Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Molecular detection of vector-borne pathogens in Greek cats

Mathios E. Mylonakis[a,1], Megan Schreeg[b,1], Manolis K. Chatzis[c], Julian Pearce[b], Henry S. Marr[b], Manolis N. Saridomichelakis[c], Adam J. Birkenheuer[b,*]

[a] Companion Animal Clinic, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 11 Stavrou Voutyra Str., 54627, Thessaloniki, Greece
[b] College of Veterinary Medicine, North Carolina State University (NCSU-CVM), 4700 Hillsborough Str., 27606, Raleigh, NC, USA
[c] Clinic of Medicine, School of Veterinary Sciences, Faculty of Health Sciences, University of Thessaly, 224 Trikalon Str., 43132, Karditsa, Greece

ARTICLE INFO

Keywords:
Vector-borne pathogens
Bartonella spp.
Haemoplasmas
Cat
Greece

ABSTRACT

Infectious diseases have been increasingly recognized in cats worldwide. The objective of this study was the molecular investigation of the prevalence of selected pathogens in healthy and sick cats from Greece, a country highly endemic for several canine vector-borne pathogens. Blood and/or bone marrow samples from 50 clinically healthy and 50 sick adult (> 1 year-old) cats were retrospectively examined for the amplification of *Bartonella* spp., haemoplasmas, *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., and *Cytauxzoon* spp. DNA. Overall, 14.9% of the cats were found to be infected or co-infected by haemoplasmas, including *Candidatus* Mycoplasma haemominutum and *M. haemofelis*. In addition, 8.5% of the cats were infected by *Bartonella henselae, Bartonella clarridgeiae* or *Bartonella koehlerae*. In contrast, DNA of *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. and *Cytauxzoon* spp. was not amplified from the blood or bone marrow of any cat. There was no significant difference in either haemoplasma or *Bartonella* infection rates when comparing healthy and sick cats. This study represents the first description of multiple zoonotic *Bartonella* spp. in Greek cats.

1. Introduction

Feline vector-borne pathogens have been increasingly recognized worldwide based on serological and/or molecular epidemiological studies (Vilhena et al., 2013; Chatzis et al., 2014). Apart from their role as primary veterinary pathogens, several feline vector-borne pathogens are also of medical importance, due to the potential role of cats as reservoirs or sentinels of these agents (Kamrani et al., 2008; Maia et al., 2014). Despite the rapidly accumulating scientific literature, limited information is currently available regarding the prevalence of feline vector-borne pathogens in Greece, although numerous vector-borne pathogens (e.g. *Ehrlichia canis*, *Leishmania infantum*, *Anaplasma phagocytophilum*, *A. platys*, *Bartonella* spp., *Rickettsia* spp.) have been documented in the canine population of the same country (Mylonakis et al., 2004, 2014).

In a previous polymerase chain reaction (PCR)-based study in Greece, 20.6% of the cats were haemoplasma-positive, with *Mycoplasma haemofelis* and *Candidatus* Mycoplasma haemominutum being the only species detected (Maher et al., 2010). In another study of 100 cats, 41 were found to be infected by *L. infantum*, while four were seropositive for *B. henselae* (Chatzis et al., 2014).

We hypothesized that by applying molecular methods, we might be able to expand the spectrum of *Bartonella* or haemoplasma species infesting felines and to document other vector-borne pathogens that could be of veterinary and/or comparative medical importance in Greece. Therefore, the objective of this study was the molecular investigation of the prevalence of selected vector-borne pathogens in healthy and sick cats from Greece, including *Bartonella* spp., haemoplasma species, *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. and *Cytauxoon* spp.

2. Materials and methods

2.1. Animals and sample collection

Archived blood and bone marrow samples from 100 adult (> 1 year-old) cats living in central (Thessaly, n = 77) or northern (Macedonia, n = 23) Greece, available from a previous study investigating the prevalence of infection by *L. infantum* were retrospectively examined (Chatzis et al., 2014). Blood and bone marrow

Abbreviations: FeLV, feline leukaemia virus; FIV, feline immunodeficiency virus; PCR, polymerase chain reaction

* Corresponding author at: North Carolina State University, College of Veterinary Medicine, 1060 William Moore Drive, Raleigh, NC, USA.
E-mail address: ajbirken@ncsu.edu (A.J. Birkenheuer).

[1] These authors contributed equally in the study.

http://dx.doi.org/10.1016/j.ttbdis.2017.08.013
Received 21 May 2017; Received in revised form 20 August 2017; Accepted 24 August 2017

samples were stored at −20 °C until analyzed (Chatzis et al., 2014).

Animal handling was in accordance with European Communities Council Directive 86/609/EEC and state laws and the experimental protocol had been approved by the State Authorities (license No 3698/31-10-08). The study population included eight purebred (six Siamese, one Birman, and one Persian) and 92 common European cats with an age range of 1–24 years (median 3.75 years). The cats lived exclusively indoors (40), exclusively outdoors (43) or both indoors and outdoors (17), in urban (40), semi-urban (57) or rural (3) areas (Chatzis et al., 2014). Cats were assigned on admission as either clinically healthy (n = 50) or sick (n = 50) presenting various dermatologic, ocular and/or systemic clinical signs compatible with feline leishmaniasis. Overall, 41% of the cats, including 21/50 healthy and 20/50 sick cats, were infected by *L. infantum* as documented by PCR, while 3/100 and 8/100 cats were seropositive for feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV), respectively (Chatzis et al., 2014). Blood samples were available from 94 cats (50 healthy and 44 sick cats, including 37 infected by *L. infantum*, 3 seropositive for FeLV and 7 seropositive for FIV), while bone marrow samples were available from all 100 cats. However, the volume of bone marrow samples was adequate only for *Ehrlichia* spp. and *Anaplasma* spp. molecular testing. Two sick cats had flea infestation on admission and 5 cats had received antiparasitic drugs during the previous 3 mo; historical evidence of recent flea or tick infestation could not be inferred for any other cat.

2.2. DNA extraction

DNA was isolated from 200 µL of whole blood using an automated work bench (Qiagen BioRobot M48 Robotic Workstation, Qiagen Inc., Valencia, CA, USA) and commercially available kit, according to manufacturer's instructions (QIAGEN MagAttract DNA Mini M48 Kit, Qiagen Inc.). DNA was isolated from bone marrow using the Roche High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to manufacturer's instructions. DNA quantity and purity was assessed by a NanoDrop spectrophotometer, and the presence of PCR inhibition in samples was excluded by amplification of mammalian glyceraldehyde 3-phosphate dehydrogenase genes.

2.3. Babesia PCR

An approximately 200 base pair fragment of the *Babesia* 18S rRNA gene was amplified by using a combination of two forward primers and one reverse primer (Primer sequences: Forward 1: 5′- GCATTTA GCGATGGACCATTCAAG-3′, Forward 2: 5′- GCCGGCGATGTATCATTCA AG-3′, Reverse: 5′- CCTGTATTGTTATTTCTTGTCACTACCTC-3′) as described previously (Birkenheuer et al., 2008). A mixture of forward primers was utilized to increase chances of detecting a broad range of *Babesia* species. Each 50 µL PCR reaction contained 25 µL of 2X SYBR Green Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 12.5 pmol of each forward primer, 25 pmol of reverse primer, and 5 µL of DNA sample. Thermal cycling conditions (MiniOpticon, Bio-Rad Laboratories, Inc., Hercules, CA, USA) consisted of an initial denaturation step at 95 °C for 3 min followed by 45 amplification cycles (95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s) and a melt curve step (65–90 °C, increasing at increments of 0.5 °C every 5 s). Positive control consisted of a *Babesia* infected canine blood sample, while negative control consisted of water (no DNA).

2.4. Cytauxzoon PCR

Primers were designed (Forward: 5′- CTTTCCGGAGTATCAATTGG-3′, Reverse: 5′- GTCCTACTTAGTTATTCCATGC-3′) to amplify a 270-base pair fragment of *Cytauxzoon* 18S rRNA gene. Primers were designed based on previously reported 18S rRNA sequences of multiple *Cytauxzoon* species (GenBank Accession Numbers: *C. felis* AF399930, *C. manul* AY485690, *Cytauxzoon* sp. Iberian lynx EF094470, *Cytauxzoon* sp. French cat EU622908, *Cytauxzoon* sp. Spanish cat AY309956, *Cytauxzoon* sp. Italian cat HM146423, *Cytauxzoon* sp. meerkat KM025200). Each 50 µL PCR reaction contained 25 µL of 2X SYBR Green Master Mix (Applied Biosystems, Life Technologies), 25 pmol of each primer, and 5 µL of DNA sample. Thermal cycling conditions (MiniOpticon, Bio-Rad Laboratories, Inc.) consisted of an initial denaturation step at 95 °C for 3 min followed by 45 amplification cycles (95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s) and a melt curve step (65–90 °C, increasing at increments of 0.5 °C every 5 s). Positive control consisted of a *C. felis* infected blood sample, while negative control consisted of water (no DNA).

2.5. Anaplasma PCR

Primers were designed (Forward: 5′- ATGTTTATGACTTCTCAAGC AC-3′, Reverse: 5′- CCCTTTTCGTATTTTTGTAC-3′) to amplify a 194-base pair amplicon of the *Anaplasma Tr-1* gene as previously described (Hegarty et al., 2015). Each 25 µL PCR reaction contained 12.5 µL of 2X SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 10 pmol of each primer, and 5 µL of DNA sample. Thermal cycling conditions (MiniOpticon, Bio-Rad Laboratories, Inc.) consisted of an initial denaturation step at 98 °C for 2 min followed by 40 amplification cycles (98 °C for 15 s, 57 °C for 15 s, and 72 °C for 15 s) and a melt curve step (65–88 °C, increasing at increments of 0.5 °C every 5 s). Positive control consisted of an *A. platys Tr-1* plasmid, while negative control consisted of water (no DNA).

2.6. Ehrlichia PCR

A 300-base pair fragment of the *Ehrlichia sodB* gene was amplified (Primer sequences: Forward: 5′- TTTAATAATGCTGGTCAAGTATG GAATCAT-3′, Reverse: 5′- AAGCGTGTTCCCATACATCCATAG-3′) as previously described (Qurollo et al., 2014). Each 25 µL PCR reaction contained 12.5 µL of 2X SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 10 pmol of each primer, and 5 µL of DNA sample. Thermal cycling conditions (MiniOpticon, Bio-Rad Laboratories, Inc.) consisted of an initial denaturation step at 98 °C for 2 min followed by 40 amplification cycles (98 °C for 15 s, 57 °C for 15 s, and 72 °C for 15 s) and a melt curve step (65–88 °C, increasing at increments of 0.5 °C every 5 s). Positive control consisted of a Panola mountain *Ehrlichia* sp. *sodB* plasmid, while negative control consisted of water (no DNA).

2.7. Bartonella PCR

A 209-base pair fragment of the *Bartonella ssrA* gene was amplified (Forward: 5′- GCTATGGTAATAAATGGACAATGAAATAA-3′, Reverse: 5′- GACGTGCTTCCGCATAGTTGTC-3′) by modifying a previously described PCR assay (Diaz et al., 2012). Each 25 µL PCR reaction contained 12.5 µL of 2X SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Inc.), 10 pmol of each primer, and 5 µL of DNA sample. Thermal cycling conditions (MiniOpticon, Bio-Rad Laboratories, Inc.) consisted of an initial denaturation step at 98 °C for 3 min followed by 40 amplification cycles (98 °C for 15 s, 62 °C for 15 s, and 72 °C for 15 s) and a melt curve step (65–88 °C, increasing at increments of 0.5 °C every 5 s). Positive control consisted of a *Bartonella henselae ssrA* plasmid, while negative control consisted of water (no DNA).

2.8. Mycoplasma PCR

Primers were designed (Forward: 5′- ACGAAAGTCTGATGGAGCA ATA-3′, Reverse: 5′- ACGCCCAATAAATCCGRATAAT-3′) to amplify a fragment of the *Mycoplasma* 16S rRNA gene (Jensen et al., 2001). Each 50 µL PCR reaction contained 25 µL of 2X SYBR Green Master Mix (Applied Biosystems, Life Technologies,), 25 pmol of each primer, and 5 µL of DNA sample. Thermal cycling conditions (MiniOpticon, Bio-Rad Laboratories, Inc.) consisted of an initial denaturation step at 95 °C for