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

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

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 ectoparasites [7]. Previous studies have reported the presence of Anaplasma 65 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 [10]. Borrelia burgdorferi s.l., the agent of Lyme disease, has also been detected in E. 71 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., Toxoplasma gondii and Cryptosporidium) have also been found in hedgehogs [7,16]. 78 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

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

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

133

134 2.4. Morphological identification of arthropods

All collected arthropods were morphologically identified to the species level initially in 135 136 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 ¹/₄ 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).

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

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

Using ChromasPro software version 1.7.7 (Technelysium Pty. Ltd., Tewantin, Australia), the
obtained sequences were assembled, analyzed, emended and then blasted against the NCBI
GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to be compared to available reference
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, Anaplasmataceae 23S and Ehrlichia spp. groEL sequenced genes, together with their

216 corresponding reference sequences available in GenBank, were performed using MEGA

version X software (Kumar, Stecher, Li, Knyaz, and Tamura 2018) prior to phylogenetic treeplotting.

219

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

231	3. Results
230	
229	phylogenetic trees.
228	examined using bootstrapping (Felsenstein, 1985) over 200 replications for all
227	(K2) +G model for the Leptospira spp. rpoB gene. Support for the topology was
226	groEL gene and Wolbachia clades Anaplasmataceae 23S gene and the Kimura 2-parameter
225	Anaplasmataceae 23S and Rickettsia spp. ompA genes, HKY +G model for the Ehrlichia spp.

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 ticks. All details regarding capture sites, hedgehog sex, and the number and species of 237 238 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*

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 <i>Hae. erinacei</i> , 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 <i>Rh. sanguineus</i> 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

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

the unengorged ticks (*Hae. erinacei* and *Rh. sanguineus* s.l.), 88.5% (23/26) of the tested fleas

and 75% (21/28) of the engorged *Rhipicephalus* spp. ticks.

280 The BLAST analysis revealed that unengorged *Rh. sanguineus* s.l. *16S* sequences had

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

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

with reference sequences of *Rhipicephalus sanguineus* IIa haplotype of the temperate lineage

and five had similarities with sequences of IIb haplotype of the same lineage. The 16S

sequences obtained for *Hae. erinacei* had a query cover ranging from 95% to 100% and

identities ranging from 97.44% to 97.97% with the *Hae. erinacei 16S* reference sequences

from GenBank (Accession Numbers: KX901846 and KU880549). As for BLAST results of

the flea *ITS1* sequences, they had an identity ranging from 99.77% to 100% with those of A.

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.

The tick (*Hae. erinacei* and *Rh. sanguineus* s.l.) *16S RNA* partial sequences and the flea (*A. erinacei*) *ITS1* partial sequences have been deposited in GenBank and the accession numbers
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

308kidney 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

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

interrogans.

325

324

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*. aeschlimannii. 335 336 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 <i>gltA</i> gene for <i>Bartonella</i> 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 <i>gltA</i> gene, a 333 base pairs fragment of
358	the <i>ftsZ</i> 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

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 from pigs in Democratic Republic of the Congo (Accession Numbers: MK942592, 386 387 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

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.

In our study, the fleas and ticks collected from hedgehogs were morphologically identified as *A. erinacei, Rh. sanguineus* s.l. and *Hae. erinacei.* Twenty-eight engorged female ticks were
identified only at the genus level as *Rhipicephalus* spp. for the reason that they were lacking
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 serological study reported the presence of antibodies to Coxiella burnetii in European 490 491 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.

erinacei or if these fleas were infected by feeding on a bacteremic hedgehog or by co-feeding
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 aerobe 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.

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

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

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 reservoirs of Bartonella species [72,73]. Several studies reported different species of 564 565 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

in muscle and skin samples collected on northern white-breasted hedgehogs *Erinaceus 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

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

mainly by arthropods [79]. It also includes *Wolbachia spp*. bacteria, which are known as anendosymbiont 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 5. Conclusion 629

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

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

637

638 **Declarations**

639

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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 674 675 [1] H.J. Temple, A. Cuttelod, (Compilers), The Status and Distribution of Mediterranean 676 Mammals, Gland, IUCN, Switzerland and Cambridge, UK, 2009. 677 [2] M. Khaldi, C. Socolovschi, M. Benyettou, G. Barech, M. Biche, T. Kernif, D. Raoult, 678 P. Parola, Rickettsiae in arthropods collected from the North African Hedgehog 679 (Atelerix algirus) and the desert hedgehog (Paraechinus aethiopicus) in Algeria, 680 Comparative Immunology, Microbiology and Infectious Diseases. 35 (2012) 117–122. 681 https://doi.org/10.1016/j.cimid.2011.11.007. 682 [3] A. Nematollahi, J.A. Helan, H. Golezardy, N. Zaboli, M. Nouruzi, M. Azari, Parasitic 683 Fauna of East European Hedgehog (Erinaceus concolor) and their pathological aspects 684 in Iran, Advances in Zoology and Botany. 2 (2014) 1-5. 685 https://doi.org/10.13189/azb.2014.020101. 686 [4] Y. Goz, A.B. Yilmaz, A. Aydin, Y. Dicle, Ticks and Fleas Infestation on East 687 Hedgehogs (Erinaceus concolor) in Van Province, Eastern Region of Turkey, Journal 688 of Arthropod-Borne Diseases. 10 (2016) 50-54. 689 [5] H. Leulmi, A. Aouadi, I. Bitam, A. Bessas, A. Benakhla, D. Raoult, P. Parola, 690 Detection of Bartonella tamiae, Coxiella burnetii and rickettsiae in arthropods and 691 tissues from wild and domestic animals in northeastern Algeria, Parasites & Vectors. 9 (2016) 1-8. https://doi.org/10.1186/s13071-016-1316-9. 692

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- 1018 Figure captions
- 1019
- 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
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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.

- Supplementary Data 1: Summary table of *Atelerix algirus* hedgehog collection sites and
 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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- MALDI-TOF MS identification Arthropod Morphological identification Molecular Identification Number of specimens used for Species ID Good quality sequence Species ID 1st Hit GenBank Blind test Tested LSV* obtained Number Tested Number Number Number Number of specimens with good quality spectra Correct of specimen selected for this of specimens used for blind test in blind test analysis of collected specin n Number (BLAST results) ۸ Species ID (ID%) specimens database study upgrade LT627362 Archaeopsylla erinacei 23/26 LT604112 24/26 20/20 1.861 - 2.485Fleas 853 119 26 A. erinacei 26 4 20 (88.46%) LT576377 (92.31%) (100%) (average: 2.172) (99.77%-100%) KX901846 12/13 10/10 1.926 - 2.226 Haemaphysalis erinacei 6/6 39 32 2 10 6 Hae. erinacei KU880549 13 (100%) (92.31%) (100%) (average: 2.068) (97.44%-97.97%) MG855662 MH018852 KX793742 MW172416 JX997393 KT382469 Rh. Rhipicephalus sanguineus s.1. 10/10 48/51 45/45 1.415 - 2.362 151 78 10 nguineus s.l. 51 3 45 (94.12%) (100%) (100%) (average: 1.916) Ticks (99.15%-100%) KX793742 KT382469 MH630344 Rhipicephalus Rh. 21/28 28/28 28/28 1.451 - 2.085 28 28 28 KT382465 28 0 28 spp sanguineus s.l. (100%) (100%) (average: 1.856) (75%) (Engorged ticks) JX997393 MH018852 (97,66%-100%) Total 60/70 112/118 103/103 1071 257 70 118 9 103 1.415 - 2.485 (85.71%) (94.91%) (100%) 1088 (*) LSV: Logarithmic Score Value 1089 1090 1091 1092 1093 1094
- 1087 **Table 1:** Arthropod morphological, molecular and MALDI-TOF MS identification results.

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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		
Real-Time PCR					
<i>Rickettsia</i> spp. (Spotted fever group)	gltA (RKND03)	F_GTGAATGAAAGATTACACTATTTAT R_GTATCTTAGCAATCATTCTAATAGC P_CTATTATGCTTGCGGCTGTCGGTTC	[89]		
Rickettsia felis	Phosphatase gene	Rfel phosp MBF_GCAAACATCGGTGAAATTGA Rfel_phosp_MBR_GCCACTGTGCTTCACAAACA Rfel_phosp_MBP_CCGCTTCGTTATCCGTGGGACC	[68]		
Rickettsia typhi	Glycosyl- transferase	Rpr331F_TGCTTCATGGGCAATGTCTG Rpr331R_TTGAGCATAAAACTGCCCTGC Rpr331P_CGCTGGATTATCAAAAGAATTAGCACG	[90]		
Rickettsia massiliae	Hypothetical protein	R.massi 9666-F_CCAACCTTTTGTTGTTGCAC R.massi 9666-R_TTGGATCAGTGTGACGGACT R.massi 9666-P_CACGTGCTGCTTATACCAGCAAACA	[91]		
Rickettsia conorii	Hypothetical protein RC0743	F_TTTTGGGGTAGCACATTTTCAA R_TTCGCCGTAACGTGCTGTAG P_GGAAGATTTGCAGCAAAATGCTTGGTG	[89]		
Rickettsia aeschlimannii	Sca1	R aesSca1 F_AAGCGGCACTTTAGGTAAAGAAA R aesSca1 R_CATGCTCTGCAAATGAACCA R aesSca1 P_TGGGGAAATATGCCGTATACGCAAGC	[91]		
Bartonella spp.	Barto ITS2	F_GATGCCGGGGAAGGTTTTC R_GCCTGGGAGGACTTGAACCT P_GCGCGCGCTTGATAAGCGTG	[92]		
Borrelia spp.	Bor ITS4	F_GGCTTCGGGTCTACCACATCTA R_ CCGGGAGGGGAGTGAAATAG P_TGCAAAAGGCACGCCATCACC	[91]		
Leptospira spp.	165	F_CCCGCGTCCGATTAG R_TCCATTGTGGCCGRACAC P_CTCACCAAGGCGACGATCGGTAGC	[93]		
Coxiella burnetii	IS1111	F_CAAGAAACGTATCGCTGTGGC R_CACAGAGCCACCGTATGAATC P_CCGAGTTCGAAACAATGAGGGCTG	[94]		
	IS30A	F_CGCTGACCTACAGAAATATGTCC R_GGGGTAAGTAAATAATACCTTCTGG P_CATGAAGCGATTTATCAATACGTGTATG	[94]		
Anaplasmataceae	23S rRNA	F_TGACAGCGTACCTTTTGCAT R_TGGAGGACCGAACCTGTTAC P_GGATTAGACCCGAAACCAAG	[95]		
Wolbachia spp.	235	F_CCAAAATTACAGCTAAGTGG R_AGTGAGCTGTTACGCTTTCT P_ AGTGAGCTGTTACGCTTTCT	[96]		
Standard PCR					
Rickettsia spp.	<i>ompA</i> (632 bp)	190-70_ F_ATGGCGAATATTTCTCCAAAA 190-701_ R_GTTCCGTTAATGGCAGCATCT	[97]		
Bartonella spp.	gltA (200 bp)	F_ACGTCGAAAAGAYAAAAATG R_GTAATRCCAGAAATARAAATC	[96]		
	ftsZ (333 bp)	F_FTSZDIR_CCGTGAATAATATGATTAATGC R_FTSZREV_TTGAAATGGCTTTGTCACAAC	[96]		
Leptospira spp.	<i>rpoB</i> (592 bp)	Lept 1900F_CCTCATGGGTTCCAACATGCA Lept 2500 R_CGCATCCTCRAAGTTGTAWCCTT	[98]		
Anaplasmataceae	23S rRNA (485 bp)	F_GTTGAAAARACTGATGGTATGCA R_TGCAAAAGGTACGCTGTCAC	[85]		
Ehrlichia spp.	groEL (590 bp)	Ehr-groEL-F_GTTGAAAARACTGATGGTATGCA Ehr-groEL-R_ACACGRTCTTTACGYTCYTTAAC	[99]		
Ticks	<i>16S</i> (460 bp)	F_CCGGTCTGAACTCAGATCAAGT 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	Archaeopsylla erinacei	Rhipicephalus	Haemaphysalis erinacei	Total
Rickettsia sp.	-	-	87/119	58/106	21/32	55.7% (166/298)
Rickettsia felis	/	/	68/87	/	/	22.8% (68/298)
Rickettsia massiliae	/	/	/	40/58	/	13.4% (40/298)
Uncultivated Rickettsia spp.	/	/	/	-	2/32	0.67% (2/298)
Leptospira sp.	1/20	7/21	-	2/78	-	3.3% (10/298)
Leptospira interrogans	-	2/7	/	-	/	0.7% (2/298)
Anaplasmataceae	5/20	2/21	62/119	3/78	4/32	25.5% (76/298)
Wolbachia sp.	/	/	62/62	-	-	20.8% (62/298)
Ehrlichia sp.	/	/	/	1/3	3/4	1.3% (4/298)
Coxiella burnetii	6/20	11/21	4/119	9/78	15/32	15.1% (45/298)
Bartonella sp.	2/20	-	6/119	-	-	2.7% (8/298)
Uncultivated Bartonella spp.	1/20	/	1/119	/	/	0.7% (2/298)

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









Leptospira interrogans

Leptospira borgpetersenii

Other Leptospira species



0.022







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0.050
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Treagenogs																					
	1 •	2	3	4	5 •	6 •	7	8	9	10	11	12	13	14 •	15	16	17	18 •	19	20 •	21 •
Coxiella burnetii	0		${\boldsymbol{\bigtriangleup}}$			${\boldsymbol{\Delta}}$	0	★	${\boldsymbol{\bigtriangleup}}$	${\boldsymbol{\bigtriangleup}}$	Δ	Δ	${\boldsymbol{\Delta}}$			${\boldsymbol{\Delta}}$		★	${\boldsymbol{\bigtriangleup}}$	$oldsymbol{\circ}$	Δ
Leptospira spp.		$\mathbf{\Delta}$				${\color{black} \bigtriangleup}$	0			$ \Delta $	Δ			Δ					${\color{black} \bigtriangleup}$		
Leptospira interrogans																	Δ			${\boldsymbol{\Delta}}$	
Bartonella spp.						\star		\circ				$ \Delta $		0				0		\circ	0
Rickettsia spp.	0	0	0	0	0	0	0	0						0						0	
Rickettsia felis		0		0	0	0	0	0						0				0		$oldsymbol{\circ}$	0
Rickettsia massiliae		0				0	0	0						0						\circ	0
Borrelia spp.																					
Anaplasmataceae				★		0	★	★	$ \Delta $									★	$ \Delta $		
Wolbachia spp.		0		0	0	0	0							0				0		\circ	0
Ehrlichia spp.				0			0														

 ▲ Only Organs (+)
 ● Only Arthropods (+)
 ★
 Both Organs and Arthropods (+)
 (•): Hedgehogs with Ectoparasites

