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Short communication

Hemoprotozoa of domestic animals in France: Prevalence and molecular characterization

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ABSTRACT

Very limited information is available on epizootiology of haematozoan infections in French domestic animals. In an attempt to address this issue, prevalence of piroplasmida was studied in carnivores and ruminants, whereas prevalence of *Hepatozoon* spp. was only investigated in carnivores. In total, 383 animals were included in the survey (namely 116 cats, 108 dogs, 91 sheep and 68 cows). Parasite diagnosis was carried out using molecular methods such as PCR and sequencing of the 18S rRNA gene. In addition, ruminant samples were analyzed with the reverse line blotting technique (RLB). Results of RLB and PCR plus sequencing were in total agreement.

In carnivores, haematozoan prevalence was close to 1%. Two cats were infected by *H. canis* (1.7% prevalence) and one of them was co-infected by *Cytauxzoon* sp. (0.8%). This represents the first finding of both pathogens in French cats. One dog was infected by *H. canis* (0.9%) and another by *Babesia canis vogeli* (0.9%).

In ruminants, haematozoan prevalence (piroplasmida) was significantly higher than in carnivores (4.8% in sheep and 8.8% in cow). *Theileria ovis* was found in 1 sheep, *Theileria* sp. in 2 sheep, *Theileria buffeli* in 5 cows and *B. major* in 1 cow.

Evidence presented in this contribution indicates that haematic protozoa are not widely distributed in domestic mammal populations of France.

1. Introduction

Members of the order Piroplasmida (like the genera *Babesia*, *Theileria* and *Cytauxzoon*) and Adeleorina (like *Hepatozoon canis*) are apicomplexan protozoa. They live in blood cells of vertebrates and are transmitted by ticks. Both piroplasmida and *Hepatozoon* are causative agents of important zoonotic diseases. Traditionally these organisms have been diagnosed by low sensitivity techniques, such as microscopic examination of blood smears. Following the advent of new molecular diagnostic techniques *Babesia*, *Theileria* and *Hepatozoon* have been diagnosed with increased frequency in domestic animals. The same is true for human babesiosis (Gratz, 2004). In addition, DNA sequencing combined with phylogenetic analysis has revealed the existence of some new haematozoans in recent years (Allsopp and Allsopp, 2006). It is therefore generally accepted among parasitologists that emergent diseases caused by piroplasmida or *Hepatozoon* pose serious health risks both for animal and human (Harrus and Baneth, 2005).

Many studies on the biogeography of tick-borne haematozoans have been published during the last decade, but precise details of their epizootiology are still lacking for

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some European countries (Anon., 2007). This is particularly true of France, where only a few studies on the presence of theileriosis, babesiosis or hepatozoonosis have been carried out (L'Hostis and Seegers, 2002; Devos and Geysen, 2004; Bourdoiseau, 2006; Criado-Fornelio et al., 2007b). With the aim of filling this gap in the knowledge of protozoa biogeography, this communication reports on a study of hematozoans (namely piroplasmida and *Hepatozoon* sp.) in France. Techniques such as PCR, RLB and sequencing of the 18S rRNA gene were employed to diagnose and identify haematozoans in a representative number of domestic animals.

2. Materials and methods

During the survey period (December 2006–July 2007), blood samples from some 116 cats, 108 dogs, 91 sheep and 68 cows were sent by veterinarians to the Scanelis laboratory (Toulouse, France) for diagnosis. Blood specimens were collected into EDTA Vacutainer® tubes (Franklin Lakes, USA) by veterinarians. Blood samples were refrigerated and sent to the laboratory in a cold pack. Samples from ruminants were taken from animals showing no signs of disease, whereas samples from cats or dogs were obtained from symptomatic animals (showing signs related to infection with pathogens such as viruses, bacteria or parasites). Thirty-five percent of the cats were from the South of France, 19% from the Centre, 42% from the North, 1% from overseas and 3% were of unknown location. In dog the proportion of samples by geographic origin was as follows: 50% South, 30% Centre, 17% North and 3% overseas. Most of the ruminant samples came from Southern France (100% of ovine and 79% of bovine).

All of the samples were studied in Spain by standard PCR (piroplasmida) or qPCR (*Hepatozoon*) and sequencing of the 18S rRNA gene. In addition, ruminant samples were analyzed at the Crabart laboratory in Italy by RLB, to obtain alternative diagnostic results by a more sensitive procedure. Small animal samples were not analyzed by RLB, due to the absence of suitable probes for identification of the diverse species of carnivore piroplasmida. DNA was extracted from 200 μl of each blood specimen. The Nucleospin blood isolation kit (Macherey-Nagel, Düren, Germany) was employed in France for DNA extraction, following manufacturer's instructions. Purified DNA samples were stored at −20 °C and finally sent to Spain/Italy for molecular diagnosis in a cold package. Standard PCR was employed for diagnosis of piroplasmida (Criado-Fornelio et al., 2003b). The piroplasmida PCR was performed with primers BT1-F (5′-GGTTGATCCTGCCAGTAGT-3′) and BT1-R (5′-GCCTGCTGCCTTCCTTA-3′). A DNA fragment of approximately 400 bp was amplified and sequenced in all positives. Whenever new protozoa isolates were found, the complete 18S rRNA gene was sequenced. Measures were taken to avoid cross-contamination (laminar flow hoods, separated work areas for reaction mixture preparation, DNA extraction, amplification and analysis of PCR products, etc.) and suitable positive and negative controls were always included in amplifications. The quantitative PCR (qPCR) technique described by Criado-Fornelio et al. (2007b) was used for diagnosis of *Hepatozoon* sp. in carnivores. The qPCR assay was performed in an ABI 7500 fast instrument (Applied Biosystems, Inc., Foster City, CA, USA). The reaction mix included primers HEP-1 (5′-CGCGCAAATT ACCCAATT-3′) and HEP-2 (5′-CAGACCGGTTACTTTYAGCAG-3′). A commercial kit (iTaq SYBR Green Supermix with ROX, from BioRad—Hercules, CA, USA) was used for all qPCR amplifications. Fragments obtained in positive qPCR assays (235 bp) were sequenced for species identification. The RLB technique was performed as described by Georges et al. (2001). Amplification mix included the primers RLB-F2 (5′-GACACAGGGAGGTA GTGACAAG-3′) and RLB-R2 (biotin-5′-CTAAGAATTTCACCTCTGACAGT-3′). DNA electrophoresis conditions and procedures for DNA purification from agarose gels can be consulted in Criado-Fornelio et al. (2003a). DNA fragments obtained in at least two separate amplifications were sequenced using an ABI 3130 (Applied Biosystems Inc., Foster City, CA, USA) automated sequencer.

Calculation of sample size was made based on the following parameters: population size (infinite), expected prevalence (10%), maximum error (5%) and confidence level (95%). A minimal sample size of 138 animals was used to estimate the prevalence of haematozoans (Thrusfield, 1995). In this respect, the total number of animals diagnosed was 383, which means that sample size could be considered more than sufficient. The same was true when samples were divided in two groups: carnivores (159 cats and dogs) and ruminants (224 cows and sheep). Statistical analysis of prevalence data was performed with the STATGRAPHIC software package (Manugistics, Rockville, MD, USA).

3. Results and discussion

Table 1 shows the results of the molecular survey. Most of the protozoa isolates genetically characterized in this work were closely related to haematozoans previously identified in Europe. BLASTN® comparison of the small ribosomal subunit gene sequences always yielded identity higher than 99%. By this reason, phylogenetic analyses were deemed unnecessary to identify protozoan isolates. The region where most positive samples appeared was southern France, albeit 1 cow was positive for *Theileria buffeli* in central France and 2 positive samples for *H. canis* (1 dog and 1 cat) came from northern France. These results are logical considering that most ruminant samples had been taken in the South of that country.

Prevalence of haematozoan infection in carnivores was close to 1% (Table 1). Two cats were infected with slightly different *H. canis* isolates. There was a single base difference between them, which may have been caused by a sequencing error or a single nucleotide polymorphism of the 18S rRNA gene. Both *H. canis* sequences were introduced in GenBank® with accession numbers EU622909 and EU622910, respectively. It is also interesting to note that one of the domestic cats infected by *H. canis* was co-infected by *Cytauxzoon* sp. as well (the sequence of the latter was introduced in GenBank® with accession number EU622908). This is the first time that these two