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1 New records of bacteria in different species of fleas from France and Spain.

2

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21 **Abstract**

22 In this study, we assessed the presence of vector-borne microorganisms in different  
23 species of fleas collected from different hosts in diverse areas of South-Western Europe  
24 by molecular methods. A total of 319 fleas belonging to eight different species was  
25 tested for the presence of eight microorganisms. *Wolbachia* spp. endosymbionts were  
26 detected in *Ctenocephalides felis*, *Pulex irritans*, *Archaeopsylla erinacei* and  
27 *Ctenophthalmus baeticus boisseauorum* specimens. *Rickettsia felis*, an emerging  
28 pathogen, was detected in *C. felis*, *A. erinacei* and *Ct. b. boisseauorum*. *Rickettsia typhi*,  
29 the agent of murine typhus was detected for the first time in *A. erinacei* and  
30 *Mycobacterium* spp. were detected for the first time in fleas (*C. felis*, *P. irritans* and *A.*  
31 *erinacei*). Lastly, five different species of *Bartonella* were detected in fleas' DNA in  
32 this study, including a possible new bacterium belonging to this genus. With this study,  
33 we updated the knowledge of the flea-borne bacteria present in the South-West of  
34 Europe reinforcing the idea about the necessity to expand and increase the current  
35 knowledge on flea-borne pathogens.

36

37 **Keywords:** Siphonaptera; flea-borne diseases; *Bartonella* spp.; *Rickettsia felis*;  
38 *Rickettsia typhi*.

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42 **1. Introduction**

43 Vector-borne diseases cause significant morbidity and mortality throughout the world.  
44 The majority of recent vector-borne zoonoses research focuses on tick- and mosquito-  
45 borne illnesses, such as Lyme disease and arboviral diseases, respectively [1].  
46 Nevertheless, the public health importance of flea-borne infections has been reported in  
47 several studies in recent years [2].

48 Several studies published in the last twenty years have warned about the possibility of  
49 emergence or reemergence of certain vector-borne diseases in several geographic areas,  
50 including flea-borne diseases [3, 4, 5, 6, 7, 8, 9]. In this sense, health care professionals  
51 and health authorities should be aware of the flea-borne pathogens circulating in their  
52 area in order to improve diagnosis and vector surveillance, and if necessary be able to  
53 manage a potential outbreak [2].

54 The role of fleas in the transmission of several pathogenic agents such as bacteria,  
55 viruses and helminths is well known [2, 10]. Therefore, in the last years, numerous  
56 studies have assessed the presence of pathogenic microorganisms in fleas using  
57 molecular methods [3] or even proteomics methods more recently [11]. Nevertheless,  
58 the role of fleas as competent vectors of several zoonotic pathogens such as rickettsiae  
59 or *Bartonella* species has been poorly studied. Moreover, epidemiological data is still  
60 missing for several flea species which may play an important role in the transmission of  
61 zoonotic vector borne pathogens.

62 Other pathogens have been detected in fleas such as *Coxiella burnetii* [12], *Leishmania*  
63 *infantum* [13], *Francisella tularensis* [14] or *Borrelia burgdorferi* [15]. As for  
64 *Wolbachia* spp., they are considered a common endosymbiont of several arthropod  
65 species including fleas [16].

66 The aim of this work was to assess the presence and prevalence of several  
67 microorganisms (*Bartonella* spp., *Rickettsia* spp., *C. burnetii*, *Borrelia* spp., *Leishmania*  
68 spp. and *Mycobacterium* spp., *Wolbachia* spp.) in different flea species collected from  
69 domestic, peridomestic and wild animals from different geographical areas of Spain and  
70 France.

## 71 **2. Material and Methods**

### 72 *2.1 Ethical statement*

73 Catching and handling procedures of animals in this study were carried out according to  
74 the guidelines provided by the Institutional Animal Care of the University of Seville  
75 based on RD 53/2013 of January 1<sup>st</sup>, 2013 (BOE number 34 of February 8<sup>th</sup>, 2013) of  
76 the Spanish government. Rodents were trapped using Sherman traps (H. B. Sherman  
77 Inc., Tallahassee, Florida) and released into their natural habitat after flea collecting  
78 procedures. Dogs belonged to private owners and public dog kennels and the consent of  
79 owners and dog kennels chairman was obtained for the inclusion of the dogs in the  
80 study. Rodents and dogs were exhaustively and manually examined for fleas through an  
81 inspection of head, neck, body, sides, tail, and ventral regions of each animal.  
82 Hedgehogs killed on roads at night were collected by hand early morning. Collected  
83 hedgehogs were taken to the laboratory and visually inspected for ectoparasites on a  
84 white sheet. No specific approval of any Institutional Animal Care and Use Committee  
85 were needed in this study since no anesthesia substances were applied for animal  
86 handling procedures.

### 87 *2.2 Sample collection and morphological identification*

88 Fleas were collected from March 2011 to April 2018 from dogs (*Canis lupus*  
89 *familiaris*), hedgehogs (*Erinaceus europaeus*) and rodents (*Rattus* sp., *Mus* sp.,

90 *Apodemus* sp. and *Arvicola terrestris*) from different localities of the South of Europe  
91 (Spain and France). Details about geographical areas, number and sex of samples,  
92 preservation methods, date of collection and hosts species tested in this study are  
93 showed in Table 1. Dogs were randomly selected by breed, sex and age in the study  
94 area. All dogs handled in this work were domestic dogs. Owners reported flea  
95 infestation in their pets to their veterinarians. Pets were subsequently included in the  
96 study after obtaining owners' permission to examined their dogs for fleas. Rodents were  
97 trapped using live traps, whereas, hedgehogs killed on roads at night were located and  
98 collected by hand early morning. Collected hedgehogs were taken to the laboratory and  
99 then placed on a white sheet of paper in order to be visually examined for ectoparasites.  
100 Dogs, hedgehogs and rodents were exhaustively examined for fleas through an  
101 inspection of head, neck, body, sides, tail, and ventral regions of each animal. All these  
102 hosts were examined by combing for a standardized period of time of 15 minutes. On  
103 the other hand, some specimens were collected from a neglected horse stable with a flea  
104 infestation. This place had been previously populated by horses, dogs and probably  
105 rodents; however, specimens were directly caught from the ground. All flea specimens  
106 were preserved in 70% and 96% ethanol at room temperature until morphological  
107 identification. Fleas were collected and morphologically identified using different  
108 taxonomic keys. Genus and species identification were carried out according to different  
109 authors and taxonomic keys [17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29]. For  
110 morphological analysis, all specimens were examined and photographed using a Nikon  
111 eclipse *Ci* microscope (Minato City, Tokyo, Japan) equipped with a camera lucid  
112 system and a photomicroscope.

### 113 2.3 Molecular detection of pathogens

114 Prior to DNA amplification, the abdomen of each specimen was cut off and transferred  
115 to a 1.5 mL tube containing 180 µL of G2 lysis buffer (Qiagen, Hilden, Germany) and  
116 20 µL of proteinase K (Qiagen), and incubated at 56° C overnight. DNA extraction was  
117 individually performed with an EZ1 DNA Tissue Kit (Qiagen) according to the  
118 manufacturer's recommendations. Flea DNAs were then eluted in 100 µL of Tris EDTA  
119 buffer using the DNA extracting EZ1 Advanced XL Robot (Qiagen). The DNA was  
120 either immediately used or stored at -20° C until molecular analysis. The DNA  
121 extracting EZI Advanced XL Robot was disinfected after each batch of extraction as per  
122 the manufacturer's recommendations, to avoid cross-contamination.

123 Quantitative PCR was individually performed according to the manufacturer's protocol  
124 using a PCR detection system: a CFX Connect™ Real-Time (Bio-Rad) with the Light  
125 Cycler 480 Probes Master Mix (Roche Diagnostics, Meylan, France). The qPCR  
126 reaction contained 10 µl of Light Cycler 480 Probes Master Mix (Roche Diagnostics,  
127 Meylan, France), 3 µl sterile distilled water, 0.5 µl of each of the primers, 0.5 µl of  
128 Uracil-DNA-glycosylase (UDG), 0.5 µl of probe and 5 µl of the DNA extract. All  
129 samples were screened using primers and probes targeting specific sequences of the  
130 following microorganisms: *Rickettsia* spp., *R. felis*, *R. typhi*, Anaplasmataceae,  
131 *Wolbachia* spp., *Borrelia* spp., *Bartonella* spp., *B. henselae*, *B. quintana*, *C. burnetii*,  
132 *Leishmania* spp. and *Mycobacterium* spp. The primers and probes references and the  
133 targeted sequences used to detect each pathogen by real-time quantitative PCR are  
134 summarized in table 2. DNA from our laboratory cultures of *R. montanensis*, *R. felis*, *R.*  
135 *typhi*, *Anaplasma phagocytophilum*, *W. pipientis*, *Borrelia crocidurae*, *B. quintana*, *B.*  
136 *henselae*, *C. burnetii* and *Mycobacterium ulcerans* were used as PCR positive controls.  
137 For *C. burnetii* two genes were tested in qPCR in order to avoid false positives, thus  
138 only the samples positive for both genes were considered positive. For all samples

139 tested by qPCR, only those with a cycle threshold (Ct) value lower than 35 were  
140 considered positive. The *Bartonella* spp.-positive samples that were negative for *B.*  
141 *henselae*- and *B. quintana*-specific qPCRs were subsequently subjected to standard PCR  
142 prior to sequencing in order to obtain identification to the species level. The same  
143 protocol was carried out for the other bacteria genera; each time we were not able to  
144 obtain a specific identification by qPCR, we used sequencing methods. Details about  
145 the primers used for standard PCR in this study are also listed in table 2. A Bio-Rad  
146 Thermocycler (Bio-Rad Laboratories, Hercules, California, USA) was used to amplify  
147 the target sequences prior to sequencing. The cycling protocol and PCR mix were  
148 described by Diarra et al. [47]. Amplificated products were visualized by  
149 electrophoresis through a 1.5 % agarose-tris-borate-EDTA gel containing SYBR Safe.  
150 PCR products were sequenced in both directions using a Big Dye Terminator kit and an  
151 ABI PRISM 3130 Genetic Analyzer (Applied BioSystems, Courtabeuf, France). The  
152 sequences were analysed using the ABIPRISM DNA Sequencing Analysis software  
153 version 3.0 (Applied BioSystems) and compared with sequences available in the  
154 GenBank database using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

#### 156 2.4 Phylogenetic analysis

157 The obtained partial 16S-23S Intergenic Spacer (ITS) sequences of *Bartonella* spp.  
158 were firstly subjected to BLAST analysis using the NCBI GenBank database.  
159 Phylogenetic analyses were then carried out using included reference sequences of  
160 different *Bartonella* species which are commonly detected in fleas. Phylogenetic trees  
161 were inferred using nucleotide data and performed using two methods: Maximum  
162 Likelihood (ML) trees were generated using the PHYML package from [48] whereas  
163 Bayesian inferences (B) were generated using MrBayes-3.2.6 [49] JMODELTEST [50]



164 program was used to determinate the best-fit substitution model for the bacteria data.  
165 Models of evolution were chosen for subsequent analyses according to the Akaike  
166 Information Criterion [51, 52]. Support for the topology was examined using  
167 bootstrapping (heuristic option) [53] over 1000 replications to assess the relative  
168 reliability of clades. For ML inference, best-fit nucleotide substitution model included  
169 general time reversible model with gamma-distributed rate variation GTR + G. The  
170 commands used in MrBayes-3.2.6 for BI were nst = 6 with gamma rates. For Bayesian  
171 inference, the standard deviation of split frequencies was used to assess if the number of  
172 generations completed was sufficient; the chain was sampled every 500 generations and  
173 each dataset was run for 10 million generations. Adequacy of sampling and run  
174 convergence was assessed using the effective sample size diagnostic in TRACER  
175 program version 1.6 [54]. Trees from the first million generations were discarded based  
176 on an assessment of convergence. Burn-in was determined empirically by examination  
177 of the log likelihood values of the chains. The Bayesian Posterior Probabilities (BPP)  
178 was percentage converted.

179 The *ftsZ* partial sequences from our *Bartonella* spp. and those obtained from GenBank  
180 database belonging to different *Bartonella* species were aligned and subjected to  
181 phylogenetic analysis in order to carry out a specific identification. Phylogenetic trees  
182 were inferred using Neighbor-joining (NJ) and Maximum Likelihood (ML) under  
183 Kimura-2-parameter +G and GTR+G+I models, respectively. The trees were plotted  
184 using the MEGA version X [55] software. Support for the topology was examined using  
185 bootstrapping [53] over 1000 and 200 replications for NJ and ML respectively.

### 186 **3. Results**

#### 187 *3.1 Morphological identification*

188 A total of 319 fleas was collected for this study. Eight different species were  
189 morphologically identified including *Ctenocephalides felis*, *Pulex irritans*, *Nosopsyllus*  
190 *fasciatus*, *Stenoponia tripectinata tripectinata*, *Archaeopsylla erinacei*, *Leptopsylla*  
191 *taschenbergi*, *Ctenophthalmus baeticus boisseauorum* and *Ct. apertus allani* (Table 1,  
192 Fig. 1). All specimens collected from dogs from Villamanrique de la Condesa, Dos  
193 Hermanas (Seville, Spain), Sanlúcar de Barrameda (Cádiz, Spain), and six samples from  
194 Seville (Spain) showed specific morphological characteristics of *C. felis*. On the other  
195 hand, *P. irritans* was only collected from Seville, while specimens collected from  
196 Corsica (France) were morphological identified as *N. fasciatus* and *S. t. tripectinata*  
197 (Table 1). *Ctenophthalmus b. boisseauorum* and *Ct. a. allani* were isolated from Asturias  
198 (Spain), *A. erinacei* was collected from hedgehogs from Dos Hermanas and Almonte  
199 localities (Spain), whereas *L. taschenbergi* species was identified among fleas collected  
200 from the Pyrenean area (Table 1).

### 201 3.2 Molecular detection of microorganisms

202 All fleas were individually tested to assess the prevalence of certain microorganisms  
203 using molecular methods (Table 3). All the samples showed qPCR negative results for  
204 the presence of *Leishmania* spp., *C. burnetii* and *Borrelia* spp. *Wolbachia* DNA was  
205 detected in *C. felis*, *P. irritans*, *A. erinacei* and *Ct. b. boisseauorum* with a prevalence of  
206 60.4 %, 95.7 %, 55.5 % and 16.7 %, respectively.

207 *Rickettsia felis* was also detected in *C. felis*, *A. erinacei* and *Ct. b. boisseauorum* with a  
208 prevalence of 28.3 %, 33.3 % and 1.6 %, respectively (Table 3). *Rickettsia typhi* was  
209 detected in one specimen of *A. erinacei* from Almonte (Spain). *Mycobacterium* DNA  
210 was detected in one specimen of three different species belonging to Pulicidae family  
211 (*C. felis*, *P. irritans* and *A. erinacei*) (2.0 %, 4.3 % and 5.5 % of prevalence,

212 respectively) targeting the internal transcribed spacer (Table 3). However, all attempts  
213 to amplify a fragment of *rpoB* gene to formally identify the *Mycobacterium* species  
214 failed. Lastly, the screening of all fleas for *Bartonella* spp. revealed that *Bartonella*  
215 DNA was present in *P. irritans*, *S. t. tripectinata*, *N. fasciatus*, *L. taschenbergi*, *Ct. b.*  
216 *boisseauorum* and *Ct. a. allani*. Using qPCR approaches, we were able to identify *B.*  
217 *henselae* in *S. t. tripectinata* and *N. fasciatus* with prevalence ranging from 9.1 % to  
218 13.0 % but also *B. quintana* in *S. t. tripectinata* with prevalence of 13.6 % (Table 3).  
219 The remaining *Bartonella* spp. positive samples were subjected to standard PCR  
220 targeting 16S-23S Intergenic Spacer Region (ITS region) for specific *Bartonella*  
221 identification using sequencing. Unfortunately, we were not able to amplify the ITS  
222 region of all qPCR-*Bartonella* spp. positive samples, thus, in these cases we did not  
223 manage to identify these pathogens at species level. BLAST analysis was carried out  
224 using the *Bartonella*-ITS sequences obtained and showed different percentages of  
225 identity for each sample. *Bartonella elizabethae* was detected in *N. fasciatus*, *S. t.*  
226 *tripectinata*, *L. taschenbergi* and *Ct. b. boisseauorum* with a percentage of identity  
227 ranging from 98 % to 99 % with a reference sequence of *B. elizabethae* (GenBank  
228 ACCN JF766266). *Bartonella taylorii* was detected with a prevalence of 6.6% in each  
229 of the 3 species *L. taschenbergi*, *Ct. b. boisseauorum* and *Ct. a. allani*, with percentages  
230 of identity ranging from 96 % to 99 % with *B. taylorii* (GenBank ACCN AJ269788).  
231 Lastly, the ITS sequences of *Bartonella* spp. obtained for two samples of *L.*  
232 *taschenbergi* were of too poor quality to be considered for further analyses and to allow  
233 species identification. Therefore, these two samples were subsequently subjected to  
234 sequencing targeting two other genes (*ftsZ* and *gltA*). One of these samples showed a  
235 percentage of identity of 97 % and 100 % with *Bartonella doshiae* for *ftsZ* and *gltA*  
236 genes, respectively (GenBank ACCN AF467754 and AF207827). The *ftsZ* gene did not

237 allow species identification of the last *Bartonella* spp. carried by *L. taschenbergi*. It  
238 showed 97,30% of identity with several *Bartonella* spp., such as *B. elizabethae*, *B.*  
239 *queenslandensis*, *B. kosoyi*, *B. tribocorum* and Candidatus *Bartonella mastomydis*.  
240 Using a fragment of the *gltA* gene revealed a percentage of identity of 98,58 % with *B.*  
241 *rochalimae* and Candidatus *Bartonella rudakovii*. All the sequences obtained in this  
242 study are now available on GenBank database (GenBank ACCN LR746174-LR746190  
243 and LR746192-LR746195) and in supplementary data 1.

244 The phylogenetic tree based on the 16S-23S Intergenic Spacer Region (ITS region) of  
245 *Bartonella* spp. showed our sequences clustering with different *Bartonella* species in  
246 two different clades with high bootstrap and BPP values (Fig. 2). Some of the sequences  
247 obtained from *N. fasciatus*, *S. t. tripectinata*, *L. taschenbergi* and *Ct. b. boisseauorum*  
248 fleas appeared clustering in politomy with *B. elizabethae* whereas 12 ITS sequences of  
249 *Bartonella* sp. detected in *Ct. a. allani*, *L. taschenbergi* and *Ct. b. boisseauorum*  
250 clustered in politomy with *B. taylorii* in the same clade (Fig. 2). On the other hand, the  
251 phylogenetic trees based on *ftsZ* gene of two *Bartonella* sequences amplified from *L.*  
252 *taschenbergi* and different *Bartonella* species, showed one sample clustered together  
253 with *B. doshiae* with high bootstrap values (Fig. 3). The second sample appeared  
254 closely related to *B. elizabethae*, *B. kosoyi*, *B. tribocorum*, Candidatus *Bartonella*  
255 *mastomydis* and *B. queenslandensis* (Fig. 3). The sequences obtained for the *gltA* gene  
256 were too short to be used for phylogenetic tree plotting. Indeed, the targeted fragment  
257 was of 200 base pair only.

#### 258 **4. Discussion**

259 Our results support the role of *Wolbachia* spp. as a common endosymbiont of *C. felis*  
260 and *P. irritans*. They are in agreement with other studies, which have shown a high

261 prevalence of this bacterium in these two flea species in Malaysia and Ecuador [56, 57].  
262 Furthermore, as far as we know, in this study we detected for the first time *Wolbachia*  
263 spp. in *A. erinacei* and *Ct. b. boisseauorum*, supporting the hypothesis of Dittmar &  
264 Whiting [58] who placed *Wolbachia* spp. as a common symbiont of Siphonaptera  
265 Order. Dittmar & Whiting [58] carried out their study in flea populations from  
266 Neotropical and Nearctic areas; therefore, based on our results we could suggest that  
267 there is a widespread distribution of *Wolbachia* spp. in Palearctic flea populations too.  
268 Although *Wolbachia* spp. is not considered a pathogenic agent, this endosymbiont has  
269 been widely studied in arthropod species in the last years for several reasons. *Wolbachia*  
270 spp. endosymbionts have been widely studied for their ability to affect the reproductive  
271 system of arthropod hosts and their capacity to modify their mtDNA genetic diversity  
272 [16]. It was recently reported that *Wolbachia* endosymbionts on fleas shape the  
273 reproductive success of their hosts according to their selective environment [59].  
274 Moreover, Zurita et al. suggested a possible introgression of *C. canis cox1* mtDNA into  
275 *C. felis* by *Wolbachia pipientis* [58]. The impact of *Wolbachia* spp. on the transmission  
276 of arthropod-borne pathogens due to a possible antagonistic effect between *Wolbachia*  
277 spp. and other bacteria – which could decrease cases of co-infection – has also been  
278 reported [60, 61]. Nevertheless, according to Dittmar & Whiting [58] and Zurita et al.  
279 [16], more exhaustive studies are needed to support this hypothesis in the context of  
280 flea-borne pathogens since the number of studies about the role of *Wolbachia* spp. in  
281 fleas is still scarce.

282 *Rickettsia felis* is an emergent bacterial pathogen infecting humans, causing fever,  
283 fatigue, headache, and generalized maculopapular rash and inoculation eschar [62].  
284 Recent studies have suggested that these rickettsiae could be a major agent of fever of  
285 unknown origin in Sub-Saharan Africa (FUO) [63]. Its main reservoir and vector are

286 known to be the cat flea (*C. felis*) [62, 64] but *R. felis* has been detected in 12 species of  
287 fleas, 8 species of ticks and 3 species of mites [64]. Further studies are still needed to  
288 confirm the role of these species as possible vectors of this infectious agent. Further  
289 epidemiological studies revealed that *R. felis* was detected in African mosquitoes [65,  
290 66], and it was later demonstrated that *Anopheles gambiae* mosquitoes may act as  
291 competent vectors for the transmission of this bacterium [67]. Since then, *R. felis* has  
292 been detected in various mosquito species from different countries [68, 69].

293 In our study, *R. felis* was detected in *C. felis*, *A. erinacei* and *Ct. b. boisseauorum*. These  
294 results support the idea that this pathogen is well established in Asturias (North of  
295 Spain) and Andalusia (South of Spain). Additional studies conducted in this region have  
296 reported the presence of this emerging flea-borne pathogen in fleas, cats and humans in  
297 the last years using serological and molecular techniques [70, 71, 72, 73]. These studies  
298 reported that *R. felis*' prevalence ranged from 6.5 % [72] to 36.8 % [73]. Our results are  
299 in agreement with Brown & Macaluso [74] who considered *C. felis* as the primary  
300 vector and reservoir of *R. felis*. These authors related the worldwide distribution of this  
301 pathogen, associated with this flea species. *Rickettsia felis* had already been reported in  
302 *A. erinacei* collected in Europe with a prevalence ranging from 11 % [75] to 100 %  
303 [76]. Further investigation is still necessary in order to assess the role of this flea and its  
304 hedgehog host in the transmission of *R. felis* [77]. De Sousa et al. [78] detected *R. felis*  
305 in one specimen of *Ctenophthalmus* sp. collected in Portugal, however these authors did  
306 not carry out the identification of this specimen to the species level. Our study would  
307 therefore constitute the first evidence of the presence of this pathogen in the species *Ct.*  
308 *b. boisseauorum*. Our data is too limited to consider a role of this flea species as a  
309 vector of *R. felis* yet; however, these results should be considered in order to carry out  
310 further epidemiological studies that assess this fact.

311 *Rickettsia typhi* is the causative agent of murine typhus or endemic typhus [1]. This  
312 disease is widespread and occurs in some areas where commensal rats (*Rattus rattus*  
313 and closely related species like *Rattus norvegicus*) and the Oriental rat flea (*X. cheopis*)  
314 are found [10]. Murine typhus in humans is often relatively mild, thus common signs  
315 and symptoms include a characteristic rickettsial rash (macular in appearance), fever,  
316 headache, chills, achiness, and prostration [1]. *Rickettsia typhi* has been widely studied  
317 in Spain by serological and molecular methods [79, 80]. It has only been detected in *C.*  
318 *felis* species in this country and most clinical cases occur in two regions: Andalusia [81]  
319 and the Canary Islands [82, 83]. Although *X. cheopis* and other *Xenopsylla* species such  
320 as *Xenopsylla astia* and *Xenopsylla brasiliensis* are considered the main vectors of *R.*  
321 *typhi* in most locations around the world, this bacterium has been detected in other flea  
322 species associated with rodents such as *N. fasciatus* and *Leptopsylla segnis* [78].  
323 Furthermore, it has been demonstrated that even the so-called human flea *P. irritans* is  
324 an efficient vector for *R. typhi* under laboratory conditions [1]. In our study, we detected  
325 for the first time the causative agent of murine typhus in the flea species *A. erinacei*,  
326 with a prevalence of 5.5 %. With this species and its hosts (*E. europaeus*) so close to  
327 humans, experimental models should be conducted to investigate the potential role of  
328 this flea in the transmission of *R. typhi*.

329 Unlike *R. felis* and *R. typhi*, which prevalence has been widely studied in fleas from  
330 several countries, to the best of our knowledge, this work constitutes the first attempt to  
331 characterize *Mycobacterium* spp. detected in fleas. Different species of this genus such  
332 as *Mycobacterium ulcerans*, the etiological agent of the Buruli ulcer, have recently been  
333 detected by qPCR in water bugs [84, 85] and mosquitoes [86]. In this study, we detected  
334 *Mycobacterium* spp. in three fleas from Seville belonging to three different species (*C.*  
335 *felis*, *P. irritans* and *A. erinacei*). Unfortunately, we were not able to amplify and

336 sequence *Mycobacterium* qPCR-positive samples by standard PCR in order to obtain an  
337 identification to the species level. This fact could be explained by the higher sensitivity  
338 of qPCR compared to standard PCR [87]. Therefore, although we could not determine  
339 which species of *Mycobacterium* we dealt with, this study constitutes the first report of  
340 the detection of *Mycobacterium* spp. in fleas. In this sense, this bacterium genus should  
341 be included in flea epidemiological studies in order to increase the knowledge about  
342 *Mycobacterium* species potentially carried by fleas. Nevertheless, experimental models  
343 would still be needed to evaluate if fleas could really play a role in *Mycobacterium* spp.  
344 transmission.

345 Nowadays, 11 species of *Bartonella* are known to cause human diseases [88]. These  
346 infections can result in significant complications such as endocarditis, bacillary  
347 angiomatosis, peliosis hepatis, chronic bacteremia, chronic lymphadenopathy, or  
348 neurological disorders [88]. Particularly, *B. henselae* and *B. clarridgeiae* are considered  
349 the main causative agents of cat scratch disease, while *B. quintana*, *B. elizabethae*, *B.*  
350 *henselae*, or *B. koehlerae* have been related to human and animal endocarditis in several  
351 countries [89, 90]. A large list of mammals is considered as reservoirs of *Bartonella*  
352 spp., out of which several species are kept as pets [91]. Arthropods are known to  
353 transmit *Bartonella* spp. either through saliva (fleas, sand flies) or through their feces  
354 (fleas and lice) [92].

355 Within these pathogenic *Bartonella* species, at least 8 (*B. henselae*, *B. clarridgeiae*, *B.*  
356 *quintana*, *B. koehlerae*, *B. tribocorum*, *B. taylorii*, *B. grahamii* and *B. elizabethae*) have  
357 been detected in different flea species worldwide by molecular methods [3]. Five  
358 different species of *Bartonella* infesting fleas were detected in this study: *B. quintana*,  
359 *B. henselae*, *B. taylorii*, *B. elizabethae* and *B. doshiae*. This genus showed a prevalence  
360 of 8.7 % in *P. irritans* isolated from Seville. Our results are in agreement with Márquez



361 et al. [93] who observed a prevalence of 31.8 % of this pathogenic bacterium in *P.*  
362 *irritans* isolated from red foxes from Andalusian region (Huelva and Jaén). On the other  
363 hand, three different species of *Bartonella* (*B. quintana*, *B. henselae* and *B. elizabethae*)  
364 were detected in *S. t. tripectinata* isolated from Corsica (France), whereas *B. elizabethae*  
365 and *B. henselae* were detected in *N. fasciatus* collected from the same island. This  
366 completes recent results from the Canary Islands reporting *B. elizabethae* as the sole  
367 *Bartonella* species found in *S. t. tripectinata* fleas [94]. As far as we know, this work is  
368 the first investigation on the prevalence of *Bartonella* species in fleas from this  
369 geographical area. Furthermore, *L. taschenbergi* was shown to harbor *B. doshiae* and *B.*  
370 *elizabethae*, the latter was also detected in *Ct. b. boisseauorum*. This work provides an  
371 updated list of flea species which could potentially play a role in the transmission of this  
372 pathogen in Europe.

373 Lice are considered the main vector in the transmission of *B. quintana* [95]; but, Kernif  
374 et al. [96] reported the fact that cat fleas (*C. felis*) can transmit this bacterium by  
375 releasing viable microorganisms into their faeces. Thus, they suggested that fleas might  
376 play a role as vectors of trench fever or other infections caused by *B. quintana*. In our  
377 study, it should be noted the presence of *B. quintana*, the causative agent of trench  
378 fever, was detected in *S. t. tripectinata* fleas.

379 Based on epidemiological data provided in this work it seems plausible that different  
380 *Bartonella* species could be circulating among diverse rodent genus (*Rattus* sp.,  
381 *Arvicola* sp., *Apodemus* sp and *Mus* sp.) and infecting different flea species in Corsica  
382 Island and North of Spain. Although the vectorial competence of these flea species  
383 needs to be confirmed with experimental studies, the zoonotic character of these  
384 infections must alert public health authorities and the veterinary community about the  
385 risk of possible diseases associated with these flea species and their rodent hosts.

386 As for *B. taylorii*, it was detected in three different species of rodent fleas: *Ct. b.*  
387 *boisseauorum*, *Ct. a. allani* and *L. taschenbergi*, with the prevalence (6.6 %). This  
388 bacterium can cause infection in animals, but the real pathogenic potential of this  
389 *Bartonella* species is yet unknown [97]. Nevertheless, the fact that rodent fleas could be  
390 substantial vectors for the transmission of *B. taylorii* among rodents has been reported  
391 in several former studies [97, 98, 99, 100]. Therefore, in the last years, *B. taylorii* has  
392 been detected in more and more flea species encouraging veterinary and medical  
393 community to assess the possible pathogenic potential of this *Bartonella* species in  
394 humans and animals and to investigate which flea species could be implicated in the  
395 transmission of this bacterium. Lastly, complementary molecular and phylogenetic  
396 analysis based on *ftsZ* and *gltA* genes allowed us to detect *B. doshiae* in one specimen of  
397 *L. taschenbergi* collected from the French Pyrenees. This bacterium was detected in  
398 fleas for the first time using rodent fleas collected from Afghanistan [100]. Our results  
399 constitutes the first evidence of the presence of this bacterium in fleas from Western  
400 Europe. It is noteworthy, that Vayssier-Taussat et al. [101] found this species in the  
401 blood of French human patients reporting tick bites. These patients exhibited non-  
402 specific symptoms, suggesting a zoonotic potential of this bacterium.

403 Phylogenetic analysis based on *ftsZ* and *gltA* genes did not allow to characterize the  
404 second *Bartonella* species detected in *L. taschenbergi*. Thus, it would be necessary to  
405 conduct complementary studies involving the isolation of the possible novel strain of  
406 *Bartonella* spp. in order to confirm a possible new species.

## 407 **5. Conclusions**

408 With this study we updated the knowledge of the flea-borne pathogens present in some  
409 regions of south of France and Spain. Additional studies are needed to expand the

410 current knowledge on flea-borne pathogens and characterize new host-vector  
411 relationships. They should provide additional data on the epidemiology the clinical  
412 features of flea-borne diseases, which would allow the establishment of appropriately  
413 targeted vector control programmes.

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724 **Figure captions:**

725 **Figure 1.** Photos of fleas isolated for this study. 1. *Ctenocephalides felis* 2. *Pulex*  
726 *irritans* 3. *Ctenophthalmus apertus allani* 4. *Archaeopsylla erinacei* 5. *Stenoponia*  
727 *triptectinata triptectinata* 6. *Leptopsylla taschenbergi* 7. *Ctenophthalmus baeticus*  
728 *boisseauorum* 8. *Nosopsyllus fasciatus*.

729 **Figure 2.** Phylogenetic tree of *Bartonella* spp. based on 16S-23S Intergenic Spacer  
730 Region (ITS region) inferred using the Bayesian (B) and Maximum-Likelihood (ML)  
731 methods and Bayesian topology. The percentage of replicate trees in which the  
732 associated taxa clustered together in the bootstrap test (1000 replicates) is shown on the  
733 branches (B/ML). Bootstrap and BPP values lower than 60 % are not shown. Bayesian  
734 posterior probabilities (BPP) are percentage-converted. (\*) A total of four ITS  
735 sequences of *Bartonella* spp., which did not, showed nucleotide differences among  
736 them. In bold those sequences, which showed low percentage of homology with *B.*  
737 *rochalimae* in BLAST analysis.

738 **Figure 3.** Phylogenetic tree of *Bartonella* spp. based on *ftsZ* partial gene inferred using  
739 Maximum-Likelihood (ML) and Neighbor-Joining (NJ) methods and ML topology. The  
740 percentage of replicate trees in which the associated taxa clustered together in the  
741 bootstrap test (1000 replicates) is shown on the branches (ML/NJ). Bootstrap values



742 **Table 1. Geographical origin, number and sex, preservation method, date of collection and hosts of eight different species of fleas used in**  
 743 **this study.**

Species	Geographical area	Number of samples (male/female)	Preservation method	Date of collection	Hosts
<i>C. felis</i>	Seville (Spain)	7 (1/6)	96 % Ethanol	May 2016	Neglected horse stable with a flea infestation
	Villamanique de la Condesa, Seville (Spain)	13 (3/10)	96 % Ethanol	August 2014	1 owned dog ( <i>Canis lupus familiaris</i> )
	Dos Hermanas, Seville (Spain)	12 (1/11)	96 % Ethanol	January-June 2016	2 owned dogs ( <i>Canis lupus familiaris</i> )
	Sanlúcar de Barrameda, Cádiz (Spain)	21 (1/20)	70 % Ethanol	March-September 2011	3 owned dogs ( <i>Canis lupus familiaris</i> )
<i>P. irritans</i>	Seville (Spain)	23 (10/13)	96 % Ethanol	May 2016	Neglected horse stable with a flea infestation
<i>N. fasciatus</i>	Corse (France)	23 (5/18)	70 % Ethanol	January-June 2016	6 rodents ( <i>Rattus</i> sp.)
<i>S. t. tripectinata</i>	Corse (France)	22 (1/21)	70 % Ethanol	January-June 2016	5 rodents ( <i>Rattus</i> sp., <i>Mus</i> sp., <i>Apodemus</i> sp.)
<i>A. erinacei</i>	Almonte, Huelva (Spain)	16 (5/2)	96 % Ethanol	April 2018	3 hedgehogs ( <i>Erinaceus europaeus</i> )
	Dos Hermanas, Seville (Spain)	2 (0/2)		March 2017	
<i>L. taschenbergi</i>	Conat, Pyrenee (France)	16 (6/10)	70 % Ethanol	June-September 2016	12 rodents ( <i>Apodemus</i> sp.)
	Py, Pyrenee (France)	44 (20/24)			
<i>Ct. b. boisseauorum</i>	Asturias (Spain)	60 (60/0)	70 % Ethanol	June 2016	13 rodents ( <i>Arvicola terrestris</i> )
<i>Ct. a. allani</i>	Asturias (Spain)	60 (60/0)	70 % Ethanol	June 2016	16 rodents ( <i>Arvicola terrestris</i> )

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749 **Table 2. Primers and probes references used for real-time quantitative and standard PCR in this study.**

<b>qPCR</b>		
<b>Microorganism</b>	<b>Targeted sequence</b>	<b>Reference</b>
<i>Rickettsia</i> spp.	<i>gltA</i>	[30]
<i>R. felis</i>	<i>Phosphatase gene</i>	[31]
<i>R. typhi</i>	Hypothetical protein	[32]
<i>Bartonella</i> spp.	ITS2	[33]
<i>B. henselae</i>	<i>Pap31</i>	[34]
<i>B. quintana</i>	<i>yopP</i>	[35]
<i>Borrelia</i> spp.	ITS4	[36]
	16S	[37]
Anaplasmataceae spp.	23S	[38]
<i>Wolbachia</i> spp.	23S	[39]
<i>Leishmania</i> spp.	Kinetoplatic DNA	[40]
<i>C. burnetii</i>	IS30A	[41]
	IS1111A	[42]
<i>Mycobacterium</i> spp.	ITS	[43]
<b>Standard PCR</b>		
<i>Bartonella</i> spp.	ITS	[44]
<i>Bartonella</i> spp.	<i>ftsZ</i>	[45]
<i>Bartonella</i> spp.	<i>gltA</i>	[46]
<i>Mycobacterium</i> spp.	<i>rpoB</i>	[47]

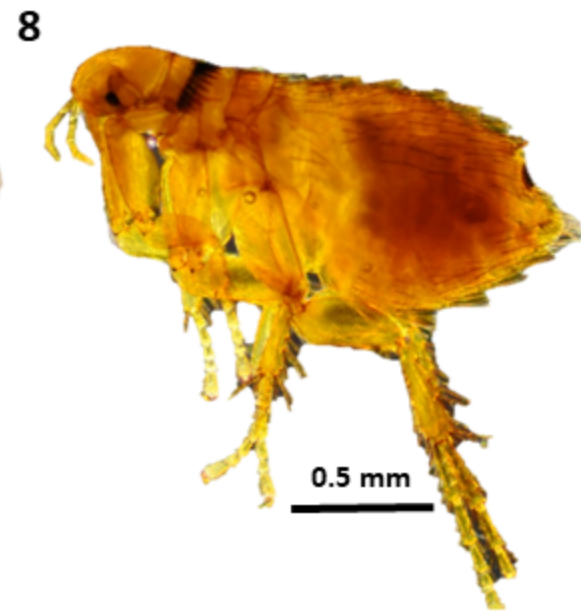
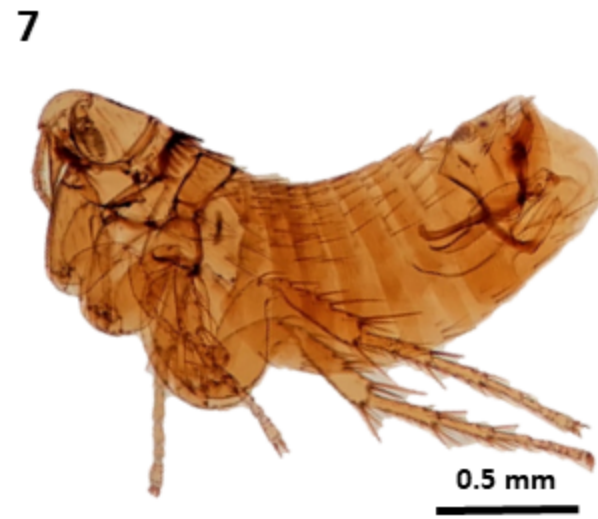
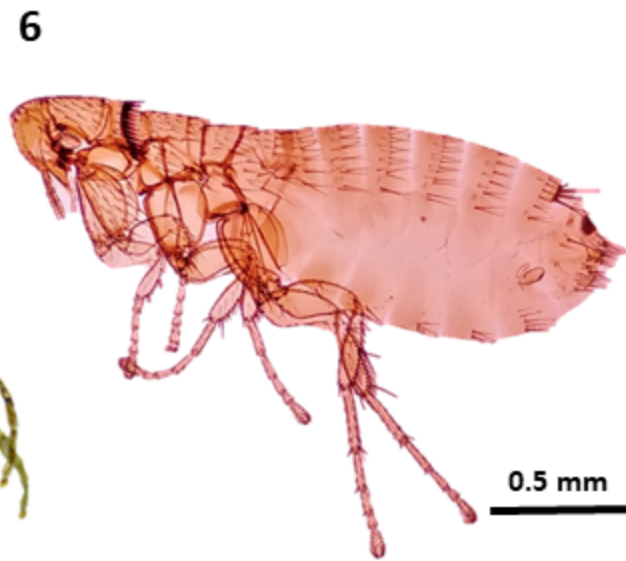
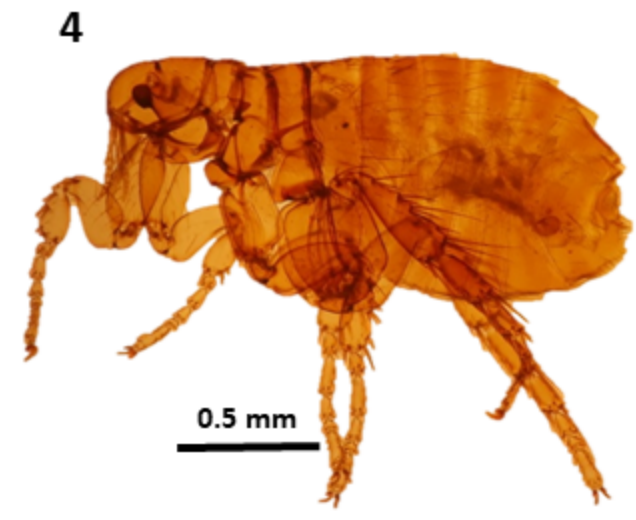
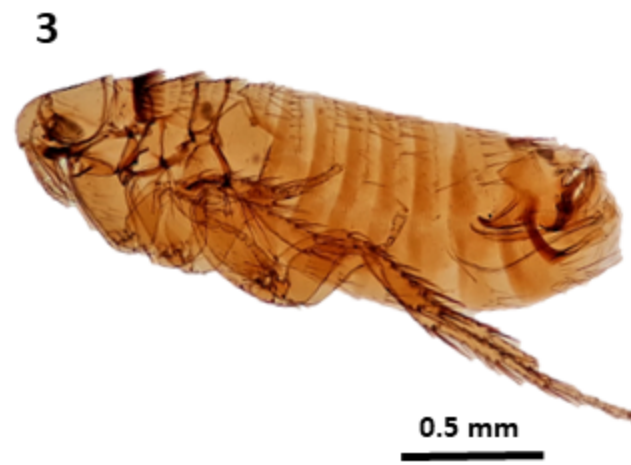
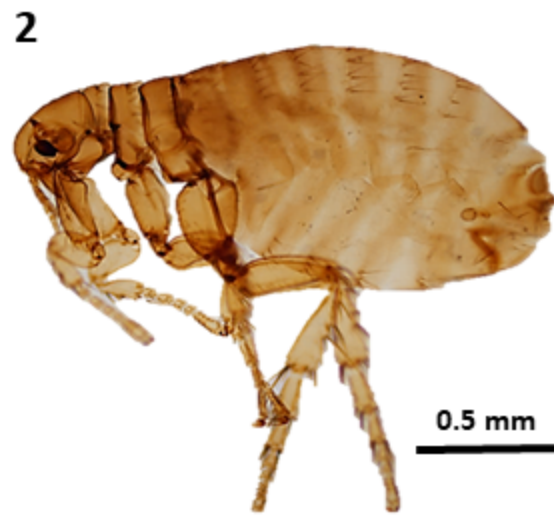
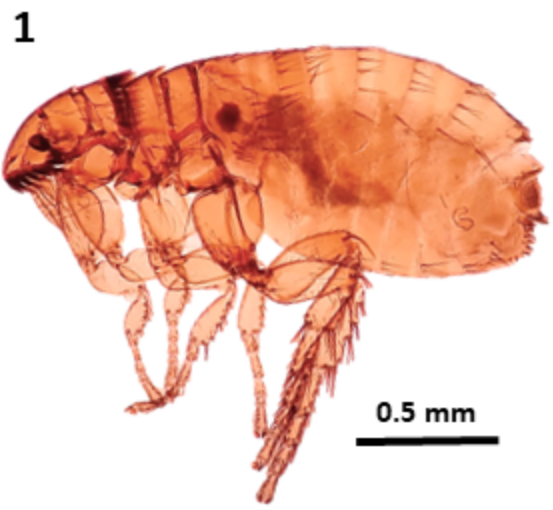
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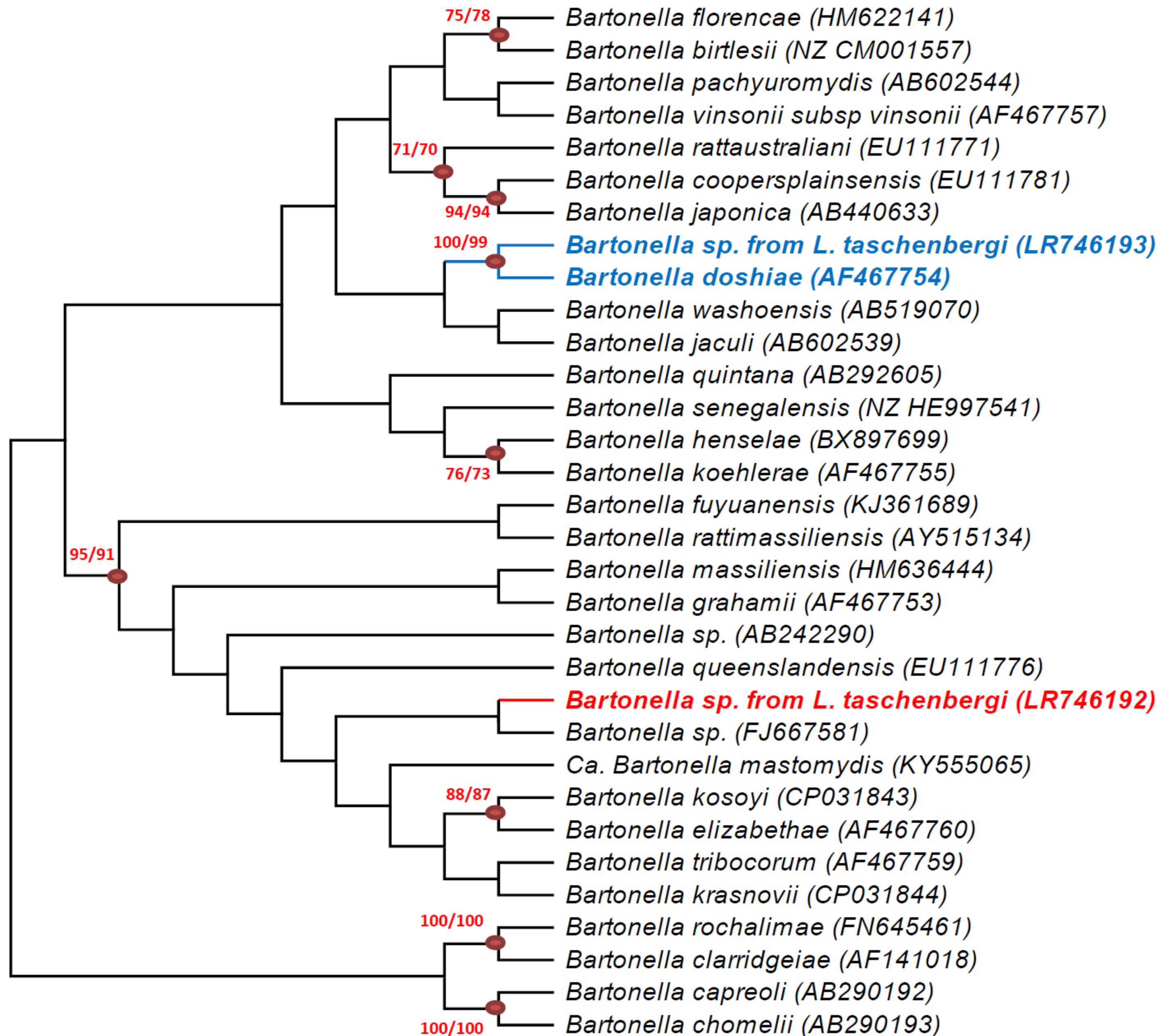
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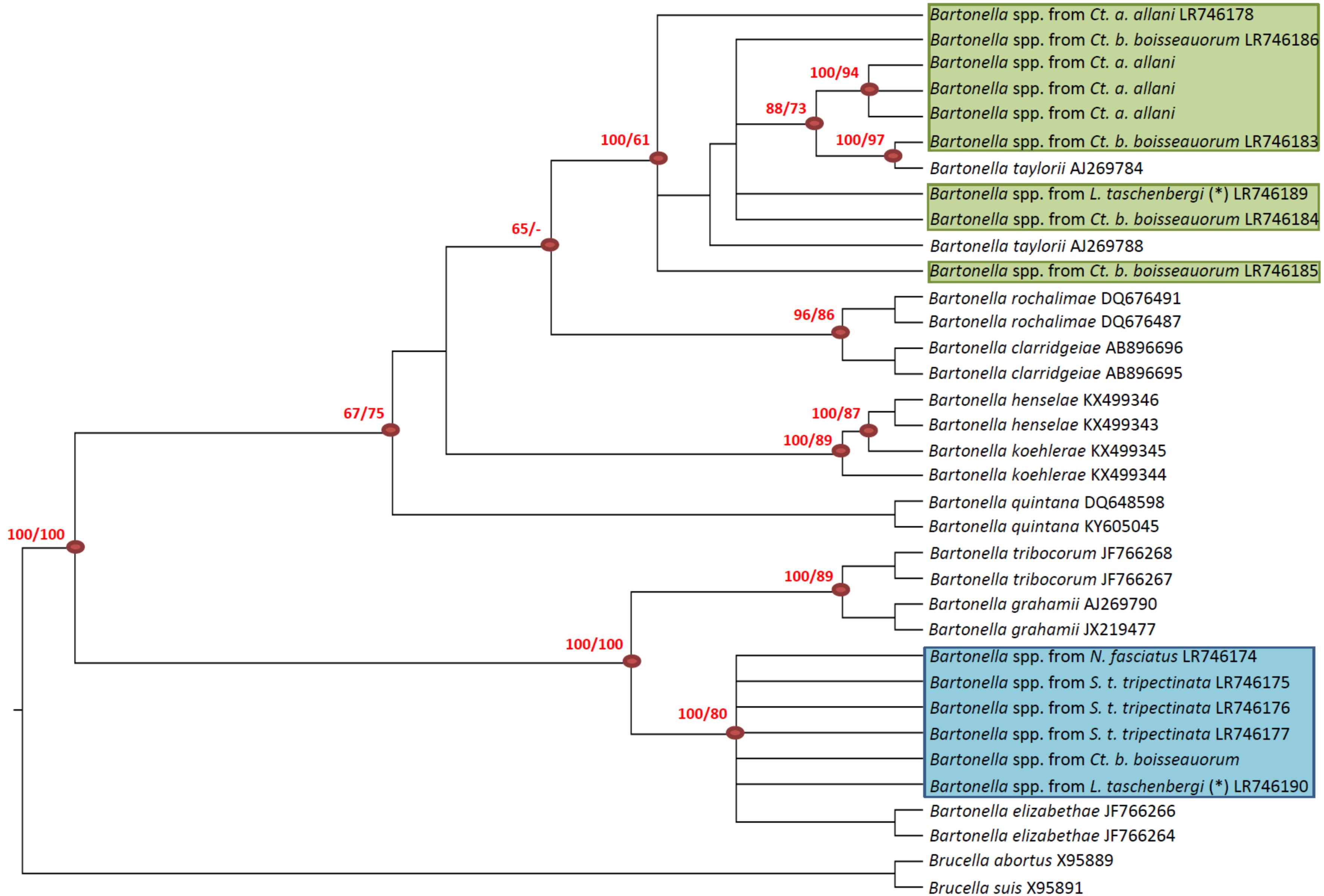
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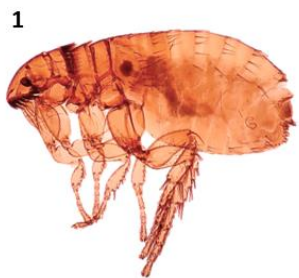
753 **Table 3. Percentage of microorganism-positive fleas detected by PCR (qPCR and standard PCR).**

	<b>Anaplasmataceae</b>	<b>Rickettsia sp.</b>	<b>R. typhi</b>	<b>C. burnetti</b>	<b>Mycobacterium spp.</b>	<b>Borrelia spp.</b>	<b>Bartonella spp.</b>	<b>Leishmania spp.</b>
<b>C. felis</b>	32/53( <i>Wolbachia</i> spp.) (60.4 %)	15/53 ( <i>R. felis</i> ) (28.3 %)	-	-	1/53 (2.0 %)	-	-	-
<b>P. irritans</b>	22/23( <i>Wolbachia</i> spp.) (95.7 %)	-	-	-	1/23 (4.3 %)	-	2/23 ( <i>Bartonella</i> spp.) (8.7 %)	-
<b>S. t. tripectinata</b>	-	-	-	-	-	-	2/22( <i>B. henselae</i> ) (9.1 %); 3/22 ( <i>B. quintana</i> ) (13.6 %) 3/22 ( <i>B. elizabethae</i> ) (13.6 %)	-
<b>N. fasciatus</b>	-	-	-	-	-	-	3/23 ( <i>B. henselae</i> ) (13.0 %); 1/23( <i>B. elizabethae</i> ) (4.3 %) 3/23 ( <i>Bartonella</i> spp.) (13.0 %)	-
<b>A. erinacei</b>	10/18( <i>Wolbachia</i> spp.) (55.5 %)	6/18 ( <i>R. felis</i> ) (33.3 %)	1/18 (5.5 %)	-	1/18 (5.5 %)	-	-	-
<b>L. taschenbergi</b>	-	-	-	-	-	-	4/60 ( <i>B. taylorii</i> ) (6.6 %); 4/60 ( <i>B. elizabethae</i> ) (6.6 %) 2/60 ( <i>Bartonella</i> spp.) (3.3 %) 1/60 ( <i>B. doshiae</i> ) (1.6 %)	-
<b>Ct. b. boisseauorum</b>	10/60( <i>Wolbachia</i> spp.) (16.7 %)	1/60 ( <i>R. felis</i> ) (1.6 %)	-	-	-	-	4/60 ( <i>B. taylorii</i> ) (6.6%); 1/60 ( <i>B. elizabethae</i> ) (1.6%)	-
<b>Ct. a. allani</b>	-	-	-	-	-	-	4/60 ( <i>B. taylorii</i> ) (6.6 %); 1/60 ( <i>Bartonella</i> spp.) (1.6%)	-

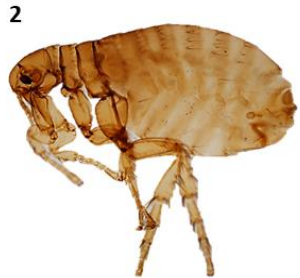








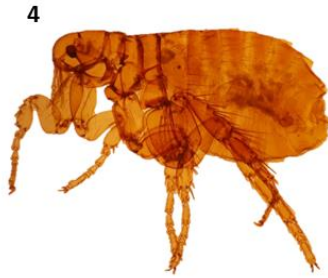
*C. felis*



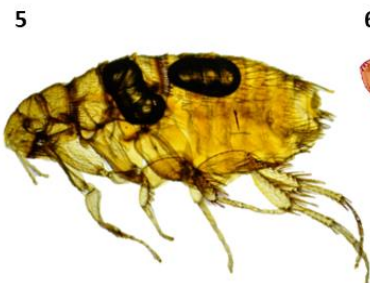
*P. irritans*



*Ct. apertus allani*



*A. erinacei*



*St. t. tripectinata*



*L. taschenbergi*



*Ct. b. boisseauorum*



*N. fasciatus*

