Bartonella Species in Fleas from Palestinian Territories: Prevalence and Genetic Diversity

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Bartonella species in fleas from Palestinian territories: Prevalence and genetic diversity

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ABSTRACT**:** Bartonellosis is an infectious bacterial disease. The prevalence and genetic characteristics of *Bartonella spp*. in fleas of wild and domestic animals from Palestinian territories are described. Flea samples (n=289) were collected from 121 cats, 135 dogs, 26 hyraxes and seven rats from northern (n=165), central (n=113), and southern Palestinian territories (n=11). The prevalent flea species were: *Ctenocephalides felis* (n=119/289; 41.2%), *Ctenocephalides canis* (n=159/289; 55%), and *Xenopsylla* sp. (n=7/289; 2.4%). Targeting the Intergenic Transcribed Spacer (*ITS*) locus, DNA of *Bartonella* was detected in 22% (64/289) of all fleas. Fifty percent of the *C. felis* and 57% of the *Xenopsylla* sp. contained *Bartonella* DNA. DNA sequencing showed the presence of *Bartonella clarridgeiae* (50%), *Bartonella henselae* (27%), and *Bartonella koehlerae* (3%) in *C. felis*. *Xenopsylla* sp. collected from *Rattus rattus* rats were infected with *Bartonella tribocorum*, *Bartonella elizabethae*, and *Bartonella rochalimae*. Phylogenetic sequence analysis using the 16S ribosomal RNA gene obtained four genetic clusters, *B. henselae* and *B. koehlerae* as subcluster 1, *B. clarridgeiae* as cluster 2, while the rat *Bartonella* species (*B. tribocorum* and *B. elizabethae*) were an outgroup cluster. These findings showed the important role of cat and rat fleas as vectors of zoonotic *Bartonella* species in Palestinian territories. It is hoped that this publication will raise awareness among physicians, veterinarians, and other health workers of the high prevalence of *Bartonella* spp. in fleas in Palestinian territories and the potential risk of these pathogens to humans and animals in this region. ***Journal of Vector Ecology* 39 (2): 261-270. 2014.**

Keyword Index: *Bartonella henselae, Bartonella clarridgeiae, Bartonella koehlerae, Ctenocephalides felis*, Intergenic Transcribed Spacer, Palestinian territories.

INTRODUCTION

Bartonella are gram-negative bacteria including more than 22 known species, of which at least 12 are reported to be infectious to humans (Guptill 2010a). They infect the host endothelial cells and erythrocytes and are transmitted by hematophagous arthropods such as fleas, sand flies, ticks, and mosquitoes (Chomel and Boulouis 2005). Several human specific and zoonotic *Bartonella* spp. causing Cat-Scratch Disease (CSD), endocarditis, Carrion´s disease, trench fever, bacillary angiomatosis, peliosis hepatis, chronic bacteremia, chronic lymphadenopathy, and neurologic disorders in humans have been identified to date (Guptill 2010a). In particular, *Bartonella henselae* and *Bartonella clarridgeiae* are known as the main causative agents for CSD, while *Bartonella quintana, Bartonella elizabethae, B. henselae,* and *Bartonella koehlerae* were reported to cause endocarditis in humans and animals (Chomel et al. 2009, Guptill 2010b). The cat flea (*Ctenocephalides felis*) is the main recognized vector of *B. henselae* and transmission among cats and humans occurs mainly through infected flea feces (Bouhsira et al. 2013). In addition, the cat flea is considered as a potential vector for *B. clarridgeiae and B. koehlerae* (Rolain et al. 2003), while *C. canis* is a suspected vector for *B. henselae* (Ishida et al. 2001). Several *Bartonella* species have been reported in wild rodents and cats in Israel (Gutierrez et al. 2014, Gutierrez et al. 2013, Morick et al. 2009, Morick et al. 2013). In addition, several studies showed that fleas are the main vectors of these bacteria. Fleas from cats and rodents have been reported to be infected with *B. hensalae* and *B. elizabethae*, (Breitschwerdt et al. 2008, Kerkhoff et al. 1999), as well as *B. koehlerae* (Avidor et al. 2004, Chaloner et al. 2013). The infection has been identified in Israeli cats and dogs (Baneth et al. 1996, Harrus et al. 2009, Ohad et al. 2010). Infections in humans have been reported in Israel, a country neighboring Palestinian territories. For example, a five-year-old boy with cat scratch disease showed a complicated symptom with a painful twisted neck and osteomyelitis of the cervical spine associated with an epidural abscess (Tasher et al. 2009). In addition, visceral disease with endocarditis was demonstrated in an immunocompetent adult (Shasha et al. 2014). To the best of our knowledge, no reports on the prevalence of bartonellosis, *Bartonella* species, and their genotypes in Palestinian territories have been published to date. Serologic and microscopic tests have a limited diagnostic value for detection of the specific *Bartonella* species causing infection due to their low sensitivity and specificity, as *Bartonella* can cross-react serologically with *Coxiella burnetii* and *Chlamydophila* spp. (Johnson et al.

2003). In addition, species-level identifications are difficult and often impossible using these tests. Moreover, *Bartonella* species identification using conventional biochemical tests is not possible (Maurin et al. 1997). DNA analysis by PCR and sequencing has been shown to be efficient in diagnosis and identification of different *Bartonella* species (Agan and Dolan 2002, Maurin et al. 1997). Different target genes can be/are employed for DNA analysis including the citrate synthase (*gltA*), 16S rRNA gene, RNA polymerase beta subunit (*rpoB*), cell division-associated protein (*ftsZ*), heat shock protein (*groEL*), and riboflavin synthase alpha chain (*ribC*) (Chaloner et al. 2011, Johnson et al. 2003, Maggi and Breitschwerdt 2005). Previous systems using direct PCR for *Bartonella* detection were unable to differentiate between close species, such as *B. hensalae* and *B. koehlerae*. Here we investigate the use of restriction fragment length polymorphism to separate these species. The goal of this study was to elucidate the presence and distribution of *Bartonella* spp. in Palestinian territories using molecular techniques.

MATERIALS AND METHODS

Animal sampling

Cats, dogs, hyraxes, and rats, captured between February, 2011 and August, 2012, were included in the study. They were mainly outdoor animals that had been captured from three geographic regions in Palestinian territories, including Nablus (135), Tamoon, and Tubas (21) from the northern region of Palestine, East Jerusalem (113) as central, and 11 fleas from Bethlehem as the southern region. The sampled population was categorized according to animal species, geographic location, host gender, flea species, flea sex, and number of fleas on the same animal. The ethnic group in the area was noted for comparisons of prevalence, vector species, and genotypes between Arab and Jewish neighborhoods. The Ethics Research Committee of the University of Al-Quds (Palestinian territories) approved all the activities involving animal subjects.

Flea collection and identification

Fleas were collected from infested animals either using a fine-toothed metal comb (11 teeth\per cm) (Lochdan, Regev, Israel) or hair forceps, and were transferred in sterile microfuge tubes (1.5 ml) (SARSTEDT, Nümbrecht, Germany) containing 70% ethanol. Samples were sent to the Laboratory of Al-Quds Nutrition and Health Research Institute (ANAHRI), Al-Quds University, Jerusalem in cool boxes (4˚ C), and then kept at −20˚ C until used. Fleas were subsequently sorted based on sex and species using the stereoscopic microscope (Zeiss) according to published identification keys (Smit 1973). Each individual flea was placed in a separate tube for DNA extraction for *Bartonella* prevalence and species identification.

DNA extraction

Each flea was removed from the alcohol tube and dried on tissue paper, placed into a new microfuge tube, digested by mechanical grinding using plastic pestles, and was then subjected to DNA extraction using the GeneJet Genomic DNA purification Kit (Thermoscientific, Lithuania) following the company manufacturer's instructions. Briefly, samples were lysed with 400 µl of the kit lysis solution that contained 20 µl Proteinase K, ground, vortexed, incubated at 56˚C overnight, purified with GeneJet columns, and finally eluted in 100 µl of kit elution buffer.

Selection of the appropriate PCR assay for *Bartonella* diagnosis

The *Bartonella gltA* gene (Morick et al. 2009) and the intergenic transcribed spacer (*ITS*) locus (Maggi and Breitschwerdt 2005) were targeted for the bacterium DNA detection. The *Bartonella gltA* gene (379 bp) was amplified as follows: BhCS.781p (5′-GGGGACCAGCTCATGGTGG-3′) and BhCS.1137n (5′-AATGCAAAAAGAACAGTAAACA-3′) (Norman et al. 1995). The *ITS* locus was amplified using the primers (321s: 5′-AGATGATGATCCCAAGCCTTCTGG and H493as: 5′-TGAACCTCCGACCTCACGCTTATC) as previously described (Gutierrez et al. 2013, Maggi and Breitschwerdt 2005). PCR reactions were performed in 25-µl syntezza PCR ready mix (Syntezza, Jerusalem), containing 0.8 µM of each set of primers and 10 µl of the extracted DNA. The PCR conditions were as described previously (Maggi and Breitschwerdt 2005, Norman et al. 1995, Renesto et al. 2001). All PCR products of the positive samples were sent for DNA sequencing. PCR grade water (No DNA) was included as negative controls.

To evaluate the best system for diagnosis based on high sensitivity and specificity, the first positive sample that was obtained and found belonging to *B. henselae* by DNA sequencing in both PCR assays, was used. A directly extracted DNA sample (10 µl) followed with serial dilution 1:5 was applied. A *Wolbachia* endosymbiont of *Nasonia longicornis* positive sample previously identified by DNA sequencing of the *gltA* was used for specificity evaluation. The *ITS* PCR previously had a high sensitivity and specificity in the amplification of *Bartonella* from flea samples (Morick et al. 2009). Where there was more than one band in the agarose gel, they were cut separately from the gel, purified and re-amplified, and submitted for DNA sequencing.

Statistical tests

Statistical analysis was done using the SPSS program v13. A two-tailed t-test was performed and a significant Pearson Correlation was considered when $p \leq 0.05$.

PCR for phylogenetic analysis

PCR products from all samples that were positive by *ITS* PCR using the above primers were sent for DNA sequencing. The *gltA* locus (379 bp) was amplified using the above-mentioned set of primers, while the *rpoB* locus (825 bp) was amplified using primers 1400F (5′-CGCATTGGCTTACTTC-GTATG-3′) and 2300R (5′-GTAGACTGATTAGAAC-GCTG-3′) (Renesto et al. 2001). All amplified products were submitted for DNA sequencing. The PCR reactions and conditions were performed as described previously (Maggi and Breitschwerdt 2005, Norman et al. 1995, Renesto et al. 2001).

A non-experimental *Rickettsia rickettsii* 16S rRNA gene, GenBank: U11021.1 ribosomal RNA intergenic spacer was used as an out-group.

The CLUSTALW program (http://www.genome.jp/tools/clustalw/) was used for the multiple sequence alignment of *Bartonella* sequences obtained in this study with those of known *Bartonella* species deposited in the GenBank/EMBL/DDBJ databases. A phylogenetic tree was drawn based on the sequences of *gltA* (379 bp), *rpoB* (825 bp), and *ITS* (190 bp), using the IUB scoring matrix used by BESTFIT for the comparison of nucleic acid sequences.

PCR for restriction fragment length polymorphism (RFLP)

A gel-based PCR-RFLP system for differentiation of *B. henselae* and *B. koehlerae* was prepared for this study. DNA sequences of the *ITS* loci of the two species were scanned for differential restriction sites using the nebcutter website, http://tools.neb.com/NEBcutter2/. A selected restriction enzyme (*PsiI*) was directly incubated for 2 h with the PCR product and then all products were subjected to electrophoresis after loading on a 2% agarose gel and visualized following ethidium bromide staining. Sequenced samples from both *Bartonella* species were used to confirm the virtual digestion.

RESULTS

Animal sampling

A total of 289 fleas was collected from 46 animals from Nablus, Tubas and East Jerusalem. Overall, 119 *C. felis* were collected from 27 cats, 133 *C. canis* and two *Xenopsylla* sp. from nine dogs, 23 *C. canis* were sampled from five hyraxes, and seven *Xenopsylla* sp., three *C. canis* and two *C. felis* were isolated from five rats (Table 1). These included 212 female and 73 male fleas. The sex of four fleas was not determined due to their damage during handling. The most prevalent species were *C. felis* (n=119/289; 41.2%), *C. canis* (n=159/289; 55%), and *Xenopsylla* sp. (n=7/289; 2.4%).

A significant correlation was observed between *Bartonella ITS* PCR positivity and the animal host (cats, $p<0.01$), flea species (*C. felis*; $p<0.01$), flea sex (female; $p=0.012$), and number of fleas on each animal ($p=0.008$). More than half of the tested cats (16/27, 59.3%) carried at least one infected flea. The cat gender, host's geographic location, and neighborhood ethnicity showed no significant correlation ($p>0.05$) with *Bartonella* presence.

Selection of appropriate diagnostic system

Of the first 30 random screened samples, four were positive for *ITS* PCR (showed a band of ~192 bp), and DNA sequencing of the four positive samples showed *B. henselae* (100% sequence identity) as the bacterial organism present in these fleas by using nucleotide blast analysis (website: http://blast.ncbi.nlm.nih.gov/Blast.cgi). While *gltA* showed eight samples to be positive (band at the level of 379 bp), four of those positive samples, all originating from *C. felis* collected from cats, were *B. henselae* (100% sequence identity). The other four *gltA* sequences matched with *Wolbachia* endosymbiont of *Nasonia longicornis* (81% sequence identity), and originated from *C. canis* fleas collected from dogs.

Sensitivity of the *ITS* PCR assay was higher than that of the *gltA* with capability of detection at the dilution of 1:125, while the *gltA* PCR system was positive with non-diluted DNA. The *gltA* was not specific because it detected *Wolbachia* DNA in addition to *Bartonella* (data not shown).

Screening all flea samples by *ITS* PCR system

Since the *ITS* PCR showed better sensitivity and specificity than the *gltA* PCR, it was used to screen all 289 flea samples. *Bartonella* DNA was detected in 22% of them (Table 1). Four band patterns were observed on agarose gel using the *ITS* locus PCR: a band at the molecular weight level of 192 bp was shown to belong to *B. henselae* or *B. koehlerae*; a band at the 182 bp level was shown by sequencing to belong to *B. clarridgeiae*, a 250 bp band was shown to belong either to *B. elizabethae* or *Bartonella tribocorum,* and a dual infection shown with the presence of a 250 bp and a 182 bp band

Table 1. Prevalence of *Bartonella* spp. in fleas from different animals in Palestinian territories.

Fleas source (flea species; number of fleas)	*ITS* PCR results (number; %)	
	Positive (%)	Species
Cats (*C. felis*; 119)	60 (50.4)	*B. clarridgeiae* (30/64; 46.7); *B. henselae* (16/64; 25); *B. koehlerae* (2/64; 3.1)
Rats (*Xenopsylla* sp.; 7)	4 (57)	*B. elizabethae* (3; 5); *B. tribocorum* (1/64; 1.6); *B. rochalimae* (1/64; 1.6)
Dogs (*C. canis;* 135)	0 (0)*	
Hyraxes (23)	0 (0)	
Unknown (5)	0 (0)	
Total (289)	64 (22)	

*By sequencing of the gltA DNA products, 18.5% (25/135) of the dog fleas were found to harbor *Wolbachia* DNA.

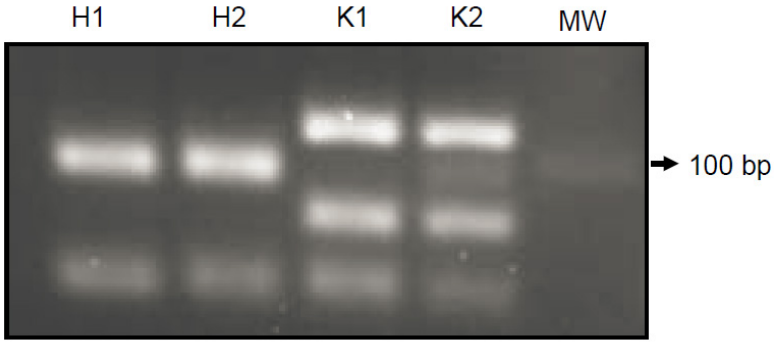


Figure 1. Restriction fragment length polymorphism (RFLP) analysis after digestion of the *ITS* entire product using PsiI enzyme. Product was loaded on 2% agarose gel. H1 and H2 represent samples proven of *B. henselae*. K1 and K2 are *B. koehlerae*, both were from fleas originated from cats and previously confirmed by DNA sequencing.

(later both proved *B. elizabethae* and *Bartonella rochalimae*, respectively, by DNA sequencing) (data not shown). BLAST analysis of the positive *Bartonella* DNA sequences showed the following species distribution: *B. clarridgeiae* 30/64 (46.7%), *B. henselae* 16/64 (25%), *B. koehlerae* 2/64 (3.1%), and *B. elizabethae* 3/64 (4.6%). In addition, both *B. tribocorum* and *B. rochalimae* were detected in one out of 64 samples (1.6%) Both were from the same sample but with two different band levels, they were extracted, purified from the gel, and then sequenced (Table 1). Since both *B. henselae* and *B. koehlerae* showed a 192 bp band, we developed restriction fragment length polymorphism for distinguishing between the two spp.

Bartonella spp. were only observed in *C. felis* and *Xenopsylla* sp. fleas, of which 60/119 (50.4%) and 4/7 (57%) were infected, respectively. *Wolbachia* endosymbionts DNA was only detected in *C. canis* fleas of which 25/135 (18.5%) were infected. *Wolbachia* endosymbionts-infected *C. canis* fleas originated only from dogs. No *Bartonella* DNA was obtained from fleas that originated from dogs and hyraxes. Out of 12 fleas which originated from rats, one was positive for *B. tribocorum*, two for *B. elizabethae*, and one showed dual infection with *B. elizabethae* and *B. rochalimae* (Billeter et al. 2013).

RFLP analysis

The digestion of the *ITS* locus was clear as the RFLP was able to differentiate between the closely related species *B. henselae* and *B. koehlerae*. *Bartonella henselae* produced bands at the DNA molecular weight level of 97 bp and 94 bp, which appeared as one band since it is difficult to show a 3 bp difference on 2% agarose gel. *B. koehlerae* showed two bands at the DNA molecular weight levels of 114 bp and 74 bp (Figure 1). These two patterns were easily distinguishable in the agarose gel (Figure 1).

DNA sequencing and genetic characterization

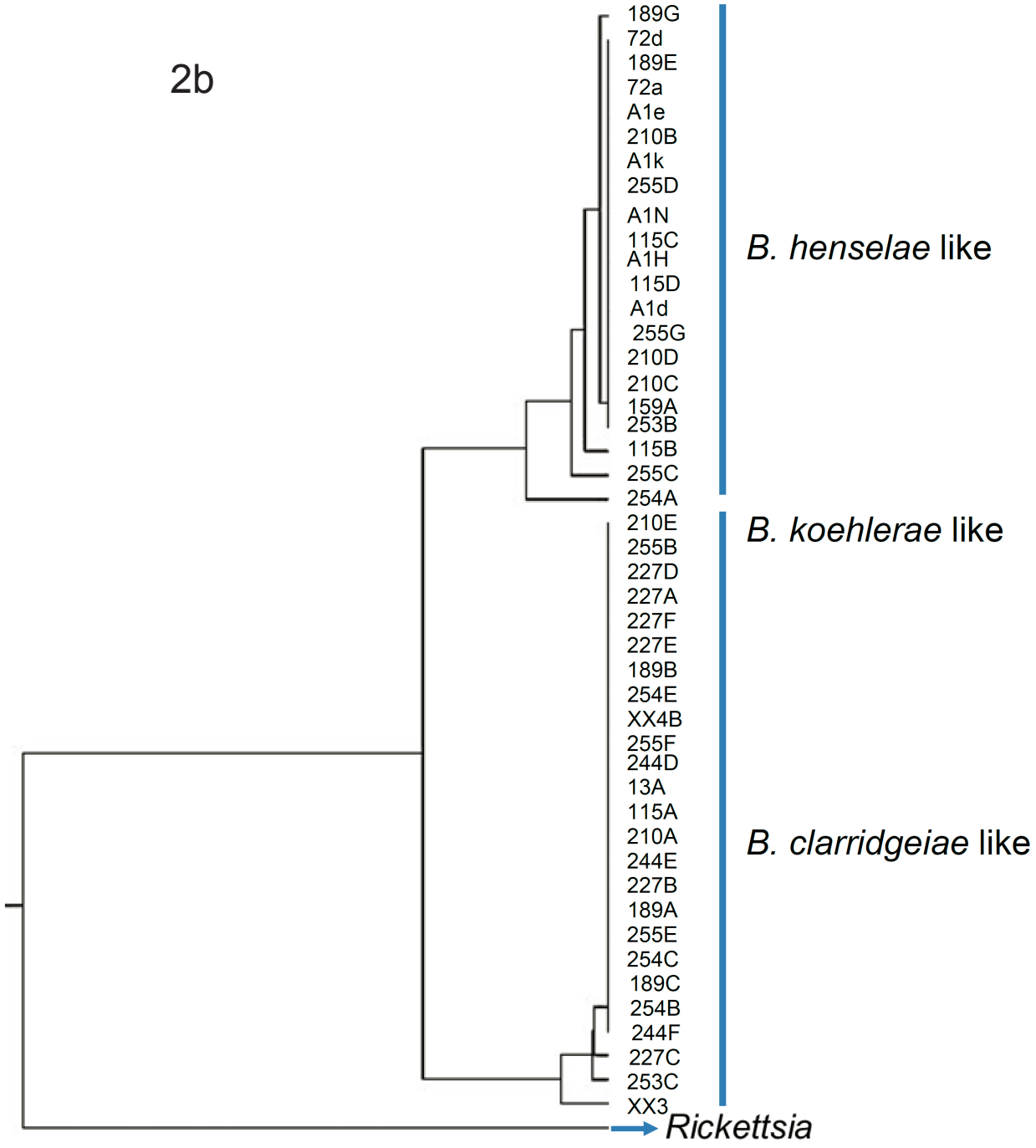
Successful DNA sequencing was obtained from 64 positive fleas as follows: *ITS* (52/64)81%, *gltA* (48/64) 75%, and *rpoB* (33/64) 52%. Not every *ITS* positive *Bartonella* gave positive PCR results for the other two genes. *ITS* phylogenetic analysis showed three main clusters, a *B. henselae* and *B. koehlerae*-like cluster, a *B. clarridgeiae*-like cluster, and a rat *Bartonella*-associated cluster (including *B. elizabethae* and *B. tribocorum*) which showed as an out-group in the *ITS* locus phylogeny analysis (Figure 2). The *B. henselae* clade apparently belonged to a separate sub-cluster (*B. koehlerae*), while the *B. clarridgeiae*-like cluster separated into three sub-clusters (Figure 2a). A non-experimental *Rickettsia rickettsii* 16S rRNA gene (GenBank: U11021.1) was used as an out-group. Previous studies (Houpikian and Raoult 2001, Sato et al. 2012) indicated that the *ITS* phylogenetic analysis was a useful tool to identify *Bartonella* species.

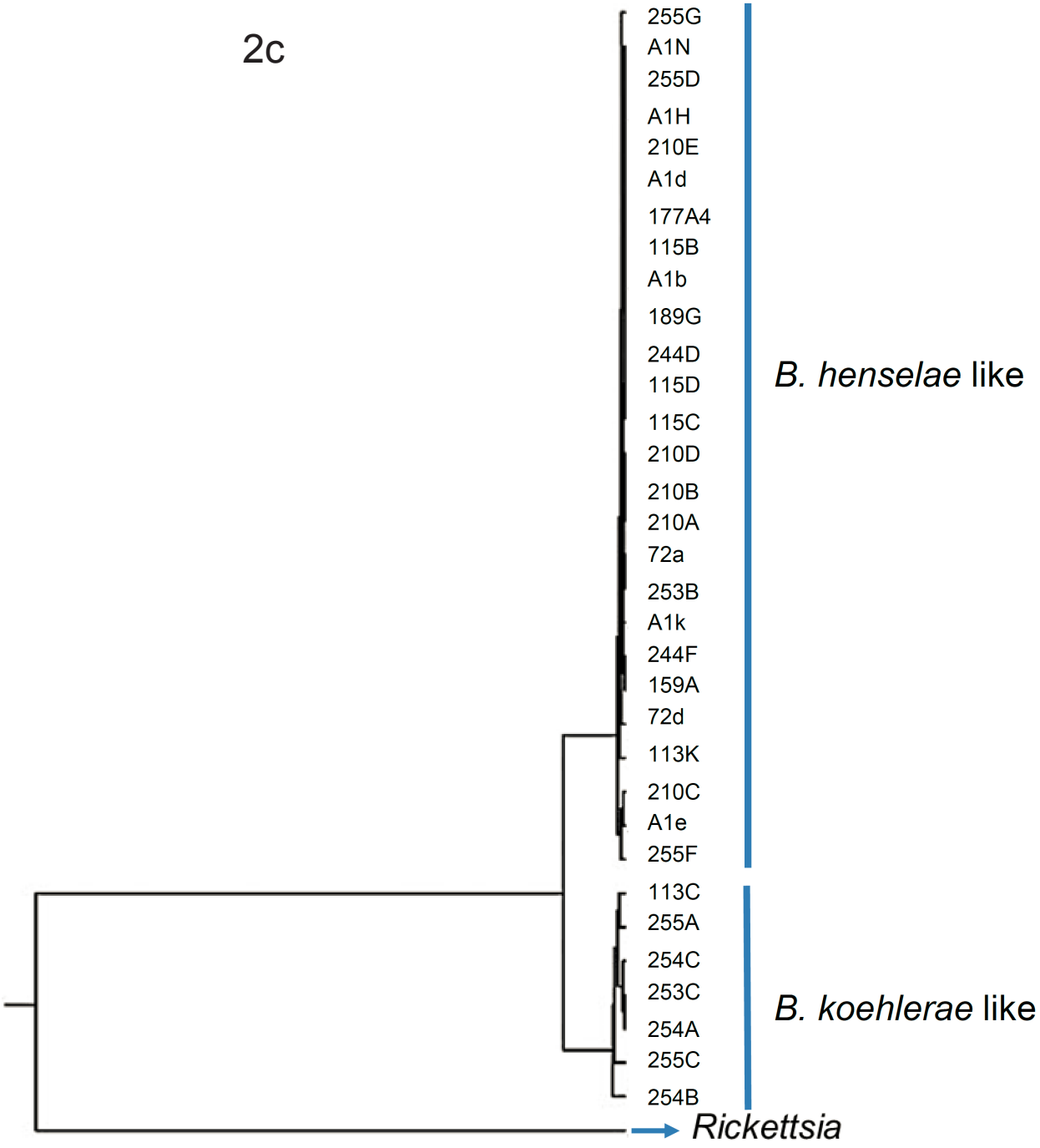
Two main clusters for *gltA* and four clusters for *rpoB* genes were observed (Figure 2b,c). Each cluster of *rpoB* correlated with one of the clusters of the *gltA* and *ITS* loci. All *Bartonella* genotypes seemed to follow a pattern in which the genotype in cluster I of the *rpoB* also belonged to cluster I of *gltA* and *ITS* (*B. henselae* group) (Figure 2a,b,c).

DISCUSSION

This study investigated the prevalence of *Bartonella* organisms in fleas from cats, dogs, rats, and hyraxes in Palestinian territories and characterized their genetic composition. The overall prevalence of *Bartonella* infection in fleas from these animals was 22% (64/289); these results were similar to those obtained from Israeli cats, according to which 25.1% were infected with *Bartonella* species (Gutierrez et al. 2013). In the present study, no *Bartonella* DNA was detected in fleas collected from dogs (n = 135) or hyraxes (n = 23). Interestingly, fleas collected from dogs in other Mediterranean countries, including Greece and Italy, were reported to be infected with *Bartonella* spp. with a prevalence of 4% and 11.7%, respectively (Diniz et al. 2009). The prevalence of different *Bartonella* spp. found in cat fleas in this study, i.e., *B. clarridgeiae* (46.7%), *B. henselae* (25%), and *B. koehlerae* (3.1%) was remarkable. It is interesting to note that the same three *Bartonella* species were detected in cats from Israel (Gutierrez et al. 2013). *Rattus rattus* rats and cats captured in the Palestinian areas were highly infested with fleas infected with *Bartonella* spp. (57% and 50.4%, respectively), suggesting that those mammals might be major reservoirs of *Bartonella* species in Palestinian territories. However, as the rat-flea sample size examined in this study was very small (n=7), further studies of rodent populations and their fleas in this region are warranted. *Rattus rattus* rats captured in this study were infected with *B. tribocorum*, *B. elizabethae*, and *B. rochalimae*. As *B. rochalimae* was reported to infect humans and animals, its potential zoonotic role is emphasized (Chomel et al. 2009, Eremeeva et al. 2007). Co-

Figure 2. Phylogenetic classification of Palestinian *Bartonella* based on sequences of a) *ITS*, b) *gltA*, and c) *rpoB* loci. The phylogenetic trees were constructed by the neighbor-joining method using the CLUSTAL_X program (http://www.genome.jp/tools/clustalw/) for the alignment of *Bartonella* sequences obtained in this study with those of known *Bartonella* species deposited in the GenBank/EMBL/DDBJ databases. They were drawn using the IUB scoring matrix used by BESTFIT for the comparison of nucleic acid sequences.


2b
189G
72d
189E
72a
A1e
210B
A1k
255D
A1N
115C
A1H
115D
A1d
255G
210D
210C
159A
253B
115B
255C
254A
210E
255B
227D
227A
227F
227E
189B
254E
XX4B
255F
244D
13A
115A
210A
244E
227B
189A
255E
254C
189C
254B
244F
227C
253C
XX3
Rickettsia
B. henselae like
B. koehlerae like
B. clarridgeiae like


2c
255G
A1N
255D
A1H
210E
A1d
177A4
115B
A1b
189G
244D
115D
115C
210D
210B
210A
72a
253B
A1k
244F
159A
72d
113K
210C
A1e
255F
113C
255A
254C
253C
254A
255C
254B
B. henselae like
B. koehlerae like
Rickettsia

infection with more than one *Bartonella* sp. was documented in this study in 6% (4/64) of the infected fleas and in 16 (59.3%) of the cats harboring at least one ITS PCR-positive flea. This was shown through sequencing of different individual fleas that originated from the same cat. The percentage of cats that hosted *Bartonella*-positive fleas was strongly associated with the number of fleas from each cat (p=0.008). Similar results were found in fleas from northeastern Thailand, where *Bartonella* DNA was detected in 59.1% (114 of 193) of the fleas examined (Billeter et al. 2013). Previous studies in other countries, including Israel, have shown that wild rats, including the species *Rattus norvegicus* as well as *R. rattus*, are reservoirs of *B. tribocorum* and *B. elizabethae* (Harrus et al. 2009, Morick et al. 2009). The latter *Bartonella* spp. were also detected in fleas collected from other rodent species from Israel (Morick et al. 2010), France (Heller et al. 1998), the United States (Gundi et al. 2012), and Indonesia (Winoto et al. 2005). The detection of *B. elizabethae*, a causative agent of human endocarditis and neuroretinitis, is of particular potential public health importance in this region (Boulouis et al. 2005, Winoto et al. 2005).

In the present study, different fleas collected from the same animal showed mixed *Bartonella* species and genotypes. Co-infection of fleas with different *Bartonella* species has already been described. A previous study conducted in Israel (Gutierrez et al. 2013) showed co-infection with two or more different *Bartonella* spp. in 2.1% of stray and domestic cats. Domestic cats from Thailand were also demonstrated to be co-infected with more than one species of *Bartonella* as described earlier (Maruyama et al. 2001).

The significant correlation between flea *Bartonella* positivity rate and the flea species examined, in particular the high infection rate of *C. felis*, was not surprising because it is well known that cats are the main reservoir of some *Bartonella* spp. and the main host of this flea species (Azzag et al. 2012, Chomel and Boulouis 2005, Chomel and Kasten 2010).

The DNA amplification and DNA sequencing of the *ITS* loci has been shown to offer a sensitive means of species and intra-species differentiation of the *Bartonella* organism as shown before (Houpikian and Raoult 2001) and confirmed by the present study. Several advantages for the *ITS* PCR system over the gltA PCR system were found in our study: higher sensitivity for *ITS* PCR (192 bp) possibly due to a shorter PCR product than the gltA (379 bp) as shown in a previous report (Meissner et al. 2007). The *ITS* locus PCR was shown to be specific for *Bartonella* spp., while the *gltA* PCR was less specific and amplified *Wolbachia* spp. in addition to *Bartonella*. The *ITS* locus RFLP was able to differentiate the closely related species *B. henselae* and *B. koehlerae* in our samples. It needs to be applied on a sufficiently large sample size for system approval. Then it can be used in the future in molecular laboratories for diagnostic and epidemiological purposes. Our study showed clustering in the phylogenetic trees, with interspecies heterogeneity, evident more in *B. clarridgeiae* than *B. henselae* (Figure 2a) based on *ITS* DNA sequence. *ITS*-based phylogeny was shown to split each species in a specific cluster and, as expected, *B. henselae* and *B. koehlerae* showed closer clusters than others as demonstrated previously (Billeter et al. 2013, Inoue et al. 2008, Sato et al. 2012). Rodent *Bartonella* spp. was shown to be an out-group as expected (Billeter et al. 2013, Inoue et al. 2008). *Bartonella clarridgeiae* presented a higher heterogeneous group than the other species. The phylogenetic tree based on DNA sequences of *gltA* divided the *Bartonella* species into two main clades: *B. clarridgeiae* and *B. henselae* with less inter-species genetic variability in both species than the *ITS* locus. The DNA sequences of *rpoB* clustered the *Bartonella* species into two main ones: *B. henselae,* and *B. koehlerae* species. Higher genetic variability of *B. henselae* was observed using this gene than the *gltA* and *ITS* loci.

In conclusion, a high infection rate with different *Bartonella* spp. was found in fleas from animal hosts in Palestinian territories. Our findings are of potential public health importance and should alert local physicians and public health authorities to the possibility of human infections with these *Bartonella* species.

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