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Molecular detection of *Anaplasma platys*, *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia monacensis* in dogs from Maio Island of Cape Verde archipelago

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ABSTRACT

Tick-borne diseases are emerging worldwide and have an important zoonotic relevance. Dogs play an important role in the epidemiology of several zoonotic tick-borne pathogens acting as sentinels and/or reservoirs. This study focused on the molecular identification of tick-borne pathogens in blood samples of 153 autochthonous asymptomatic dogs in Maio Island, Cape Verde archipelago. Eighty-four (54.9%) dogs were positive for one or more pathogens. Fifty-five (35.9%) dogs were infected with *Hepatozoon canis*, 53 (34.6%) with *Anaplasma platys*, five (3.3%) with *Ehrlichia canis* and *Rickettsia monacensis*, an emerging human pathogen, was also identified in a single dog (0.7%). The former three pathogens cause important canine tick-borne diseases that are transmitted or potentially transmitted by *Rhipicephalus sanguineus* s.l., the only hard tick identified in Cape Verde. Furthermore, *Wolbachia* spp. was amplified from the blood of one dog. None of the dogs were positive for *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, *Midichloria mitochondrii*, *Bartonella* spp., *Babesia* spp. or *Theileria* spp. Fifty-four (35.3%) animals showed single infections and 30 (19.6%) co-infections, with *A. platys* and *H. canis* co-infection being the most frequent (28 dogs, 18.3%). The frequency of *E. canis* infection was statistically different among age groups ($P = 0.017$), being higher among dogs older than 4 years compared to younger dogs. Infection by *A. platys* was also statistically different among age groups ($P = 0.031$), being higher in dogs younger than 2 years compared to older dogs. The statistical analyses showed no significant association of PCR positivity with gender or location. The frequency of tick-borne pathogens detected in dogs in Maio Island, including *R. monacensis*, highlights the need to improve diagnosis and control in order to prevent the risk of transmission of these pathogens among dogs and humans living in or travelling to this touristic island.

1. Introduction

Tick-borne diseases (TBDs) are recognized as important emerging diseases worldwide in humans and animals and have an important zoonotic relevance (Chomel, 2011). Zoonotic TBDs shared between humans and dogs, such as anaplasmosis, babesiosis, ehrlichiosis, Lyme borreliosis and rickettiosis are known for decades, and a One Health approach is recommended for their management (Dantas-Torres et al., 2012). To our knowledge, no zoonotic risk has been reported up to now for canine *Hepatozoon* spp. infections. Recently, *Ehrlichia canis* and *Anaplasma platys*, two typical canine tick-borne diseases, have emerged as human pathogens in Venezuela (Arraga-Alvarado et al., 2014; Perez et al., 2006). *Midichloria mitochondrii*, the agent responsible for an emerging tick-borne zoonosis, and a potential new zoonotic *Bartonella* species have also been identified in dogs (Bazzocchi et al., 2013; Chomel et al., 2012). Considering the close association with humans and the susceptibility to tick bites and tick-borne agents, dogs can act as sentinels for numerous human tick-borne infections and for

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other zoonotic pathogens potentially transmitted by ticks, such as bartonelloses (Chomel, 2011; Hornok et al., 2013).

In recent years, several factors have been linked to the emergence of these diseases, including climate changes and increase in international travel (Kilpatrick and Randolph, 2012). An increasing number of TBDs, especially rickettioses, have been reported in European and North American travelers and dogs exposed to tick bites while travelling during warmer months in foreign countries (Delord et al., 2014; Leschnik et al., 2008). *Rickettsia conorii* sensu lato, the agent of Mediterranean spotted fever (MSF) transmitted by the brown dog tick *Rhipicephalus sanguineus* s.l., is endemic in all Mediterranean areas, with sporadic cases reported in sub-Saharan Africa, northern and central Europe and Asia (Parola et al., 2013). Apart from *R. conorii* sensu lato, other *Rickettsia* species of the spotted fever group (SFG) cause MSF-like illness: *R. helvetica*, *R. monacensis*, *R. massiliae* or *R. aeschlimannii* (Parola et al., 2013). MSF is the most emerging rickettiosis among European travelers (Delord et al., 2014). Recently, an eschar, typical finding in MSF or MSF-like illness, was observed in a veterinary colleague returning to Europe after an animal welfare campaign conducted in early summer 2012 in the touristic Maio Island, Cape Verde and rickettiosis was confirmed (Pereira C., personal communication).

Despite the presence of the tick vector *R. sanguineus* s.l. and the report of MSF-like illness in this traveler, no data is available on the presence of rickettioses or other TBDs in Maio Island, to the best of our knowledge. *R. sanguineus* s.l. is the only hard tick reported on Cape Verde archipelago, being prevalent throughout the year, and pathogens transmitted or potentially transmitted by this tick species, such as *Babesia canis*, *Babesia gibsoni*, *Hepatozoon canis*, *A. platys* and *E. canis,* have been reported in dogs in Santiago Island of this archipelago (Duarte, 2013; Götsch et al., 2009; Kirchner et al., 2008). The aim of this work was molecular detection and identification of tick-borne pathogens in canine blood from free-roaming private dogs from Maio Island.

2. Materials and methods

2.1. Animals and sample collection

Dogs from Maio Island were included in this study by random sampling. Autochthonous dogs, aged ≥ 6 months were included after owner's consent to participate in the study. All the animals included in the study were private dogs with an outdoor or mixed indoor-outdoor lifestyle and were therefore considered free-roaming. All dogs were apparently healthy, but detailed clinical examinations were not performed. Owners were not aware of TBDs and no tick control measures had been used in these dogs. Sampling was performed in July 2012 and data on age, gender and locality were recorded for each dog.

Blood samples were collected by jugular venipuncture in ethylene diamine tetraacetic acid (EDTA) and 200 μl of whole blood from each animal were spotted onto Whatman filter paper into four separate 50 μl dots and dried completely for 1 day and kept at 4 °C to be used later on for molecular analyses. The packed cell volume (PCV) was also measured on whole blood collected in EDTA and transferred to microhematocrit capillary tubes, using a portable microhematocrit centrifuge (Heraeus Pico 17Haematocrit, Heraeus Kulzer GmbH, Germany), at 12,000 rpm for 10 min.

2.2. PCR and sequencing

DNA was extracted using a commercial kit, following the kit manufacturer's instructions (NucleSpin Tissue, Macherey-Nagel, Germany). Firstly, a portion of the gene coding for canine GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was amplified to confirm DNA extraction following a published protocol (Bazzocchi et al., 2003).

Extracted DNAs were analyzed through specific PCR protocols, for the presence of bacteria of the Anaplasmataceae family (Parola et al., 2000), *Rickettsia* genus (Labruna et al., 2004; Roux et al., 1996), *Borrelia burgdorferi* sensu lato complex (Marconi and Garon 1992), *Midichloria mitochondrii* (Epis et al., 2008) and *Bartonella* genus (Jensen et al., 2000). Piroplasms (*Babesia/Theileria*) (Beck et al., 2009) and *Hepatozoon* species (Ujvari et al., 2004) were also screened by PCR. In order to characterize the bacterial species of the Anaplasmataceae family detected by PCR in positive samples, species-specific PCRs for *A. phagocytophilum* (Massung et al., 1998), *A. platys* (Inokuma et al., 2000), and *E. canis* (Stich et al., 2002) were also performed. DNAs extracted from blood of naturally infected dogs with *A. phagocytophilum*, *A. platys*, *E. canis, H. canis* or *Babesia vogeli*, were used as positive controls in the corresponding PCR reaction. DNAs extracted from infected *I. ricinus* ticks with *R. helvetica*, *B. burgdorferi* sensu lato or *M. mitochondrii* were included as positive controls in the *Rickettsia* genus PCR, *B. burgdorferi* sensu lato complex PCR and *M. mitochondrii* PCR, respectively. DNA extracted from the blood of a naturally infected cat with *B. henselae* was used as positive control in the *Bartonella* genus PCR. A negative control without DNA was also included in all PCR reactions. PCR products were visualized under UV after electrophoresis migration on a 1.5% agarose gel stained with ethidium bromide.

For *Hepatozoon* spp. and for bacteria belonging to the genus *Rickettsia,* the amplicons of the expected sizes from PCR positive samples were purified and sequenced using the forward and reverse primers used for DNA amplification (Labruna et al., 2004; Ujvari et al., 2004). One PCR positive sample for Anaplasmataceae family that was negative for the species-specific PCR protocols (targeting *A. phagocytophilum*, *A. platys* and *E. canis*) was also sequenced. Sequencing was performed using a Big Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) and an ABI PRISM 3130 sequencing device, as well as sequenced by a commercial sequencing facility (Macrogen Inc.). The sequence data were assembled and manually corrected using BioEdit software version 7.0 (freely available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Geneious version 6.1 (Biomatters Ltd). The sequences were then compared with those available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences obtained in this study were deposited in the GenBank under accession numbers: *H. canis* (KU961914- KU961968), *R. monacensis* (KU961970), and *Wolbachia* spp. (KU961969).

2.3. Data analysis

In the presence of negative results for the pathogens tested, the maximum possible prevalence in the total dog population was calculated using WinEpi (http://www.winepi.net). A Person's Chi-square test was used to assess the relationship between presence of pathogens and independent variables such as gender, age and location. The presence of at least one pathogen was also treated as single entity. The PCV values recorded in dogs with and without pathogens were compared using a non-parametric *t*-test (Mann-Withney *U* test), with 95% confidence interval (CI) as a measure of uncertainty. A p value <0.05 was considered as statistically significant. Statistical analysis was performed in an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it 2.30 software (Analyse-it Software Ltd, Leeds, UK).

3. Results

A total of 153 dogs of private owners were analysed in this study, which represent approximately a quarter of the dog popu-