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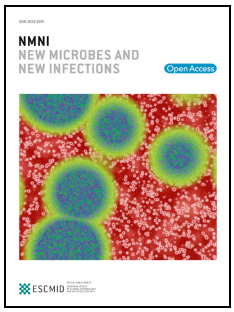

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Title page

Bartonella vinsonii sub. *arupensis* infection in animals of veterinary importance, ticks and biopsy samples.

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Running title: *Bartonella* infection in animals, ticks and biopsy samples.

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Abstract

Testing for vector-borne pathogens in livestock is largely reliant upon blood and tissue. The role of biopsy samples remains poorly explored for detecting tick-borne bacteria in animals.

In a 2-year survey, animals of veterinary importance from farms throughout the Northern part of Greece were routinely checked for the presence of biopsy samples. Where detected, either a portion or biopsy was collected together with whole blood samples and any ticks at the site of the biopsy sample. Molecular testing was carried out by real-time PCR targeting the ITS gene of *Bartonella* species.

A total 68 samples [28 blood samples, 28 biopsy samples and 12 ticks (9 *Rhipicephalus bursa* and 3 *R. turanicus*)] were collected from goats (64 samples) and bovine (4 samples).

Eight (11.8%) of the 68 samples were positive for *Bartonella* species. Of the biopsy sample and whole blood samples, four (14.3%) of each type were positive for *Bartonella* species. None of the ticks was tested positive for Bartonella species. All pairs of positive biopsy samples/whole blood samples originated from the same animals. Positive samples were identified as *B. vinsonii* sub. *arupensis*.

Although many more samples from a much wider spectrum of animal species is required before concluding upon the merit of biopsy samples on the study of tick-borne diseases, the significance of our finding warrants further study, both for clinical consequences in small ruminants and for those humans farming infected animals.

Keywords: Animals of veterinary importance, Bartonella, biopsy sample, tick.

Introduction

Bartonella are considered as emerging pathogens, being increasingly associated with a number of diseases both in humans (trench fever, Carrion's disease, bacillary angiomatosis, endocarditis, cat scratch disease and neuroretinitis) (1), as well as, in animals (including ruminants, cattle, cats, rodents, dogs and a wide range of wild animals) (2). Whilst in vertebrates, *Bartonella* parasitize erythrocytes and endothelial cells (3), typically for protracted periods (4).

Established and proposed new members of *Bartonella* species have increased exponentially over recent years. Over 30 species have been recognized with some having global distribution and infecting a wide variety of vertebrates (5). A wide variety of vectors are involved in transmission of *Bartonella* species including body lice, fleas, ticks, mites and sandflies (6). Examples of bacteria of the genus of *Bartonella* associated with vector transmission are *B. bacilliformis* that is transmitted by sand flies, *B. henselae* (transmitted by cat fleas) and *B. quintana* (transmitted by the human body louse). The role of ticks in the ecology of *Bartonella* is hypothesized (7-9), despite their notable ability to serve as arthropod vectors/reservoirs of various agents posing medical and veterinary health significance (10), and upsurge in the incidence of tick-borne diseases in many regions of the world (11).

The association between *Bartonella* and their mammalian hosts is varied, with some strictly limited whereas others are less restricted (12). Cats play the role of the main reservoir for *B. henselae* causing cat-scratch disease. Furthermore, several strains have been isolated from various rodent (13, 14) and ruminant (15, 16) species throughout the world. Ruminants can also become infected with *B. schoenbuchensis, B. chomelii* and *B. bovis* have been isolated from blood in Europe, Africa and North America (15, 17, 18). Amongst cattle, *B. bovis* has been implicated in causing bovine endocarditis (19), while *B. chomelii*, has, also, been isolated from

the same animal species (20), although no clinical consequence has been demonstrated for the latter species. Moreover, *B. rochalimae* causes infection in domestic animals, wild carnivores and in humans (21).

In cases where vertebrate hosts, vectors and wild animal species interact with each other, deciphering the transmission cycles of zoonotic agents seems quite challenging (22). Proper sampling plays a crucial role in the accurate approach of the study of a zoonotic disease. Serological analysis has been used extensively especially in epidemiological studies but is limited in its ability to discriminate closely related pathogen genotypes. Moreover, detection of antibodies does not necessarily conclude bacteraemia or even infection of the host; whereas detection of the pathogen in the host's blood or from a direct sample (biopsy sample for example) would seem a more secure approach.

The purpose of the current study was to compared biopsy sample (removed scab) with whole blood or tick vectors for detection of tick-borne bacteria in livestock in order to assess the diagnostic merits of various sample types for the detection of *Bartonella* species.

Materials and methods

Sampling

In a 2-year survey carried out in the laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of Crete (Greece) in conjunction with the Veterinary department of the Aristotle University of Thessalonica (Greece), (AUT) animals of veterinary importance (sheep, goats, bovine) from farms throughout the Northern part of Greece were routinely checked for the presence of biopsy samples. Where detected, either a portion or biopsy was collected together with whole blood samples and any ticks at the site of the biopsy sample. Data on animal species, farm location, time of collection, etc. were recorded.

Ticks removed from animals were placed in separate 1.5ml tubes with 70% ethanol and were uniquely coded according to individual animal, livestock, and region; then transported to AUT where they were kept at -80°C prior to testing. Each tick was identified by species using existing taxonomic keys (23) at the laboratory of Clinical Bacteriology of the University of Crete, in Greece.

Blood samples and biopsy samples were similarly removed, transferred into individual 1.5ml tubes, labeled and stored frozen until assessed.

Molecular analysis

DNA extraction from whole blood samples (QIAamp DNA blood mini kit, Hilden, Germany) or biopsy samples and ticks (QIAamp Tissue extraction kit, Hilden, Germany) was undertaken according to the manufacturers' instructions at the laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of Crete. Each tick and biopsy sample was washed in 70% alcohol, rinsed in sterile water and dried on sterile filter paper. Consequently,

84 samples were triturated individually into sterile tubes and a portion of them was used for further
85 DNA extraction. Once extracted, DNA samples were kept at -20^{o}C until further analysis.
86 Molecular testing was undertaken at University of East London using an initial real-time PCR
87 targeting the ITS gene of *Bartonella* species to screen as previously described (24). Master mix
88 was prepared containing PCR buffer, dNTPs (0.2mM each), $MgCl_2$ (5mM), Taq DNA
89 polymerase (0.06mM; Invitrogen), as well as, primers (1µM each) and probe (0.1µM; (Sigma
90 Genosys) at a final volume of 25µl. Agilent 96 well plates and cap strips were used. Nucleotide-
91 free sterile H_2O was used as negative control. At least four randomly selected wells in each plate
92 were used as negative controls. A single well was used as positive control each assay, the
93 positive control being a verified positive *B. quintana* DNA isolated from human blood. The
94 master mix preparation room, the DNA addition room and the amplification room were all
95 separated from each other to avoid any chance of contamination. All positive and/or ambiguous
96 samples were re-tested at least once in order to demonstrate reproducibility using similar
97 conditions as those described above. Only samples producing cT values of less than 35 were
98 considered to be positive. All amplifications were performed using an Agilent Aria Mx cycler.
99 Positive samples were further tested by conventional PCR (targeting ITS) to get amplicons that
100 were further used for sequencing as previously described (25). All primers and probes used both
101 for Real-time PCR and for the conventional PCR are summarized at Table 1. Amplicons were
102 purified using the PCR product purification kit (QIAquick Qiagen) and sequenced in both
103 directions by Sanger sequencing (Durham) using the same primers used for PCR. All sequences
104 obtained were aligned using ClustalW. Sequences were compared for similarity with those at
105 GenBank using the nucleotide BLAST program (National Centre for Biotechnology Information)

106 http://www.ncbi.nlm.nih.gov/BLAST) the ClustalW online software
107 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the MEGA v. X software.
108

109 **Results**

110 A total of 68 samples (n=28 blood samples; n=28 biopsy samples; and n=12 ticks) were collected
111 and tested for *Bartonella* species. Livestock included goats (12 ticks, 26 eschars, 26 blood
112 samples) and bovine animals (2 eschars and 2 blood samples) .

113 Of the 12 ticks collected, nine (9) were characterized as *Rhipicephalus bursa* and three (3) as *R.*
114 *turanicus*. Ticks were collected from goats only.

115 Eight (11.8%) of the 68 samples revealed presence of *Bartonella* species with Ct values ranging
116 from 29.07 – 34.44 (see Table 2). All positive samples were verified by a 2nd amplification. Of
117 the biopsy sample and whole blood samples, eight (four from each sample type; 14.3%) were
118 positive for *Bartonella* species. All pairs of positive biopsy samples/whole blood samples
119 originated from the same animals. All remaining samples were negative. Of the eight positive
120 samples, we amplified and sequence a 408 bps portion of ITS from six (6) samples (sample
121 numbers 11-16) that revealed identical sequence in both directions. All positive samples despite
122 their origin were identified as *B. vinsonii* sub. *arupensis* showing 100% (408/408 bp) similarity
123 to the already published sequence AF312504 and 99% (404/408) similarity to the already
124 published sequence AF442952. To further explore the extent of the relatedness of our sequences
125 with published ones, partial ITS sequences for another 32 *Bartonella* species were aligned to
126 construct a phylogenetic tree (Figure 1) in which, the position of our sequences against other
127 *Bartonella* species' sequences was demonstrated.

128 All bovine samples and all ticks tested were negative for *Bartonella* species. The results are
129 summarized at Table 2.

130

131 **Discussion**

132 An increasing interest in zoonotic tick-borne diseases has been revealed during the last few
133 decades, since these are considered as important zoonoses in Europe (26); among them are
134 Bartonellaceae.

135 *Bartonella vinsonii* was described as the Canadian vole agent back in 1946 (27), while almost
136 four decades (1982) later Weiss and Dasch further characterized the agent and named it after
137 *Rochalimaea vinsonii* (28). Fifteen years later (1999), its first isolation from a 62-year-old
138 bacteraemic man was recorded (29).

139 A number of genes are used as targets for the identification of *Bartonella* species, including the
140 16S rRNA and citrate synthase (*gltA*) (30), the 16S/23S rRNA intergenic spacer region (ITS)
141 (31), which shows a high degree of interspecies variability among *Bartonella* species, the *ftsZ*
142 (32) and the *GroEL* (33) genes. In our case, we did not have enough DNA to go through the
143 amplification of further genes, nevertheless, the successful detection of *Bartonella* in four
144 animals, both in biopsy sample and blood samples, demonstrates robustness of our findings.
145 Control samples were included in all assays and verified correct performance of the tests
146 reported. Sanger sequencing revealed that in all cases we had detected *B. vinsonii* subsp.
147 *arupensis*, close to *B. vinsonii* subsp. *vinsonii*, which is rodent-associated, and to *B. vinsonii*
148 subsp. *berkhoffii*, which has been described in dogs.

149 Rodent infections caused by *Bartonellae* tend to be asymptomatic, however whether they could
150 serve as a pathogen in other vertebrates is a cause for concern. As far as ruminants (including
151 water buffalo, several deer species, cattle, camels and moose) and animals of veterinary
152 importance are concerned, a number of *Bartonella* species have been associated with these
153 animal species, such as *B. bovis*, *B. capreoli*, *B. chomelii*, *B. dromedarii* and *B. schoenbuchensis*

154 (15, 16, 34). Contrary to large ruminants above, the isolation of *Bartonella* species from small
155 ruminants (including sheep and goats which we studied herein) has been more puzzling. Indeed,
156 several studies have failed to detect any *Bartonella* species from sheep or goats (35, 36), while
157 others have detected *B. melophagi* from domestic sheep samples (37) despite the great
158 difficulties on the isolation of this group of bacteria.
159 The natural reservoirs of *Bartonella vinsonii* subsp. *arupensis* are small rodents with mice
160 believed to show persistent infection (34). Further reports have detected this agent in deer mice
161 in North America (38), in rodents in Mexico (39, 40), in Brazil (2) and in the USA (California)
162 (41). Its zoonotic potential was revealed by its isolation from a human suffering from
163 endocarditis (42), in pre-enriched blood of four patients in Thailand (43) and in child where it
164 caused hepatic granulomatous lesions (44). *Bartonella vinsonii* subsp. *berkhoffii* is now
165 established as a canine pathogen with ability to cause endocarditis (45). Interestingly, *B. vinsonii*
166 subsp. *arupensis* has, also, been detected in the blood of stray dogs in Thailand (46). The role of
167 this organism as a pathogen in other vertebrate species remains to be clarified. Our detection of
168 *B. vinsonii* subsp. *arupensis* in goats is intriguing. Whether it has pathogenic potential in the
169 small ruminant is worthy or further exploration.
170 Importantly, this study reports the validity of biopsy samples for detection of *Bartonella*
171 infection in livestock. Infection was confirmed by demonstration of *Bartonella* in the blood of all
172 biopsy sample-positive animals. To the best of our knowledge, this is the first time that the
173 presence of *Bartonella* DNA in veterinary biopsy samples has been recorded; on the other hand
174 simultaneous detection of the same *Bartonella* species in ruminants and in the vectors they carry
175 (deer keds and cattle tail louse), has been described (37). A biopsy sample or cutaneous necrosis
176 is caused by vasculitis at the tick-bite site of inoculation, known as tache noire ("black spot") and

177 usually it is pathognomonic for infection by *Rickettsia*. The presence of an eschar plays a
178 significant role in both human clinical and laboratory diagnosis (47-50). Contrary to humans, the
179 role of biopsy samples in animals of veterinary importance has not been studied.
180 Epidemiological surveys for tick-borne diseases infecting animals are generally restricted to use
181 of serum and whole blood alone. The limitation presented with serum antibodies is that, if
182 present, they might correspond to past infection; furthermore, only IgG antibodies can be used as
183 a screening method. Furthermore, whole blood often fails to yield a positive PCR since
184 bacteraemia is rare in the case in animals and is not always a feature of vector-borne pathogens.
185 It seems that ticks may have the potential to act as vectors of *Bartonella* species (51). *Bartonella*
186 has been detected in questing ticks (*I. pacificus, Dermacentor*, and *R. sanguineus*) in the USA
187 (16), while other European studies (Netherlands, France, Poland, and Austria) have demonstrated
188 the presence of *Bartonella* in *I. ricinus* ticks obtained from vegetation either by molecular means
189 (52) or following isolation of the pathogen (*B. henselae* in *I. ricinus*) (53).
190 Although *R. turanicus* is considered as the species frequently associated with sheep (54), it is *R.*
191 *bursa* ticks that is considered a major ectoparasite of sheep in the Mediterranean basin (54). In
192 our study, although we collected ticks belonging to both these species, we failed to detect any
193 *Bartonella* DNA in any of those ticks. Nevertheless, although the total number of ticks collected
194 in the current survey was low (12 samples), our finding agrees with previous studies (54-59) that
195 failed to detect pathogenic species in *R. turanicus*. In an earlier study carried out in Palestine,
196 DNA of *Bartonella* species was detected in *R. sanguineus* collected from dogs and from camels,
197 however all ticks collected from sheep or goats were negative (60). A study of *R. bursa* ticks
198 removed from goat reported limited detection of *Bartonella* species from Sardinia (54).

199 The limitations of our study are that our numbers and range of livestock and ticks tested was
200 small. Furthermore, insufficient material was available to enable exhaustive molecular typing to
201 confirm the identity of the *Bartonella vinsonii* subsp. *arupensis* present in small ruminants.
202
203 **Conclusion**
204 We report the presence of *Bartonella vinsonii* subsp. *arupensis* species in goats from Greece,
205 with four animals showing positive blood and biopsy samples. The significance of this finding
206 warrants further study, both for clinical consequences in small ruminants and for those humans
207 farming infected animals. Certainly, many more samples from a much wider spectrum of animal
208 species is required before concluding upon the merit of biopsy samples on the study of tick-
209 borne diseases; however, we provide valuable proof-of-concept data that should promote future
210 research.
211
212 **Acknowledgements**
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214
215 Declarations of interest: none.

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386

387 **Tables and Figures**

		Gene targeted
Real-time PCR	**Sequence**	ITS
Primer forward	GGGGCCGTAGCTCAGCTG	
Primer reverse	TGAATATATCTTCTCTTCACAATTTC	
Probe	6-carboxyfluorescein-CGATCCCGTCCGGCTCCACCA-6-carboxytetramethylrhodamine	
PCR		ITS
Primer forward (438s)	GGTTTTCCGGTTTATCCCGGAGGGC	
Primer reverse (1100as)	GAACCGACGACCCCCTGCTTGCAAAGC	

388 **Table 1:** Primers and probes used to target the ITS gene either by Real-time PCR or by
389 conventional PCR.

390

Animals		Ticks		Biopsy samples		Blood samples		Blood sample and eschar (pairs)*		Blood sample, tick and eschar (triad)^	
Species	No	No	Pos (%)	No	Pos (%)	No	Pos (%)	No	Pos (%)	No	Pos (%)
Bovine	2	0	0	2	0	2	0	2	0	0	0
Goat	26	12	0	26	4 (15.4)	26	4 (15.4)	26	4 (15.4)	12	0
Total	28	12	0	28	4 (14.3)	28	4 (14.3)	28	4 (14.3)	12	0

391 **Table 2: S**ample types and origins tested for *Bartonella* species.

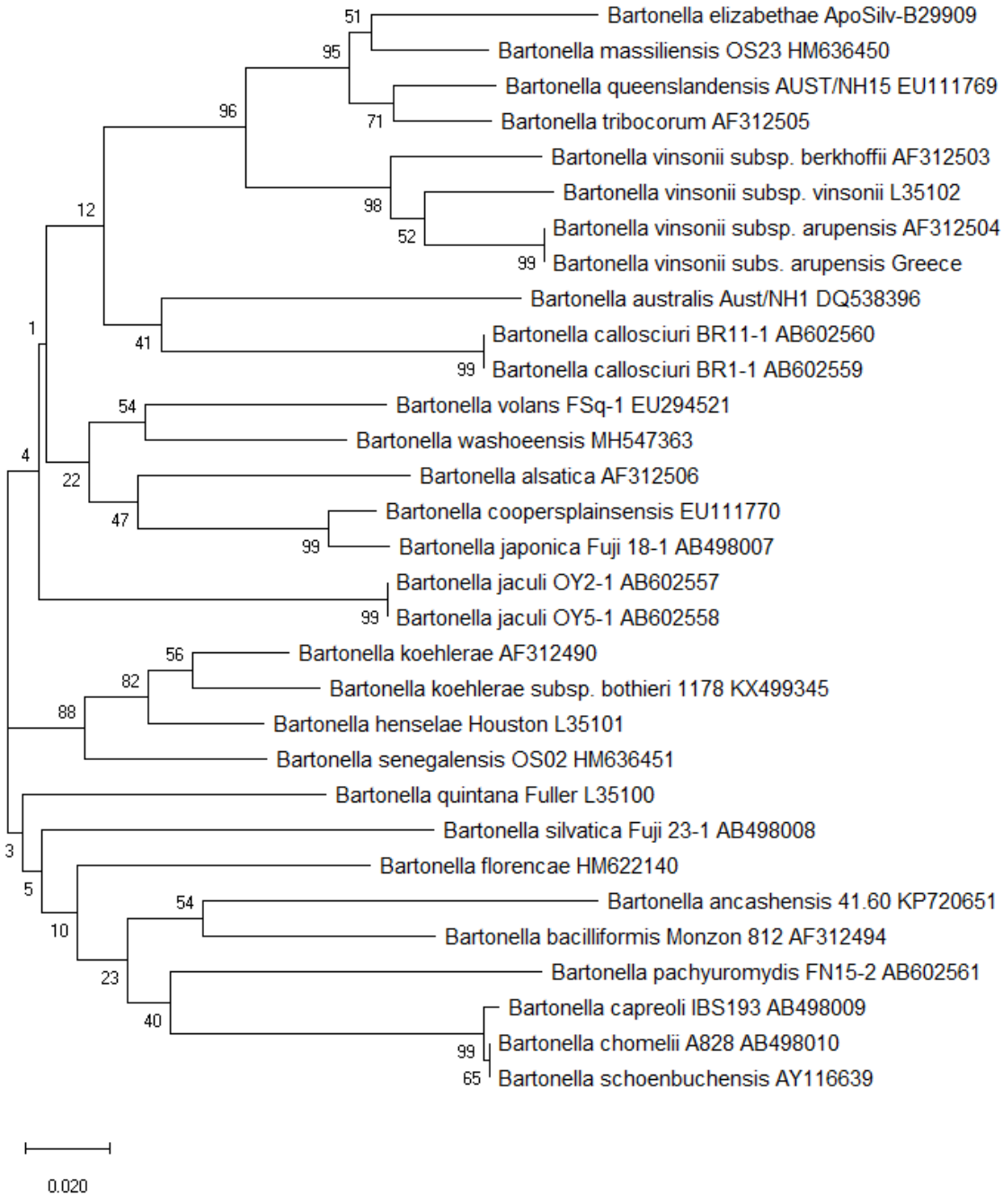
392 *: corresponds to cases where both eschar and whole blood samples were collected from the
393 same animal.

394 ^: corresponds to cases where biopsy sample, whole blood sample and a tick were collected from
395 the same animal.

396

397 **Figure 1:** ITS phylogeny for a 408 bp fragment of the 16S-23S intergenic linker region of 33
398 *Bartonella* species. The evolutionary history was inferred using the Neighbor-Joining method.

399 The optimal tree with the sum of branch length = 1.67495836 is shown. The percentage of
400 replicate trees in which the associated taxa clustered together in the bootstrap test (1000
401 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the
402 same units as those of the evolutionary distances used to infer the phylogenetic tree. The
403 evolutionary distances were computed using the Maximum Composite Likelihood method and
404 are in the units of the number of base substitutions per site. Codon positions included were
405 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated
406 (complete deletion option) (61).
407


Bartonella elizabethae ApoSilv-B29909
Bartonella massiliensis OS23 HM636450
Bartonella queenslandensis AUST/NH15 EU111769
Bartonella tribocorum AF312505
Bartonella vinsonii subsp. berkhoffii AF312503
Bartonella vinsonii subsp. vinsonii L35102
Bartonella vinsonii subsp. arupensis AF312504
Bartonella vinsonii subs. arupensis Greece
Bartonella australis Aust/NH1 DQ538396
Bartonella callosciuri BR11-1 AB602560
Bartonella callosciuri BR1-1 AB602559
Bartonella volans FSq-1 EU294521
Bartonella washoeensis MH547363
Bartonella alsatica AF312506
Bartonella coopersplainsensis EU111770
Bartonella japonica Fuji 18-1 AB498007
Bartonella jaculi OY2-1 AB602557
Bartonella jaculi OY5-1 AB602558
Bartonella koehlerae AF312490
Bartonella koehlerae subsp. bothieri 1178 KX499345
Bartonella henselae Houston L35101
Bartonella senegalensis OS02 HM636451
Bartonella quintana Fuller L35100
Bartonella silvatica Fuji 23-1 AB498008
Bartonella florencae HM622140
Bartonella ancashensis 41.60 KP720651
Bartonella bacilliformis Monzon 812 AF312494
Bartonella pachyuromydis FN15-2 AB602561
Bartonella capreoli IBS193 AB498009
Bartonella chomelii A828 AB498010
Bartonella schoenbuchensis AY116639
0.020