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Short communication

Non-pet dogs as sentinels and potential synanthropic reservoirs of tick-borne and zoonotic bacteria

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ABSTRACT

Blood samples were collected from 100 shepherd dogs, 12 hunting dogs and 14 stray dogs (apparently healthy) in southern Hungary to screen for the presence of emerging tick-borne pathogens. Based on real-time PCR results, 14 dogs (11%) had single or dual haemoplasma infection, and a same number of samples were positive for *Anaplasma phagocytophilum*. In one sample *Coxiella burnetii* was molecularly identified, and 20.3% of dogs seroconverted to the Q fever agent. Rickettsaemia (sensu stricto) was also detected in one animal. This is the first molecular evidence of autochthonous infection of dogs with the above pathogens in Hungary. The relatively high prevalence of haemoplasma and anaplasma infection among non-pet dogs is suggestive of a prolonged carrier status and bacteraemia of these animals rendering them epidemiologically significant as potential reservoirs and sentinels for tick-borne infections.

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1. Introduction

The number of reported cases of tick-borne diseases and the variety of tick-borne agents have been increasing worldwide in recent years (Paddock and Telford, 2010) in part because they represent emerging problems but also because the recently developed high-sensitivity molecular tools allow for more effective detection (Telford and Goethert, 2004). The significance of tick bites and tick-borne infections is a rapidly increasing concern in both veterinary medicine (Fritz, 2009) and human health (Parola and Raoult, 2001).

Living in close association, humans and dogs play a particularly intertwined role in the epidemiology of pathogens transmitted by ticks. In addition to being susceptible to tick-borne agents, dogs may serve as reservoirs of tick-borne human pathogens, as a source of infection for vector ticks, as mechanical transporters of ticks, and as sentinel indicators of regional infection risk (Fritz, 2009). Conversely, pet dogs in developed countries are not only well cared for but are also usually treated against tick bites using preventative measures. Apart from this care, they are usually only sporadically taken on walks and may have less access to alternative infectious sources

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of tick-borne pathogens. Consequently, although the majority of studies on tick-borne and zoonotic canine infections focus on pet dogs (e.g., Kohn et al., 2011), data obtained from this type of sample source may not reflect the real epidemiological situation or actual veterinary-medical health hazards associated with dog-keeping in an endemic region.

To compensate for this inconsistency in the literature, it was decided to molecularly investigate tick-borne and zoonotic pathogens in blood samples from dogs that are either kept extensively (shepherd dogs) or are exposed to tick bites and other infectious sources more often than pet dogs (i.e., hunting and stray dogs).

2. Materials and methods

EDTA-anticoagulated and non-treated blood samples were collected by cephalic venipuncture from 100 shepherd dogs, 12 hunting dogs and 14 stray dogs from 24 locations in south Hungary, during the mid-summer of 2012. All dogs were selected randomly and appeared to be healthy but none were clinically evaluated. Animal data (sex, age; the latter for stray dogs estimated from their dentition) were recorded. EDTA blood samples were frozen at −20 °C until further processing. Sera were separated from non-anticoagulated blood samples after an overnight storage at 4 °C.

DNA was obtained using the QIAamp Mini Kit (Qiagen Inc., Hilden, Germany) individually from 200 μl of blood per sample (adding extraction controls) following the manufacturer's instruction. The quality and quantity of extracted DNA was examined with a TaqMan real-time PCR, which amplifies the canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as described previously (Boretti et al., 2009). Samples were screened for haemoplasma infection with a universal SYBR Green real-time PCR adapted from Willi et al. (2009) using an ABI 7500 Fast Sequence Detection System (Life Technologies, Zug, Switzerland) and the KAPA SYBR® FAST qPCR Kit (KAPABiosystems, Boston, USA) with 200 nM of primers. This was followed by species-specific TaqMan real-time PCRs, which detect part of the 16S rRNA gene (Wengi et al., 2008) with dilutions of plasmid DNA of known copy number for quantification purposes.

For comparison with haemoplasma prevalence, *Ehrlichia canis* was also evaluated in the blood samples using a TaqMan real-time PCR that amplifies a portion of the 16S rRNA gene of *E. canis* as described previously (Foley et al., 2007). The presence of *A. phagocytophilum* was investigated with a TaqMan real-time PCR, which detects part of the major surface protein (*msp*) -2 gene as reported (Courtney et al., 2004), but with a modified probe (FAM instead of HEX). The target for *C. burnetii* was the IS1111a gene in a TaqMan real-time PCR (Loftis et al., 2006). Evaluation of *Rickettsia* spp. was conducted using two real-time TaqMan PCRs based on the detection of the 23S gene of *R. helvetica* and detection of the citrate synthase (gltA) gene for other rickettsiae (Boretti et al., 2009).

To detect antibodies to *C. burnetii*, serum samples were diluted at 1:400 and examined by the commercial CHEKIT Q-Fever Antibody ELISA Test Kit (IDEXX, Liebefeld-Bern, Switzerland) using inactivated *C. burnetii* phase 1 and phase 2 antigens. The ELISA was carried out according to the manufacturer's instructions with the anti-ruminant immunoglobulin conjugate replaced with an anti-dog IgG (H + L) HRP-conjugate (1:40,000; Jackson ImmunoResearch Laboratories, Inc.,West Grove, PA, USA). Control samples were chosen and validated in advance with the complement fixation test (current gold standard) as previously reported (Gyuranecz et al., 2012). The ELISA was optimised by measuring linearity, intra-run precision, inter-run precision, analytical sensitivity, recovery, dilution verification and reference range. The optical density (OD) of samples was measured using a plate reader (Labsystems Oy, Helsinki, Finland) at a wavelength of 450 nm. The results were expressed as a percentage of the OD reading of the test sample (value), which was calculated as value = 100 × (S − NC)/(PC − NC) where S, NC, and PC are the OD of the test sample, the negative control, and the positive control, respectively. Serum samples were considered to be positive if they had a value of 40% or more, suspect if the value was between 30% and 40%, and negative if the value was <30%.

Exact confidence intervals (CI) for prevalence rates at the 95% level were calculated according to Sterne's method (Reiczigel, 2003). Prevalence rates were compared with the Fisher's exact test and differences were considered significant when $P < 0.05$.

3. Results and discussion

Except for *E. canis*, all evaluated tick-borne pathogens were found in non-pet dogs in the present study. Considering the haemoplasmas, *A. phagocytophilum* and *C. burnetii*, these were detected with high prevalence (Table 1). PCR and seropositivity did not correlate with the breed, sex or age groups of dogs (data not shown). Moreover, none of the evaluated infections was associated with geographical regions (i.e., positive samples were found from south-western to south-eastern Hungary). Co-infections with bacteria belonging to different genera were seldom detected (there were two dogs with concurrent haemoplasma and *A. phagocytophilum* PCR positivity and two with either simultaneous haemoplasma or *A. phagocytophilum* and *Coxiella* positivity), which is most likely due to the different routes (tick vectors) of infection and the epidemiology of the relevant agents. The prevalence rates of the evaluated agents were not significantly different between the three categories of non-pet dogs (Table 1).

3.1. Haemotropic Mycoplasma spp.

Altogether 14 dogs (11.1%, CI: 6.2–18%) had haemoplasma infection: all of them harboured '*Candidatus* M. haematoparvum', and 8 dogs (6.3%, CI: 2.8–12.1%) were co-infected with *M. haemocanis* (i.e., the latter species was not detected in single infection dogs). For *M. haemocanis* the copy numbers of DNA (reflecting bacterial loads) reached higher values (Table 1), which implies that in 7 out of 8 dual-PCR positive samples *M. haemocanis* predominated. The high prevalence of canine haemoplasmas is an unexpected finding because the geographical distribution

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