by applying 50 μl of each sample to FTA® Mini Cards (Whatman International Ltd., UK). Circles were punched out of cards with a 1.2 mm Harris Micro Punch (Whatman International Ltd., UK) and treated as recommended by the manufacturer, then transferred into PCR tubes for later processing.

2.2.2. DNA amplification

All DNA samples were analyzed by diagnostic PCR using primers Piro-A1 (5′AGGGAGCCTGAGAGACGGCTACC 3′) and Piro-B (5′TTAAATACGAATGCCCCCAAC 3′) that amplify about 450-bp of the 18S rRNA gene of *Babesia* sp. as previously described (Jefferies et al., 2003). Each FTA® punch was added to a 24 μL reaction mixture containing 100 μM of each deoxyribonucleotide, 12.5 pmol of each primer and 0.625 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) in the reaction buffer provided by the manufacturer. The following thermocycle conditions were used: 95 °C for 15 min; 62 °C for 1 min; 72 °C for 2 min; 30 cycles at 94 °C for 30 s, 62 °C for 20 s and 72 °C for 30 s; and 72 °C for 7 min. Positive controls containing *B. canis* or *B. vogeli* DNA and negative controls without DNA were included in parallel. An aliquot (6 μl) of each amplified product was separated on a 1.5% agarose gel stained with ethidium bromide at 130 V for 1 h and visualized under ultraviolet illumination.

2.2.3. RFLP-PCR analysis

DNA amplified from dog blood samples (10 μL of the PCR reaction) was analyzed for restriction fragment length polymorphisms using *Taq*I and *Hin*fI enzymes (Promega, Madison, WI, USA) according to the manufacturer's instructions using a method (Carret et al., 1999) adapted for amplification of 450-bp fragments (Jefferies et al., 2003). Products were then separated on 2% agarose gels stained with ethidium bromide at 130 V for 1 h and visualized under ultraviolet illumination.

RFLP-PCR profiles of samples were compared to profiles of *B. canis* and *B. vogeli* genomic DNA, as these were the only two species that had been detected at a molecular level in dog blood samples from France in 2007 (Cacciò et al., 2002; Shaw et al., 2003). The specificity of the original RFLP-PCR was tested *a posteriori* against RFLP-PCR profiles of *B. gibsoni* and *T. annae* genomic DNA.

2.3. Statistical analysis

Only babesiosis cases confirmed by observation of typical *Babesia* forms in blood smears or suspected cases confirmed by PCR were included in the study. Statistical analyses were performed using R software (R Development Core Team, 2011). Seasonal case frequencies from December 2006 to November 2007 were compared to theoretical frequencies ($f = 0.25$ for each of the four seasons) using a Chi-squared test. When possible, data obtained from babesiosis cases reported in questionnaires were