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Research Article

Molecular identification and evaluation of *Coxiella*-like endosymbionts genetic diversity carried by cattle ticks in Algeria

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Abstract

Coxiella-like bacteria are a large group of yet-to-isolate and characterize bacteria phylogenetically close to the agent of Q fever, *Coxiella burnetii*, and often associated with ixodid ticks worldwide.

This study was designed to assess the presence of *Coxiella*-like endosymbionts (CLE) in ticks and to describe their genetic diversity in different tick species infesting cattle in Algeria. A total of 765 ticks were collected from three locations. The screening of 20 % of sampled ticks (147/765) exhibited the presence of *Coxiella*-like in 51.7 % (76/147). The sequencing of partial 16S rRNA and the G*roEl* genes showed an identity higher than 98 % with different *Coxiella*-like endosymbionts. The phylogenetic analysis based on the 16S rRNA gene showed the positions of identified *Coxiella* bacteria. Eleven of the 13 sequences from *Rhipicephalus*, *Dermacentor* and *Hyalomma* ticks were grouped in a distinct clade, the other two each represent an independent clade.

This study reported that CLE are prevalent in cattle ticks. Most of the identified *Coxiella*-like bacteria, from different species of ticks found on cattle, were identical. This may mean that, unlike the currently accepted paradigm, *Coxiella*-like bacteria are not only tick host-associated, but rather can be transmitted from one tick species to another via the vertebrate host.

Key words: *Coxiella*-like, Ticks, Cattle, qPCR, sequencing, Algeria.

1. Introduction

Ticks and mosquitoes are considered the most important vectors of pathogenic microorganisms for both humans and terrestrial vertebrate animals in the world (Dehhaghi et al., 2019). A high number of infectious agents, such as viruses, bacteria and protozoans, are transmitted by ticks (Brinkmann et al., 2019).

All tick-borne diseases (TBDs) might be considered zoonotic diseases that represent one of the most rapidly expanding arthropod-borne diseases (Pfäffle et al., 2013), since several tick-borne infections with a significant impact on human and animal's health have been observed in recent decades (Brinkmann et al., 2019).

To date, about 900 tick species have been identified and classified worldwide, while in Africa, 223 tick species have been described, 180 of which are classified as hard and 43 as soft ticks (Diarra et al., 2017). Besides, the pathogens carried by these ticks, diverse commensal and symbiotic microorganisms are also present, such as *Coxiella*-like endosymbionts (CLEs) (Špitalská et al., 2018; Zhong, 2012).

Coxiella burnetii, the Q fever agent distributed worldwide, has already been reported many times in Algeria (Angelakis et al., 2014; Khaled et al., 2016). Multispacer type 20 was determined to be the circulating genotype in cattle in the northern Algeria (Rahal et al., 2018).

According to Duron et al. (2015), all strains of *C. burnetii* are the descendants of a *Coxiella*-like progenitor. Consequently, they originated from this group hosted by ticks. No strains of *Coxiella*-like are yet cultured in the environment of vertebrate cells, suggesting that their metabolic needs are different from those of *C. burnetii.* In addition, the recent evolvement of *C. burnetii* from an inherited symbiont of ticks allowed it to infect vertebrates after acquisitions of novel virulence factors (Duron et al., 2015).

According to the recent sequencing of *Coxiella*-like bacterium found in *Amblyomma americanum* (Smith et al., 2015), recognizable virulence genes have not been found and the main biosynthesis pathways of vitamins and cofactors are encoded by its genome. Thus, it may be a vitamin-provisioning endosymbiont (Duron et al., 2015). Such bacterial functions might be involved in a mutualistic interaction with the tick host by compensating nutritional deficiencies. In fact Guizzo et al. (2017) and Gottlieb et al. (2015) reported that the biosynthetic pathways for the vitamins Biotin (B7), Riboflavin (B2), Pyridoxine (B6), Folic acid (B9) and Pantothenate (B5) are found in all CLE. In addition, the coding genes for the cofactors Flavin

adenine dinucleotide (FAD) and Coenzyme A (CoA) are found in all CLEs A hypothesis is supported with the observed decreases in ticks fecundity and life-history traits successive to the symbiont elimination (René-Martellet et al., 2017). The study conducted by Guizzo et al. (2017) and (Zhong et al., 2007) showed that tetracycline treatment of ticks led to the reduction of levels of bacteria in progeny in 74 % for eggs and 90 % for larvae, and hampered the tick to reach the adult life stage, as no tick was able to progress beyond the metanymph stage. While Lalzar et al. (2012), suggested vertical transmission with a high fidelity of CLE since they identified it in eggs and larvae of *Rhipicephalus sanguineus* sensu lato-associated (*R. sanguineus* SL-A). This vertical transmission showed 98 % of the 16S rRNA sequence identity in both eggs and larvae (Guizzo et al., 2017), which may explain the high frequency of *Coxiella*-like in the tick populations.

However, *Coxiella*-like was confirmed to be a new agent of human infections. These bacteria can possibly lead to an atypical scalp eschar and neck lymphadenopathy syndrome with delayed evolution to crust eschar in the area of the tick-bite (Guimard et al., 2017).

A genetic variant of *R. sanguineus* SL-A *Coxiella*-like bacterium was detected in skin biopsy samples and ticks collected from patients with eschar (Angelakis et al., 2016). This is evidence that at least this strain might be responsible for scalp eschar and neck lymphadenopathy after a tick bite (Guimard et al., 2017). Furthermore, a multiorgan infection with a *Coxiella*-like organism was determined to be the cause of death of a female eclectus parrot *Eclectus roratus* (Vapniarsky et al., 2012).

Although *Coxiella*-like bacteria and *C. burnetii* share a certain degree of identity, it is not high enough to be clustered in the same species (Angelakis et al., 2016). All *Coxiella*-like bacteria undoubtfully belong to the genus *Coxiella* in the gamma-subgroup of the *Proteobacteria.* This taxonomy was based on the 16S rRNA nucleotide sequences. The sequence identity of the 16S rRNA gene among the *Coxiella* -like bacteria varies from species to species. The assessment of 16S rRNA nucleotide sequencing between *Coxiella*-like strains showed that the maximal identity between them reached 98 %, while the minimal declined to 91 % (Zhong, 2012).

The aim of this study was to assess the occurrence and genetic diversity of *Coxiella*-like ticks in cattle in Algeria, where no studies on *Coxiella*-like have been conducted to date.

2. Material and Methods

2.1. Period and area of the study:

Seven hundred sixty-five (765) ticks were collected from dairy cattle between 2013 and 2015, mainly in spring and summer. All ticks (engorged and non-engorged) were collected manually with forceps, as described before (Diarra et al., 2017). The ticks were counted, pooled in tubes and kept frozen at -20 C until morphological, molecular and MALDI-TOF MS analyses. Samples were transported from Algeria to the IHU-Méditerranée Infection, Marseille, France to perform analysis.

The samples collection was carried out in three provinces in northern Algeria, two of which were in the central northern region named Blida (36° 33′ 3.258″ N, 2° 54′ 31.113″ E) and Medea (36° 15′ 43.54″ N, 2° 45′ 18.947″ E), and the third location is Batna (35° 39′ 34.671″ N, 6° 19′ 42.839″ E) located in northeastern of Algeria (Fig. 2).

In Blida, 469 ticks were collected, 238 of which were collected from cattle living in the wild in the mountains and brought to the farm only once a month by the farmer. The climate in Blida province is Mediterranean. In winter, precipitation is more abundant than in summer, with about 791 millimeters of precipitation per year. The average annual temperature in Blida is 17.9 °C. However, in Medea (130 ticks), the climate is distinguished by its position above the Tellian Atlas mountains, its altitude of 1,240 meters and its exposed to winds and waves of westerly currents, Blida and Medea are characterized by Mediterranean climate with dry and hot summers (Kottek et al., 2006). While Batna is located in the Aurès Mountains (part of the Atlas Mountains), at 1,048 meters above sea level. Batna (166 ticks) has a cold steppe climate (Kottek et al., 2006), with Mediterranean influences and average annual precipitation of 326 mm. Summers are moderately hot (by Saharan standards) and dry. Winters are chilly and wetter, with the possibility of snowfall, more details about the long-term mean temperature of the coldest and the warmest month are shown in the Fig. 1.

2.2. Morphological identification

This task was performed down to species level if possible, while engorged ticks were identified at the genus level only. The tick identification was performed twice by two expert tick entomologists and checked using previously established taxonomic identification keys

(Walker and Bouattour, 2003). This task was performed under a microscope at a magnification of ×56 (Zeiss Axio Zoom.V16, Zeiss, Marly le Roi, France).

2.3. DNA extraction

To complete the molecular identification of ticks and the screening for the presence of *Coxiella*-like bacteria, 147 of the 735 ticks were selected according to their previous morphological identification. The selection was accomplished randomly conferring to their diversities. Thus the selection concerned all species identified morphologically (15 ticks maximum for each specie, in case we had low number of species type e.g. *Ixodes ricinus* 5/735 all of them were chosen). Moreover the selected ticks belonged to both sexes (male and female), two life stages (nymph, adult), and different engorgement states (engorged or unfed).

DNA extraction was carried out according to the manufacturer's instructions. Briefly, each half tick without legs was incubated at 56°C overnight with 180 µL of G2 lysis buffer (Qiagen, Hilden, Germany) and 20 µL proteinase K (Hilden, Germany). Successively, the extraction using an EZ1 DNA Tissue Kit (Hilden, Germany) and EZ1 BioRobot extraction device was realized. Finally, 200 µL total genomic DNA was eluted in Tris-EDTA (TE) buffer (Hilden, Germany) and kept at −20 °C until use.

2.4. *Coxiella*-like qPCR screening

All selected ticks (20 %; 147/735) were screened for the presence of *Coxiella*-like endosymbionts, using a qPCR assay targeting the rrs gene (Angelakis et al., 2016). Reaction mix for qPCR assay contained 5 µl of the DNA template, 10 µl of Eurogentec Master Mix Roche, 0.5 µl UDG, 0.5 µl of each primer (at 20 µM concentration), and 0.5 µl of the FAM-labeled probe (at 5 µM concentration), (Table 1) and 3 µl of distilled water DNAse and RNAse free, for a final volume of 20 µl. The qPCR amplification was performed in a CFX96 Real-Time system (Bio-rad Laboratories, Foster City, CA, USA). with the following thermal profile: the first step consisted in an incubation at 50°C for two min and an initial denaturation step at 95°C for five min, followed by 40 cycles of denaturation at 95°C for 5 s and annealing-extension at 60°C for 30 s. We included master mixture in two wells as negative controls for

each test, while DNA from *Rhipicephalus bursa* reared in the laboratory, was used as positive control. Samples were considered positive when the cycle threshold (Ct) was lower than 35 Ct.

2.5. Molecular identification of ticks by 16S rRNA gene sequencing

The morphological identification of ticks showed positive results during the screening (76 ticks) was confirmed by 16S rRNA gene sequencing for 58/76 of them. All selected samples were subjected to standard PCR in an automated DNA thermal cycler. Details about the systems used in the standard PCR are summarized in Table 1.

The standard PCR assays consisted of a volume of 25 µl, including 12.5 µl of Amplitaq Gold Master Mix, 0.75 µl of each primer (at 20 µM concentration), 5 µl of DNA model and water. The thermal cycling conditions were: incubation step at 95°C for 15 min, 40 cycles of 1 min at 95°C, 30 s annealing at hybridization temperature and one min at 72°C followed by a final extension for five min at 72°C (Table 1). PCR amplification was performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA). The results of amplification were visualized by electrophoresis on 2 % agarose gel. The purification of PCR products was performed using Nucleo Fast 96 PCR plates (Macherey Nagel EURL, Hoerdt, France) according to the manufacturer's instructions. The amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). The sequences obtained were assembled and analyzed using the ChromasPro software (version 1.34) (Technelysium Pty. Ltd., Tewantin, Australia). Finally, assembled sequences were compared with those available in GenBank using the BLAST server from the National Centre for Biotechnology (http://www.ncbi.nlm.nih.gov/blast/).

2.6. *Coxiella*-like typing

Following the precise molecular identification of ticks, two strains samples belonging to the same species were selected for the typing by PCR / sequencing of the *Coxiella*-like 16S rRNA and *GroEL* partial genes, since *Coxiella*-like bacteria are hypothetically specific endosymbionts of ticks according to the species (Duron et al., 2015).

196 The experimental protocol for the conventional PCRs, purification of PCR products,
197 sequencing, assembly and sequences analysis were performed as described above. These tasks
198 were repeated four times for each system (*GroEL* and 16S) to support the finding and avoid all
199 possibility of contamination between samples.

200

201 **2.7. Phylogenetic analyses**

202 To carry out the construction of the phylogenetic tree, the *Coxiella*-like obtained sequences
203 in the present study were aligned with reference sequences available in the GenBank database
204 using ClustalW multiple sequence alignment algorithm. A maximum-likelihood method
205 (Kimura 2-parameter model) was used to infer the phylogenetic analyses and tree
206 reconstruction was performed using MEGA6.06 software (Tamura et al., 2013). Bootstrap
207 analyses were conducted using 500 replicates.

208

209 **2.8. Statistical analysis**

210 After set of databases Microsoft Excel® program (Microsoft Corp. Redmont, USA), a
211 descriptive study of ticks' population and of the CLE carriage was performed. The statistical
212 analysis was conducted using XLSTAT Addinsoft version 2018.7 (Data Analysis and
213 Statistical Solution for Microsoft Excel, Paris, France). The comparison of CLE infection's
214 prevalence according to the regions was accomplished using Khi-2 test, for that statistical
215 signification was considered at P-value < 0.05.

216

3. Results

3.1. Tick identification

In this study, 735 hard ticks were collected from cattle. Thirteen species belonging to five genera were identified as cattle ticks in the three northern provinces of Algeria. Almost all of ticks in north-central Algeria (Blida and Medea) belonged to the genus *Rhipicephalus*, while in Batna, in the northeast, species belonging to genus *Hyalomma* were the most often identified ticks. More details are provided in Fig. 2.

3.2. Prevalence of *Coxiella*-like in ticks collected in northern of Algeria

In this study, we performed an extensive screening of *Coxiella*-like on 147 selected ticks (20 % of all ticks collected were selected for DNA extraction). Using qPCR, the prevalence revealed in this study was 51.7 % (76/147) among all the selected ticks for the screening. The prevalence of *Coxiella*-like in hard ticks in Medea province of 80 % (21/26) was higher than in Blida province, 47 % (47/100) (value P = 0.001); traditional breeding cattle, 55 % (27/49) and wild cattle, 39 % (20/51). Prevalence in Medea was also higher than Batna province, 24 % (8/33) (P-value <0.0001). No differences were observed in *Coxiella*-like carriage in ticks between Blida and Batna (P-value= 1.000). *Coxiella*-like DNA was detected in (2/2) of *Dermacentor marginatus* and *Haemaphysalis sulcata*, in 36 % (8/22) of *Hyalomma excavatum,* in (1/4) of *Hyalomma detritum*, in (1/3) of *Hyalomma lusitanicum*, in 14 % (4/28) of *Hyalomma marginatum* and in 15 % (2/13) of unidentified *Hyalomma* spp.

Regarding the genus *Rhipicephalus*; *Coxiella*-like DNA was detected in 81 % (26/32) of *R. bursa,* in 61 % (11/18) of *R. sanguineus* SL-A and in 64 % (16/25) of unidentified *Rhipicephalus* spp. Finally, 50 % (3/6) of *I. ricinus* were found to be carriers of *Coxiella*-like.

3.3. Phylogenetic analysis

Sequencing of the positive samples using the *Coxiella*-like specific primers 16S rRNA failed in 10 of 23 samples, as a result 13 samples were successfully sequenced, and provided a BLAST identity percentage greater than 98 % for better identification (Table 2).

Sequencing using the specific *GroEL* primers also failed in 19/23 sequenced with these primers, so only four could be sequenced. The best BLAST hit oscillated between 98.38 % and 99.63 %. Of the 4 sequences, two (samples 111 and 118) exhibited 98.38 and 98.63 % of identity with C. burnetii str. Schperling (CP014563). The other samples exhibited 98.49 and 98.92 % of identity with Candidatus Coxiella mudrowiae isolate CRS-CAT (CP024961) (Table 2).

The phylogenetic analysis, based on the 16S rRNA gene, showed that *Coxiella* bacteria sequences from *Rhipicephalus*, *Dermacentor, Ixodes* and *Hyalomma* species (11 of 13 successfully sequenced) are grouped into an independent and distinct clad. While the sequences from a female *R. sanguineus* SL-A from Blida remained distinct to others and share the same common ancestor with *Coxiella* endosymbiont of *R. sanguineus* SL-A isolate Rhsa 2 from Brazil (KP994844). In addition, the 16S rRNA sequence of *Coxiella*-like of a male *H. sulcata* from Blida in this study clustered with the *Coxiella* endosymbiont of *Rhipicephalus* from Italy (KP994831) (Fig. 3).

Phylogenetic analysis based on *GroEL* partial gene of *Coxiella*-like bacteria was carried out. Two sequences gave similarity with *C. burnetii* by BLAST. They are grouped with *C. burnetii* Nine Mile strain and other *C. burnetii* strains. However, other sequences of *Coxiella*-like endosymbionts identified in *R. sanguineus* SL-A and *H. excavatum* were grouped with endosymbionts of *R. sanguineus* SL-A isolate Rhsa1 chaperonin (*GroEL*) gene and share the same common ancestor (Fig. 4).

4. Discussion

This study is to the best of our knowledge the first report on the occurrence and diversity of *Coxiella*-like in cattle ticks from Algeria. The study highlighted the presence of two genera of ticks that constitute the dominant populations of ticks infesting livestock in northern Algeria. In Blida and Medea, *Rhipicephalus* spp. are dominant, especially, *R. bursa* and *R. sanguineus* SL-A (Fig. 2). *R. bursa* is a tick of the Mediterranean climatic region, which is the characteristic climate of the concerned provinces. In Africa, it is confined to the coastal areas from Morocco to Libya including Algeria (Walker and Bouattour, 2003). *R. sanguineus* SL-A, is specialized to domestic dogs, but it may be found on cattle (Walker and Bouattour, 2003). These details support our findings. In Batna, northeastern Algeria, the dominant tick genus was *Hyalomma* especially *H. excavatum.* This species is adapted to the Mediterranean and steppe climatic regions of North Africa and to steppe climatic regions elsewhere in its wide range (Walker and Bouattour, 2003).

For the first time in Algeria, we report the occurrence of *Coxiella*-like in ixodid ticks. The prevalence was 51.7 % (76/147) among all the selected ticks for the screening. *Coxiella*-like DNA was detected in all tick species identified in the present study with different percentages. The highest prevalence was reported in the genus *Rhipicephalus* 70 % (53 out of 75), while it was lower in other genera such as *Hyalomma* with 37 % (26 out of 70). This prevalence in Algeria seems to be lower than those reported worldwide. Actually, Guizzo et al. (2017) reported that the treatment of cattle with tetracycline (frequently used in Algeria), leads to the reduction of bacteria levels in the progeny of ticks. These facts might explain the low prevalence reported in this study.

In the study conducted by Duron et al. (2015) *Coxiella*-like was detected in >90 % of tested ticks, which is extremely high. The screening was performed in 916 tick specimens from 58 species belonging to the two main tick families, Ixodidae (36 species) and Argasidae (22 species). *Coxiella* was found in most of the tested genera of hard ticks (*Rhipicephalus, Ixodes, Amblyomma, Dermacentor, Haemaphysalis*) and soft ticks (*Ornithodoros, Argas*).

Actually, the prevalence of *Coxiella*-like varies widely in different tick species. In Europe and North America, it rates from 6.25 % (3/48) in *R. sanguineus* SL-A to 100 % (50/50) in *A. americanum* (Zhong, 2012). Machado-Ferreira et al. (2016) reported that *Coxiella* DNA was detected in 37 % (10/27) of *Rhipicephalus microplus* (5/15 adult females, 4/11 adult males and 1/1 nymph), and in 61 % (29/47) of the *R. sanguineus* SL-A (15/24 adult females 13/18 adult

297 males 1/5 nymph). These details support our findings in Algeria where the prevalence ranged
298 from 14 % (4/28) for *H. marginatum* to 81 % (26/32) for *R. bursa,* to 2/2 for *D. marginatus.*

299 In Tunisia, Selmi et al. (2019) reported that *Coxiella*-like DNA was detected in 10 ± 0.04
300 % (16/158) of *Hyalomma impeltatum* and 6% (10/160) of *Hyalomma dromedarii* (partially
301 engorged adult ticks) with an overall prevalence of 8 % (26/327). This is close to our results
302 regarding *Hyalomma* genus with 37.1 %. Khoo et al. (2016) reported a prevalence of 89%
303 (49/55) of tested ticks (*Haemaphysalis hystricis*: 6/6 unfed adult females, 13/13 unfed adult
304 males; *Dermacentor steini*: 0/1 unfed adult females, 2/6 unfed adult
305 males; *Dermacentor compactus*: 1/2 unfed adult males; *Amblyomma* sp.: 5/5 engorged adult
306 females, 1/1 unfed adult male; *Haemaphysalis bispinosa*: 18/18 adult females, among them five
307 engorged, 2/2 unfed adult males and 1/1 nymph). In South Korea, Seo et al. (2016) reported the
308 prevalence of 52.4 % (121/231) of adult *Haemaphysalis longicornis*. These different data
309 suggest that the *Coxiella*-like prevalence in ticks may depend, not only on the tick's species,
310 but also on the bioclimatic area. Our results report a higher prevalence in Blida and Medea,
311 characterized by Mediterranean climate, than in Batna with a cold semi-arid climate. In
312 addition, in Malaysia, which is characterized by an equatorial climate, a higher prevalence (89
313 %) than reported in this study was found by Khoo et al. (2016). According to the study carried
314 out by Selmi et al. (2019) the desertic areas reduce the ticks activity to the minimum, especially
315 during summer, regarding the high average of temperature as well as the scarcity of animal
316 species (Selmi et al., 2019).

317 However, the prevalence of *Coxiella*-like bacteria reported here or in other studies, where
318 ticks were collected on host animals, are much higher than in studies reported in ticks collected
319 by flagging over vegetation. Environmental *H. longicornis* ticks, collected in the Chungbuk
320 province (middle of the Korean), showed a *Coxiella*-like prevalence of 2 % (2 /100) of *H.*
321 *longicornis* (Lee et al., 2004); in Queensland, Australia, the prevalence reached 6 % (3/45) (Seo
322 et al., 2016), 0.9 % (1 of 109) in *Haemaphysalis punctata* ticks from Spain (Barandika et al.,
323 2008). Thus, *Coxiella*-like infection in ticks might be originated from host animals since its
324 prevalence in environmental ticks is much lower than that described in ticks collected on
325 animals.

326 The gender of the ticks does not appear to be a factor of genetic variability of the *Coxiella*-
327 like since sequences belonging to the same clade were found in both male and female ticks.
328 According to the 16S rRNA phylogenetic analysis, three different clades of *Coxiella*-like
329 bacteria occur in ticks from Algerian cattle. The dominant one, regrouping 11 of the 13

sequences identified seems to be genetically homogenous with few differences among each other (based on 16S rRNA gene). However, *Coxiella*-like bacteria from this genetic group were found in our study in different tick species (*R. bursa, R. sanguineus* SL-A*, H. marginatum, H. excavatum, D. marginatus, H. sulcata* and *I. ricinus*) and from different regions. This goes against the previous concept that each genetic variant of *Coxiella*-like bacteria is associated with specific tick species. The second 16S-based genetic group was identified in a female of *R. sanguineus* SL-A (it is close to *Coxiella* endosymbiont of *R. sanguineus* SL-A from Brazil (Duron et al., 2015) and *Coxiella*-like endosymbiont of *H. excavatum* from Anatolia, Turkey (Brinkmann et al., 2019). The third genetic clade clustered with *Coxiella* endosymbiont of *Rhipicephalus* from Italy (Duron et al., 2015) (Fig. 3). Actually, the studies proofed the vertical transmission of CLE nevertheless; they do not deny the possible horizontal transmission, between ticks' species via infested cattle (the common point between them). For that, we hypothesized that the genetic diversities of the CLE don't only depend on the tick's species but also on the vertebrates' hosts.

Only four sequences were obtained for *GroEL* gene. This could be due to the primer's specificity and / or to the use of non-nested PCR protocol to detect *Coxiella*-like DNA which probably decreased, the PCR sensitivity. However, in our study, CLE DNA were successfully sequenced but some of sequences were impossibly assembled with the ChromasPro software due to their poor quality. We avoided the inclusion of these poor sequences in the analysis.

Two sequences from female *R. bursa* ticks were almost identical to *C. burnetii* str. Schperling detected on *R. sanguineus* SL-A female tick from Blida province. Two other sequences from *Hyalomma* ticks showed high similarity (>98 %) with *Candidatus* Coxiella mudrowiae isolate CRS-CAT detected on *R. sanguineus* SL-A and *H. excavatum* ticks from Blida and Batna successively. Whenever we were able, in 3 ticks, to amplify both genes of *Coxiella*-like bacteria (16S rRNA gene and *GroEL*), we may suggest that *GroEL* sequences from ticks 11 and 118 (Fig. 4) represent the same first dominant 16S clade and the sequences from ticks 110 and 146 are the sequences corresponding to the second 16S clade. Unfortunately, we were not able to amplify the *GroEL* gene from the only *H. sulcata* tick where the third 16S-based clade of *Coxiella*-like bacteria was identified.

5. Conclusion

In our study, we report for the first time in Algeria that *Coxiella*-like 16S rRNA gene sequences from different tick species grouped into three clades. Beside the tick species specificities, the genetic diversity of CLE seems to be related to the vertebrate animal hosts, which support the possibility of horizontal transmission between ticks via the vertebrate animals.

Conflict of interest

The authors declare no conflicts of interest.

Ethical considerations

The collection of samples from cows was carried out in accordance with the Algerian legislation. Permission was obtained from the Ministry of Higher Education and Scientific Research of Algeria, in collaboration with the University Yahya Fares of Medea, and the National Veterinary High School, Algiers, Algeria.

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Supplementary data

No.

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504 **Table 1.** PCR systems used in the present study, their conditions and sources.

Target	Target Gene	Orientation	Nucleotide sequence (5´–3´)	Fragment length	Tm °C	References
***Coxiella*-like**	rrs	Forward	ACCTACCCTTGACATCCTCGGAA			(Angelakis et al., 2016)
		Reverse	GCAACTAAGGACGAGGGTTG			
		Probe	6FAM-CAGCTCGTGTCGTGAGATGT-TAMRA			
***Coxiella*-like**	*GroEL*	Forward 660f	GGCGCICARATGGTTAARGAA	550 bp	54	(Angelakis et al., 2016)
		Reverse 1320r	AACATCGCTTTACGACG			
	16S	Forward Cox1	CAGAGAGTGCAAGCGTTAATC	1066 bp	50	(Mediannikov et al., 2003)
		Reverse Cox2r	CTCCTTAGAGTTCCCGGCCAACCG			
ticks	16S	Forward 16S-F	TTAAATTGCTGTRGTATT3	454 bp	54	(Diarra et al., 2017)
		Reverse 16S-R	CCGGTCTGAACTCASAWC			

505 **Tm**: Annealing temperature.

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512 **Table 2.** BLAST analysis of specimens sequenced with 16S rRNA and *GroEL* partial
513 genes and selected to create the phylogenetic trees.

Primers	Localities	No of sequenced / tested	DNA samples ID	Gender of tick	Ticks species	Best BLAST hit	The Percent identity	Accession Numbers
16S	Medea	1/3	5	Female	*R. bursa*	Coxiellaceae bacterium CR07	99.39 %	KM079623
	Blida	12 /16	34	Female	*R. bursa*	Coxiellaceae bacterium CR07	99.60 %	KM079623
			69	Male	*H. sulcata*	Uncultured Coxiella sp. clone 195C	98.64 %	MK164020
			74	Male	*R. bursa*	Uncultured Coxiella sp. clone 195C	98.64 %	MK164020
			75	Male	*D. marginatus*	Coxiellaceae bacteriumCR07	98.31 %	KM079623
			90	Male	*R. bursa*	Coxiellaceae bacterium CR07	99.37 %	KM079623
			106	Female	*H. marginatum*	Coxiellaceae bacterium CR07	99.80 %	KM079623
			110	Male	*R. sanguineus* SL-A	Coxiellaceae bacterium CR07	100 %	KM079623
			111	Female	*R. bursa*	Coxiellaceae bacterium AL09	100 %	KM079626
			113	Female	*R. bursa*	Coxiellaceae bacterium CR07	100 %	KM079623
			118	Male	*R. bursa*	Coxiellaceae bacterium CR07	100 %	KM079623

			122	Female	*I. ricinus*	Coxiellaceae bacterium CR07	100 %	KM079623
			148	Male	*R. sanguineus* SL-A	Coxiellaceae bacterium CR07	99.80 %	KM079623
	Batna	0/ 4						
	Medea	0/3						
GroEL	Blida	3/16	110	Female	*R. sanguineus* SL-A	Candidatus Coxiella mudrowiae isolate CRS-CAT	98.49 %	CP024961
			111	Female	*R. bursa*	C. burnetii str. Schperling chromosome	99.63 %	CP014563
			118	Female	*R. bursa*	C. burnetii str. Schperling chromosome	98.38 %	CP014563
	Batna	1/3	146	Male	*H. excavatum*	Candidatus Coxiella mudrowiae isolate CRS-CAT	98.92 %	CP024961

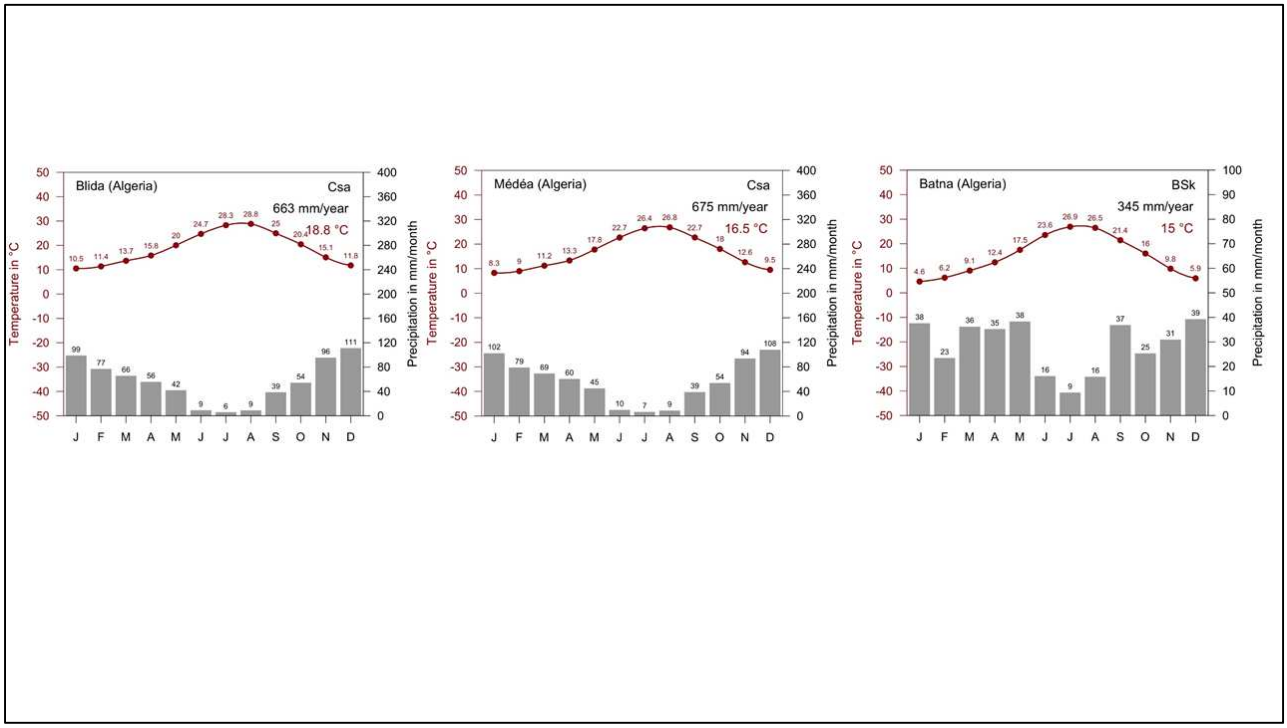

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Fig. 1. Climate diagrams for the three Algerian provinces concerned by the tick collection, showing the long-term mean temperature of the coldest and the warmest month (Rubel, 2020, pers. communication).

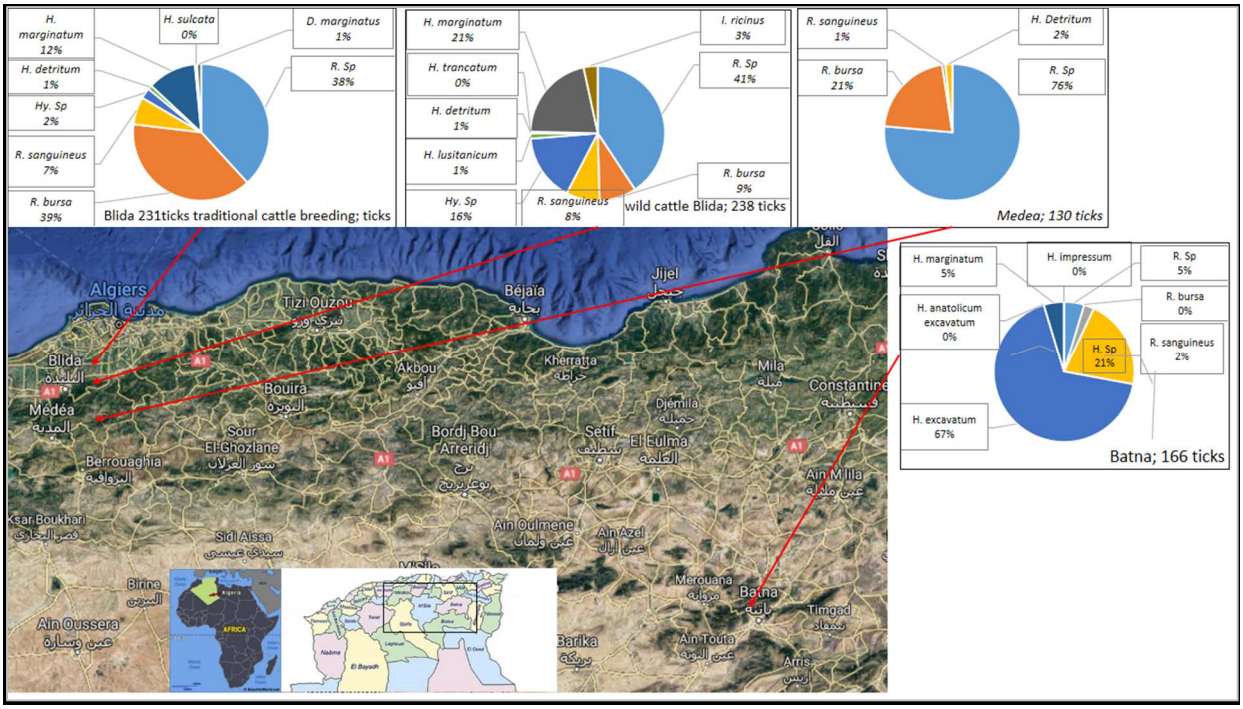

Fig. 2. A map of northern Algeria showing different provinces from which the sample collections were carried out, with percentage of each tick species found and identified. (https://www.google.com/maps/@36.2736941,4.221111,230025m/data=!3m1!1e3?hl=en).

H: *Hyalomma / Haemaphysalis;* **R:** *Rhipicephalus;* **D:** *Dermacentor;* **I:** *Ixodes.*

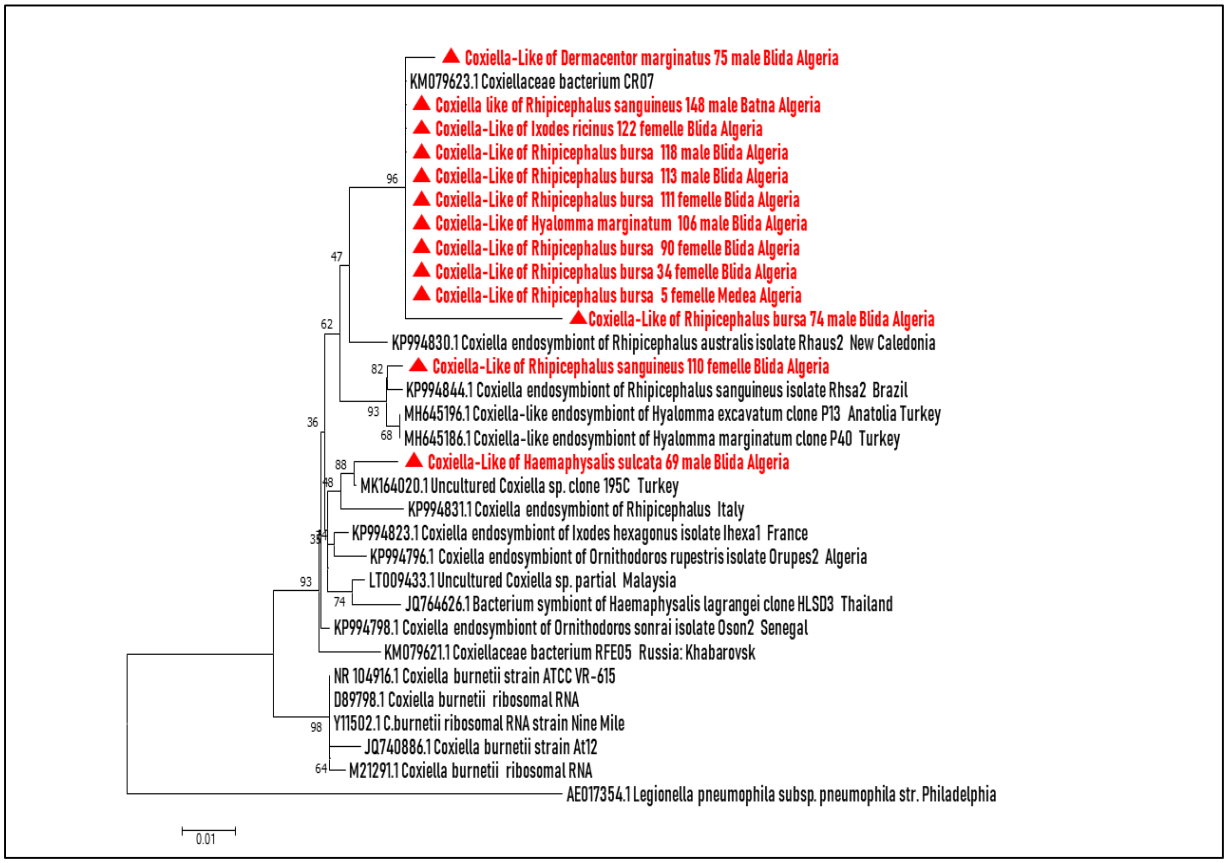

Fig. 3. Maximum-likelihood phylogenetic analysis of the *Coxiella*-like based on 16S rRNA gene sequences. Sequences characterized in this study are given in bold and indicated with the identification (number) of DNA sample, host tick species, its gender and the geographical origin, respectively. *Legionella pneumophila* subsp. *pneumophila* str. Philadelphia was included as the outgroup. Sequences were aligned using CLUSTALW, and phylogenetic inferences was conducted in MEGA6.06 using the maximum likelihood method based on the

Tamura-Nei model for nucleotide sequences. The GenBank accession numbers are indicated at the beginning. Statistical support for the internal branches of the trees was evaluated by bootstrapping with 5,00 iterations. Bootstrapping under 40 were removed.

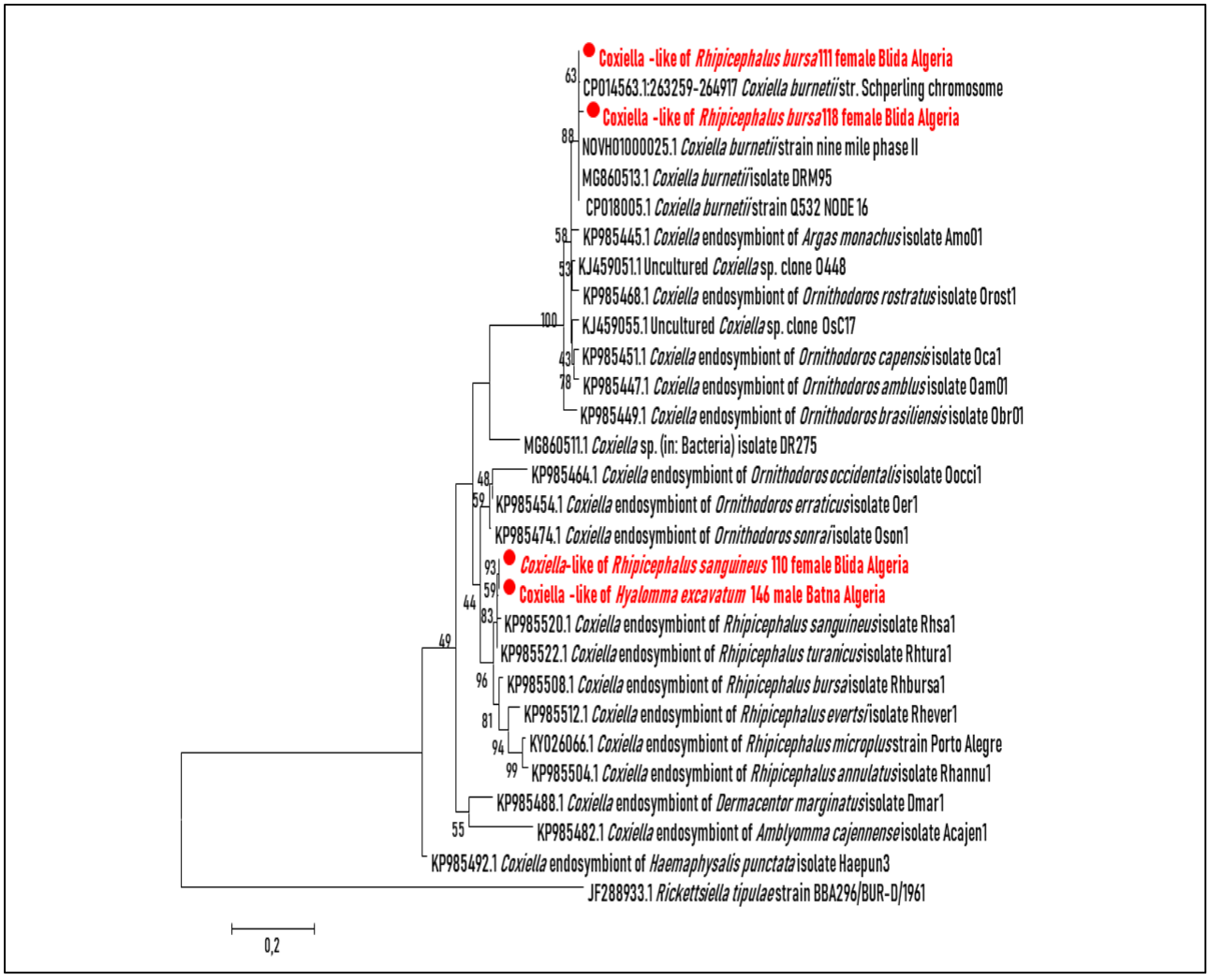

Fig. 4. Maximum-likelihood phylogenetic tree of the *Coxiella*-like based on *GroEL* gene sequences. Sequences characterized in this study are given bold and indicated with the identification (number) of DNA sample, host tick species, its gender and the geographical

origin, respectively. *Rickettsiella tipulae* strain BBA296/BUR-D/1961 23S ribosomal RNA (rrl) gene was included as the outgroup. Sequences were aligned using CLUSTALW, and phylogenetic inferences was conducted in MEGA6.06 using the maximum likelihood method based on the Tamura-Neimodel for nucleotide sequences. The GenBank accession numbers are indicated at the beginning. Statistical support for the internal branches of the trees was evaluated by bootstrapping with 5,00 iterations. Bootstrapping under 40 were removed.