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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 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 l of whole blood from each animal were spotted onto Whatman filter paper into four separate 50 l 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 population in Maio Island (Antunes, 2013). Dogs came from the 10 most important municipalities of the island (Table 1) and data on age and gender are reported in Table 2.

Based on molecular analysis, 84 (54.9%) dogs were positive for at least one of the tested pathogens (Table 1). Fifty-five (35.9%) dogs were positive for H. canis, as confirmed by BLAST analysis showing 98%-100% of identity to H. canis sequences available in GenBank. Fifty-three (34.6%) dogs were positive for A. platys and five (3.3%) for E. canis. One (0.7%) dog was positive for R. monacensis, as confirmed by BLAST analysis showing 100% of identity with R. monacensis isolate SK1 from I. ricinus (GenBank accession no. KC996728). Fifty-four (35.3%) dogs were infected with a single pathogen and 30 (19.6%) were co-infected with two pathogens.

H. canis and A. platys were the most frequently detected either as single infections or co-infections (Table 3). One Wolbachia spp., confirmed by BLAST analysis as 100% identical to a Wolbachia endosymbiont of Dirofilaria repens (GenBank accession no. AJ276500), was also identified in the sample of the only dog that was PCR positive in the Anaplasmataceae family PCR but not in the species-specific PCRs (A. phagocytophilum, A. platys or E. canis). DNA from A. phagocytophilum, B. burgdorferi sensu lato, M. mitochondrii, Bartonella spp., Babesia spp or Theileria spp was not detected in the dogs tested. A 1.8% maximum possible prevalence in the total dog population of Maio Island was calculated for these pathogens with negative PCR results in all the samples tested.

Results on the presence of tick-borne pathogens in dogs among dog age groups and gender are shown in Table 2. E. canis infection was statistically different among age groups ($P = 0.017$), with a higher number of infections in dogs older than 4 years compared to younger dogs ($P = 0.029$ vs 2-4 years of age, and $P = 0.018$ vs <2 years of age). Positivity of A. platys was statistically different among age groups ($P = 0.031$). In particular, a significant difference was observed in dogs younger than 2 years compared to dogs older than 4 years ($P = 0.019$). No significant difference was observed in dogs younger than 2 years compared to 2-4 years old dogs. No significant association was observed for positivity to H. canis, R. monacensis or for the presence of at least one pathogen and dog age groups. The statistical analyses showed no significant association of PCR positivity with gender or location. Dogs infected with at least one pathogen had a significantly lower PCV (P

= 0.019) compared to non-infected dogs, although the mean values of infected dogs were within the accepted reference intervals of 37-55 PCV adopted in our laboratory.

4. Discussion

Our results showed the presence of four tick-borne pathogens in dogs of Maio Island characterized by different prevalences. H. canis and A. platys were the most frequently detected pathogens (35.9% and 34.6%, respectively), while E. canis was sporadically detected (3.3%) and R. monacensis rarely detected (0.7%).

H. canis and A. platys were also the most frequently detected either as single infections (17% and 16.3%, respectively) or as co-infections (18.3%). This is not surprising given that the co-occurrence of H. canis and other canine tick-borne pathogens are commonly reported, especially in Europe (Baneth, 2011). The presence of these pathogens in asymptomatic dogs in Maio Island is in accordance with previous reports of both infections in apparently healthy dogs because these pathogens often cause subclinical to mild disease, even if laboratory abnormalities can be observed (Baneth, 2011; Harrus et al., 1997). H. canis and A. platys are the agents of canine hepatozoonosis and infectious canine cyclic thrombocytopenia, respectively, and are reported worldwide (Baneth, 2011; Harvey, 2012). Dogs are important in the life cycle of these pathogens, serving as natural hosts for H. canis and A. platys (Baneth, 2011; Harvey, 2012). H. canis is transmitted and A. platys is potentially transmitted by R. sanguineus s.l. (Dantas-Torres and Otranto, 2015), the only hard tick reported up to now in Cape Verde archipelago (Duarte, 2013).

E. canis is the primary etiologic agent of canine monocytic ehrlichiosis, an important canine disease that is also transmitted by R. sanguineus s.l. It is a severe disease of dogs and it is divided into acute, subclinical and chronic phases, with minimal clinical signs observed only during the subclinical disease phase (Waner et al., 1997). The low prevalence observed in our study in apparently healthy dogs may reflect the persistence of the subclinical phase of disease in the

sampled animals (Harrus et al., 1998) and is in accordance with a study conducted in asymptomatic dogs from Turkey (Aktas et al., 2015).

Interestingly, R. monacensis was the only rickettsiae identified in our study, whereas R. conorii sensu lato was not detected, despite the fact that dogs may act as sentinels for human infections and have been recently indicated as probable reservoir hosts for R. conorii subsp. conorii (Levin et al., 2012). To our knowledge, R. monacensis, an emerging human pathogen of the SFG rickettsiae, has never been reported in dogs up to now (Wächter et al., 2015). The pathogen has been detected in ticks from Europe, North Africa, and Asia and is reported in humans with MSF-like illness from Spain and Italy and in lizards from Madeira Island (Portugal) (Benredjem et al., 2014; De Sousa et al., 2012; Jado et al., 2007; Madeddu et al., 2012; Parola et al., 2013; Sun et al., 2015). Recently, lizards have been proposed as potential or transitory reservoir for this pathogen (De Sousa et al., 2012). R. monancensis is mainly transmitted by I. ricinus but it has also been found in other ticks and mites, suggesting that many vector species are involved in the zoonotic cycles and wide geographic distribution (Schreiber et al., 2014; Ye et al., 2014; Mi'ková et al., 2015). The invertebrate hosts were not included in our study, but the fact that R. monacensis has been reported in R. sanguineus s.l. (Pennisi et al., 2015) could explain the presence of this pathogen in Maio Island. This assumption is in accordance with previous findings that suggest that when ticks of the genus Rhipiephalus are prominent, these may act as vectors for R. monacensis (Madeddu et al., 2012), but further investigations are needed on the prevalence of tick-borne pathogens in R. sanguineus s.l. in the Cape Verde archipelago.Regarding the other tick-borne pathogens tested in this study, the fact that all our samples were negative for these pathogens is indicative, in the total dog population of Maio Island, of the absence of infection or of a very low (1.8%) maximum possible prevalence. The negative PCR results for piroplasms transmitted byR. sanguineus s.l. are in accordance with the low prevalence recently reported for B. canis and the absence of B. gibsoni in dogs from Santiago, the closest island to Maio on the Cape Verde archipelago (Dantas-Torres and Otranto 2015; Götsch et al., 2009). The negative results for A. phagocytophilum, B. burgdorferi

sensu lato and M. mitochondrii is not surprising because these pathogens are normally transmitted by hard ticks other than R. sanguineus s.l. that have never been reported in Maio Island (Duarte, 2013). Furthermore, the absence of bartonellosis in this island should be further confirmed by analyzing cats that are known to be the reservoir of different Bartonella species, including zoonotic B. henselae, the causative agent of cat scratch disease in humans (Chomel et al., 2006).

One dog was found with Wolbachia spp. DNA identical to a Wolbachia endosymbiont of D. repens. Wolbachia spp. are intracellular endosymbionts of filarial nematodes and their involvement in canine febrile illness has been proposed (Unver et al., 2003).

The finding of Wolbachia spp. DNA in canine blood is considered presumptive of dirofilariosis (Landum et al., 2014), which is in accordance with the fact that D. repens was identified in this dog in a recent study (Marcos et al., 2016).

The overall prevalence of tick-borne pathogens presented in our study for dogs in Maio Island (54.9%) was lower than the prevaence reported for dogs from Santiago Island (77.7%) (Götsch et al., 2009), whereas A. platys infection was more frequent in Maio Island (34.6% compared to 7.7% in Santiago Island). R. monacensis was only reported in Maio Island. Interestingly, dogs from Maio Island were apparently healthy, while animals analysed in Santiago Island were presented at the veterinary centre in Praia and therefore the clinical status of the dogs might explain the different prevalences of tick-borne pathogens in the two islands. Moreover, the differences between both studies in Cape Verde may reflect a different epidemiological situation in the two islands, which could be associated with the ecological features of both islands and distribution of vectors, or different analytical sensitivities of the molecular protocols used (Aktas et al., 2015).

In this study, the absence of a significant association between pathogen infection and location suggests that the infections are distributed on the whole island, probably reflecting the wide distribution of the vector R. sanguineus s.l. (Duarte, 2013). No significant association between age and TBDs was generally reported in dogs up to now (Maia et al., 2015). A higher frequency of tick-borne infections was recently observed in Turkey in adult dogs compared to dogs younger than 1

year in endemic areas, where older dogs have a higher probability of exposure to infected ticks than young animals (Aktas et al., 2015). Our results did not show a significant association between age and overall tick-borne infection but showed that animals aged over 4 years were more likely to be infected by E. canis compared to younger dogs, probably reflecting the persistence of subclinical disease in older animals (Harrus et al., 1998) and in agreement with the findings of Sainz et al. (2015). Furthermore we found a significantly higher frequency of A. platys infection in dogs less than 2 years old compared to dogs older than 4 years. Considering that no age predisposition for A. platys infections have been described, further studies including a more homogenous composition of age groups are needed, because sampling size and limitation in the detection methods used could bias the estimation of pathogen infection (Jovani and Tella, 2006).

Previous studies showed that dogs infected by TBDs may have severe anemia and thrombocytopenia, especially in symptomatic E. canis infections (Harrus et al., 1997). However, even if in our study infections with at least one pathogen were associated with significantly lower PCV values compared to uninfected dogs, PCV mean values within the accepted reference interval in all the sampled animals were indicative of the absence of anemia in dogs from Maio Island. This finding, together with the apparent absence of clinical signs of disease in these dogs, supports that PCV may not be a reliable indicator of the presence of tick-borne infection in dogs.

Regarding the impact of the results of this study for human health, three of the four pathogens detected in dogs from Maio Island have a zoonotic relevance. While A. platys and E. canis have rarely been reported in humans (Arraga-Alvarado et al., 2014; Perez et al., 2006), R. monacensis is an important emerging human pathogen (Parola et al., 2013). Concerning the risk of rickettiosis for human travelers to this touristic island, further investigations are needed.

5. Conclusions

The prevalence of tick-borne pathogens, some of them zoonotic, in apparently healthy dogs in Maio Island is high. This is probably because private dogs are free-roaming and are at high risk of tick

infestations, particularly because owners are not aware of these diseases and prophylactic/control measures (acaricides) are not used.

Our results should encourage a campaign of TBDs monitoring and control in this island, with special emphasis on the investigation in humans, animals and vectors, to obtain a wider epidemiological perspective on tick-borne pathogens and to understand R. monacensis infection dynamics. Our results should also reinforce the importance to alert the veterinary community, owners and public health authorities to prevent the risk of transmission of tick-borne pathogens among dogs and humans in this touristic island.

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Table 1

Frequency of tick-borne pathogens in dogs at municipalities in Maio Island.

Municipality	Dogs tested	Positive dogs No.				
	No.	H. canis	A.platys	E. canis	R. monacensis	Total infected dogs (≥1 pathogen)
Morro	13	4	5	-	-	6
Calheta	42	20	14	5	1	29
Praia Gon ¸ alo	4	3	1	-	-	3
Pedro Vaz	13	4	5	-	-	6
Alcatraz	13	2	5	-	-	6
Pilão Cão	11	4	8	-	-	8
Ribeira D. João	17	5	2	-	-	6
Figueira da Horta	8	1	1	-	-	2
Barreiro	8	4	2	-	-	5
Cidade do Porto Inglês		24	8	10	-	- 13
Total	153	55 (35.9%)	53 (34.6%)	5 (3.3%)	1 (0.7%)	84 (54.9%)

Table 2

Number and percentage of dogs positive for tick-borne pathogens according to age and gender

.

	Age (years)			Gender	
	<2	2-4	>4	Male	Female
No. tested (%)	69 (45.1)	61 (39.9)	23 (15)	101 (66.1)	52 (33.9)
H. canis (%)	27 (39.1)	21 (34.4)	7 (30.4)	38 (37.6)	17 (34.7)
A. platys (%)	31 (44.9)[a]	18 (29.5)	4 (17.4)[a]	35 (34.7)	18 (34.6)
E. canis (%)	1 (1.4)[a]	1 (1.6)[b]	3 (13)[a,b]	2 (2)	3 (5.8)
R. monacensis (%)	0 (0)	0 (0)	1 (4.3)	1 (1)	0 (0)
Total infected dogs (≥1 pathogen) (%)		43 (62.3)	29 (47.5)	12 (52.2)	53 (52.5) 31 (59.6)

[a,b] Significant difference for the same pathogen between categories of the same variable ($p < 0.05$).

Table 3

Frequency of tick-borne pathogens in dogs.

No. of pathogens	Pathogen	Dogs No. (%)
Single infection	H. canis	26 (17)
	A. platys	25 (16.3)
	E. canis	3 (2)
Co-infection	H. canis/A. platys	28 (18.3)
	H.canis/E. canis	1 (0.7)
	E.canis/R.monacensis	1 (0.7)
Negative		69 (45.1)
Total		153 (100)