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Short communication

Identification of *Babesia* species infecting dogs using reverse line blot hybridization for six canine piroplasms, and evaluation of co-infection by other vector-borne pathogens

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ABSTRACT

Canine infection by vector-borne hemoparasites is frequent in tropical and sub-tropical areas where exposure to hematophageous ectoparasites is intensive. A reverse line blot (RLB) assay was designed to improve the simultaneous detection of all named canine piroplasm species combined with other vector-borne pathogens of dogs including *Ehrlichia canis*, *Hepatozoon canis* and *Leishmania infantum* common in the Mediterranean basin. Blood samples of 110 dogs from Spain ($n = 21$), Portugal ($n = 14$) and Israel ($n = 75$) were analyzed. The study evaluated 2 groups of dogs, 49 dogs with piroplasm infection detected by blood smear microscopy from Portugal, Spain and Israel, and 61 dogs surveyed from rural areas in Israel, for which infection status with vector-borne pathogens was unknown. Among the dogs previously diagnosed with piroplasmosis, infection with *Babesia canis*, *Babesia vogeli*, *Babesia gibsoni* and *Theileria annae* was detected in the Iberian dogs while only *B. vogeli* was found in Israeli dogs. These differences are attributed to the absence of tick vectors for some piroplasm species such as *Dermacentor reticulatus* in Israel. Eleven (79%) of the *Babesia*-positive dogs from Portugal were co-infected with other pathogens including *L. infantum*, *H. canis* and *E. canis*. Eight of 61 (13%) rural Israeli dogs were co-infected with two or more pathogens including *B. vogeli*, *L. infantum*, *E. canis*, and *H. canis*. Triple infections were demonstrated in 2 dogs. The RLB detection limit for *Babesia* was 50-fold lower than that of PCR. This study presents a RLB to simultaneously detect and separate the major vector-borne dog pathogens in southern Europe and the Middle East.

1. Introduction

Vector-borne infections of dogs such as babesiosis, leishmaniosis, and ehrlichiosis are among the most common canine diseases in tropical, sub-tropical and temperate climate regions of the world. Co-infection with more than one pathogen is common due to the abundance of hematophageous disease vectors, such as ticks and sand flies, and the ability of arthropod vectors to host and transmit several pathogens simultaneously.

Although sensitive and specific molecular techniques have been developed for the detection of canine vector-borne pathogens, most assays are able to detect only one pathogen in a single run, and unable to identify co-infection of closely related pathogens. Most PCR assays designed to amplify the DNA of a certain pathogen will not detect the

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presence of more than one closely related pathogens and tend to amplify the DNA of the more abundant organism in the sample. Multiplex PCR assays are not efficient in detecting more than two or three organisms simultaneously (Peleg et al., 2010).

The reverse line blot (RLB) utilizes the hybridization of biotinylated PCR products to specific oligonucleotide probes immobilized on a membrane. The RLB technique is sensitive enough to distinguish between single nucleotide polymorphisms and has been used extensively for the detection of infections (Gubbels et al., 1999; Georges et al., 2001).

Piroplasms are tick-borne apicomplexan parasites that infect erythrocytes of domestic and wild animals. *Babesia* infections in dogs are caused by at least six genetically distinct named species. The large piroplasms previously considered as sub-species of *Babesia canis* are currently categorized as *B. canis*, *Babesia vogeli* and *Babesia rossi*, and the smaller canine piroplasms include *Babesia gibsoni*, *Babesia conradae* and *Theileria annae* (synonym *Babesia microti*-like). Accurate identification of the piroplasm species causing canine infection is important for the selection of specific anti-babesial therapy; prediction of the animals prognosis and follow up for disease recurrence after initial therapy; epidemiological surveillance for the introduction of *Babesia* species into previously non-endemic regions and the threat of emergence of infection in these areas.

Co-infection of canine piroplasms with other tick-borne pathogens such as *Ehrlichia canis*, *Hepatozoon canis*, and sand fly borne *Leishmania infantum* are frequent in areas where exposure to ectoparasites is extensive (Otranto et al., 2009). Co-infection may induce more severe clinical manifestations than single infections, and also present a diagnostic and therapeutic challenge (Otranto et al., 2009).

In this context, the main purpose of this study was to develop a molecular assay for improved simultaneous detection of piroplasm species and additional vector-borne co-infections common in dogs in the Mediterranean region, allowing comparison with other related pathogen species belonging to the same or close genera from other areas of the world.

2. Materials and methods

2.1. Samples

The study included blood samples from 110 domestic dogs from Israel, Spain and Portugal of which 49 were diagnosed with clinical disease in which *Babesia* merozoites were visually detected by light microscopy of giemsa-stained blood smears at the Hebrew University of Jerusalem in Israel ($n = 14$), the University of Trás-os-Montes e Alto Douro in Portugal ($n = 14$) or the Autonomous University of Barcelona in Spain ($n = 21$). The additional 61 samples were from rural domestic dogs examined as part of a *Babesia* infection survey in central Israel and not positive for *Babesia* on blood smear examination. All blood samples were collected in EDTA tubes, spotted on sterile filter paper, and stored at −80 °C until DNA was extracted.

2.2. DNA extraction, PCR amplification and sequencing

DNA was extracted from blood spotted on filter paper. Samples spotted onto filter paper, corresponding to ~20 μL of fluid, were cut out using individual sterile scalpel blades and transferred into sterile tubes. DNA was extracted by the phenol–chloroform technique as previously described (Cardoso et al., 2010). Amplification of the 18S SSU rRNA gene (460–520 bp fragment) of piroplasms and *Hepatozoon* species was carried out using the forward primer, RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′), and a modified reverse primer, RLB-R3 (Biotin-5′ CTT TAA CAA ATC TAA GAA TTT CAC CTC TGA CAG T-3′) (Georges et al., 2001). The reaction mixture consisted of PCR-Ready High Specificity (PCR-S-192, Synteza Bioscience Ltd., Israel) containing 1.5 mM $MgCl_2$, 20 μM of each primer (IDT, Integrated DNA Technologies, Inc, Coralville, IA, USA) and 40 ng purified DNA sample in 25 μl of double distilled water (DDW). PCR conditions were as follows: an initial step 2 min at 94 °C followed by 40 cycles of denaturation 45 s at 94 °C, annealing 45 s at 59 °C, and extension 45 s at 72 °C. A final extension step of 5 min at 72 °C completed the program.

PCRs targeting the *Leishmania* ribosomal internal transcribed spacer 1 (ITS1) region (Nasereddin et al., 2008), and *Ehrlichia* and *Anaplasma* spp. 16S rRNA genes were carried out as described (Schouls et al., 1999). PCR amplicons were purified with Exo-Sap (USB, USA) prior to DNA sequencing. Sequencing was carried out at the Center for Genomic Technologies, Institute of Life Sciences, Hebrew University of Jerusalem and was performed on all the PCR products in this study. The sequences were compared to GenBank database National Center for Biotechnology Information (NCBI) using the BLAST 2.2.9 Programme (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed using the program ClustalX (1.83) with an engine based on the ClustalW algorithm.

2.3. RLB – reverse line blot hybridization

2.3.1. Specific and "catch all" RLB oligonucleotide probes

The sequences of the oligonucleotide probes used in this study are presented in Table 1. "Catch all" and some specific probes for *Babesia*/*Theileria*, *Hepatozoon*, *Leishmania* and *Ehrlichia*/*Anaplasma* were adapted from previous publications by Georges et al. (2001), Matjila et al. (2008), Nasereddin et al. (2008) and Schouls et al. (1999), respectively. New specific probes to the hypervariable V4 region of the 18S rRNA gene (supp. Fig. 1) were designed based on DNA sequences deposited in the NCBI GenBank for *B. gibsoni* (AY278443); *B. conradae* (AF158702); *T. annae* (AF188001, AY457974.1, AY150068), and *H. canis* (AY150067.2). In addition, probes that anneal specifically to the amplified region of the 16S rRNA gene sequences from *E. canis* (AY394465.1); *Anaplasma platys* (AF156784.1) and *Anaplasma phagocytophilum* (AY055469.3) were also developed. These sequences were aligned using the Clustal W algorithm online interface (http://www.ebi.ac.uk/clustalw/index.html). All the probes were synthesized with a C6 amino linker (IDT, Integrated DNA Technologies, Inc, Coralville, IA, USA).