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RESEARCH ARTICLE

Molecular screening of cat and dog ectoparasites for the presence of *Bartonella* spp. in Attica, Greece

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ABSTRACT

The purpose of this study was the molecular detection of *Bartonella* spp. in fleas and ticks parasitizing cats and dogs from 39 locations in Attica, Greece. One hundred and forty five ectoparasites (104 fleas and 41 ticks) from 92 cats and 53 dogs were investigated individually using PCRs targeting the 16S–23S ribosomal RNA intergenic spacer (ITS) and the citrate synthase (*gltA*) genetic loci. *Bartonella* spp. were detected in 14 out of 104 fleas (13.5%) and in none of the ticks examined. Consequent sequence analysis of the amplicons from the two loci identified 3 strains as *Bartonella henselae*, and 11 as *Bartonella clarridgeiae*. Our study demonstrates the presence of *B. henselae* and *B. clarridgeiae* in *Ctenocephalides felis* fleas from cat and dog in Greece. We also report a novel ITS sequence for *B. clarridgeiae*. Considering that fleas could pose a risk for human bartonellosis from their infected hosts, further studies on the public health risk of *Bartonella* presence in animal ectoparasites are warranted.

KEYWORDS

Bartonella, cat, dog, *Ctenocephalides felis*, PCR, Greece

INTRODUCTION

Bartonella is a Gram-negative fastidious bacterial genus, which currently includes 45 named species, many potentially zoonotic. *Bartonellae* infect erythrocytes and endothelial cells, causing clinical disease in animals and humans (Lamas et al., 2008; Okaro et al., 2017; Álvarez-Fernández et al., 2018). Human disease can be caused by *B. henselae*, *B. bacilliformis*, *B. quintana, B. clarridgeiae, B. rochalimae* and *B. vinsonii*, the first three most frequently reported (Kaiser et al., 2011; Okaro et al., 2017).

The domestic cat is considered the natural reservoir for *B. henselae* and *B. clarridgeiae* and *Ctenocephalides felis* serves as competent vector for *B. henselae*, *B. clarridgeiae* and *B. koehlerae* (Lamas et al., 2008; Chomel and Kasten, 2010). However, *Bartonella* spp. have been detected in arthropods that occasionally feed on human and mammalian blood, like keds, ticks, biting flies and mites although transmission to humans has not been proved

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(Billeter et al., 2008). *Bartonella* spp. have been detected by PCR in up to 40% of *Ixodes ricinus* populations tested in Europe (Dietrich et al., 2010).

The presence of *Bartonellae* in mammalian hosts in Greece has been reported, but there haven't been investigations concerning their arthropod vectors. Stray dogs and free-roaming cats, common in urban neighborhoods, are more susceptible to parasitic infestation than owned dogs or cats kept indoors, due to increased chances of contact with infection sources and lack of veterinarian attention in case of stray animals. Therefore, they could represent a source of bacterial dispersal to public areas and hence to animals and humans through their ectoparasites and the potentially carried pathogens. The aim of this study was to investigate the presence of *Bartonella* spp., particularly potential human pathogens, in fleas and ticks parasitizing cats and dogs in the broader Attica region, including the city of Athens.

MATERIALS AND METHODS

From September 2016 to October 2017, fleas and ticks were collected in five veterinary clinics from cats and dogs originating from 39 different areas in the Attica region (Fig. 1). The majority were stray animals brought to the clinics by volunteers or animal protection organizations for neutering. Ectoparasites were collected in the context of non-experimental clinical veterinary practice. In case of owned animals, the owner's consent for parasite collection was obtained. Fleas and ticks were collected by using sterile forceps, placed in tubes containing 95% alcohol and stored at 4°C until

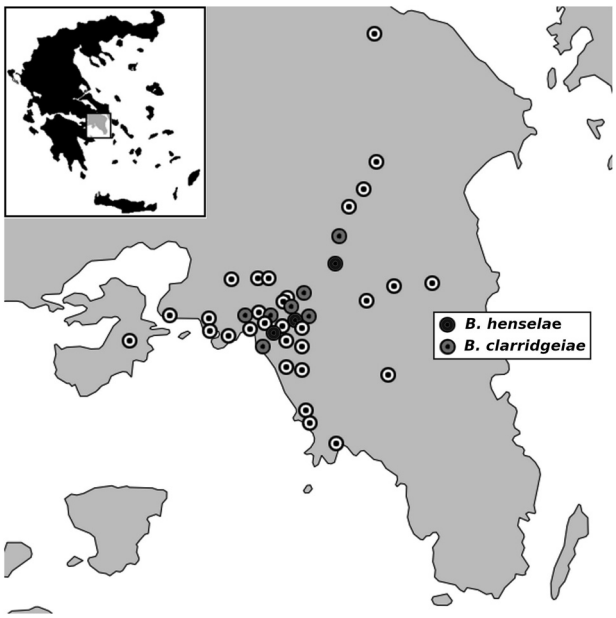

Fig. 1. Sampling sites within the Attica region are represented by circles. Grey circles indicate locations where fleas positive for *Bartonella* spp. were collected. Darker grey circles correspond to *B. henselae* and lighter grey circles to *B. clarridgeiae*

DNA extraction. The arthropods were photographed and identified, using standard morphological identification keys (Lane and Crosskey, 1993; Estrada-Peña et al., 2004).

The ectoparasites were washed twice in sterile distilled water for 10 min then dried on a sterile filter paper. The DNA extraction was performed using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH, Germany) with modification. Specifically, each arthropod was cut into pieces with a sterile scalpel and placed in a microcentrifuge tube containing T1 buffer, proteinase K. The tubes were incubated at 56°C overnight. The remaining procedure was carried out according to the manufacturer's instructions. The extracted DNA was stored at −20°C until testing. All samples were tested for the absence of PCR inhibitors by the amplification of a fragment of the cytochrome oxidase subunit I (COI) gene, using previously described primers (Duron et al., 2015).

Detection of *Bartonella* spp. was performed using primers 325a and 1100as, targeting the 16S–23S ribosomal RNA intergenic spacer (ITS), in 25 µL volume reactions containing 3 µL of isolated DNA, under previously described conditions (Maia et al., 2014). Positive samples were also tested by PCR with primers CSH1f and BhCS.1137 targeting the citrate synthase gene (*gltA*) in 25 µL volume reaction mixtures containing 3 µL of extracted DNA, as previously described (Corduneanu et al., 2018). All PCR experiments were performed twice and negative controls, containing sterile water instead of the sample DNA, were included. A BioRad S1000 thermocycler was used and the PCR products were visualized using Bio-Rad GelDoc XR+ Gel Documentation System (Chemidoc XRS+ Gel Imaging System) after electrophoresis on 2% agarose gels, stained with ethidium bromide.

All amplicons were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel GmbH, Germany) according to manufacturer's instructions and were sequenced in both directions using the PCR primers (CeMIA Larissa, Greece). Base calling was performed manually using the FinchTV 1.3.1. software. All sequences were compared for homology to sequences deposited in GenBank using the blastn suite of the BLAST program (www.ncbi.nlm.nih.gov/BLAST). The sequences were aligned using the MUSCLE (Madeira et al., 2019) and a Maximum Likelihood phylogenetic tree was constructed using the MEGA 11.0 software (Tamura et al., 2021). Parameters used were 100 bootstrap replications, Tamura-Nei substitution model, use of all sites, uniform rates and Nearest-Neighbor-Interchange option.

RESULTS

One hundred forty-five animals (92 cats and 53 dogs) were examined. One ectoparasite from each animal was used: 104 fleas (86 from cats and 18 from dogs) and 41 ticks (6 from cats and 35 from dogs). All fleas were identified as *C. felis* (97♀/7♂) and the ticks were identified as *Rhipicephalus sanguineus* s.l. (34♀/4♂), *Rhipicephalus* sp. (2 nymphs) and *Ixodes* sp. (1♀).

Bartonella spp. were detected in 14/104 (13.5%) of the fleas examined. Based on the sequence analysis of the amplicons from the two loci, the presence of *B. henselae* and *B. clarridgeiae* was determined in 3 and 11 samples, respectively (Table 1). In the case of 8 samples, the species determination was straightforward, as the obtained sequences from the ITS and/or *gltA* fragments were >99% identical with the corresponding sequences deposited in GenBank from characterized isolates. When both sequences were available, there was no inconsistency in species definition. The ITS sequences from 6 samples however, were only 96.4% identical with that of *B. clarridgeiae* due to a 19-bp deletion. The amplified *gltA* fragment from two of these afore-mentioned samples could not be sequenced, probably due to the presence of non-specific DNA fragments that were co-amplified in the PCR. The *gltA* sequences of the other 4 of these samples shared >99% identity with the reported sequences of *B. clarridgeiae*. As a result, all these 6 isolates were classified as *B. clarridgeiae*. This was confirmed by the results of the phylogenetic analysis that showed clustering of the new sequence with a known *B. clarridgeiae* sequence (Fig. 2).

Selected sequences were deposited in GenBank with accession numbers MN170534-MN170544 for the 16S–23S rRNA ITS locus, and MN170987-MN170998 for the *gltA* gene.

In our study, the prevalence of *Bartonella* spp. was found to be 14% (12/86) and 11.1% (2/18) in cat and dog fleas, respectively. Half of the positive samples (7/14) were from stray kittens of 2–6 months old. None of the ticks tested was positive for *Bartonella* spp.

DISCUSSION

Our study provides molecular evidence for the presence of two *Bartonella* species, *B. henselae* and *B. clarridgeiae,* in fleas from cats and dogs in Attica, Greece including the Athens greater area. The prevalence of *Bartonella* found in fleas in our study (13.5%) is comparable with that reported from similar studies conducted worldwide (Rolain et al., 2003; Just et al., 2008; Kamani et al., 2015; Persichetti et al., 2016). *B. henselae* has been reported from Greece, and recently *B. clarridgeiae* as well, both species in dog and cat blood samples (Diniz et al., 2009; Mylonakis et al., 2014; Diakou et al., 2017; Mylonakis et al., 2017). In the present study, we detected these bacterium species in cat and dog fleas.

Also, we report a novel ITS sequence for *B. clarridgeiae* which is 19 bp shorter than that of the *B. clarridgeiae* strain 73 (GenBank Ac. No. FN645454.1, bases 1,269,279–1,269,806) (Engel et al., 2011). The conclusion that this sequence can be attributed to *B. clarridgeiae* with a high degree of confidence is supported by the fact that the sequence of the *gltA* fragment from this particular sample was 100% identical with that of other *B. clarridgeiae* strains. We postulate that the observed sequence difference of approximately 4% for the ITS locus is likely the result of a single evolutionary event, i.e. a 19-bp deletion. In addition, the sequence of the *gltA* amplicons from our study had 100% identity with a *B. clarridgeiae* sequence (MG384320) isolated from a human case of aortic root abscess and endocarditis in Great Britain (Logan et al., 2019). The *B. henselae* sequences were 100% identical to the sequence L38987, corresponding to strain Houston-1 (Norman et al., 1995).

In our study, the majority of the *Bartonella* spp. from the fleas were identified as *B. clarridgeiae* (10.6%) vs 2.9% as *B. henselae*. This is in accordance with several studies, where *B. clarridgeiae* has been found to be more prevalent than *B. henselae* (Rolain et al., 2003; Just et al., 2008; Kamani et al., 2015; Persichetti et al., 2016). It is also in accordance with a study from Taiwan in which *B. clarridgeiae* has been more frequently detected in cat fleas than in their mammalian hosts (Tsai et al., 2010). This observation has led Tsai et al. (2010) to formulate the assumption that *B. clarridgeiae* may be better adapted to the flea than to its

Table 1. Results of the comparative BLAST analyses of our query sequences for the two loci

Sample ID	Area of collection	Host	% identity		*Bartonella* species
			ITS genomic fragment	gltA fragment	
ML0061	Athens-Center	Cat	99.8% *B. henselae* (545/546 bp)	100% *B. henselae* (317/317 bp)	*B. henselae*
ML0082	Nea Smyrni	Cat	99.8% *B. henselae* (545/546 bp)	100% *B. henselae* (317/317 bp)	*B. henselae*
ML0035	Marousi	Dog	———*	100% *B. henselae* (317/317 bp)	*B. henselae*
ML0053	Athens-Center	Cat	100% *B. clarridgeiae* (528/528 bp)	100% *B. clarridgeiae* (317/317 bp)	*B. clarridgeiae*
ML0054	Athens-Center	Cat	100% *B. clarridgeiae* (528/528 bp)	100% *B. clarridgeiae* (317/317 bp)	*B. clarridgeiae*
ML0059	Athens-Pangrati	Dog	100% *B. clarridgeiae* (528/528 bp)	100% *B. clarridgeiae* (317/317 bp)	*B. clarridgeiae*
ML0094	Palaio Phaliro	Cat	100% *B. clarridgeiae* (528/528 bp)	100% *B. clarridgeiae* (317/317 bp)	*B. clarridgeiae*
ML0110	Halandri	Cat	100% *B. clarridgeiae* (528/528 bp)	100% *B.* clarridgeiae (317/317 bp)	*B. clarridgeiae*
ML0086	Athens-Petralona	Cat	96.4% *B. clarridgeiae* (509/528 bp)	100% *B. clarridgeiae* (317/317 bp)	*B. clarridgeiae*
ML0089	Palaio Phaliro	Cat	96.4% *B. clarridgeiae* (509/528 bp)	100% *B. clarridgeiae* (317/317 bp)	*B. clarridgeiae*
ML0025	Rentis	Cat	96.4% *B. clarridgeiae* (509/528 bp)	99.7% *B. clarridgeiae* (316/317 bp)	*B. clarridgeiae*
ML0045	Athens-Ampelokipi	Cat	96.4% *B. clarridgeiae* (509/528 bp)	99.7% *B. clarridgeiae* (316/317 bp)	*B. clarridgeiae*
ML0046*	Kaisariani	Cat	96.4% *B. clarridgeiae* (509/528 bp)	———*	*B. clarridgeiae*
ML0096c*	Nea Smyrni	Cat	96.4% *B. clarridgeiae* (509/528 bp)	———*	*B. clarridgeiae*

*Not available due to unsuccessful sequencing of the PCR fragments.

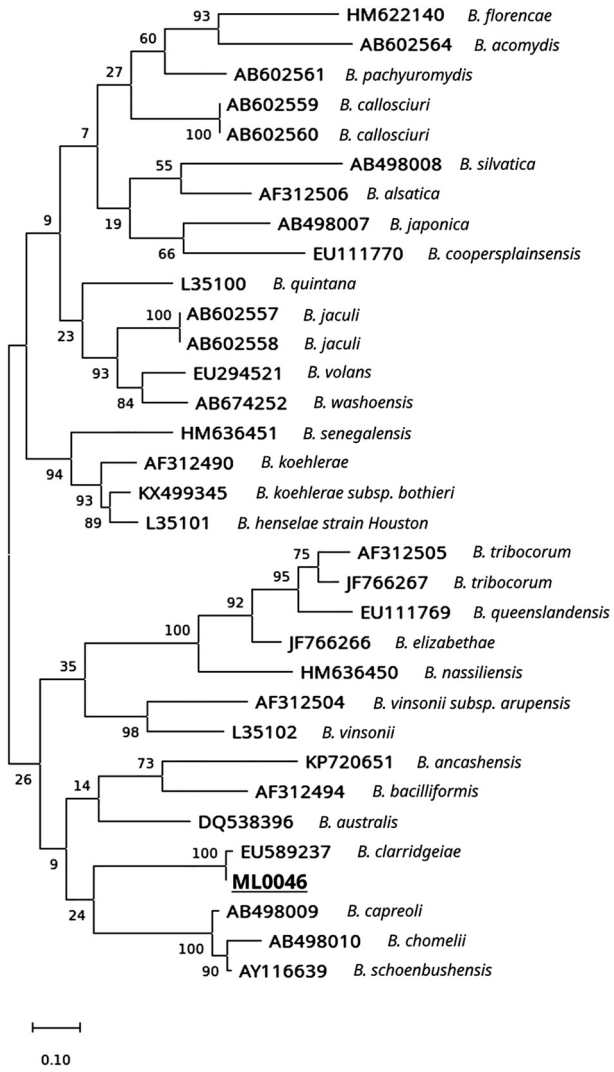

Fig. 2. Phylogenetic tree of *Bartonella* species, based on 16S–23S ribosomal RNA intergenic spacer (ITS) sequences, inferred using the Maximum Likelihood Method. Numbers on the branches represent bootstrap values obtained by using 100 replicates. The ML0046 ITS genomic fragment (in bold underlined) that clusters with the EU589237 *Bartonella clarridgeiae* strain, is derived from a *Ctenocephalides felis* flea from cat host

mammalian host. The same pattern seems to apply to our study, where *B. clarridgeiae* was the commonest species detected in fleas, whereas in other studies conducted in Greece, *B. henselae* was the most frequent species demonstrated from dogs and cats (Diakou et al., 2017; Mylonakis et al., 2017).

Several studies from various geographical areas have detected *Bartonella* spp. in *Rhipicephalus* and *Ixodes* ticks (Satta et al., 2011; Billeter et al., 2012; Wikswo et al., 2015). Although there is indirect evidence for the possibility of transmission of *Bartonella* spp. by ticks to vertebrate hosts (Billeter et al., 2008), the potential vector competency of ticks has not been confirmed by experimental transmission studies (Angelakis et al., 2010). All ticks examined in the present study were negative for *Bartonella* spp., probably due to the small number of samples available.

Both *B. henselae* and *B. clarridgeiae* have been detected in humans in several countries including Greece (Kordick et al., 1997; Chondrogiannis et al., 2012; Vieira-Damiani et al., 2015; Oteo et al., 2017; Kalogeropoulos et al., 2019). Since serological testing does not distinguish antibody responses to specific *Bartonella* spp., it is possible that *B. clarridgeiae* may be the cause of the disease at least in some cases.

Bartonellae were found in arthropods collected from ten different urban localities in the Attica prefecture, where Athens is located. Within the city of Athens, they were found in four localities (the Center, Ampelokipi, Pangrati and Petralona) and in several city suburbs: two in the North (Marousi and Chalandri), two in the South (Palaio Phaliro and Nea Smyrni), one located in the East (Kaisariani) and one in the North-West of Athens (Rentis). Both species were found in two localities, the center and the suburb of Nea Smyrni. Most cat fleas with *Bartonella* spp. were found within the greater Athens area, without any particular variability between different municipalities, reflecting the fact that most samples were collected there.

In conclusion, our study shows the presence of two *Bartonella* species, *B. henselae* and *B. clarridgeiae* in cat and dog fleas from the greater Athens urban region. Half of the positive samples were from stray kittens and most of them were found in the city of Athens. Given the fact that infections due to *Bartonella* spp. have been reported in Greece, fleas could pose a risk for humans through their infected hosts.

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