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1 **Microorganisms associated with the North African hedgehog *Atelerix algirus* and its**  
2 **parasitizing arthropods in Algeria**

3 Nawal Aouadi<sup>a,◇</sup>, Linda Benkacimi<sup>b,c,◇</sup>, Adama Zan Diarra<sup>b,c</sup>, Maureen Laroche<sup>b,c</sup>, Jean-Michel  
4 Bérenger<sup>b,c</sup>, Idir Bitam<sup>c,d</sup>, Philippe Parola<sup>b,c,\*</sup>

5  
6  
7 <sup>a</sup> Laboratoire de Valorisation et Conservation des Ressources Biologiques (VALCOR),  
8 Faculté des Sciences, Université M'Hamed Bougara, Boumerdes, Algeria.

9 <sup>b</sup> Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France.

10 <sup>c</sup> IHU Méditerranée Infection, 13005 Marseille, France.

11 <sup>d</sup> École Supérieure en Sciences de l'Aliment et des Industries Agroalimentaires (ESSAIA), El  
12 Harrach, Alger, Algeria.

13  
14  
15  
16  
17 <sup>◇</sup>Equal contributors

18  
19 \*Corresponding author: Prof. Philippe PAROLA, VITROME, Institut Hospitalo-Universitaire  
20 Méditerranée Infection, 19-21 Boulevard Jean Moulin 13005 Marseille, France. Phone: + 33  
21 (0) 4 13 73 24 01. Fax: + 33 (0) 4 13 73 24 02. Email address: [philippe.parola@univ-amu.fr](mailto:philippe.parola@univ-amu.fr)

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26 **Abstract**

27 Hedgehogs are small mammals. They are potential reservoirs of various zoonotic agents. This  
28 study was conducted in Bouira, a north-central region of Algeria. A total of 21 *Atelerix*  
29 *algirus* corpses were picked up on roadsides and gardens. Hedgehog kidneys, spleens and  
30 ectoparasites were collected. Twelve hedgehogs were infested with ectoparasites, including  
31 *Archaeopsylla erinacei*, *Rhipicephalus sanguineus* s.l. and *Haemaphysalis erinacei*.  
32 Hedgehog organs and randomly selected arthropods were screened for microorganisms using  
33 molecular methods. *Coxiella burnetii* was detected in kidneys, spleens, *A. erinacei*, *Hae.*  
34 *erinacei* and *Rh. sanguineus* s.l. *Leptospira interrogans* was detected in kidneys. *Rickettsia*  
35 *felis* and *Rickettsia massiliae* were detected respectively in *A. erinacei* and in *Rh. sanguineus*  
36 s.l. DNA of an uncultivated *Rickettsia* spp. was found in *Hae. erinacei*. *Wolbachia* spp. DNA  
37 was detected in fleas. The DNA of potential new *Bartonella* and *Ehrlichia* species were found  
38 respectively in fleas and ticks. This study highlights the presence of DNA from a broad range  
39 of microorganisms in hedgehogs and their ectoparasites that may be responsible for zoonoses  
40 in Algeria.

41 **Keywords:** Hedgehogs; ticks; fleas; microorganisms; zoonoses; Algeria.

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## 51        **1. Introduction**

52 Hedgehogs are small spiny-coated mammals. They are a common synanthropic species living  
53 in burrows, not only in rural environments, but also in urban and suburban ones [1–3]. They  
54 are solitary, nocturnal and omnivorous animals belonging to the Erinaceidae family [1–3].  
55 Five genera and about seventeen species are distributed on different continents, including six  
56 species living in the Mediterranean basin: *Atelerix algirus*, *Erinaceus concolor*, *Erinaceus*  
57 *europaeus*, *Erinaceus roumanicus*, *Hemiechinus auratus* and *Paraechinus aethiopicus*. They  
58 are all classified as Least Concern (LC) in the list of species threatened with extinction except  
59 *P. aethiopicus* which is classified as Data Deficient (DD) [1–3]. Hedgehogs are often  
60 parasitized by fleas, including *Archaeopsylla erinacei*, *Ctenocephalides canis*,  
61 *Ctenocephalides felis* and *Leptopsylla segnis* [2–5] and ticks, including *Rhipicephalus*  
62 *sanguineus* s.l., *Haemaphysalis erinacei*, *Haemaphysalis parva*, *Ixodes hexagonus*, *Ixodes*  
63 *ricinus* and *Hyalomma excavatum* [2–4,6]. In recent decades, the development of molecular  
64 tools has made it possible to identify microorganisms infecting hedgehogs and their  
65 ectoparasites [7]. Previous studies have reported the presence of *Anaplasma*  
66 *phagocytophilum*, the agent of Granulocytic Anaplasmosis in European hedgehogs (*Erinaceus*  
67 *europaeus*), northern white-breasted hedgehogs (*Erinaceus roumanicus*) and their ticks  
68 [6,8,9]. Furthermore, *Candidatus Neoehrlichia mikurensis*, an emerging tickborne pathogen  
69 responsible for a severe atypical infection in humans and hitherto associated only with rodent  
70 reservoirs, has been detected in urban *E. roumanicus* hedgehogs from Budapest, Hungary  
71 [10]. *Borrelia burgdorferi* s.l., the agent of Lyme disease, has also been detected in *E.*  
72 *roumanicus* and their ticks and in *E. europaeus* [9,11]. Recently, spotted fever group  
73 rickettsial agents (*Rickettsia conorii*, *R. massiliae*, *R. siberica* and *R. helvetica*) have been  
74 detected in hedgehogs and their ticks [2,9,12–14] and *R. felis* has been reported in fleas  
75 collected from hedgehogs in different countries [2,12,14,15]. Other bacteria, such as *Coxiella*

76 *burnetii*, *Salmonella* spp., *Mycobacterium marinum*, *Yersinia pestis*, *Yersinia*  
77 *pseudotuberculosis* and *Chlamydia psittaci* [7] as well as some protozoa (*Leishmania* spp.,  
78 *Toxoplasma gondii* and *Cryptosporidium*) have also been found in hedgehogs [7,16].  
79 In Algeria, two different species of hedgehogs have been recorded: The North African  
80 hedgehog *Atelerix algirus* and the desert hedgehog *Paraechinus aethiopicus*. They have a  
81 different distribution; *A. Algirus* lives mainly in the north of the country while *P. aethiopicus*  
82 is found most often in the highlands area [17]. Nevertheless, both species can sometimes  
83 coexist in the same area, especially in the north of the country. Indeed, in a study conducted  
84 by Khaldi et al, 2012 both species have been captured in M'sila a North-eastern region of  
85 Algeria. [2,18]. A previous study reported the presence of DNA and antibodies against  
86 *Leishmania major*, the agent of cutaneous leishmaniasis in *A. algirus* and *P. aethiopicus*,  
87 suggesting that these hedgehogs could be a natural reservoir of this zoonosis in Algeria [16].  
88 In addition, *Rickettsia felis* DNA has been detected in *A. erinacei* and *C. felis* collected from  
89 *A. algirus* and in *A. erinacei* from *P. aethiopicus* [2,5,19], and that of *R. massiliae* and *R.*  
90 *conorii* in *Rh. sanguineus* s.l. sampled from hedgehogs [2,20]. Moreover, *Bartonella*  
91 *elizabethae* and *Bartonella clarridgeiae* have been reported in *A. erinacei* collected on  
92 hedgehogs from western Algeria (Oran and Mascara) [21].  
93 Algeria being an endemic region for rickettsioses of the spotted fever group (SFG) as well as  
94 for various other zoonoses [2,22], the identification of potential reservoirs and arthropod  
95 vectors therefore represents an important step in the prevention of these diseases.  
96 The aim of this work was to investigate the presence of various zoonotic microorganisms in  
97 hedgehog organs and in their associated hematophagous arthropods in a north-central region  
98 of Algeria.

99

100

101       **2. Materials and Methods**

102       *2.1. Ethical statement*

103       Consent for this study was provided by the University of M'Hamed Bougara, Boumerdes,  
104       Algeria. No specific approval of any Institutional Animal Care and Use Committee was  
105       needed for this study since the hedgehogs were already dead when picked up and no  
106       anesthetic substances were used for animal handling procedures. Prior import authorization of  
107       animal samples intended for research from outside the European Union (Algeria) to France  
108       has been obtained under the number ER21/17.

109

110       *2.2. Study areas and collection period*

111       The study was carried out over a period of approximately 3 years (from August 2014 to June  
112       2017) in several villages located in 4 different municipalities of Bouira province in the North-  
113       central region of Algeria: Ahl -El-Ksar (Ahl-el-ksar center, Thiliwa, Thigmit, Thalamine and  
114       Ighil Mehallah) (36°15'11"N, 4°2'22"E), Ouled Rached (H'lassa and Assif el Khemis)  
115       (36°12'43"N, 4°6'38"E), Bechloul (Bechloul center) (36°19'00"N, 4°04'00"E) and Bouira  
116       (Oued eddous) (36°22'0"N, 3°52'60"E) (**Figure 1**). These municipalities are located at  
117       different altitudes (414 m for Bechloul, 489 m for Oued Eddhous, 612 m for Ahl-el-ksar and  
118       918 m for Ouled Rached) and are characterized by significant agricultural activity and the  
119       presence of forests. Bouira province has a Mediterranean climate (Köppen climate  
120       classification: Csa) which is characterized by hot, dry summers and mild, wet winters.

121

122       *2.3. Hedgehog collection and sampling of organs and ectoparasites*

123       Hedgehog corpses were collected by hand either on roadsides for those which were hit by  
124       vehicles, in forests or gardens for those which died accidentally poisoned by farmers. They  
125       were transported to the biology laboratory of the University Akli Mohand Oulhadj of Bouira

126 where they were identified and thoroughly inspected for ectoparasites by separating their  
127 quills. Arthropods were collected from hedgehogs using tweezers in a large, light-colored  
128 plastic basin. They were sorted by group, counted, and each group of arthropods from the  
129 same hedgehog was immediately placed in the same tube containing 70% ethanol. The spleen  
130 and kidney of each hedgehog were collected and immediately placed separately in sterile vials  
131 containing 70% ethanol. All samples were stored at room temperature before being sent to the  
132 VITROME laboratory at the IHU Méditerranée Infection, (Marseille, France) for analysis.

133

#### 134 *2.4. Morphological identification of arthropods*

135 All collected arthropods were morphologically identified to the species level initially in  
136 Algeria, using the OPTIKA ST-30, FX-EU 20X binoculars (Via Rigla, Ponteranica BG,  
137 Italia) and identification keys [23–25]. Morphological identification was verified at the IHU  
138 Méditerranée Infection (Marseille, France) using a Zeiss Axio Zoom V16 stereomicroscope  
139 (Zeiss, Marly le Roi, France), a VHX-7000 digital microscope (Keyence, Osaka, Japan) and  
140 identification keys [25,26]. Some randomly selected flea specimens were submitted to KOH  
141 clearing and a bath of Amman's lactophenol before being mounted in Euparal mounting  
142 medium in order to make the morphological criteria more visible. All the information about  
143 arthropod species, hedgehog number, gender, collection site and date were codified, marked  
144 on sample tubes and summarized in a table (**Supplementary Data 1**).

145

#### 146 *2.5. DNA extraction*

147 All hedgehog kidney and spleen samples, 138/218 ticks (including the 28 engorged  
148 specimens) and 119/853 fleas have been randomly selected, rinsed in a bath of ethanol  
149 followed by distilled water, dissected and subjected to DNA extraction. A piece of  
150 approximately 25 mg from each organ, the flea abdomens, half of the unengorged and

151 approximately ¼ of the engorged tick bodies have been transferred individually into 1.5 ml  
152 Eppendorf tubes and incubated overnight at 56°C in 180 µl G2 lysis buffer and 20 µl  
153 proteinase K (Qiagen, Hilden, Germany) to allow tissue lysis [27]. DNA was extracted from  
154 200 µl of each sample in a 100 µl eluate using the EZ1 DNA tissue kit (Qiagen) together with  
155 the EZ1 robot (Qiagen) according to the manufacturer's instructions. The DNA was either  
156 used immediately or stored frozen at -20°C until use.

157

#### 158 *2.6. MALDI-TOF MS identification of collected arthropods*

159 In order to support the morphological identification of arthropods, 92/138 ticks (including 28  
160 engorged specimens) and 26/119 fleas were randomly selected. Tick legs and flea heads and  
161 thoraxes with legs were subjected to MALDI-TOF MS analysis, applying previously  
162 described protocols for specimens preserved in alcohol [27,28]. 1µL of the supernatant of  
163 each sample was deposited in quadruplicate on a MALDI-TOF MS steel plate (Bruker  
164 Daltonics, Wissembourg, France) and covered after drying with 1µL of a matrix solution [29].  
165 The target plate was then introduced into the Microflex LT MALDI-TOF mass spectrometer  
166 (Bruker Daltonics) to obtain spectra according to the parameters already described [30]. The  
167 quality of the obtained spectra (reproducibility, overall intensity and absence of background  
168 noise) was evaluated using Flex analysis v.3.3 (Bruker Daltonics, Germany).  
169 The high-quality spectra were blind tested using MALDI Biotyper v.3.0 software against our  
170 in-lab database containing reference spectra of several arthropod species [31] and updated  
171 with 2 to 4 selected reference spectra of each arthropod species [30]. The log score values  
172 (LSVs) and MALDI-TOF MS species identification resulting from the database queries were  
173 recorded for each tested specimen, to be compared to morphological identification results  
174 (**Table 1**).

175



176        2.7. *Molecular identification of arthropods*

177        The DNA from twenty-eight engorged and sixteen unengorged tick specimens and twenty-six  
178 fleas was randomly selected and subjected to conventional PCR, followed by sequencing in  
179 an automated thermal cycler (Applied Biosystems, Foster City, CA, USA), amplifying a  
180 fragment of approximately 1000 base pairs of the *ITS1* (internal transcribed spacer) gene and  
181 a fragment of 460 base pairs of the tick *16S RNA* gene respectively for the identification of  
182 fleas and ticks, as previously described [32,33]. DNA from unambiguously identified  
183 specimens of *Rh. sanguineus* s.l. and *Ctenocephalides felis* bred in our laboratory were used  
184 as positive controls. A DNA-free PCR mixture was used as a negative control.

185

186        2.8. *Molecular screening of microorganisms in hedgehogs and their ectoparasites*

187        DNA previously extracted from hedgehog kidneys and spleens, 119 fleas and 138 ticks  
188 randomly selected were subjected to quantitative polymerase chain reactions (qPCR) using  
189 primers targeting *gltA* for *Rickettsia* spp., *ITS2* for *Bartonella* spp., *ITS4* for *Borrelia* spp.,  
190 *16S RNA* for *Leptospira* spp., *IS30A* for *Coxiella burnetii* and *23S* for Anaplasmataceae and  
191 performed in a CFX96 Touch™ Detection system (Bio-Rad Laboratories, Foster City, CA,  
192 USA) according to the manufacturer's instructions. The reaction mixture composition and the  
193 qPCR conditions were the same as those previously described [34]. Each reaction contained  
194 negative controls (DNA-free PCR mixture and DNA from a laboratory-bred flea or tick  
195 specimen) and a positive control (DNA from bacterial strains cultured in our laboratory).  
196 Samples were considered positive when the cycle threshold (Ct) cut-off values were  $\leq 35$ . All  
197 positive fleas for the *Rickettsia* spp. *gltA* gene were submitted to a specific qPCR using  
198 primers and probe targeting *R. felis* and others targeting *R. typhi*. All *Rickettsia* spp. positive  
199 ticks were submitted to specific qPCRs targeting either *R. aeschlimannii* or *R. massiliae*  
200 followed by *R. conorii* (depending on the tick species). Similarly, all positive arthropods for

201 the Anaplasmatataceae 23S gene were tested for *Wolbachia* spp., and *C. burnetii* IS30A positive  
202 samples were subjected to a second test targeting the *C. burnetii* IS1111 specific gene  
203 fragment. Positive samples whose bacterial species could not be confirmed by specific qPCR  
204 were subjected to conventional PCR and sequencing for species determination as previously  
205 described [31]. Negative and positive controls were used as described above. The sequences  
206 of the used primers and probes are listed in **Table 2**.

207 Using ChromasPro software version 1.7.7 (Technelysium Pty. Ltd., Tewantin, Australia), the  
208 obtained sequences were assembled, analyzed, emended and then blasted against the NCBI  
209 GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to be compared to available reference  
210 sequences.

211

### 212 2.9. Phylogenetic analysis

213 In order to carry out a specific identification, multiple sequence alignments analysis of the  
214 obtained partial sequences for *Leptospira* spp. *rpoB*, *Bartonella* spp. *ftsZ*, *Rickettsia* spp.  
215 *ompA*, Anaplasmatataceae 23S and *Ehrlichia* spp. *groEL* sequenced genes, together with their  
216 corresponding reference sequences available in GenBank, were performed using MEGA  
217 version X software (Kumar, Stecher, Li, Knyaz, and Tamura 2018) prior to phylogenetic tree  
218 plotting.

219

220 The Models program of MEGA version X software (Kumar) was used to find the best DNA  
221 model for each alignment data. The model with the lowest Bayesian information criterion  
222 (BIC) was chosen for each phylogenetic tree plotting. The phylogenetic trees were inferred by  
223 using the maximum likelihood (ML) method under the Hasegawa-Kishino-Yano (HKY)  
224 +G+I model for *Bartonella* spp. *ftsZ* gene, Tamura 3-parameter (T92) +G model for the

225 Anaplasmataceae 23S and *Rickettsia* spp. *ompA* genes, HKY +G model for the *Ehrlichia* spp.  
226 *groEL* gene and *Wolbachia* clades Anaplasmataceae 23S gene and the Kimura 2-parameter  
227 (K2) +G model for the *Leptospira* spp. *rpoB* gene. Support for the topology was  
228 examined using bootstrapping (Felsenstein, 1985) over 200 replications for all  
229 phylogenetic trees.

230

### 231 3. Results

#### 232 3.1. Hedgehog collection and sampling of organs and ectoparasites

233 A total of 21 dead hedgehogs (12 males and 9 females) were picked up by hand and were all  
234 morphologically identified as *A. algirus*. Twelve of the twenty-one hedgehogs (57.1%) were  
235 parasitized by arthropods; among them 2/12 (16.67%) were parasitized only by ticks, 0/12  
236 (0%) were parasitized only by fleas and 10/12 (83.33%) were parasitized by both fleas and  
237 ticks. All details regarding capture sites, hedgehog sex, and the number and species of  
238 arthropods collected for each hedgehog are summarized in **Figure 1** and in a table in  
239 **Supplementary Data 1**. Spleens and kidneys were retrieved from all hedgehogs (20 and 21  
240 respectively), except for one hedgehog for which spleen was in a state of putrefaction too  
241 advanced to be considered for further analysis.

242

#### 243 3.2. Morphological identification of arthropods

244 A total of 853 fleas and 218 ticks were collected from twelve hedgehogs. All fleas were  
245 morphologically identified as *A. erinacei*. Among the ticks, 151/218 (69.3%) were  
246 morphologically identified as *Rh. sanguineus* s.l., 39/218 (17.9%) as *Hae. erinacei* and 28  
247 (12.8%) as *Rhipicephalus* spp. (engorged females lacking characteristics for definitive  
248 identification). Pictures of morphologically identified fleas and ticks are shown in the **Figures**

249 **2** and **3**. A total of 10/21 (47.6%) hedgehogs were parasitized by *Hae. erinacei*, 7/21 (33.3%)  
250 by *Rh. sanguineus* and 5/21 (23.8%) were infested by *Rhipicephalus* spp. Eleven (91.7%) of  
251 the twelve parasitized hedgehogs were co-infested by at least 2 arthropod species  
252 **(Supplementary Data 1)**.

253

### 254 3.3. MALDI-TOF MS identification of collected arthropods

255 In order to support morphological species identification, as well as to test the ability of  
256 MALDI-TOF MS to identify ticks and fleas, 26/853 *A. erinacei*, 13/39 *Hae. erinacei* and  
257 51/151 *Rh. sanguineus* s.l. have been randomly selected from different hedgehogs for  
258 MALDI-TOF MS analysis. Similarly, so as to determine the species of the 28 engorged ticks  
259 morphologically identified to the genus level as *Rhipicephalus* spp., they have been also  
260 submitted to MALDI-TOF MS analysis. Twenty-four (92.3%) *A. erinacei*, 12 (92.3%) *Hae.*  
261 *erinacei*, 48 (94.1%) *Rh. sanguineus* s.l. and 28 (100%) engorged *Rhipicephalus* spp.,  
262 provided high quality and reproducible spectra within each species **(Supplementary Data 2)**.  
263 Blind test analysis of these spectra against our laboratory database containing several  
264 arthropod species [31] and updated with reference spectra of 2 *Hae. erinacei*, 3 *Rh.*  
265 *sanguineus* and 4 *A. erinacei* showed that 28/28 (100%) engorged *Rhipicephalus* spp. have  
266 been identified as *Rhipicephalus sanguineus* s.l. with log-score values (LSVs) ranging from  
267 1.451 to 2.085 (average: 1.856). All the other specimens tested have been correctly identified  
268 at the species level with LSVs ranging from 1.926 to 2.226 (average: 2.068) for *Hae. erinacei*,  
269 1.415 to 2.362 (average: 1.916) for *Rh. sanguineus* s.l. and 1.861 to 2.485 (average: 2.172) for  
270 *A. erinacei* **(Table 1)**.

271

### 272 3.4. Molecular identification of the collected arthropods

273 In order to confirm the identification of the arthropods, the DNA of six *Hae. erinacei*, ten *Rh.*  
274 *sanguineus* s.l. and twenty-six *A. erinacei* previously identified morphologically and using  
275 MALDI-TOF MS has been randomly selected for molecular identification. To determine the  
276 species of the 28 engorged females *Rhipicephalus* spp., they have been also submitted to  
277 molecular identification. Good quality partial sequences were obtained for 100% (16/16) of  
278 the unengorged ticks (*Hae. erinacei* and *Rh. sanguineus* s.l.), 88.5% (23/26) of the tested fleas  
279 and 75% (21/28) of the engorged *Rhipicephalus* spp. ticks.

280 The BLAST analysis revealed that unengorged *Rh. sanguineus* s.l. *16S* sequences had  
281 identities ranging from 99.15% to 100% with *Rh. sanguineus* reference sequences available in  
282 GenBank (Accession Numbers: MG855662, MH018852, MW172416, JX997393 and  
283 KT382469) including sequences of *Rhipicephalus sanguineus* IIa haplotype of the temperate  
284 lineage from Algeria, Malta and Croatia (Accession Numbers: KX793742, KX793735,  
285 KX793726 and KX793724) [35]. Those of *Rhipicephalus* spp. engorged ticks showed  
286 identities ranging from 97.66% to 100% with *Rhipicephalus sanguineus* reference sequences  
287 (Accession Numbers: KX793742, KT382469, MH630344, KT382465, JX997393,  
288 MH018852). Among these *Rhipicephalus* spp. engorged specimens, sixteen had identities  
289 with reference sequences of *Rhipicephalus sanguineus* IIa haplotype of the temperate lineage  
290 and five had similarities with sequences of IIb haplotype of the same lineage. The *16S*  
291 sequences obtained for *Hae. erinacei* had a query cover ranging from 95% to 100% and  
292 identities ranging from 97.44% to 97.97% with the *Hae. erinacei 16S* reference sequences  
293 from GenBank (Accession Numbers: KX901846 and KU880549). As for BLAST results of  
294 the flea *ITS1* sequences, they had an identity ranging from 99.77% to 100% with those of *A.*  
295 *erinacei* available in GenBank (Accession Numbers: LT627362, LT604112 and LT576377).  
296 These molecular results support the morphological and MALDI-TOF MS identification

297 previously performed (**Table 1**). All the sequences obtained for arthropods are available in  
298 **Supplementary Data 3**.

299 The tick (*Hae. erinacei* and *Rh. sanguineus* s.l.) *16S RNA* partial sequences and the flea (*A.*  
300 *erinacei*) *ITS1* partial sequences have been deposited in GenBank and the accession numbers  
301 are summarized in a table in **Supplementary Data 4**.

302

### 303 3.5. Molecular screening for microorganism detection

304 Overall, 45 samples, including eleven (52.4%) kidneys, six (30%) spleens, fifteen (46.9%)  
305 *Hae. erinacei*, nine (11.5%) *Rh. sanguineus* s.l. and four (3.4%) *A. erinacei* collected from 15  
306 hedgehogs were positive in qPCR for *Coxiella burnetii*, targeting both genes (*IS1111* and  
307 *IS30A*). Nine hedgehogs had positive organs only (1 had positive spleen only, 5 had positive  
308 kidney only and 4 had both positive organs), three for which only the collected arthropods  
309 were positive and three others for which both arthropods and organs were positive (1 had both  
310 organs and *Rh. sanguineus* positive, 1 had both organs, *A. erinacei* and *Hae. erinacei* positive  
311 and 1 had kidney, *A. erinacei* and *Rh. sanguineus* positive).

312

313 Ten samples (7 kidneys, 1 spleen and 2 *Rh. sanguineus* s.l. ticks) were positive for *Leptospira*  
314 spp. qPCR. These positive samples belonged to 9 hedgehogs, among them 8 for which only  
315 the organs tested positive (1 had positive spleen only and 7 had positive kidney only) and 1  
316 for which only the *Rh. sanguineus* s.l. ticks tested positive. All the tested fleas were negative.

317 The ten positive samples were then subjected to conventional PCR amplification of a 592  
318 base pairs fragment of the *Leptospira* spp. *rpoB* gene. Only 3 kidneys were positive, and good  
319 quality sequences were obtained for two of them. The results of BLAST analysis showed  
320 100% similarity of our sequences with *Leptospira interrogans* detected in a human from  
321 China (Accession number: CP044513). The phylogenetic analysis using published reference

322 sequences only revealed that our sequences clustered with the group of *Leptospira*  
323 *interrogans* reference sequences (**Figure 4**), confirming their identification as *Leptospira*  
324 *interrogans*.

325

326 A total of 166 arthropods (87 *A. erinacei*, 56 *Rh. sanguineus* s.l., 21 *Hae. erinacei* and 2  
327 engorged *Rhipicephalus* spp.) sampled from twelve hedgehogs were positive for *Rickettsia*  
328 spp. by qPCR. However, all the tested organs (kidneys and spleens) were negative. Following  
329 a specific qPCR, 68/87 (78.2%) of the *Rickettsia* spp. found in *A. erinacei* fleas were  
330 confirmed to be *Rickettsia felis*. These fleas were collected on 10/21 hedgehogs. *Rickettsia*  
331 spp. qPCR positive fleas which were negative for *R. felis* were submitted to *R. typhi* specific  
332 qPCR and were all negative. Thirty-eight *Rh. sanguineus* s.l. and the two *Rhipicephalus* spp.  
333 engorged ticks, collected from 7/21 hedgehogs, were positive for *R. massiliae* by specific  
334 qPCR, and all were negative for *R. conorii*. All positive *Hae. erinacei* tested negative for *R.*  
335 *aeschlimannii*.

336 The 58 qPCR positive specimens for which *Rickettsia* species was not confirmed by specific  
337 qPCR, (19 *A. erinacei*, 18 *Rh. sanguineus* s.l. and 21 *Hae. erinacei*) were subjected to  
338 conventional PCR and sequencing targeting a 630 base pairs fragment of the *ompA* gene for  
339 *Rickettsia* spp. Only six samples were positive (1 *Rh. sanguineus* s.l. from one hedgehog and  
340 5 *Hae. erinacei* from 4 other hedgehogs) and good quality sequences were obtained for 2/6  
341 samples (2 *Hae. erinacei*).

342 BLAST analysis indicated that the two obtained sequences were identical and showed 100%  
343 similarity with an uncultured *Rickettsia* spp. (Accession Number: MG228270) previously  
344 detected in *Dermacentor nuttalli* ticks from China. Phylogenetic analysis (**Figure 5**) did not  
345 provide additional information on the identification of this species of *Rickettsia*, since it  
346 clustered with other uncultured *Rickettsia* spp.

347

348 *Bartonella* spp. was detected in 8/298 (2.7%) tested samples (2/20 (10%) spleens and 6/119  
349 (5.04%) *A. erinacei* fleas) from 7/21 (33.3%) different hedgehogs. All the kidneys and tick  
350 specimens tested negative. These qPCR positive samples were afterward subjected to  
351 conventional PCR, amplifying a 200 base pairs fragment of the *gltA* gene for *Bartonella* spp.  
352 Four samples were successfully amplified, and two good quality sequences were obtained (1  
353 from a spleen and 1 from an *A. erinacei* flea). BLAST analysis results indicated 98.02%  
354 identity with an uncultivated *Bartonella* spp., previously detected in a plateau pika (*Ochotona*  
355 *curzoniae*) from China (Accession Number: MN056885) for both sequences obtained from  
356 the spleen and *A. erinacei*.

357 Given the low identity percentages obtained for the *gltA* gene, a 333 base pairs fragment of  
358 the *ftsZ* gene was also amplified and sequenced for the qPCR positive samples. Four  
359 sequences (*A. erinacei* from 4 hedgehogs) were obtained. BLAST analysis of the sequences  
360 indicated for all of them 98% query cover and 97.22% identity with reference *Bartonella*  
361 *clarridgeiae* isolated from cats in France (Accession Number: FN645454).

362 The sequencing results based on two genes together with the phylogenetic analysis of the *ftsZ*  
363 gene suggest that the *Bartonella* detected in this spleen and these *A. erinacei* fleas is  
364 potentially a new species (**Figure 6**).

365

366 A total of 7 organs sampled from 6 hedgehogs (4 had positive spleen only, 1 had positive  
367 kidney only and 1 had both positive organs), 7/138 ticks (4 *Hae. erinacei* collected from 2  
368 hedgehogs and 3 *Rh. sanguineus* collected from 2 other hedgehogs) and 62/119 *A. erinacei*  
369 fleas sampled from 9 hedgehogs were positive in qPCR targeting the Anaplasmataceae 23S  
370 gene. All (62/62) positive fleas for Anaplasmataceae bacteria were positive for *Wolbachia*  
371 *spp.* by qPCR and all the ticks were negative. All the positive organs and ticks for



372 Anaplasmataceae 23S qPCR and 42 randomly selected flea specimens positive for *Wolbachia*  
373 spp. qPCR were subjected to conventional PCR and sequencing targeting the  
374 Anaplasmataceae 23S gene amplifying a 485 base pairs fragment. All the tested organs (7/7)  
375 were negative. Good quality 23S gene sequences were obtained for 37/42 *Wolbachia* qPCR-  
376 positive fleas and were all identical. BLAST analysis of these sequences revealed identities of  
377 99.56% with *Wolbachia pipientis* (Accession Number: CP037426) and *Wolbachia*  
378 endosymbiont of different arthropods, including *Drosophila simulans* and *Drosophila*  
379 *melanogaster* (Accession Numbers: CP003884 and AE017196). Phylogenetic analysis  
380 revealed that our *Wolbachia* sequences detected in fleas are located within the *Wolbachia* “A”  
381 clade and would seem to belong to this clade (**Figure 7**).

382 DNA from five ticks was successfully amplified (3 *Hae. erinacei* and 2 *Rh. sanguineus* s.l.)  
383 and 4 good quality sequences were obtained (3 *Hae. erinacei* and 1 *Rh. sanguineus* s.l.).  
384 BLAST results revealed 96.65% to 96.66% identity of the obtained sequences with uncultured  
385 *Ehrlichia* spp. detected in *Rhipicephalus complanatus* and *Haemaphysalis muhsami* collected  
386 from pigs in Democratic Republic of the Congo (Accession Numbers: MK942592,  
387 MK942591 and MK942590). Using primers amplifying a 590 base pairs fragment of the  
388 *Ehrlichia* spp. *groEL* gene, three good quality sequences were obtained (2 *Hae. erinacei* and  
389 1 *Rh. sanguineus* s.l.). BLAST results revealed 94.89% to 95.08% identity with uncultivated  
390 *Ehrlichia* spp. detected in *Haemaphysalis concinna* from Russia (Accession Number:  
391 JX092091). The phylogenetic trees (**Figure 8**) showed for both sequenced genes that our  
392 *Ehrlichia* spp. sequences clustered separately from the other *Ehrlichia* species, thus  
393 suggesting that they probably belong to a potential new *Ehrlichia* species.

394

395 All the tested samples were negative for *Borrelia* spp. *ITS4* gene fragment targeted in qPCR  
396 analysis. Microorganism detection results are summarized in **Table 3** and **Figure 9**.

397 Consensus sequences obtained for the different microorganisms detected in hedgehogs and  
398 their associated arthropods have been deposited in GenBank database and all the accession  
399 numbers are detailed in a table in **Supplementary Data 4**. All FASTA sequences of the  
400 amplified gene fragments for the detected microorganisms are made available in  
401 **Supplementary Data 5**.

402

#### 403 **4. Discussion**

404 In this study conducted in a north-central region of Algeria, the DNA of various  
405 microorganisms, including *C. burnetii*, *L. interrogans*, *Leptospira* spp., *R. felis*, *R. massiliae*,  
406 *Rickettsia* spp., *Wolbachia* spp., Anaplasmataceae bacteria and potential new strains of  
407 *Bartonella* and *Ehrlichia* were detected in hedgehog organs and/or their ticks and fleas.

408 In the last decades, the development of new technologies, including molecular tools such as  
409 polymerase chain reaction (PCR) and sequencing, has enabled considerable progress in the  
410 study of infectious diseases and in the identification of their reservoirs. In this study, these  
411 methods allowed us using appropriate negative and positive controls to identify different  
412 microorganisms infecting hedgehogs and their ectoparasites.

413 The North African hedgehog (*A. algirus*) is an endemic species in the Mediterranean basin  
414 countries and islands, including North Africa (Algeria, Tunisia, Morocco and Libya) and  
415 southern Europe (Spain and France) [36]. In Algeria, these small mammals are on the  
416 protected species list (Presidential decree No. 06-405 of 22 Chaoual 1427, corresponding to  
417 the November 14, 2006 ratification of the protocol relating to specially protected areas and  
418 organic diversity in the Mediterranean, signed in Barcelona on June 10, 1995). Even though  
419 they have been suggested as a potential reservoir of various zoonotic diseases, they remain  
420 relatively understudied [7].

421 In this study, 57.1% (12/21) of the hedgehogs were infested with ectoparasites. This  
422 infestation rate is similar to that reported in a study on dead hedgehogs (*Erinaceus concolor*)  
423 from Turkey [37], but lower than that reported in a northeastern region of Algeria with the  
424 same hedgehog species captured alive [2]. This large difference in the infestation rate could  
425 be explained by the fact that in our study, hedgehogs were recovered dead, and it is known  
426 that when body temperature drops, arthropods tend to detach themselves to look for a new  
427 host. Nevertheless, two of the hedgehogs picked up in Ahl-El-Ksar were heavily infested by  
428 fleas with 500 and 236 specimens collected respectively on a hedgehog from Thiliwa village  
429 and another from Thigmit.

430 In our study, the fleas and ticks collected from hedgehogs were morphologically identified as  
431 *A. erinacei*, *Rh. sanguineus* s.l. and *Hae. erinacei*. Twenty-eight engorged female ticks were  
432 identified only at the genus level as *Rhipicephalus* spp. for the reason that they were lacking  
433 characteristics for definitive morphological species identification.

434  
435 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-  
436 TOF MS) is an innovative proteomic tool which has been successfully applied for  
437 identification of arthropods of medical interest. With an appropriate database, this tool  
438 represents a good alternative to the tedious methods habitually used for arthropod  
439 identification [42]. This inexpensive method allows to test readily a large number of samples  
440 compared to molecular biology which is a more expensive and time-consuming method. In  
441 the present study, this technology was used as a complementary tool to support the  
442 morphological and molecular identification of the collected arthropods. Our in-lab database  
443 did not contain any reference spectrum for *Hae. erinacei*, thus we had to implement it in order  
444 to allow for identification. The selected arthropod specimens were all identified to the species  
445 level using this tool. However, the obtained log scores values for a few specimens were low

446 (<1.7). This may be explained by the fact that our samples were kept in alcohol for more than  
447 three years, and it has been demonstrated that this method of preservation alters the protein  
448 composition of the samples [43]. In order to perform molecular identification of the selected  
449 arthropods, *ITS1* and tick *16S RNA* gene fragments were amplified. The tick and flea obtained  
450 sequences all showed high similarity with reference sequences of *Rh. sanguineus* s.l., *Hae.*  
451 *erinacei* and *A. erinacei* available in GenBank. Regarding the engorged tick specimens, both  
452 MALDI-TOF MS and molecular biology identification results allowed their assignation to the  
453 *Rhipicephalus sanguineus* s.l. species. The number of selected ectoparasites to confirm their  
454 identification was randomly chosen, considering potential species diversity and the number of  
455 specimens. Fleas are small insects; thus, they are challenging to identify. The morphological  
456 criteria for identifying and distinguishing species are sometimes very subtle and difficult to  
457 observe even for people with entomological skills. Their identification often requires a KOH  
458 clearing followed by mounting in a medium between slide and coverslip in order to make the  
459 morphological criteria more visible. This mounting method makes the sample unusable for  
460 further analysis. Consequently, it cannot be applied on all the specimens. MALDI-TOF MS  
461 and molecular methods help in unambiguous species identification, for this reason, all the flea  
462 specimens selected for MALDI-TOF MS analysis have been also submitted to molecular  
463 identification. Overall, in this study we used three different methods for arthropod  
464 identification (morphology, molecular biology and MALDI-TOF MS) and the identification  
465 results were concordant for these three methods. Nevertheless, the main purpose of this study  
466 was not to compare the performance of different identification methods, but we used them  
467 only to confirm and support the morphological identification. The number of selected  
468 specimens is therefore not important, it must only be sufficient to allow unambiguous  
469 identification of the arthropods.

470 *Archaeopsylla erinacei* and *Hae. erinacei*, commonly called the hedgehog flea and the  
471 hedgehog tick, are often associated with hedgehogs [14,38], but this flea and tick species can  
472 also be found on other animals, such as, felids, canids, foxes, hares, kestrel nests and birds  
473 respectively [14,39]. *Rhipicephalus sanguineus* s.l., the brown dog tick, is amply distributed  
474 worldwide. It is commonly associated with dogs; however, it can occasionally bite humans  
475 and other animal hosts [40]. The same arthropod species (*A. erinacei*, *Hae. erinacei* and *Rh.*  
476 *sanguineus* s.l.) have previously been collected from *A. algirus* hedgehogs in another study  
477 conducted in Algeria [2]. These arthropod species have been also reported on other hedgehog  
478 species in Algeria and other countries, including the desert hedgehog (*Paraechinus*  
479 *aethiopicus*) [2,14,37,41].

480 Using molecular tools, DNA of all tested bacteria - except for *Borrelia* spp. - were detected in  
481 the hedgehog organs and/or their arthropods, therefore indicating that hedgehogs could be  
482 significant reservoirs of various zoonotic agents. Interestingly, 2 hedgehogs and/or their  
483 ectoparasites were co-infected with 8 different microorganisms at the same time, and 17 were  
484 co-infected with at least 2 microorganisms (**Figure 9**).

485 *Coxiella burnetii*, the etiological agent of Q fever, is an obligate intracellular gram-negative  
486 bacterium. This neglected zoonotic disease can affect wild and domestic animals as well as  
487 humans. This bacterium spreads through the aerosol route and contact with infected animals  
488 or their products. Prior studies showed that *C. burnetii* can infect different arthropod species,  
489 mainly ticks and also fleas (*Xenopsylla cheopis* and *Ctenocephalides felis*) [44,45]. A  
490 serological study reported the presence of antibodies to *Coxiella burnetii* in European  
491 hedgehogs from Austria [46], and it has recently been reported in *Erinaceus amurensis*  
492 hedgehogs and their *Haemaphysalis flava* ticks from China [47,48]. Herein, we report the  
493 presence of *C. burnetii* for the first time in *A. algirus* hedgehogs and their *A. erinacei* fleas  
494 and *Hae. erinacei* ticks in Algeria. Interestingly, in our study, *Coxiella burnetii* was detected

495 in both hedgehog organs and all their associated arthropod species (*A. erinacei*, *Rh.*  
496 *sanguineus* s.l. and *Hae. erinacei*), suggesting that these arthropods may be potentially  
497 involved in the life cycle of this bacterium. However, this hypothesis cannot be confirmed,  
498 especially since this is not the usual mode of transmission of the disease, even if it has already  
499 been suggested in a previous study that tick species could facilitate the transmission  
500 of *Coxiella burnetii* [49]. Also, it cannot be confirmed if this bacterium is associated with *A.*  
501 *erinacei* or if these fleas were infected by feeding on a bacteremic hedgehog or by co-feeding  
502 with infected ticks. Experimental studies are needed to clarify this.

503 Leptospirosis is a widespread zoonotic disease affecting humans and domestic and wild  
504 animals, caused by obligate aerobic spirochete bacteria belonging to the *Leptospira* genus.  
505 Human contamination occurs most often either after direct contact with the urine of infected  
506 animals or indirectly through contaminated water or soil [50]. In the present study, *Leptospira*  
507 spp. was detected in *Rh. sanguineus* s.l. ticks and in hedgehog kidneys and spleens. BLAST  
508 analysis of the sequences obtained from hedgehog kidneys revealed that they were similar to  
509 the reference sequences of two different species. Indeed, they have the same identity  
510 percentage with several reference sequences of *Leptospira interrogans* and two sequences of  
511 *Leptospira borgpetersenii*. However, phylogenetic analysis based on the *rpoB* gene (**Figure**  
512 **4**) using only published reference sequences showed that our sequences clustered within the  
513 *Leptospira interrogans* group. Accordingly, the identification of our sequences was validated  
514 as *Leptospira interrogans*. In Algeria, there are few studies on leptospirosis, and the  
515 prevalence of this disease is still unknown. Nevertheless, this bacterium has been detected in  
516 the environment as well as in humans and certain animals, including dogs, cats and cattle [51–  
517 60]. Previous studies have already reported *Leptospira interrogans* in hedgehogs from China,  
518 and also from different countries of the Mediterranean basin, including France and Italy [61].  
519 *Leptospira* spp. was detected in *Ixodes ricinus* from Poland [62] and in *Rh. sanguineus* s.l.

520 collected on dogs in Lao PDR [63]. However, to our knowledge this bacterium has never been  
521 reported, neither in *A. algirus* hedgehogs nor in *Rh. sanguineus* ticks from Algeria. Our  
522 results suggest that hedgehogs and their ticks might potentially be considered a source of  
523 *Leptospira* spp. in Algeria. Humans and domestic animals could easily become infected by  
524 handling infected hedgehogs. Nevertheless, there is no evidence of the tick's ability to  
525 transmit this bacterium.

526 *Rickettsiae* are small obligate intracellular gram-negative bacteria belonging to the  
527 *Rickettsiaceae* family and the *Rickettsiales* order. Rickettsioses are emergent or re-emergent  
528 diseases. The main vectors are hematophagous arthropods [64,65]. Humans are considered  
529 accidental hosts [66]. At least 11 *Rickettsia* species have been detected in arthropods from  
530 Algeria [22].

531 *Rickettsia felis* is a causative agent of an emerging spotted fever group rickettsiosis [67]. This  
532 bacterium has been associated with many species of fleas. The cat flea (*Ctenocephalides felis*)  
533 is a recognized vector. Mosquitoes have been shown to be competent vectors in laboratory  
534 settings, while some booklice species were found to be naturally infected [67,68]. In this  
535 study, *R. felis* was detected in *A. erinacei* fleas collected from *A. algirus*. These results  
536 support the presence of this flea-borne spotted fever agent in Algerian fleas, including *A.*  
537 *erinacei* [14].

538 *Rickettsia massiliae* is also a causative agent of spotted fever group rickettsiosis in humans.  
539 The reservoir has not been clearly identified yet, but this bacterium is transmitted by *Rh.*  
540 *sanguineus* s.l. [69]. In this study, *R. massiliae* was detected in *Rh. sanguineus* s.l.,  
541 confirming its presence in Algerian ticks. This rickettsial agent is commonly associated with  
542 ticks and was previously detected in *Rh. sanguineus* s.l. sampled from hedgehogs in Algeria  
543 [2,20] and in other countries [70].

544 In the present study, an uncultivated *Rickettsia* spp. was detected in 2 *Hae. erinacei*. This  
545 uncultivated *Rickettsia* spp. was similar to that found in *Dermacentor nuttalli* from China  
546 (unpublished); and close to the potential new *Rickettsia* spp. sequences found in *Hae. erinacei*  
547 and *Rh. sanguineus* s.l. ticks sampled from *A. algirus* hedgehogs reported in another study in  
548 Algeria [2]. All hedgehog organs tested negative for *Rickettsia* spp. by qPCR. Accordingly,  
549 this uncultivated *Rickettsia* spp. seems to be associated only with ticks, and there is no  
550 evidence of a potential transmission to hedgehogs or that these hedgehogs could be reservoirs.  
551 Some qPCR-positive fleas for *Rickettsia* spp. (*gltA* gene) were negative to *R. felis* and *R. typhi*  
552 specific qPCR and also to the conventional PCR targeting the *ompA* gene (contained in  
553 Spotted Fever Group *Rickettsia*). Thus, these samples have not been identified to the species  
554 level. Nevertheless, in a previous study, *Rickettsia felis*-Like organism has been detected in *A.*  
555 *erinacei* fleas sampled on hedgehogs but has not been amplified by the usual primers  
556 targeting the *ompA* gene because of variations in this gene in *Rickettsia felis* strains [71].  
557 Therefore, considering the cited study as well as our PCR results, these *Rickettsia* spp.  
558 detected in fleas seem to be *Rickettsia felis*-Like organisms. Sequencing of other genes is  
559 necessary for confirmation.

560 Bartonelloses are worldwide emerging and re-emerging diseases characterized by  
561 intraerythrocytic bacteremia and caused by fastidious intracellular bacteria belonging to the  
562 *Bartonella* genus. Hematophagous arthropods such as sandflies, lice, louse flies and fleas are  
563 considered as vectors. Various mammals as well as humans have been reported to be  
564 reservoirs of *Bartonella* species [72,73]. Several studies reported different species of  
565 *Bartonella* in hedgehogs. In urban and suburban areas of the Czech Republic, *Bartonella*  
566 *washoensis* and *B. melophagi* have been detected in *Erinaceus europaeus* hedgehogs [74].  
567 The causative agent of cat scratch disease in humans *B. henselae* and a *Bartonella* spp.  
568 phylogenetically close to *B. taylorii* have been detected respectively in an *A. erinacei* flea and



569 in muscle and skin samples collected on northern white-breasted hedgehogs *Erinaceus*  
570 *roumanicus* from Budapest, Hungary [9,75].  
571 In Algeria, different *Bartonella* species have already been reported, including *B. tribocorum*  
572 in *A. algirus* hedgehogs [76], *B. elizabethae* in hedgehogs and their *A. erinacei* fleas and *B.*  
573 *clarridgeiae* in *A. erinacei* fleas sampled from hedgehogs [21,76]. In this study, we found  
574 *Bartonella* spp. DNA in hedgehog spleens as well as in their associated fleas (*A. erinacei*).  
575 The one detected in the fleas could potentially be a new species of *Bartonella*,  
576 phylogenetically close to *B. clarridgeiae*. Other studies in the world reported the presences of  
577 potential new *Bartonella* species in hedgehogs. In Italy, a new clone of *Bartonella* spp. have  
578 been reported in spleen samples of 2 European hedgehogs *E. europaeus* from Basilicata  
579 region (Matera province) [77]. This clone presented phylogenetic proximity to *B.*  
580 *clarridgeiae*. In a study carried out in Israel, the DNA of a *Bartonella* spp. was detected in a  
581 blood sample of a Southern white-breasted hedgehog *E. concolor*. The sequence obtained for  
582 this *Bartonella* spp. was different from all the known species. However, in phylogenetic  
583 analysis it clustered in the same clade with *B. clarridgeiae* and *B. rochalimae* [78]. A  
584 Potential new species *Bartonella* spp. ERIN phylogenetically close to *B. clarridgeiae* was also  
585 reported in 2 hedgehog species from Czech Republic (*E. europaeus* and *E. roumanicus*) and  
586 another species (*Bartonella* spp. SCIER) was detected in the same hedgehog species  
587 aforementioned [74]. The sequences obtained in these studies could not be compared to those  
588 we obtained for the reason that either they were from a different gene or because the primers  
589 used target different fragments of the same gene.

590

591 Bacteria belonging to the Anaplasmataceae family are the cause of emerging and endemic  
592 zoonotic diseases that have a great impact primarily on veterinary public health, such as  
593 *Anaplasma* spp. and *Ehrlichia* spp. [79]. They are obligate intracellular bacteria transmitted

594 mainly by arthropods [79]. It also includes *Wolbachia* spp. bacteria, which are known as an  
595 endosymbiont of many arthropods [80].

596 In our study, the DNA of the Anaplasmataceae bacteria family was detected in hedgehog  
597 organs and their ectoparasites by qPCR. All qPCR-positive organs were negative in  
598 conventional PCR; thus, the bacterial species could not be confirmed. Those detected in fleas  
599 were all confirmed by qPCR and sequencing to be *Wolbachia* spp. These bacteria can be  
600 transmitted by transovarial and trans-stadial means through generations of invertebrates [81],  
601 and this could explain the significant number of positive fleas. Several studies have detected  
602 the presence of *Wolbachia* spp. in different flea species [82], including *A. erinacei* fleas  
603 collected on *E. europaeus* hedgehogs from Spain [83]. However, to our knowledge, this study  
604 represents the first report of this endosymbiont bacteria in *A. erinacei* fleas from Algeria.

605 Furthermore, our results highlight the presence of a potential new *Ehrlichia* species detected  
606 in *Rh. sanguineus* s.l. and *Hae. erinacei* ticks. Previous studies have reported the presence of  
607 *Ehrlichia* spp. in *Hae. erinacei* sampled from goats in Iran [84]. *Ehrlichia ewingii* has been  
608 detected in *Haemaphysalis flava* ticks sampled on hedgehogs from China [48]. In Algeria,  
609 previous studies reported *Ehrlichia* spp. in different animal hosts, including dogs [85,86] and  
610 in ticks [87]. However, to our knowledge this study represents the first report of *Ehrlichia*  
611 spp. in hedgehogs and *Hae. erinacei* ticks in this country.

612 Some qPCR-positive samples for *Leptospira* spp., *Rickettsia* spp., *Bartonella* spp. and  
613 Anaplasmataceae were not identified to the species level either because they were not  
614 successfully amplified by conventional PCR or because the quality of the obtained sequences  
615 was too poor to be used for further analyses. This could be explained by the fact that real-time  
616 PCR may be more sensitive than conventional PCR [88].

617 Several studies have shown the presence of various pathogens in hedgehogs and their  
618 associated bloodsucking arthropods highlighting their role as potential reservoirs for various

619 infectious diseases, including some vector-borne ones. Despite this, their involvement in  
620 public health issues has rarely been investigated. Indeed, little is known about the role of  
621 hedgehogs in the transmission of these infectious diseases to humans even if in recent years  
622 they have become popular as pets and are also eaten as a traditional meal by some populations  
623 [3]. Added to that, the various interventions of humans in nature, such as intensive  
624 agriculture, deforestation and urbanization, are leading to the disturbance and degradation of  
625 hedgehog burrows and ecosystems [1]. This brings them to take refuge in gardens, looking for  
626 food and shelter, consequently living close to humans and exposing them to an increased  
627 transmission risk of various zoonotic agents.

628

## 629 **5. Conclusion**

630 Our results confirm the presence of several microorganisms associated with hedgehogs and  
631 their ectoparasites in Algeria. We report here two potentially new bacterial species, and also  
632 made it possible to expand the repertoire of the pathogens associated with hedgehogs and  
633 arthropods in this area.

634 Epidemiological investigations are lacking in Algeria and North Africa. Further studies are  
635 needed for better understanding the role of hedgehogs and their hematophagous arthropods in  
636 the transmission of zoonotic diseases to humans and their impact on public health.

637

## 638 **Declarations**

639

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645

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652

#### 653 *Authors' contributions*

654 Conceptualization: PP and IB; Data curation: LB, AZD, ML and NA; Formal analysis: LB,  
655 AZD and ML; Funding acquisition: PP; Investigation: LB and NA; Methodology: LB, NA,  
656 AZD, ML, JMB and PP; Project administration: PP; Resources: NA, PP; Software: LB, AZD  
657 and ML; Supervision: PP, AZD, ML and IB; Validation: PP, AZD and ML; Writing - original  
658 draft: LB and NA; Writing - review & editing: LB, AZD, ML and PP. All authors reviewed  
659 and approved the final version.

660

#### 661 *Conflict of interest*

662 The authors declare that they have no conflicts of interest.

663

#### 664 *Data availability*

665 All data regarding this study are included in the manuscript and its supplementary data.  
666 GenBank accession numbers of the sequences obtained in this study for arthropods and  
667 microorganisms are made available in **Supplementary Data 4**.

668

669 The FASTA sequences of all sequenced arthropod specimens and bacteria are available as  
670 **Supplementary Data 3** and **5**, respectively.

671

672

### 673 **References**

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1018 **Figure captions**

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1020 **Figure 1:** Hedgehog capture sites in Bouira province (Algeria).

1021 **Figure 2:** Photographs of *Archaeopsylla erinacei* fleas collected from an *Atelerix algirus*  
1022 hedgehog taken with a Zeiss Axio Zoom V16 stereomicroscope (Zeiss, Marly le Roi, France).

1023 **a)** Overall view of female *A. erinacei* flea mounted in Euparal mounting medium after KOH  
1024 clearing; **b)** Head of *A. erinacei* flea specimen mounted in Euparal mounting medium after  
1025 KOH clearing: The spines of the genal and pronotal ctenidia are indicated by arrows; **c)**  
1026 Overall view of female *A. erinacei* flea without KOH clearing.

1027 **Figure 3:** Photographs of the dorsal and ventral sides of each tick species taken with a Zeiss  
1028 Axio Zoom V16 stereomicroscope (Zeiss, Marly le Roi, France) and a VHX-7000 digital  
1029 microscope (Keyence, Osaka, Japan): **a), b), c)** and **d)** a male specimen of *Haemaphysalis*  
1030 *erinacei*; **e), f)** and **j)** a female specimen of *Rhipicephalus sanguineus* s.l.; **g), h), i)** and **k)** a  
1031 male specimen of *Rhipicephalus sanguineus* s.l.

1032 **Figure 4:** Phylogenetic tree of *Leptospira* spp. based on *rpoB* partial gene inferred using  
1033 maximum-likelihood (ML) method under Kimura 2-parameter (K2) +G model. Support for  
1034 the topology was examined using bootstrapping (Felsenstein, 1985) over 200  
1035 replications. Bootstrap values lower than 60% are not shown. The sequences obtained in this  
1036 study are indicated by circles.

1037 **Figure 5:** Phylogenetic tree of *Rickettsia* spp. based on *gltA* partial gene inferred using  
1038 maximum-likelihood (ML) method under Tamura 3-parameter (T92) +G model. Support for  
1039 the topology was examined using bootstrapping (Felsenstein, 1985) over 200

1040 replications. Bootstrap values lower than 60% are not shown. The sequences obtained in this  
1041 study are indicated by circles.

1042 **Figure 6:** Phylogenetic tree of *Bartonella* spp. based on *ftsZ* partial gene inferred using  
1043 maximum-likelihood (ML) method under Hasegawa-Kishino-Yano (HKY) +G+I model.  
1044 Support for the topology was examined using bootstrapping (Felsenstein, 1985)

1045 over 200 replications. Bootstrap values lower than 60% are not shown. The sequences  
1046 obtained in this study are indicated by rhombuses.

1047 **Figure 7: A)** Phylogenetic tree of *Wolbachia* spp. based on *Anaplasmataceae 23S* partial  
1048 gene inferred using maximum-likelihood (ML) method under Tamura 3-parameter (T92) +G  
1049 model. **B)** Phylogenetic tree based on *Anaplasmataceae 23S* partial gene representing  
1050 *Wolbachia* clades inferred using Maximum-Likelihood (ML) method under Hasegawa-  
1051 Kishino-Yano (HKY) +G model. Clade F and H are not represented. Support for the  
1052 topology was examined using bootstrapping (Felsenstein, 1985) over 200

1053 replications. Bootstrap values lower than 60% are not shown. The sequences obtained in this  
1054 study are indicated by circles.

1055 **Figure 8: A)** Phylogenetic tree of *Ehrlichia* spp. based on *Anaplasmataceae 23S* partial gene  
1056 inferred using maximum-likelihood (ML) method under Tamura 3-parameter (T92) +G  
1057 model. **B)** Phylogenetic tree based on *Ehrlichia groEL* partial gene inferred using maximum-  
1058 likelihood (ML) method under Hasegawa-Kishino-Yano (HKY) +G model. Support for the  
1059 topology was examined using bootstrapping (Felsenstein, 1985) over 200

1060 replications. Bootstrap values lower than 60% are not shown. The sequences obtained in this  
1061 study are indicated by circles.

1062 **Figure 9:** Graphical table summarizing the microorganisms detected in each hedgehog,  
1063 allowing visualization of the existing co-infections.

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1065 **Supplementary Data 1:** Summary table of *Atelexis algirus* hedgehog collection sites and  
1066 dates, gender and ectoparasites collection and identification results.

1067 **Supplementary Data 2:** Representative MS profiles obtained for each arthropod species  
1068 using the Flex analysis 3.3 software. a.u.: arbitrary units; m/z: mass-to-charge ratio; **(A)**  
1069 *Haemaphysalis erinacei*, **(B)** *Rhipicephalus sanguineus*, **(C)** *Archaeopsylla erinacei*.

1070 **Supplementary Data 3:** FASTA sequences of the amplified *16S RNA* and *ITS1* gene  
1071 fragments obtained from ticks (*Haemaphysalis erinacei* and *Rhipicephalus sanguineus* s.l.)  
1072 and fleas (*Archaeopsylla erinacei*) sampled from hedgehogs.

1073 **Supplementary Data 4:** GenBank accession numbers of all sequences obtained from  
1074 arthropods and bacteria.

1075 **Supplementary Data 5:** FASTA sequences of the amplified gene fragments obtained from  
1076 the different bacteria detected in hedgehog organs and arthropod samples.

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1085 **Tables**

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1087 **Table 1:** Arthropod morphological, molecular and MALDI-TOF MS identification results.

Arthropod	Morphological identification			Molecular Identification				MALDI-TOF MS identification					
	Species ID	Number of collected specimens	Number of specimens selected for this study	Tested specimens	Good quality sequences	Species ID (BLAST results)	1 <sup>st</sup> Hit GenBank Accession Number (ID%)	Tested samples	Number of specimens with good quality spectra	Number of specimens used for database upgrade	Number of specimens used for blind test	Blind test Correct Species ID	LSV* obtained in blind test analysis
<b>Fleas</b>	<i>Archaeopsylla erinacei</i>	853	119	26	23/26 (88.46%)	<i>A. erinacei</i>	LT627362 LT604112 LT576377 (99.77%-100%)	26	24/26 (92.31%)	4	20	20/20 (100%)	1.861 – 2.485 (average: 2.172)
<b>Ticks</b>	<i>Haemaphysalis erinacei</i>	39	32	6	6/6 (100%)	<i>Hae. erinacei</i>	KX901846 KU880549 (97.44%-97.97%)	13	12/13 (92.31%)	2	10	10/10 (100%)	1.926 – 2.226 (average: 2.068)
	<i>Rhipicephalus sanguineus</i> s.l.	151	78	10	10/10 (100%)	<i>Rh. sanguineus</i> s.l.	MG855662 MH018852 KX793742 MW172416 JX997393 KT382469 (99.15%-100%)	51	48/51 (94.12%)	3	45	45/45 (100%)	1.415 – 2.362 (average: 1.916)
	<i>Rhipicephalus</i> spp. (Engorged ticks)	28	28	28	21/28 (75%)	<i>Rh. sanguineus</i> s.l.	KX793742 KT382469 MH630344 KT382465 JX997393 MH018852 (97.66%-100%)	28	28/28 (100%)	0	28	28/28 (100%)	1.451 – 2.085 (average: 1.856)
<b>Total</b>		1071	257	70	60/70 (85.71%)			118	112/118 (94.91%)	9	103	103/103 (100%)	1.415 – 2.485

1088 (\*) LSV: Logarithmic Score Value

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1101 **Table 2:** Primers and probes used for real-time and conventional PCR.

Microorganisms	Targeted fragment	Primers F, R (5'-3') and Probes P (5'-6FAM-3'-TAMRA)	References
<b>Real-Time PCR</b>			
<i>Rickettsia</i> spp. (Spotted fever group)	<i>gltA</i> (RKND03)	F_GTGAATGAAAGATTACACTATTTAT R_GTATCTTAGCAATCATTCTAATAGC P_CTATTATGCTTGCGGCTGTCGGTTC	[89]
<i>Rickettsia felis</i>	<i>Phosphatase gene</i>	Rfel phosp_MBF_GCAAACATCGGTGAAATTGA Rfel_phosp_MBR_GCCACTGTGCTTCACAAACA Rfel_phosp_MBP_CCGCTTCGTTATCCGTGGGACC	[68]
<i>Rickettsia typhi</i>	<i>Glycosyl-transferase</i>	Rpr331F_TGCTTCATGGGCAATGTCTG Rpr331R_TTGAGCATAAAACTGCCCTGC Rpr331P_CGCTGGATTATCAAAAAGAATTAGCAGC	[90]
<i>Rickettsia massiliae</i>	<i>Hypothetical protein</i>	R.massi 9666-F_CCAACCTTTTGTGTTGCAC R.massi 9666-R_TGGATCAGTGTGACGGACT R.massi 9666-P_CACGTGCTGCTTATACCAGCAAACA	[91]
<i>Rickettsia conorii</i>	<i>Hypothetical protein RC0743</i>	F_TTTTGGGGTAGCACATTTTCAA R_TTCGCCGTAACGTGCTGTAG P_GGAAGATTTGCAGCAAAAATGCTTGGTG	[89]
<i>Rickettsia aeschlimannii</i>	<i>Sca1</i>	R_aesSca1_F_AAGCGCACTTTAGGTAAAGAAA R_aesSca1_R_CATGCTCTGCAAATGAACCA R_aesSca1_P_TGGGGAAATATGCCGTATACGCAAGC	[91]
<i>Bartonella</i> spp.	<i>Barto ITS2</i>	F_GATGCCGGGAAGGTTTTC R_GCCTGGGAGGACTTGAACCT P_GCGCGCGCTTGATAAGCGTG	[92]
<i>Borrelia</i> spp.	<i>Bor ITS4</i>	F_GGCTTCGGGTCTACCACATCTA R_CCGGGAGGGGAGTGAAATAG P_TGCAAAAAGGCACGCCATCACC	[91]
<i>Leptospira</i> spp.	<i>16S</i>	F_CCCGCGTCCGATTAG R_TCCATTGTGGCCGRACAC P_CTCACCAAGGCGACGATCGGTAGC	[93]
<i>Coxiella burnetii</i>	<i>IS1111</i>	F_CAAGAAACGTATCGCTGTGGC R_CACAGAGCCACCGTATGAATC P_CCGAGTTTCGAAACAATGAGGGCTG	[94]
	<i>IS30A</i>	F_CGCTGACCTACAGAAATATGTCC R_GGGGTAAGTAAATAATACCTTCTGG P_CATGAAGCGATTTATCAATACGTGTATG	[94]
<i>Anaplasmataceae</i>	<i>23S rRNA</i>	F_TGACAGCGTACCTTTTGCAT R_TGGAGGACCGAACCTGTAC P_GGATTAGACCCGAAACCAAG	[95]
<i>Wolbachia</i> spp.	<i>23S</i>	F_CCAAAATTACAGCTAAGTGG R_AGTGAGCTGTTACGCTTTCT P_AGTGAGCTGTTACGCTTTCT	[96]
<b>Standard PCR</b>			
<i>Rickettsia</i> spp.	<i>ompA</i> (632 bp)	190-70_F_ATGGCGAATATTTCTCCAAAA 190-701_R_GTTCCGTTAATGGCAGCATCT	[97]
<i>Bartonella</i> spp.	<i>gltA</i> (200 bp)	F_ACGTCGAAAAGAYAAAAATG R_GTAATRCCAGAAATARAAAATC	[96]
	<i>ftsZ</i> (333 bp)	F_FTSZDIR_CCGTGAATAATGATTAATGC R_FTSZREV_TTGAAATGGCTTTGTCAAC	[96]
<i>Leptospira</i> spp.	<i>rpoB</i> (592 bp)	Lept 1900F_CCTCATGGGTTCCAACATGCA Lept 2500 R_CGCATCCTCRAAGTTGTAWCCTT	[98]
<i>Anaplasmataceae</i>	<i>23S rRNA</i> (485 bp)	F_GTTGAAAARACTGATGGTATGCA R_TGCAAAAAGGTACGCTGTAC	[85]
<i>Ehrlichia</i> spp.	<i>groEL</i> (590 bp)	Ehr-groEL-F_GTTGAAAARACTGATGGTATGCA Ehr-groEL-R_ACACGRTCTTTACGYTCYTAAAC	[99]
Ticks	<i>16S</i> (460 bp)	F_CCGTCTGAACTCAGATCAAGT R_GC TCAATGATTTTTTAAATTGCTGT	[33]
Fleas	<i>ITS1</i> (1000 bp)	F_GTA CAC ACC GCC CGT GCG TAC T R_GCT GCG TTC TTC ATC GAC CC Sen-ITS2	[32]

1102 **Table 3:** Summary results of microorganism detection in hedgehog organs and their  
 1103 associated arthropods (ticks and fleas).

Bacterium % (Positives/Tested)	Spleen	Kidney	<i>Archaeopsylla erinacei</i>	<i>Rhipicephalus sanguineus</i>	<i>Haemaphysalis erinacei</i>	Total
<b><i>Rickettsia</i> sp.</b>	-	-	<b>87/119</b>	<b>58/106</b>	<b>21/32</b>	<b>55.7% (166/298)</b>
<i>Rickettsia felis</i>	/	/	68/87	/	/	22.8% (68/298)
<i>Rickettsia massiliae</i>	/	/	/	40/58	/	13.4% (40/298)
Uncultivated <i>Rickettsia</i> spp.	/	/	/	-	2/32	0.67% (2/298)
<b><i>Leptospira</i> sp.</b>	<b>1/20</b>	<b>7/21</b>	-	<b>2/78</b>	-	<b>3.3% (10/298)</b>
<i>Leptospira interrogans</i>	-	2/7	/	-	/	0.7% (2/298)
<b>Anaplasmataceae</b>	<b>5/20</b>	<b>2/21</b>	<b>62/119</b>	<b>3/78</b>	<b>4/32</b>	<b>25.5% (76/298)</b>
<i>Wolbachia</i> sp.	/	/	62/62	-	-	20.8% (62/298)
<i>Ehrlichia</i> sp.	/	/	/	1/3	3/4	1.3% (4/298)
<b><i>Coxiella burnetii</i></b>	<b>6/20</b>	<b>11/21</b>	<b>4/119</b>	<b>9/78</b>	<b>15/32</b>	<b>15.1% (45/298)</b>
<b><i>Bartonella</i> sp.</b>	<b>2/20</b>	-	<b>6/119</b>	-	-	<b>2.7% (8/298)</b>
Uncultivated <i>Bartonella</i> spp.	1/20	/	1/119	/	/	0.7% (2/298)

1104 (-): Negative sample; (/) Untested.

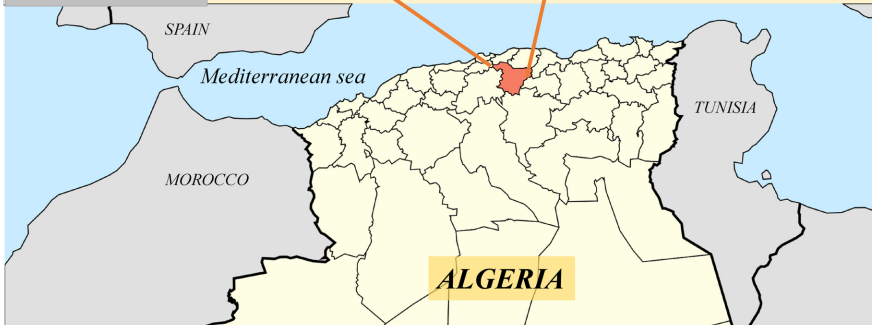
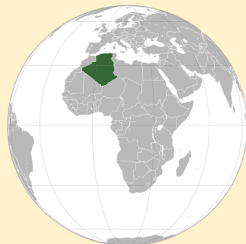
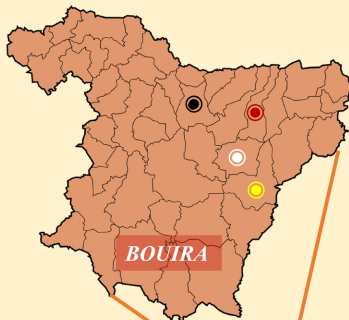


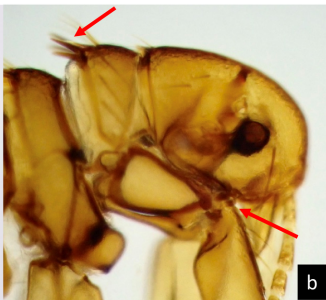
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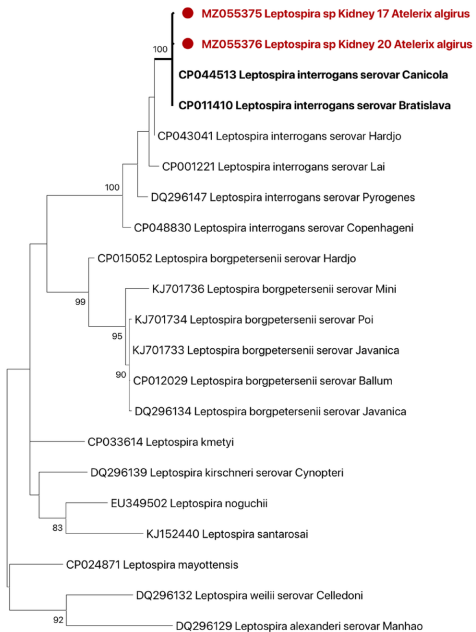
● *Bechloul*

○ *Ahl-El-Ksar*





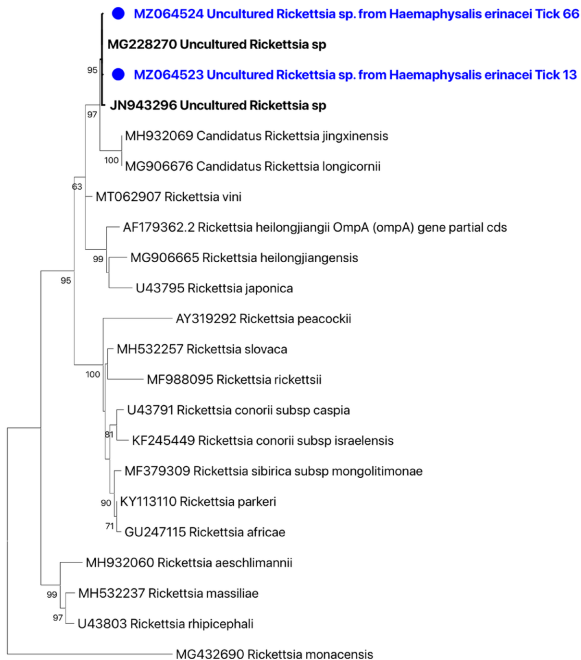


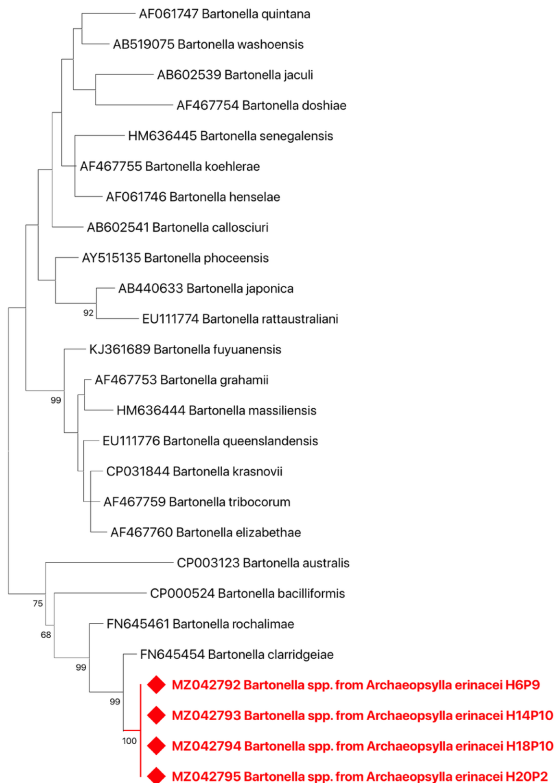


*Leptospira interrogans*

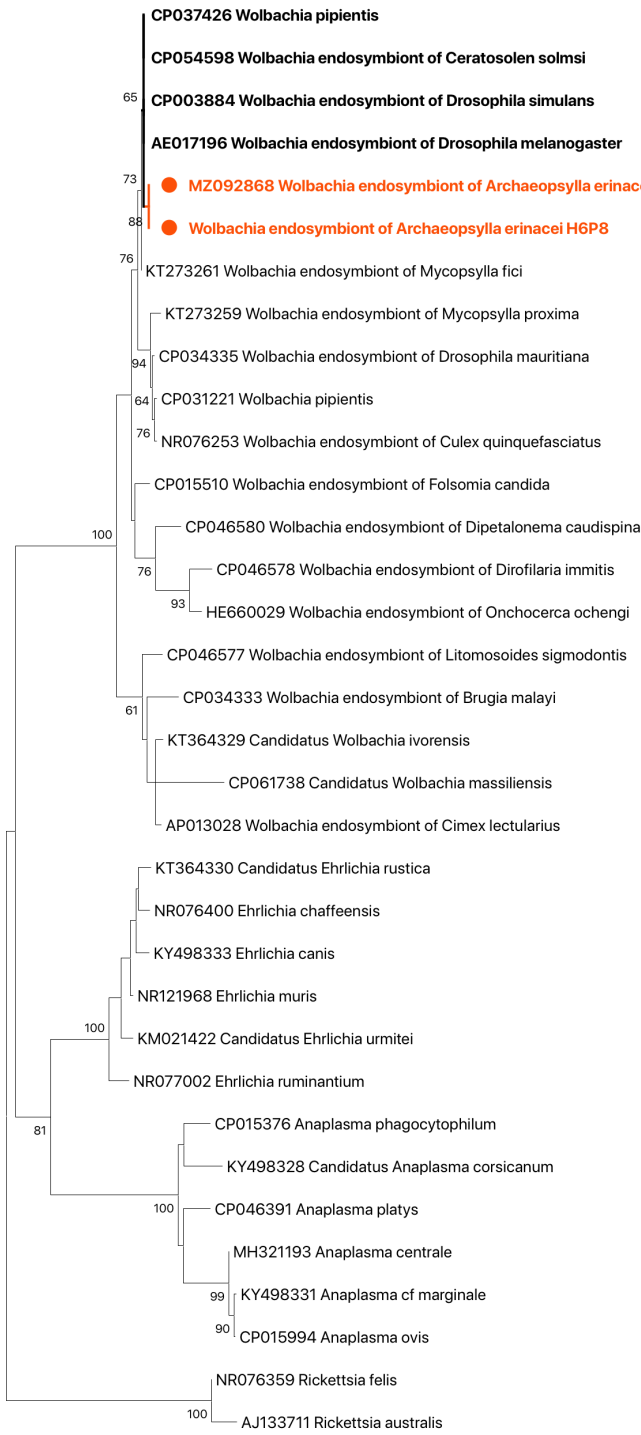
*Leptospira borgpetersenii*

Other *Leptospira* species





A



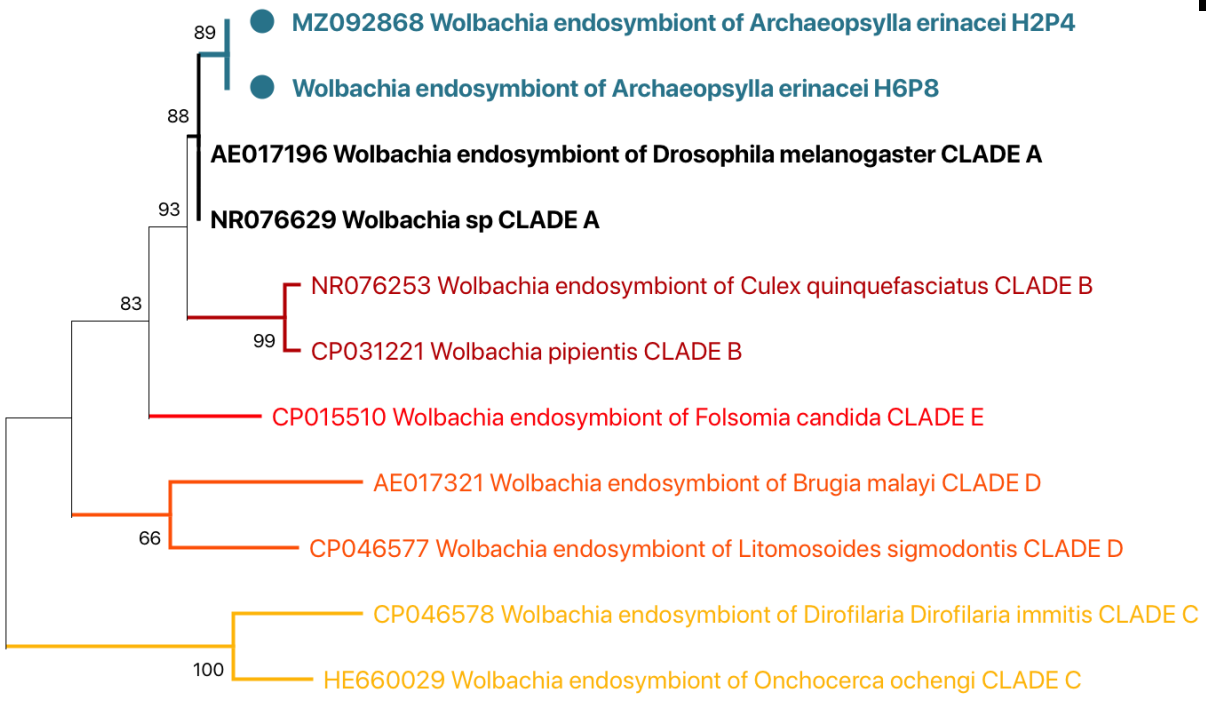
Wolbachia

Ehrlichia

Anaplasma

0.050

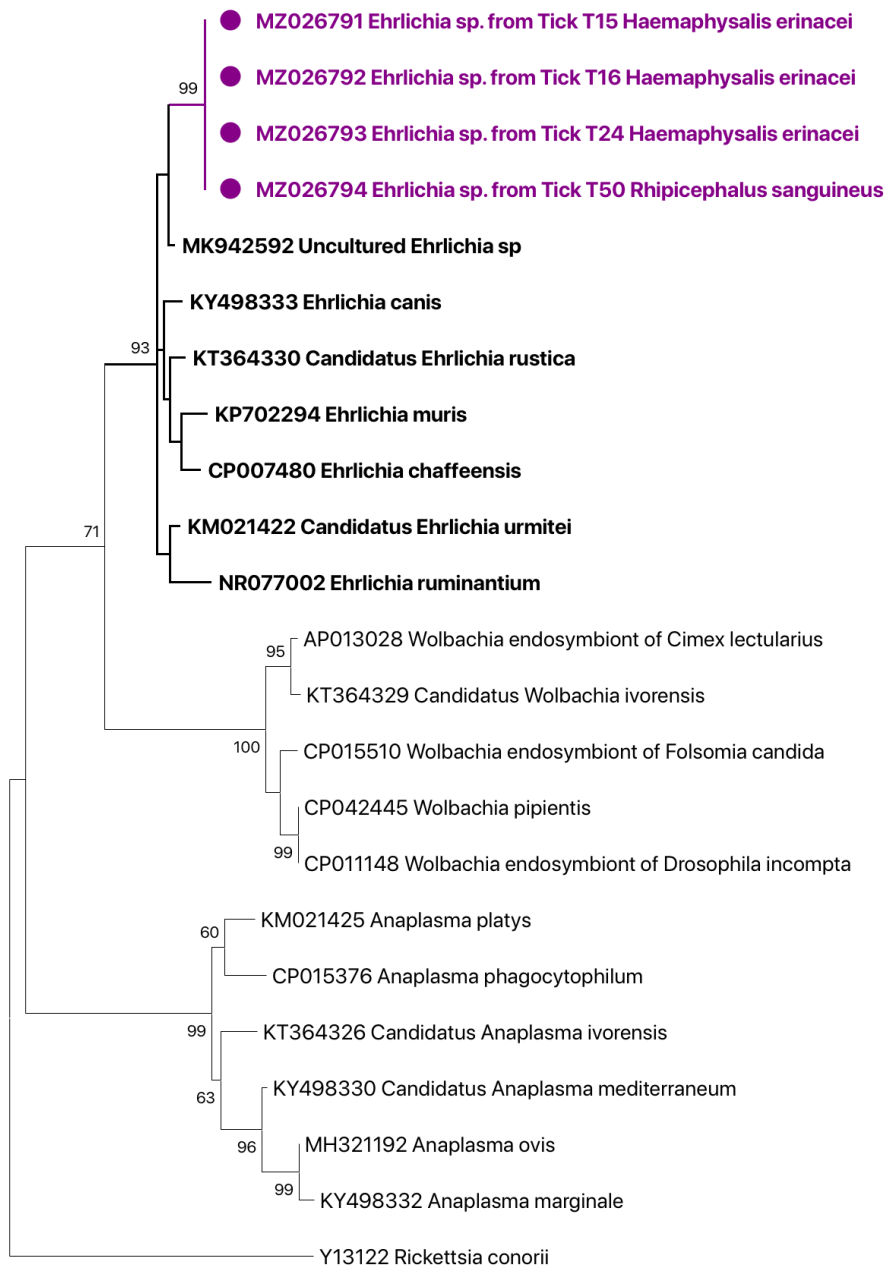
B



} A  
 } B  
 } E  
 } D  
 } C

0.010

A



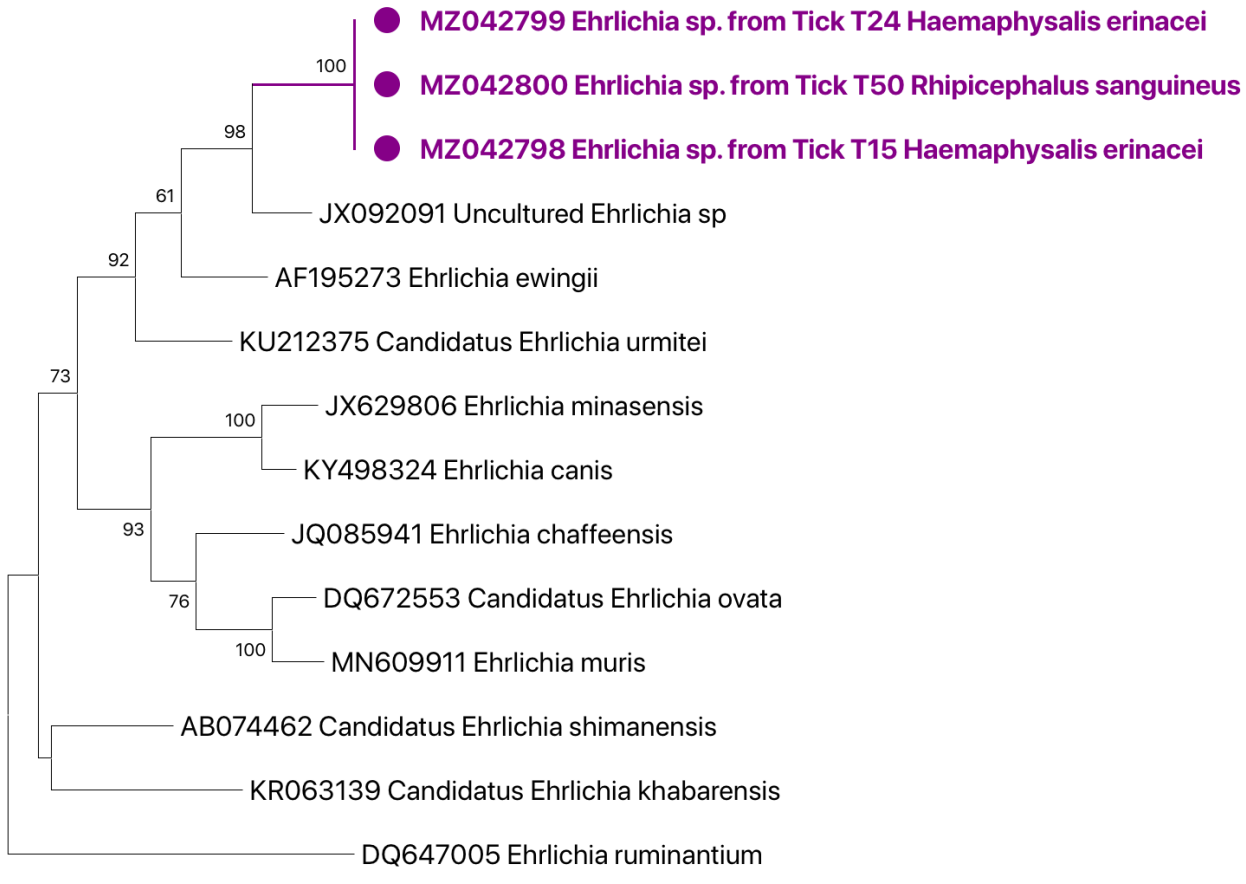
Ehrlichia

Wolbachia

Anaplasma

0.050

B



0.020



### Hedgehogs

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
<i>Coxiella burnetii</i>	●		▲			▲	●	★	▲	▲	▲	▲	▲			▲		★	▲	●	▲
<i>Leptospira spp.</i>		▲				▲	●			▲	▲			▲					▲		
<i>Leptospira interrogans</i>																	▲			▲	
<i>Bartonella spp.</i>						★	●				▲			●				●		●	●
<i>Rickettsia spp.</i>	●	●	●	●	●	●	●	●							●					●	
<i>Rickettsia felis</i>		●		●	●	●	●	●							●				●	●	●
<i>Rickettsia massiliae</i>		●				●	●	●							●					●	●
<i>Borrelia spp.</i>																					
Anaplasmataceae				★		●	★	★	▲										★	▲	
<i>Wolbachia spp.</i>		●		●	●	●	●								●				●		●
<i>Ehrlichia spp.</i>				●			●														



Only Organs (+)



Only Arthropods (+)



Both Organs and  
Arthropods (+)

(●): Hedgehogs with  
Ectoparasites



Algeria (Bouira)

21 dead hedgehogs  
(*Aterix algirus*)

12 hedgehogs parasitized  
by arthropods



+



21  
Kidneys

20  
Spleens

## Arthropod identification

Morphology      Molecular biology

MALDI-TOF MS

*A. erinacei*      *Hae. erinacei*      *Rh. sanguineus* s.l.

N= 853      N= 39      N= 179

## Detection of microorganisms

qPCR

*Rickettsia* spp.  
*Borrelia* spp.  
*Bartonella* spp.  
*Leptospira* spp.  
Anaplasmataceae  
*Coxiella burnetii*

138    119    21    20

Specific qPCR  
OR  
Standard PCR & Sequencing

*R. massiliae*  
*Rickettsia* sp.  
*Ehrlichia* sp.\*

*R. felis*  
*Rickettsia* sp.  
*Wolbachia* sp.

*Leptospira* sp.    *C. burnetii*    *Bartonella* sp.\*

*Leptospira interrogans*  
Anaplasmataceae bacteria

\*Potential new species