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SHORT COMMUNICATION

Molecular detection of zoonotic bartonellae (*B. henselae*, *B. elizabethae* and *B. rochalimae*) in fleas collected from dogs in Israel

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Abstract. Fleas represent an acknowledged burden on dogs worldwide. The characterization of flea species infesting kennel dogs from two localities in Israel (Rehovot and Jerusalem) and their molecular screening for *Bartonella* species (Rhizobiales: Bartonellaceae) was investigated. A total of 355 fleas were collected from 107 dogs. The fleas were morphologically classified and molecularly screened targeting the *Bartonella* 16S–23S internal transcribed spacer (ITS). Of the 107 dogs examined, 80 (74.8%) were infested with *Ctenocephalides canis* (Siphonaptera: Pulicidae), 68 (63.6%) with *Ctenocephalides felis*, 15 (14.0%) with *Pulex irritans* (Siphonaptera: Pulicidae) and one (0.9%) with *Xenopsylla cheopis* (Siphonaptera: Pulicidae). Fleas were grouped into 166 pools (one to nine fleas per pool) according to species and host. Thirteen of the 166 flea pools (7.8%) were found to be positive for *Bartonella* DNA. Detected ITS sequences were 99–100% similar to those of four *Bartonella* species: *Bartonella henselae* (six pools); *Bartonella elizabethae* (five pools); *Bartonella rochalimae* (one pool), and *Bartonella bovis* (one pool). The present study indicates the occurrence of a variety of flea species in dogs in Israel; these flea species are, in turn, carriers of several zoonotic *Bartonella* species. Physicians, veterinarians and public health workers should be aware of the presence of these pathogens in dog fleas in Israel and preventive measures should be implemented.

Key words. *Bartonella bovis*, *Bartonella elizabethae*, *Bartonella henselae*, *Bartonella rochalimae*, *Ctenocephalides canis*, *Ctenocephalides felis*, *Pulex irritans*, *Xenopsylla cheopis*, zoonosis.

Fleas (Siphonaptera) are major ectoparasites of dogs, to which they cause a constant physical burden. They are known vectors of several bacterial pathogens such as *Rickettsia*, *Mycoplasma*, *Bartonella* and *Yersinia* species (Dobler & Pfeffer, 2011). *Ctenocephalides felis* is the most common flea species to infest cats, dogs and humans globally (Bitam *et al.*, 2010). Moreover, the domestic dog (*Canis lupus familiaris*) has been reported to be parasitized by 15 additional flea species worldwide (Dobler & Pfeffer, 2011). In Israel, 37 flea species have been reported to parasitize several animal hosts (Theodor & Costa, 1967), but the cat flea (*Ct. felis*) is the most commonly found flea in dogs in this country (K. Y. Mumcuoglu, unpublished data, 1993).

Bartonellae are pleomorphic, Gram-negative and facultative intracellular bacteria transmitted mainly by haematophagous arthropods such as fleas, ticks and lice. They are maintained in nature within a wide variety of reservoirs including felines, rodents, ruminants and canines (Chomel *et al.*, 2014). This bacterial genus includes more than 30 species and many more

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strains and genotypes, at least 13 of which have been associated with human diseases (Chomel *et al.*, 2014).

In Israel, a diverse range of *Bartonella* species has been detected in fleas and lice from cats, ruminants and wild rodents (Morick *et al.*, 2009, 2010; Gutiérrez *et al.*, 2013, 2014). As dogs come into close contact with humans and other animals, an exploration of the flea species infesting dogs and the *Bartonella* species they carry may provide insight into their clinical significance and relevance to public health.

Fleas were collected manually from kennel dogs held in Society for the Prevention of Cruelty to Animals (SPCA) kennels in two different geographic localities in Israel: Rehovot in the Shfela coastal plain, and the Jerusalem Mountains. Fleas were collected from January to December 2010 and kept in 70% ethanol until tested. Fleas were classified taxonomically using morphological keys (Rothschild & Hopkins, 1953; Lewis, 1967) and grouped into pools of up to nine fleas according to species and host.

Fleas were washed and minced on a slide using a sterile scalpel blade. DNA was extracted using a commercial DNA extraction kit (Ilustra Tissue & Cell Genomic Prep Mini Spin Kit; GE Healthcare UK Ltd, Little Chalfont, U.K.) according to the manufacturer's instructions, as previously described (Morick *et al.*, 2011).

Molecular screening for *Bartonella* DNA was performed by high-resolution melt (HRM) real-time polymerase chain reaction (PCR) analysis using the Rotor Gene 6000 cycler (Corbett Research Pty Ltd, Sydney, NSW, Australia). Accordingly, a fragment of approximately 200 bp of the 16S–23S internal transcribed spacer (ITS) was amplified using primers H493as (TGAACCTCCGACCTCACGCTTATC) and 321s (AGATGATGATCCCAAGCCTTCTGG) (Maggi & Breitschwerdt, 2005). Reactions were carried out in a 20-μL final volume containing 1 μL of 10 μM solution of each primer, 0.6 μL of 50 μM solution of Syto9 (Invitrogen Corp., Carlsbad, CA, U.S.A.), 3.4 μL of double distilled water (DDW), 10 μL of Maxima Hot-Start PCR Master Mix 2X (Thermo Fisher Scientific Group, Epsom, U.K.), and 4 μL of each genomic DNA, as described elsewhere (Gutiérrez *et al.*, 2013). Amplification products were obtained using the following protocol: 4 min at 95 °C followed by 50 cycles of 5 s at 95 °C, 30 s at 60 °C (fluorescence acquisition on HRM channel), and 1 s at 72 °C. The melt, hybridization and HRM phases were performed as previously described (Morick *et al.*, 2009). Any amplification with a threshold cycle (Ct) value lower than 40 cycles with an HRM melt curve similar to that of *Bartonella* was considered as a positive result. A *Bartonella*-positive DNA sample (*Bartonella henselae* from culture), a *Bartonella*-negative DNA sample, and non-template DNA control (NTC) were used in each run. All positive samples were confirmed by sequencing as described below.

Positive samples for the ITS real-time PCR screening were further tested with three additional HRM real-time PCR assays. Consequently, fragments of the *ssrA*, *rpoB* and *gltA* genes were amplified. A 300-bp *ssrA* gene fragment was amplified using the primers ssrAF (GCTATGGTAATAAATGGACAATGAAATAA) and ssrAR (GCTTCTGTTGCCAGGTG), as described by Diaz *et al.* (2012). The reaction was performed according to the previously reported protocol (Gutiérrez *et al.*, 2014). A 200-bp *rpoB* gene fragment was amplified using primers 600f (GAAAATGATGATGCGAATCG) and 800r (GATCTAAATCTTCTGTTGCACG), according to previously published conditions and reagent volumes (Morick *et al.*, 2009).

A 340-bp *gltA* gene fragment was amplified using primers 443F (GCTATGTCTGCATTCTATCA) (Birtles & Raoult, 1996) and 781R (CCACCATGAGCTGGTCCCC) [this study; based on the Bhcs.781F from Norman *et al.* (1995)]. The *gltA* real-time PCR was performed in a 20-μL reaction volume containing 0.5 μL of 10 μM solution of each primer, 0.6 μL of 50 μM solution of Syto9 (Invitrogen Corp.), 10 μL of MAXIMA Hot-Start PCR Master Mix 2X (Thermo Fisher Scientific Group), 4.4 μL DDW and 4 μL of each genomic DNA. Amplification products were obtained using the following protocol: 4 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 30 s at 62 °C (fluorescence acquisition on Green channel), and 2 s at 72 °C.

All positive PCR products were purified using the NEB Exo-SAP PCR purification kit (New England Biolabs, Inc., Ipswich, MA, U.S.A.) and sequenced using both reverse and forward primers with the BigDye Terminator cycle sequencing chemistry and an ABI 3700 DNA Analyzer (Applied Biosystems, Inc., Carlsbad, CA, U.S.A.). Sequences were further analysed using BioEdit Sequence Alignment Editor Version 7.2.5 (Ibis Biosciences, Inc., Carlsbad, CA, U.S.A.) and MEGA (Molecular Evolutionary Genetics Analysis) Version 6 (Tamura *et al.*, 2013).

A total of 355 fleas were collected from 107 dogs from the two localities in Israel (Jerusalem and Rehovot). Four flea species were found to infest the examined dogs. Three of the flea species, *Ctenocephalides canis* (189 adults), *Ct. felis* (146 adults) and *Pulex irritans* (19 adults), were collected from both localities (Rehovot and Jerusalem). The fourth species, *Xenopsylla cheopis* (one adult), was collected from one dog at the Jerusalem kennel. The occurrence of the flea species on the dogs examined varied: *Ct. canis* infested 74.8% of the dogs; *Ct. felis* infested 63.6% of the dogs; *P. irritans* infested 14.0% of the dogs, and *X. cheopis* infested 0.9% of the dogs (Fig. 1). Of the 107 dogs investigated, 54 (50.5%) were infested with one flea species and 53 (49.5%) were infested with two or more species (Table 1). One dog was infested with four flea species, and two dogs hosted three flea species (*Ct. felis*, *Ct. canis*, *P. irritans*).

Bartonella DNA was detected in 13 of the 166 flea pools (7.8%) screened by the ITS assay (Table 2), all of which were *Ct. felis* and *Ct. canis* flea pools. *Bartonella* DNA was not detected in any of the *X. cheopis* or *P. irritans* pools. Two of the *Bartonella* DNA-positive flea pools were collected from the same dog (Table 2, pools 4 and 5). The *Bartonella* DNA sequences detected showed similarities to four *Bartonella* species (100% coverage; 99–100% identity with specific *Bartonella* species DNA, according to GenBank database reference sequences): *B. henselae*, *Bartonella elizabethae*, *Bartonella bovis* and *Bartonella rochalimae*. Despite several attempts, only two of the 13 samples found to be positive by the ITS assay were also found positive and to match the same *Bartonella* species with additional real-time PCR assays (*ssrA* or *rpoB* HRM real-time PCR assays). The confirmatory sequences of the *ssrA* locus (fragments > 200 bp) were deposited

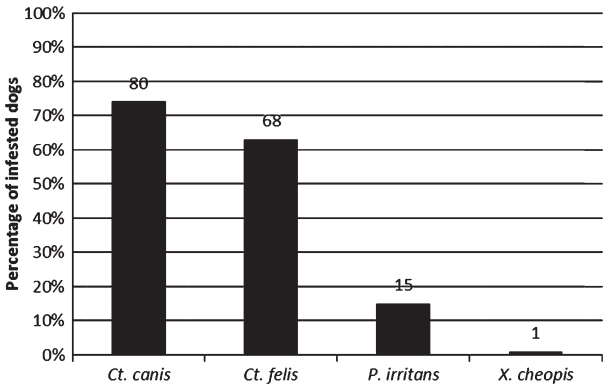

Fig. 1. Percentage of dogs infested with each flea species. Numbers above bars indicate the total number of dogs infested with each flea. *Ct. canis*, *Ctenocephalides canis*; *Ct. felis*, *Ctenocephalides felis*; *P. irritans*, *Pulex irritans*; *X. cheopsis*, *Xenopsylla cheopis.*

Table 1. Flea infestations in the study dogs ($n = 107$).

	Dogs, *n*	Dogs, %	Fleas per dog, mean ± SD
Single infestations			
Ct. canis	27	25.2	5.6 ± 4.1
Ct. felis	25	23.4	3.7 ± 1.7
P. irritans	2	1.9	1.0 ± 0.0
Co- infestations			
Ct. canis and *Ct. felis*	40	37.4	7.0 ± 5.6
Ct. canis and *P. irritans*	9	8.4	7.4 ± 2.7
Ct. felis and *P. irritans*	1	0.9	10.0
Ct. felis, *Ct. canis* and *P. irritans*	2	1.9	10.0 ± 7.1
Ct. felis, *Ct. canis*, *P. irritans* and *X. cheopis*	1	0.9	23.0

Ct. canis, *Ctenocephalides canis*; *Ct. felis*, *Ctenocephalides felis*; *P. irritans*, *Pulex irritans*; *X. cheopsis*, *Xenopsylla cheopis*; SD, standard deviation.

in GenBank under the accession numbers KP263369 (256 bp) and KP263370 (253 bp).

This study presents the detection of four different flea species (*Ct. canis*, *Ct. felis*, *P. irritans* and *X. cheopis*) on dogs from two geographically different urban regions in central Israel (Fig. 1, Table 1). Moreover, the *Ctenocephalides* spp. fleas were shown to carry DNA of four different *Bartonella* species, including the three zoonotic species *B. henselae*, *B. rochalimae* and *B. elizabethae* (Table 2). *Bartonella henselae* is the aetiological agent of cat scratch disease and is the most acknowledged zoonotic *Bartonella* species. Reported clinical manifestations caused by *B. henselae* include fatigue, fever, lymphadenomegaly, bacillary angiomatosis, peliosis hepatis, meningitis, encephalitis, neuroretinitis and endocarditis (Chomel & Kasten, 2010). *Bartonella rochalimae* has been isolated from a tourist travelling in Peru who displayed bacteraemia, fever and splenomegaly (Eremeeva *et al.*, 2007), and *B. elizabethae*, the rat-adapted *Bartonella*, was isolated from a human patient with endocarditis (Daly *et al.*, 1993). To date, the main *Bartonella* species known to infect canids are *Bartonella*

Table 2. Pools of fleas collected from dogs in two localities in Israel (Jerusalem and Rehovot), positive for *Bartonella* DNA.

Pool number	Dog location	Flea species (number of fleas in pool)	*Bartonella* species	Positive locus/loci
1	Jerusalem	*Ct. felis* (1)	*B. henselae*	ITS
2	Jerusalem	*Ct. felis* (3)	*B. henselae*	ITS
3	Jerusalem	*Ct. canis* (1)	*B. elizabethae*	ITS
4	Jerusalem	*Ct. felis* (5)	*B. elizabethae*	ITS
5	Jerusalem	*Ct. canis* (3)	*B. elizabethae*	ITS
6	Jerusalem	*Ct. canis* (1)	*B. henselae*	ITS
7	Jerusalem	*Ct. felis* (1)	*B. elizabethae*	ITS
8	Jerusalem	*Ct. felis* (2)	*B. henselae*	ITS
9	Jerusalem	*Ct. felis* (3)	*B. elizabethae*	ITS, *ssrA*
10	Rehovot	*Ct. felis* (1)	*B. henselae*	ITS
11	Rehovot	*Ct. canis* (1)	*B. henselae*	ITS
12	Rehovot	*Ct. canis* (3)	*B. bovis*	ITS
13	Rehovot	*Ct. canis* (5)	*B. rochalimae*	ITS, *ssrA*, *rpoB*

Ct. canis, *Ctenocephalides canis*; *Ct. felis*, *Ctenocephalides felis*; ITS, internal transcribed spacer.

vinsonii subsp. *berkhoffii* and *B. rochalimae* (Chomel *et al.*, 2014). Interestingly, our study demonstrated a great diversity of *Bartonella* species in dog fleas. In a similar study performed in Florida, U.S.A., DNA fragments of *B. rochalimae*, *Bartonella clarridgeiae* and *B. vinsonii* subsp. *berkhoffii*, all three of which are known to be zoonotic pathogens, were detected in *Ct. felis* and *Pulex* spp. fleas, (Yore *et al.*, 2014). In another study conducted in France, *B. clarridgeiae* and *B. henselae* DNA was detected in fleas collected from dogs (Just *et al.*, 2008). As these pathogens can cause life-threatening disease in dogs and humans, both should be monitored and flea prevention programmes should be implemented. Further larger-scale studies are required to determine the prevalences of infection of pathogenic *Bartonella* species in dogs from other geographic locations in Israel.

In this study, *B. bovis* DNA was detected in one pool of three *Ct. canis* fleas. Interestingly, this *Bartonella* species is commonly detected in cattle (Bai *et al.*, 2013). Thus, this particular dog may have been infested with *Bartonella*-infected fleas before it entered the kennel as no other fleas found in the same shelter presented a similar *Bartonella* infection. *Bartonella bovis* DNA has been previously detected in dog's blood from North Carolina, U.S.A. (Perez *et al.*, 2011). Whether our finding was incidental, which seems to be the case, or an indication that *Ct. canis* plays a role in the transmission of this pathogen from ruminants to dogs requires further investigation.

In our study, *Bartonella* DNA was detected in 7.8% of the flea pools tested. This result is close to those reported in earlier studies conducted in Florida, U.S.A. and France, in which *Bartonella* DNA was found in 11.3% of flea pools and 9.5% of fleas, respectively, collected from dogs (Just *et al.*, 2008; Yore *et al.*, 2014). The lower percentage of infected pools found in the present study may be explained by differences in the size of flea pools and/or differences in pathogen infection rates that result in varying amounts of *Bartonella* DNA within the samples, which may affect the sensitivity of the ITS HRM real-time PCR assay applied. Despite several attempts to amplify loci other than the

ITS, the repeatability of positive results was low. This may be attributable to a potentially lower sensitivity of the other assays used (as a result of lower primer sensitivity or lower gene copy numbers, per bacterium, for the other loci). One dog in this study was infested with both *Ct. felis* and *Ct. canis*, both of which were positive for DNA of *B. elizabethae*, a rodent-associated zoonotic *Bartonella* species. One explanation for this finding may be that the dog was infected with this pathogen and was the source of infection in both fleas. Another possible explanation may be that both flea species had been exposed to infected rodents. Although *B. elizabethae* DNA was previously detected in dog blood (Mexas *et al.*, 2002), to the best of our knowledge this is the first report of the detection of DNA of this *Bartonella* species in fleas collected from dogs. As this pathogen was detected in fleas from dogs from the Jerusalem SPCA kennel only, and a dog infested with *X. cheopis* was detected in the same kennel, we can speculate that a close interaction between dogs and rats in this kennel may have led to the sharing of ectoparasites and pathogens.

The detection on dogs of *P. irritans* and *X. cheopis*, which are human- and rodent-associated fleas, respectively, may be explained by the close proximity of dogs to the original host of the fleas. *Pulex irritans* has been previously detected on dogs in several countries, including Albania, Chile, France, Germany, Greece, Hungary, Iran, Nigeria, Spain, the U.K. and the U.S.A., with infestation rates ranging from 0.1% to 19% (Müller & Kutschmann, 1985; Harman *et al.*, 1987; Chesney, 1995; Koutinas *et al.*, 1995; Franc *et al.*, 1998; Alcaíno *et al.*, 2002; Ugbomoiko *et al.*, 2008; Farkas *et al.*, 2009; Marquez *et al.*, 2009; Xhaxhiu *et al.*, 2009; Tavassoli *et al.*, 2010). *Xenopsylla cheopis* fleas have been detected on dogs only in Greece and Iran, at infestation rates of 1% and 0.5%, respectively (Koutinas *et al.*, 1995; Tavassoli *et al.*, 2010). Because dogs are in close contact with humans, health care authorities should be alerted to the potential risk to humans of pathogens carried by these ectoparasites.

By contrast with the notion that the cat flea (*Ct. felis)* is the flea found most prevalently on dogs worldwide (Dobler & Pfeffer, 2011), we found the occurrence of the dog flea (*Ct. canis*) in kennel dogs from these two regions in Israel to be higher than the occurrence of the cat flea (*Ct. felis*). Similar results have been documented in other countries, including Albania, Greece and New Zealand (Guzman, 1984; Koutinas *et al.*, 1995; Xhaxhiu *et al.*, 2009). The fact that Greece, Albania and Israel share similar Mediterranean weather conditions may explain the similarities in results in these three countries. The occurrence of *P. irritans* in the present study (14%) was similar to rates reported from Chile (19%), Spain (16%), Germany (10%), and Florida, U.S.A. (18%) (Müller & Kutschmann, 1985; Alcaíno *et al.*, 2002; Marquez *et al.*, 2009; Yore *et al.*, 2014). This flea species has greater implications for human health in comparison with those of the *Ctenocephalides* genus because it is easily transferred from dogs to humans (Harman *et al.*, 1987).

This study reveals a wide diversity of flea species on dogs and a high rate of infestation of dogs in kennels with more than one flea species (49.6%), which may increase the chances of transmission of several bacterial pathogens from different animal origins. Previous studies have shown the occurrence of co-infection with more than one *Bartonella* species in dogs (Yore *et al.*, 2014) and also co-infection of *Bartonella* with other pathogens, such as *Ehrlichia* and *Babesia* spp. (Breitschwerdt *et al.*, 1998; Breitschwerdt & Kordick, 2000). Co-infections with more than one pathogen were suggested to alter the pathophysiology and clinical manifestations of diseases in affected dogs. As Israel is endemic for ehrlichiosis, babesiosis, anaplasmosis (*Anaplasma platys*) and hepatozoonosis (Baneth & Weigler, 1997; Fishman *et al.*, 2004; Harrus *et al.*, 2011) future studies should investigate the clinical importance of the *Bartonella* spp. detected in fleas in this study as primary or co-infecting organisms, and their implications for the health of dogs.

In conclusion, this study presents findings of four flea species infesting dogs in Israel. It was interesting to note that *Ct. canis* was more prevalent than *Ct. felis* and that human- and rodent-associated fleas (*P. irritans* and *X. cheopis*, respectively) were also detected. DNA of three zoonotic *Bartonella* species was detected in *Ctenocephalides* fleas collected from the dogs, highlighting the potential roles of these fleas as vectors of these zoonotic pathogens. Veterinary officials and public health workers should be aware of these findings, and preventive measures to protect both dogs and humans from these infectious agents should be implemented.

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