

Molecular typing and diagnosis of *Anaplasma* spp. closely related to *Anaplasma phagocytophilum* in ruminants from Tunisia

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1 **Molecular typing and diagnosis of *Anaplasma* spp. closely related to *A. phagocytophilum* in**
2 **ruminants from Tunisia.**

3

4 Mourad Ben Said¹, Hanène Belkahia¹, Narjesse El Mabrouk¹, Mariem Saidani¹, Mariem Ben Hassen¹,
5 Alberto Alberti², Rosanna Zobba^b, Ali Bouattour³, Lilia Messadi¹

6

7 ¹ Laboratoire de Microbiologie, Ecole Nationale de Médecine Vétérinaire, Institution de la Recherche et
8 de l'Enseignement Supérieur Agricoles, Université de La Manouba, 2020 Sidi Thabet, Tunisia

9 ² Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Sassari, Italy

10 ³ Laboratoire d'Entomologie, Institut Pasteur de Tunis, Tunisie

11

12 **Corresponding author:**

13 Pr. Lilia Messadi

14 Laboratoire de Microbiologie, Ecole Nationale de Médecine Vétérinaire, 2020 Sidi Thabet, Tunisie

15 Tel: +216 71 552 200

16 Fax: +216 71 552 441

17 E-mail: lilia_messadi@yahoo.fr

18

19 **Abstract**

20 Accurate diagnosis of animal and zoonotic diseases, such as granulocytic anaplasmosis, is crucial to
21 estimate risk during control programs. In this study, 16S rRNA nested PCR and RFLP assay were
22 combined to investigate the presence of *Anaplasma phagocytophilum* and genetically related strains
23 (namely *A. phagocytophilum*-like 1 and 2) in 936 Tunisian ruminants. By using this method, *A.*
24 *phagocytophilum* was not detected in any of the tested animals, while *A. phagocytophilum*-like 1 and *A.*
25 *phagocytophilum*-like 2 were detected at variable prevalence rates in sheep, goats and cattle at coinfection
26 rates respectively of 3.9, 2.5 and 0.5 %. Sequence analysis validated RFLP data, and confirmed the co-
27 occurrence of two potentially novel species closely related to *A. phagocytophilum* in Tunisian ruminants.
28 Phylogeny indicated the presence of genetic variants shared by different ruminant species for each type of
29 *A. phagocytophilum*-like strains. Results raise concern on the use and interpretation of indirect and direct
30 tests traditionally employed for detecting pathogenic *A. phagocytophilum* strains in ruminants and in
31 other vertebrates' species, and provide additional background to improve classification of bacterial
32 species closely related to *A. phagocytophilum*, and to reconstruct their evolutionary history.

33

34 **Keywords:** *Anaplasma phagocytophilum*-like 1 and 2; Molecular discrimination; Co-occurrence;
35 Ruminants; 16S rRNA gene; Tunisia

36 1. Introduction

37 Microorganisms belonging to the *Anaplasmataceae* family (order *Rickettsiales*) are obligate
38 intracellular Gram-negative bacteria of veterinary and public health importance (Dumler et al., 2001).
39 Several *Anaplasma* species are indeed cause of a range of diseases and conditions in humans and other
40 vertebrates, at worldwide distribution (Dumler et al., 2001). Among them, *A. phagocytophilum* is
41 zoonotic and infects neutrophil granulocytes of many host species (Stuenkel, 2007); including domestic
42 ruminants in which it causes tick-borne fever (TBF) (Woldehiwet, 2010). TBF most common symptoms
43 are high fever, anorexia, dullness, and reduced milk production (Tuomi, 1967).

44 In Japan, strains related to *A. phagocytophilum* (*A. phagocytophilum*-like 1 in this study), have been
45 identified in cattle, sika deer, and in some ticks species infesting ruminants (*Ixodes persulcatus*, *Ixodes*
46 *ovatus*, *Haemaphysalis megaspinosa*) (Ohashi et al., 2005; Jilintai et al., 2009; Yoshimoto et al., 2010).
47 Phylogenetic analysis based on 16S rRNA, *groEL*, and *gltA* genes clustered these strains in a
48 monophyletic clade distinct but closely related to *A. phagocytophilum* (Ybañez et al., 2012). Peculiar tick
49 vectors respect to *A. phagocytophilum* and the absence of clinical signs in infected animals provide
50 additional data for species designation of these potentially new *Anaplasma* strains initially found in Japan
51 (Ybañez et al., 2012). More recently in China, Kang et al. (2014) detected *Anaplasma* sp. strains (*A.*
52 *phagocytophilum*-like 2 in this study) in *Hyalomma asiaticum* ticks infesting sheep and cattle, which
53 differ from the Japanese strains (*A. phagocytophilum*-like 1) and from all other classified and unclassified
54 *Anaplasma* strains. *A. phagocytophilum*-like 2 strains cluster in an independent separated clade in
55 phylogenetic trees obtained from 16S rRNA, *gltA* and *groEL* genes, indicating the occurrence of a
56 potential novel *Anaplasma* species closely related to *A. phagocytophilum* in Chinese ruminant related
57 ticks.

58 In 2015, we detected these two types of strains (*A. phagocytophilum*-like 1 and 2) by the analysis of
59 16S rRNA sequences after DNA amplification of some *A. phagocytophilum*-like positive goat samples
60 without estimating the prevalence rate of each type of strains (Ben Said et al., 2015). Hence, the
61 development of a fast tool as restriction enzyme fragment length polymorphism (RFLP) assay is required

62 to validate the infection by these strains genetically related to *A. phagocytophilum* without being forced to
63 sequence PCR positive samples. Additionally, this approach has the intrinsic potential of revealing *A.*
64 *phagocytophilum* and related strains co-infections in vertebrate hosts and in tick vectors, reducing the
65 time and costs associated to cloning and sequencing during molecular diagnosis.

66 In this study, we develop a diagnostic approach based on 16S rRNA PCR combined to restriction
67 enzyme digestions with the potential of discriminating among *A. phagocytophilum*, *A. phagocytophilum*-
68 like 1, and *A. phagocytophilum*-like 2. This method is applied to establish presence and prevalence of
69 these strains in goats, sheep and cattle from northern Tunis. Molecular typing and phylogeny of the
70 genetic variants of the novel *A. phagocytophilum* related strains are also investigated.

71

72 **2. Materials and methods**

73 *2.1. Ruminants' populations and study regions*

74 Blood samples were collected in 2015 (between May and June) from 963 apparently healthy
75 ruminants (367 cattle, 355 sheep and 241 goats) spread in farms belonging to five governorates: Bizerte
76 (latitude 36°18' N, longitude 10°27' E), Tunis (latitude 35°0' N, longitude 9°29' E), Beja (latitude 36°73'
77 N, longitude 9°18' E), Ariana (latitude 36°51' N, longitude 10°11' E) and Nabeul (latitude 36°45' N,
78 longitude 10°73' E) (Supplementary files 1). A total of twenty-two delegations situated in these five
79 governorates from Northern Tunisia have been investigated in this cross-sectional study (Supplementary
80 files 1). They belong to three different bioclimatic areas: higher semi-arid area with a mean annual
81 rainfall from 400 to 600 mm, sub-humid area with a mean annual rainfall from 600 to 800 mm and lower
82 humid area with a mean annual rainfall from 800 to 1200 mm (Supplementary file 1).

83

84 *2.2. Sampling and DNA extraction*

85 Blood was collected from the animals' jugular vein into EDTA tubes (Becton Dickinson). DNA
86 was extracted from 300 µl volumes of EDTA-preserved whole blood using the Wizard® Genomic DNA
87 purification kit (Promega, Madison, USA) according to manufacturer's instructions. DNA yields were

88 determined with a spectrophotometer (Jenway, Genova, Italy). DNA samples were stored at -20 °C until
89 use.

90

91 2.3. Nested-PCR detection

92 Nested PCR was performed with outer primers EE1 and EE2, and inner primers SSAP2f and
93 SSAP2r to amplify 641-642 bp sequence of the 16S rRNA gene (Barlough et al., 1996; Kawahara et al.,
94 2006) (Supplementary files 2). According to Ybañez et al. (2012) and Ben Said et al. (2015), inner
95 primers allow the detection of *A. phagocytophilum* and related strains. For specific detection of *A.*
96 *phagocytophilum*, positive 16S rRNA samples were tested by hemi-nested PCR using outer primers
97 EphplgroEL-F and EphplgroEL-R, and inner primers EphplgroEL-F and EphgroEL-R amplifying 573 bp
98 sequence of the *groEL* gene (Alberti et al., 2005, Supplementary file 2). Each reaction was performed in a
99 final volume of 50 µl containing 0.125 U/µl Taq DNA polymerase (Biobasic Inc, Canada), 1x PCR
100 buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 µl genomic DNA and 0.5 µM of primers. Thermal cycling
101 reactions were performed in an automated DNA thermal cycler (Applied Biosystems 2720 Thermal
102 Cycler, Foster City, California, USA). One microliter of each amplicon was used for PCR reaction with
103 specific primers at the same conditions as for the first PCR. PCR products were electrophoresed in 1%
104 agarose gels containing 0.5 µg/ml of ethidium bromide. Distilled water and DNA samples positive to
105 specific *A. phagocytophilum* (Zobba et al., 2014) and to *A. phagocytophilum*-like 1 and 2 (Ben Said et al.,
106 2015) were used as negative and positive controls in each PCR experiment.

107

108 2.4. Selection of restriction enzymes and subtyping by RFLP assays

109 The 16S partial sequences of 598-599 bp (PCR product sequences of 641-642 bp without primer
110 sequences) derived from 15, 12 and 9 strains of specific *A. phagocytophilum*, *A. phagocytophilum*-like 1
111 and *A. phagocytophilum*-like 2, respectively, were aligned to select 2 restriction enzymes permitting the
112 subtyping of *A. phagocytophilum* and two different ruminant-adapted related strains. In particular, XcmI
113 and BsaI restriction enzymes were selected to discriminate between *A. phagocytophilum* and *A.*

114 *phagocytophilum*-like strains and between *A. phagocytophilum*-like 1 (*Anaplasma* sp.-Japan) and *A.*
115 *phagocytophilum*-like 2 (*Anaplasma* sp.-China), respectively. Both restriction reactions were done in a
116 final volume of 20 µl containing 10 µl PCR product, 2 µl buffer (10x), 1µl XcmI or BsaI enzyme (10000
117 u/ml) (Biolabs, New England, UK) and 7 µl distilled water. Incubation was done 1 hour at 37°C. The
118 restricted fragments were separated on a 3% high-resolution agarose gel by electrophoresis in TAE buffer
119 (0.04 M Tris, 0.4 mM EDTA, pH 7.7–8.8) at 100 V for 60 min and visualized under UV illumination
120 after staining with ethidium bromide.

121

122 2.5. DNA sequencing and data analysis

123 Selected positive PCR products from primers SSAP2f/SSAP2r of specific *A. phagocytophilum*, *A.*
124 *phagocytophilum*-like 1 and 2 were purified with the GF-1 Ambi Clean kit (Vivantis technologies,
125 Malaysia) according to manufacturer instructions. Purified DNA fragments were sequenced in both
126 directions, using the same primers as for the PCR amplifications (Supplementary file 2). The reactions
127 were performed using a conventional Big Dye Terminator cycle sequencing ready reaction kit (Perkin
128 Elmer, Applied Biosystems, Foster City, USA) and an ABI3730XL automated DNA sequencer. The
129 chromatograms were evaluated with Chromas Lite v 2.01. The DNAMAN software (Version 5.2.2;
130 Lynnon Biosoft, Que., Canada) was used to perform multiple 16S rRNA sequence alignment. BLAST
131 analysis of GenBank was used to assess the level of similarity with previously reported sequences
132 (<http://blast.ncbi.nlm.nih.gov/>, Altschul et al., 1997). Neighbor-Joining (NJ) phylogenetic trees were
133 constructed using the DNAMAN software based on the Saitou and Nei (1987) distance method with
134 bootstrap analysis of 1,000 reiterations.

135

136 2.6. GenBank accession numbers

137 The 16S rRNA partial sequences of *A. phagocytophilum*-like 1 (Aplike1BvCp1 and Aplike1Ov1
138 variants), *A. phagocytophilum*-like 2 (Aplike2OvCp1 and Aplike2Cp1variants) and specific *A.*

139 *phagocytophilum* isolated from dog (ApDg variant) have been deposited under GenBank accession
140 numbers from KX702974 to KX702986, respectively.

141

142 2.7. Statistical analyses

143 To study the possible influence of host, geographic location and bioclimatic area on the molecular
144 prevalence of *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2, chi square test or Fisher's exact
145 test were performed using Epi Info 6.01 (CDC, Atlanta, USA). Five percent significance level was
146 considered significant. Exact confidence intervals (CI) for prevalence rates at the 95% level were
147 calculated.

148

149 3. Results

150 3.1. Strains' molecular identification

151 A first discrimination between *A. phagocytophilum* and *A. phagocytophilum*-like strains was
152 accomplished by digesting 16S rRNA amplicons (641-642pb) with XcmI. *A. phagocytophilum* amplicons
153 were cut at position 1141 and generated two fragments, 344 and 297 bp in size, while *A.*
154 *phagocytophilum*-like strains were not cut by the same enzyme (Figure 1B, lanes 1, 2 and 3 to 6,
155 respectively). Results were confirmed by detection of *A. phagocytophilum* with *groEL* hemi-nested PCR.
156 Digestion of the same amplicons with BsaI differentiated *A. phagocytophilum*-like 1 (*Anaplasma* sp.-
157 Japan) from *A. phagocytophilum*-like 2 (*Anaplasma* sp.-China). More specifically, after restriction
158 reaction, *A. phagocytophilum*-like 2 generated two fragments of 422 (or 423) and 219 bp (Figure 1C,
159 lanes 1 to 3 and 4 to 6, respectively) while *A. phagocytophilum*-like 1 was not digested by the same
160 enzyme. *A. phagocytophilum*-like 1 and 2 co-infections were easily detectable after restriction enzyme
161 digestions, and coincided with the appearance of the three bands with sizes of 641 (or 642), 422 (or 423)
162 and 219 bp (Figure 1C, lanes 7 to 8).

163

164 3.2. *Anaplasma* spp. prevalence and co-infection rates

165 Nested 16S rRNA PCR-RFLP and *groEL* hemi-nested PCR failed to detect *A. phagocytophilum* in all
166 ruminant species, indicating that none of the analysed animals were positive to this zoonotic species.
167 In goats, *A. phagocytophilum*-like 1 and 2 overall prevalence rates were respectively 13.1 and 5%, with a
168 co-infection rate estimated at 2.5% (Table 1). Goats from lower humid area were statistically more
169 infected by *A. phagocytophilum*-like 1 (21.8%) than those from higher semi-arid and sub-humid areas
170 (9.1 and 0% respectively; $p = 0.009$). Beja, Ariana and Bizerte governorates showed similar *A.*
171 *phagocytophilum*-like 1 prevalence rates (25, 21.1, and 19.7%), while rate of infection was statistically
172 lower or absent in goats of other governorates (2.8 and 0% in Tunis and Nabeul respectively; $p < 0.001$)
173 (Supplementary file 3). Within the Ariana and Beja governorates, the delegations of Cebalet Ben Ammar
174 and Jouza Hamra showed higher infection rates respect to other delegations belonging to the same
175 governorate (80 and 40.9%, $p < 0.001$ and $p = 0.014$ respectively) (Supplementary file 3). Goats from
176 Tunis were statistically more infected by *A. phagocytophilum*-like 2 (11.8%) than those from other
177 governorates ($p = 0.017$), with Hrairia Sejoumi delegation showing the highest infection rates of this
178 governorate (26.9%, $p = 0.030$) (Supplementary file 3).

179 In sheep, *A. phagocytophilum*-like 1 and 2 overall prevalence rates were 7 and 5.4%, respectively,
180 with a co-infection rate estimated at 3.9% (Table 2). As shown in Table 2, sheep from higher semi-arid
181 area were statistically more infected by *A. phagocytophilum*-like 1 (11.2%) and *A. phagocytophilum*-like
182 2 (9.6%) than those from lower humid and sub-humid areas ($p = 0.003$ and $p = 0.002$, respectively). No
183 statistically significant differences in *A. phagocytophilum*-like 1 and 2 prevalence rates were found in the
184 different governorates (Supplementary file 4). Within Bizerte and Beja governorates, Utique and Medjez
185 El Bab delegations were statistically more infected by *A. phagocytophilum*-like 1 and 2 than the other
186 delegations belonging to the same governorate (40%, $p < 0.001$ each one for *A. phagocytophilum*-like 1
187 and 33.3 and 30%, $p < 0.001$ and $p = 0.019$, respectively, for *A. phagocytophilum*-like 2). Accordingly,
188 these same delegations were statistically the most co-infected by *A. phagocytophilum*-like 1 and 2 with
189 co-infection rates estimated at 26.7 and 30% respectively ($p < 0.001$) (Supplementary file 4).

190 In cattle, overall *A. phagocytophilum*-like 1 and 2 prevalence rates were 1.9 and 0.5%, respectively,
191 with co-infection rate estimated at 0.5% (Table 3). No statistically significant differences in *A.*
192 *phagocytophilum*-like 1 and 2 prevalence rates were found according to bioclimatic areas (Table 3). Also,
193 *A. phagocytophilum*-like 2 prevalence rates did not significantly vary among governorates
194 (Supplementary file 5). Cattle from Bizerte were statistically more infected by *A. phagocytophilum*-like 1
195 (5.8%) than those from other governorates ($p = 0.014$), and animals from Sejnane delegation were the
196 most infected in this governorate (10.2%, $p = 0.031$) (Supplementary file 5).

197

198 3.3. Molecular characterization of *Anaplasma* sp. 16S rRNA variants

199 RFLP data were confirmed by sequencing 598-599 bp of the *A. phagocytophilum*-like 1 16S rRNA
200 obtained from 6 randomly selected ruminant samples (2 goats, 2 sheep, and 2 cattle). Similarly, 6 *A.*
201 *phagocytophilum*-like 2 positive ruminant samples were randomly selected and sequenced (3 goats, 3
202 sheep) (Table 4). Nucleotide alignment allowed to assign the sequences to 2 distinct *A. phagocytophilum*-
203 like 1 variants Aplike1BvCp1 and Aplike1Ov1; GenBank accession numbers KX702974- KX702977 and
204 KX702978-KX702979, respectively) and to 2 *A. phagocytophilum*-like 2 variants (Aplike2OvCp1 and
205 Aplike2Cp1; GenBank accession numbers KX702980-KX702984 and KX702985, respectively). Notably
206 a novel variant was also obtained (Table 4). Genetic variants were distributed in single or double hosts
207 (goat and sheep or goat and cattle), and in one or more sampling sites (Bizerte and Beja) (Table 4).
208 Identity within *A. phagocytophilum*-like 1 genotypes and within *A. phagocytophilum*-like 2 genotypes
209 was 99.8%, while identity among *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2 genotypes
210 ranged from 98.5 to 98.8%, with a total of 9 SNPs observed (Tables 5 and 6).

211 *A. phagocytophilum*-like 1 genotypes shared 99.3 to 100% similarity with *Anaplasma* spp.
212 genotypes isolated from other Tunisian small ruminants (Aplike1GGo1-3 and Aplike1GOv1-3;
213 KM285226, KM285227, KM285229 and KM285230-KM285232, respectively) and from Japanese deer
214 (Clone 1; JN055357), published earlier in the GenBank (Tables 5 and 6). *A. phagocytophilum*-like 2
215 genotypes shared 99.3 to 100% similarity with *Anaplasma* sp. genotypes isolated from other Tunisian

216 goats (Aplike2GGo1; KM285228), from Chinese sheep (YC38; KJ782381), and from *Hyalomma*
217 *asiaticum* ticks feeding on ruminants (BL099-6; KJ410247), published earlier in the GenBank (Tables 5
218 and 6). When *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2 were compared to the *A.*
219 *phagocytophilum* sequence (HN strain; KC470064), identities were 98.9 and 97.5-97.7%, respectively
220 (Tables 4, 5 and 6).

221 Phylogenetic analysis of the partial 16S rRNA gene was performed by aligning *A.*
222 *phagocytophilum*-like sequences obtained in this study with selected *Anaplasma* spp. sequences isolated
223 from ruminants and ticks, and available in the GenBank (Figure 2). All *Anaplasma* spp. strains obtained
224 in this study were closely related to *A. phagocytophilum* but clustered independently in two different
225 clades. Aplike1BvCp1 and Aplike1Ov1 variants were classified in *A. phagocytophilum*-like 1 cluster.
226 However, Aplike1BvCp1 variant was identical to that of *Anaplasma* sp. (Japan) strains isolated from deer
227 (AB96720, registered as *A. phagocytophilum*, JN055357, AB588974 and AB588976) and *Anaplasma* sp.
228 strain isolated from cattle in Turkey (GU223365), while Aplike1Ov1 variant was closely related to that of
229 Aplike1GGo2 and 3 strains isolated from Tunisian goats (KM285227 and KM285229) (Figure 2).
230 Aplike2OvCp1 and Aplike2Cp1 variants were classified in *A. phagocytophilum*-like 2 cluster. Notably,
231 Aplike2Cp1 variant was identical to that of *Anaplasma* sp. (China) isolated from sheep (KJ782381-5,
232 registered as *A. phagocytophilum*, while Aplike2OvCp1 variant was closely related to that of
233 Aplike2GGo1 isolated from Tunisian goat (KM285228) (Figure 2).

234

235 **4. Discussion**

236 Accurate diagnosis of pathogenic and zoonotic diseases like granulocytic anaplasmosis is crucial for
237 estimating risk in tick-borne diseases control programs. Therefore, it is essential to differentiate between
238 pathogenic *A. phagocytophilum* and genetically related strains, which have not, until now, caused clinical
239 signs in infected animals and are therefore considered non-pathogenic. In 2006, Kawahara *et al.* have
240 developed a nested PCR based on the 16S rRNA for specific detection of *A. phagocytophilum*. More
241 recently, Ybañez *et al.* (2012) and Ben Said *et al.* (2015) have shown that, in ruminants, these primers

242 allow detection of *A. phagocytophilum*, and of one or two types of strains genetically related. Thus, the
243 identification of these *A. phagocytophilum*-like strains depends on sequencing of the nested PCR products
244 with species-specific primers, which to date is the method used for confirmation of *A. phagocytophilum*-
245 like strains infection.

246 In this study, SSAP2f and SSAP2r were used to investigate the presence of *A. phagocytophilum*
247 and/or related strains in Tunisian ruminants (Kawahara et al., 2006; Ybañez et al., 2012; Ben Said et al.,
248 2015). PCR was combined to XcmI and BsaI digestions (Figure 1) to develop a RFLP assay able to
249 discriminate *A. phagocytophilum* and related strains (XcmI), and to identify the presence of *A.*
250 *phagocytophilum*-like 1 and 2 (BsaI). These two restriction enzymes can be used sequentially or
251 simultaneously and as a consequence, there is no need for sequencing PCR products to estimate strains
252 prevalence and co-infection rates. This direct, specific, and less expensive method has important
253 advantages when used in routinely field surveys, especially in resource-poor countries that generally
254 suffer for the lack of resources and technical training. Also, fast simultaneous detection of *A.*
255 *phagocytophilum* and related strains is particularly useful in epidemiological studies based on tick
256 vectors.

257 With this novel PCR/RFLP approach we establish co-circulation of *A. phagocytophilum*-like 1 and
258 2 in Tunisian ruminants. The highest *A. phagocytophilum*-like 1 prevalence rate was estimated in goats
259 (13.3%) while sheep (5.4%) showed the highest *A. phagocytophilum*-like 2 infection rate (Tables 1 and
260 2). The two *A. phagocytophilum*-like variants have been individually detected in previous surveys
261 conducted in Asia. Particularly, *A. phagocytophilum*-like 1 (*Anaplasma* sp.-Japan) has been identified in
262 Japanese cattle, deer, and some species of ticks infesting ruminants (*Ixodes persulcatus*, *I. ovatus*,
263 *Hyalomma megaspinosa*) (Ohashi et al., 2005; Jilintai et al., 2009; Yoshimoto et al., 2010; Ybañez et al.,
264 2012) while *A. phagocytophilum*-like 2 (*Anaplasma* sp.-China) has been recently detected in *H. asiaticum*
265 ticks infested Chinese ruminants by Kang et al. (2014).

266 In this study, ruminants of the higher semi-arid area (sheep especially) were the most infected with
267 *A. phagocytophilum*-like variants (Table 2). This area is known to suffer of an important infestation by

268 *Hyalomma* and *Rhipicephalus* ticks (Bouattour et al., 2002). Animals from sub-humid and lower humid
269 areas, that are known to be almost exclusively infested by *Ixodes* ticks were less infected by these two
270 types of strains. This may suggest that vectors of these *A. phagocytophilum*-like strains could be different
271 from the one transmitting *A. phagocytophilum* that is *Ixodes* species such as *I. ricinus* and *I. scapularis*
272 (Sarih et al., 2005; Woldehiwet, 2010). This assumption is supported by (i) the absence of *A.*
273 *phagocytophilum* in all analyzed animals, (ii) the infection of *Hyalomma asiaticum* ticks infesting
274 Chinese ruminants by *A. phagocytophilum*-like 2 (Kang et al., 2014) and (iii) the presence of *A.*
275 *phagocytophilum*-like 1 DNA in one *Rhipicephalus turanicus* tick infesting a Tunisian goat (*unpublished*
276 *data*). Additionally, low co-infection rates found between *A. phagocytophilum*-like 1 and 2 in analyzed
277 sheep (3.9%), goats (2.5%) and cattle (0.5%) suggest that even these two types of strains may be
278 associated to different tick species like vectors. Further studies are needed to confirm these hypotheses
279 and to determine the main vectors involved in the transmission of each type of strains genetically related
280 to *A. phagocytophilum* in Tunisia. Furthermore, we failed to detected pathogenic *A. phagocytophilum* in
281 all investigated animals. It can be postulated that ruminants are not relevant reservoirs for this zoonotic
282 species in these studied regions, and alternative domestic animals like dogs and horses could act as
283 reservoir hosts in these areas (M'ghirbi et al., 2009, 2012). This is in agreement with what reported in the
284 North Mediterranean area (Torina et al., 2008; Zobba et al., 2014). In contrast, Dahmani *et al.* (2015)
285 reported *A. phagocytophilum* infection in cattle from Algeria.

286 In this study, sequencing of 598-599 bp of the *A. phagocytophilum*-like 16S rRNA gene isolated
287 from randomly selected ruminants revealed four distinct variants (two from each type of strains). RFLP
288 data (Figure 1 and Table 4), nucleotide alignments (Table 5) and percent sequence identity comparison
289 (Table 6) of the 16S rRNA sequence variants obtained in this study confirm that all analysed Tunisian
290 ruminant species are infected by two potential non pathogenic novel species genetically related to *A.*
291 *phagocytophilum*. This hypothesis is reinforced by the fact that infected ruminants do not shown any
292 clinical signs of active disease while for instance Algerian cattle can be infected by pathogenic *A.*
293 *phagocytophilum*, and develop hyperthermia, decreased milk production, cough, and (in some animals)

294 distal edema (Dahmani et al., 2015). The absence of clinical signs was previously reported in Japanese
295 and Tunisian ruminants infected by *A. phagocytophilum*-like 1 (Jilintai et al., 2009; Yoshimoto et al.,
296 2010; Ybañez et al., 2012; Ben Said et al., 2015), and in Tunisian ruminants infected by *A.*
297 *phagocytophilum*-like 2 (Ben Said et al., 2015).

298 Phylogenetic analysis based the alignment of 16S rRNA sequences of *A. phagocytophilum*-like
299 strains isolated from Tunisian ruminants with selected *Anaplasma* sequences obtained from the GenBank
300 confirmed the conclusions obtained from sequence identity comparisons (Figure 2). In agreement with
301 Ooshiro et al. (2008), Liu et al. (2012), Ybañez et al. (2012), Zobba et al. (2014) and Ben Said et al.
302 (2015), phylogenetic trees show two main clusters, one containing *A. marginale*, *A. centrale* and *A. ovis*
303 sequences, and another containing *A. phagocytophilum* and related strains, *A. platys* and *A. bovis*
304 sequences. *Anaplasma* sp. variants isolated from Tunisian ruminants cluster with two divergent clades
305 relatively distant from the *A. phagocytophilum* strains infecting humans, horses and rodents (Figure 2).

306 Furthermore, the most important result consists in the perfect homology found between some *A.*
307 *phagocytophilum*-like 1 sequence variants isolated from Japanese and/or Tunisian goats, sheep, cattle and
308 deer. The same trend was observed for *A. phagocytophilum*-like 2 variants isolated from Chinese and
309 Tunisian goats and sheep. These findings suggest that several ruminant species could be incriminated in
310 the transmission cycle for each type of strains.

311 Concluding, molecular discrimination between *A. phagocytophilum* and related strains allowed
312 confirming the occurrence and the co-circulation of two potentially novel species closely related to *A.*
313 *phagocytophilum* in Tunisian ruminants and the absence of *A. phagocytophilum*. For each type of *A.*
314 *phagocytophilum* related strains, phylogenetic analysis indicated the presence of variants isolated shared
315 by different ruminant species. These findings have to be considered when using indirect and direct tests to
316 detect pathogenic *A. phagocytophilum* in ruminants, and provide additional molecular background to
317 trace the evolutionary tree of bacterial species closely related to *A. phagocytophilum*. Further studies are
318 needed to (i) investigate if these *A. phagocytophilum*-like strains infect other animal species in Tunisia,
319 (ii) characterize these different strains by using additional discriminative genes and (iii) identify vectors

320 implicated in the transmission of these potentially novel *Anaplasma* spp. genetically related to *A.*
321 *phagocytophilum*.

322

323 **Competing interests**

324 The authors declare that they have no competing interests.

325

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336

337 **References**

338 Alberti, A., Zobba, R., Chessa, B., Addis, M.F., Sparagano, O.A., Pinna Parpaglia, M.L., Cubeddu, T.,
339 Pintori, G., Pittau, M., 2005. Equine and canine *Anaplasma phagocytophilum* strains isolated on the
340 island of Sardinia (Italy) are phylogenetically related to pathogenic strains from the United States.
341 Appl. Environ. Microbiol. 71, 6418–6422.

342 Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997.
343 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic
344 Acids Res. 25, 3389–3402.

345 Barlough, J.E., Madigan, A., DeRock, E., Bigornia, L., 1996. Nested polymerase chain reaction for
346 detection of *Ehrlichia equi* genomic DNA in horses and ticks (*Ixodes pacificus*). *Vet. Parasitol.* 63,
347 319–329.

348 Ben Said, M., Belkahia, H., Alberti, A., Zobba, R., Bousrih, M., Yahiaoui, M., Daaloul-Jedidi, M.,
349 Mamlouk, A., Gharbi, M., Messadi, L., 2015. Molecular survey of *Anaplasma* species in small
350 ruminants reveals the presence of novel strains closely related to *A. phagocytophilum* in Tunisia.
351 *Vector Borne Zoonotic Dis.* 15, 580–590.

352 Bouattour, A., 2002. [Dichotomous identification keys of ticks (Acari: Ixodidae), livestock parasites in
353 North Africa]. *Arch. Inst. Pasteur Tunis* 79, 43–50.

354 Chen, S.M., Dumler, J.S., Bakken, J.S., Walker, D.H., 1994. Identification of a granulocytotropic
355 Ehrlichia species as the etiologic agent of human disease. *J. Clin. Microbiol.* 32, 589–595.

356 Dahmani, M., Davoust, B., Benterki, M.S., Fenollar, F., Raoult, D., Mediannikov, O., 2015. Development
357 of a new PCR-based assay to detect *Anaplasmataceae* and the first report of *Anaplasma*
358 *phagocytophilum* and *Anaplasma platys* in cattle from Algeria. *Comp. Immunol. Microbiol. Infect.*
359 *Dis.* 39, 39–45.

360 Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa,
361 F.R., 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the
362 order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with
363 *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and
364 designation of *Ehrlichia equi* and ‘HGE agent’ as subjective synonyms of *Ehrlichia*
365 *phagocytophilum*. *Int. J. Syst. Evol. Microbiol.* 51, 2145–2165.

366 Jilintai, Seino, N., Hayakawa, D., Suzuki, M., Hata, H., Kondo, S., Matsumoto, K., Yokoyama, N.,
367 Inokuma, H. 2009. Molecular survey for *Anaplasma bovis* and *Anaplasma phagocytophilum*
368 infection in cattle in a pastureland where sika deer appear in Hokkaido, Japan. *Jpn J. Infect. Dis.* 62,
369 73–75.

370 Kang, Y.J., Diao, X.N., Zhao, G.Y., Chen, M.H., Xiong, Y., Shi, M., Fu, W.M., Guo, Y.J., Pan, B., Chen
371 X.P., Holmes, E.C., Gillespie, J.J., Dumler, S.J., Zhang, Y.Z., 2014. Extensive diversity of
372 Rickettsiales bacteria in two species of ticks from China and the evolution of the Rickettsiales.
373 BMC Evol. Biol. 14, 167.

374 Kawahara, M., Rikihisa, Y., Lin, Q., Isogai, E., Tahara, K., Itagaki, A., Hiramitsu, Y., Tajima, T., 2006.
375 Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and
376 a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. Appl. Environ.
377 Microbiol. 72, 1102–1109.

378 Lin, M., Kikuchi, T., Brewer, H.M., Norbeck, A.D., Rikihisa, Y., 2011. Global proteomic analysis of two
379 tick-borne emerging zoonotic agents: *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*. Front
380 Microbiol. 2, 24.

381 Liu, Z., Ma, M., Wang, Z., Wang, J., Peng, Y., Li, Y., Guan, G., Luo, J., Yin, H., 2012. Molecular survey
382 and genetic identification of *Anaplasma* species in goats from central and southern China. Appl.
383 Environ. Microbiol. 78, 464–470.

384 M'ghirbi, Y., Ghorbel, A., Amouri, M., Nebaoui, A., Haddad, S., Bouattour, A., 2009. Clinical,
385 serological, and molecular evidence of ehrlichiosis and anaplasmosis in dogs in Tunisia. Parasitol.
386 Res. 104, 767–774.

387 M'ghirbi, Y., Yaïch, H., Ghorbel, A., Bouattour, A., 2012. *Anaplasma phagocytophilum* in horses and
388 ticks in Tunisia. Parasit. Vectors 30, 180.

389 Ohashi, N., Inayoshi, M., Kitamura, K., Kawamori, F., Kawaguchi, D., Nishimura, Y., Naitou, H., Hiroi,
390 M., Masuzawa, T., 2005. *Anaplasma phagocytophilum*-infected ticks, Japan. Emerg. Infect. Dis. 11,
391 1780–1783.

392 Ooshiro, M., Zakimi, S., Matsukawa, Y., Katagiri, Y., Inokuma, H., 2008. Detection of *Anaplasma bovis*
393 and *Anaplasma phagocytophilum* from cattle on Yonaguni island, Okinawa, Japan. Vet. Parasitol.
394 154, 360–364.

- 395 Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic
396 trees. *Mol. Biol. Evol.* 4, 406–425.
- 397 Torina, A., Alongi, A., Naranjo, V., Estrada-Peña, A., Vicente, J., Scimeca, S., Marino, A.M., Salina, F.,
398 Caracappa, S., de la Fuente, J., 2008. Prevalence and genotypes of *Anaplasma* species and habitat
399 suitability for ticks in a Mediterranean ecosystem. *Appl. Environ. Microbiol.* 74, 7578–7584.
- 400 Tuomi, J., 1967. Experimental studies on bovine tick-borne fever .1. Clinical and haematological data,
401 some properties of the causative agent, and homologous immunity. *Acta Pathol. Microbiol. Scand.*
402 70, 429–445.
- 403 Woldehiwet, Z., 2010. The natural history of *Anaplasma phagocytophilum*. *Vet. Parasitol.* 167, 108–122.
- 404 Ybañez, A.P., Matsumoto, K., Kishimoto, T., Inokuma, H., 2012. Molecular analyses of a potentially
405 novel *Anaplasma* species closely related to *Anaplasma phagocytophilum* detected in sika deer
406 (*Cervus nippon yesoensis*) in Japan. *Vet. Microbiol.* 157, 232–236.
- 407 Yoshimoto, K., Matsuyama, Y., Matsuda, H., Sakamoto, L., Matsumoto, K., Yokoyama, N., Inokuma, H.,
408 2010. Detection of *Anaplasma bovis* and *Anaplasma phagocytophilum* DNA from *Haemaphysalis*
409 *megaspinosa* in Hokkaido, Japan. *Vet. Parasitol.* 168, 170–172.
- 410 Sarih, M., M'ghirbi, Y., Bouattour, A., Gern, L., Baranton, G., Postic, D., 2005. Detection and
411 identification of *Ehrlichia* spp. in ticks collected in Tunisia and Morocco. *J. Clin. Microbiol.* 43,
412 1127–1132.
- 413 Zobba, R., Anfossi, A.G., Pinna Parpaglia, M.L., Dore, G.M., Chessa, B., Spezzigu, A., Rocca, S., Visco,
414 S., Pittau, M., Alberti, A., 2014. Molecular investigation and phylogeny of *Anaplasma* spp. in
415 Mediterranean ruminants reveal the presence of neutrophil-tropic strains closely related to *A. platys*.
416 *Appl. Environ. Microbiol.* 80, 271–280.

417

418 **Figure legends**

419 **Figure 1**

420 RFLP strategy and results of restriction analysis using in this study. (A) RFLP strategy developed and
421 validated during this study, used after published 16S rRNA nested PCR, for the detection of and the
422 differentiation between pathogenic *A. phagocytophilum* and *A. phagocytophilum*-like strains and between
423 *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2 strains. Numbers in parentheses indicate
424 positions of primers and XcmI and BsaI restriction sites calculated using the sequence of HZ strain of *A.*
425 *phagocytophilum* as a reference. (B) Results of restriction analysis using XcmI enzyme on DNA analyzed
426 samples. Lines 1 and 9: 100 bp ladder; lines 2 and 3: PCR products after RFLP assay of pathogenic *A.*
427 *phagocytophilum* (344 and 297 bp) isolated from human and dog, respectively; lines 4, 5 and 6: PCR
428 products after RFLP assay of *A. phagocytophilum*-like strains (641-642 bp) isolated from two goats, one
429 sheep and one cattle, respectively and line 8: PCR results of negative control after RFLP assay. (C)
430 Results of restriction analysis using BsaI enzyme on DNA analyzed samples. Lines 1 and 12: 100 bp
431 ladder; lines 2, 3 and 4: PCR products after RFLP assay of *A. phagocytophilum*-like 2 (422-423 and 219
432 bp) isolated from goat, sheep and cattle, respectively; lines 5, 6 and 7: PCR products after RFLP assay of
433 *A. phagocytophilum*-like 1 (641-642 bp) isolated from goat, sheep and cattle, respectively; lines 8, 9 and
434 10: PCR products after RFLP assay of *A. phagocytophilum*-like 1 and 2 (641-642, 422-423 and 219 bp)
435 co-infecting goat, sheep and cattle, respectively and line 12: PCR results of negative control after RFLP
436 assay.¹: The presence of two sizes 1515-1516, 1438-1439, 641-642 or 422-423 is caused by a deletion at
437 the position 1113 that was found in some genetic variants of *A. phagocytophilum* and related strains. ²:
438 The first number indicates the position of the mutation involved in the XcmI restriction enzyme site and
439 the second number indicates the position where the restriction was made by the XcmI enzyme. ³: The first
440 number indicates the position of the mutation involved in the BsaI restriction enzyme site and the second
441 number indicates the position where the restriction was made by the BsaI enzyme.

442

443 **Figure 2**

444 Phylogenetic tree inferred with partial sequences (598-599 bp) of the 16S rRNA gene of *Anaplasma* sp.
445 closely related to *A. phagocytophilum* isolated from ruminants and ticks, and classified *Anaplasma*
446 species found in GenBank using the neighbor-joining method. Numbers associated with nodes represent
447 the percentage of 1000 bootstrap iterations supporting the nodes (only percentages greater than 50% were
448 presented). The host or vector, the strain or isolate name, the country of origin and the GenBank
449 accession number are indicated. The novel sequences of *A. phagocytophilum*-like 1 and 2, and canine
450 related *A. phagocytophilum* obtained in the present study are represented in bold.

451

452 **Supplementary file legends**

453 **Supplementary file 1**

454 Map of the Tunisian studied regions. (A) Map of Tunisia showing investigated governorates and (B) Map
455 of the five governorates showing the twenty-two studied delegations belonging to three bioclimatic areas.

456

457 **Supplementary file 2**

458 Primers used for detection and/or characterization of *Anaplasma phagocytophilum* and/or related species
459 in cattle, sheep and goats in the present study

460

461 **Supplementary file 3**

462 Prevalence and co-infection rates of potentially *Anaplasma* spp. closely related to *A. phagocytophilum* in
463 goats according to governorates and delegations.

464

465 **Supplementary file 4**

466 Prevalence and co-infection rates of *Anaplasma* spp. closely related to *A. phagocytophilum* in sheep
467 according to governorates and delegations.

468

469 **Supplementary file 5**

470 Prevalence and co-infection rates of *Anaplasma* spp. closely related to *A. phagocytophilum* in cattle
471 according to governorates and delegations.

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ECOLE NATIONALE DE MEDECINE VETERINAIRE
DE SIDI THABET

SERVICE DE MICROBIOLOGIE, IMMUNOLOGIE ET PATHOLOGIE GENERALE

Tunis, October the 24th 2016

Dear Editor,

Please find enclosed our manuscript, “**Molecular typing and diagnosis of *Anaplasma spp.* closely related to *A. phagocytophilum* in ruminants from Tunisia**”, by Mourad Ben Said et al., which we would like to submit for publication as a research paper to “*Ticks and Tick-borne Diseases*” journal. In this paper two Restriction Enzyme Fragment Length Polymorphism (RFLP) assays coupled to nested *16S rRNA* PCR published earlier, were developed to create a novel diagnostic protocol, and to discriminate among *Anaplasma phagocytophilum* and genetically related strains and among two different types of *A. phagocytophilum*-like strains. This approach was used for investigating the occurrence and the co-circulation of two potentially novel species closely related to *A. phagocytophilum* for the first time in goats, sheep, and cattle from North of Tunisia. Data open new concerns about the specificity of indirect and molecular methods usually used to recognize different *Anaplasma* species in ruminants, and provide molecular information to clarify the evolutionary history of bacterial strains genetically related to this zoonotic and tick-borne pathogen.

For these reasons, we believe our findings would appeal to the readership of “*Ticks and Tick-borne Diseases*” journal. We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with its submission to this journal. We look forward to hearing from you at your earliest convenience.

Best regards.

On behalf of authors,

Prof. Lilia Messadi

Figure 1 Ben Said et al., for TTBD
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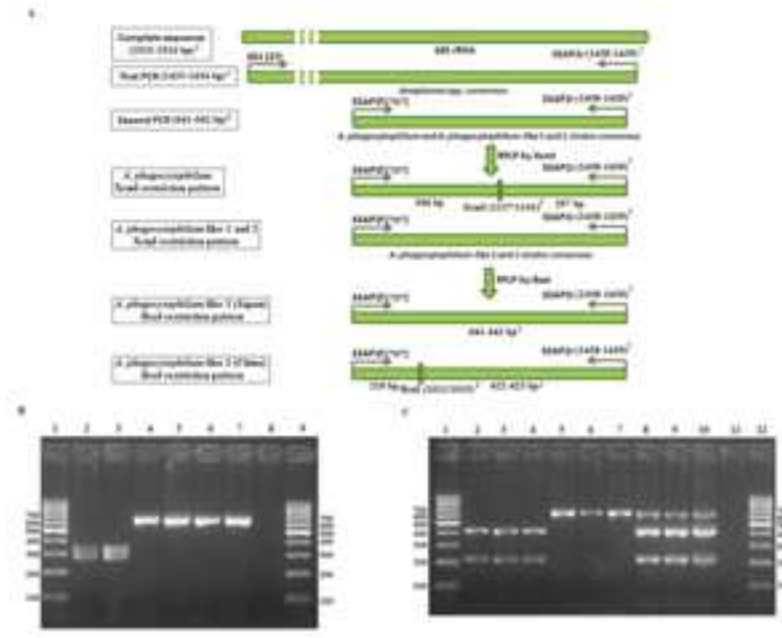


Figure 1

Table 1: Prevalence and co-infection rates of *Anaplasma* spp. closely related to *A. phagocytophilum* in goats according to bioclimatic zones and delegations.

Bioclimatic zone	Delegation	Number	Positive (%±C.I. ¹)				Co-infected (%±C.I. ¹)		
			<i>A. ph</i> -like 1	P-value ²	<i>A. ph</i> -like 2	P-value ²	<i>A. ph</i> -like 1/ <i>A. ph</i> -like 2	P-value ²	
Lower humid		87	19 (21.8±0.09)	0.009*	1 (1.2±0.02)	0.063	1 (1.2±0.02)	0.467	
	Sejnane	49	10 (20.4±0.11)	0.009*	0 (0)	0.224	0 (0)	0.224	
	Jouza Hamra	22	9 (40.9±0.21)		1 (4.5±0.09)		1 (4.5±0.09)		
	Amdoun	16	0 (0)		0 (0)		0 (0)		
Sub-humid		11	0 (0)		0 (0)		0 (0)		
	Oued Abid	11	0 (0)	-	0 (0)	-	0 (0)	-	
Higher semi-arid		143	13 (9.1±0.05)		11 (7.7±0.04)		5 (3.7±0.03)		
	Utique	12	2 (16.7±0.21)	0.000*	1 (8.3±0.15)	0.006*	1 (8.3±0.16)	0.396	
	Hrairia Agba	20	2 (10±0.13)		2 (10±0.13)		2 (10±0.13)		
	Ghdir Golla	10	0 (0)		0 (0)		0 (0)		
	Sidi Hassine	20	0 (0)		0 (0)		0 (0)		
	Hrairia Sejoumi	26	2 (7.7±0.10)		7 (26.9±0.17)		1 (3.8±0.07)		
	Medjez El Bab	10	3 (30±0.28)		1 (10±0.19)		1 (10±0.19)		
	Soliman	20	0 (0)		0 (0)		0 (0)		
	Beni Khaled	06	0 (0)		0 (0)		0 (0)		
	Cebalet Ben Ammar	05	4 (80±0.35)		0 (0)		0 (0)		
	Chorfech	04	0 (0)		0 (0)		0 (0)		
	Hessiane	10	0 (0)		0 (0)		0 (0)		
Total		241	32 (13.3±0.04)		12 (5.0±0.03)		6 (2.5±0.02)		

¹: C.I.: 95% confidence interval; ²: P-value represented in the first line is calculated according to bioclimatic areas and P-values represented in the other lines are calculated according to delegation of each appropriated bioclimatic areas; *: Statistically significant test; *A.ph*-like: *Anaplasma phagocytophilum*-like.

Table 2: Prevalence and co-infection rates of *Anaplasma* spp. closely related to *A. phagocytophilum* in sheep according to bioclimatic zones and delegations.

Bioclimatic zone	Delegation	Number	Positive (%±C.I. ¹)		Co-infected (%±C.I. ¹)			
			<i>A. ph-like 1</i>	P-value ²	<i>A. ph-like 2</i>	P-value ²	<i>A. ph-like 1/ A. ph-like 2</i>	P-value ²
Lower humid		126	2 (1.6±0.02)	0.003*	0 (0)	0.002*	0 (0)	0.347
	Sejnane	48	2 (4.2±0.06)	0.347	0 (0)	-	0 (0)	-
	Bezina	22	0 (0)		0 (0)		0 (0)	
	Jouza Hamra	22	0 (0)		0 (0)		0 (0)	
	Amdoun	34	0 (0)		0 (0)		0 (0)	
Sub-humid		32	1 (3.1±0.06)		0 (0)		0 (0)	
	Oued Abid	32	1 (3.1±0.06)	-	0 (0)	-	0 (0)	-
Higher semi-arid		197	22 (11.2±0.04)		19 (9.6±0.04)		14 (7.1±0.04)	
	Agba	20	0 (0)	0.000*	0 (0)	0.000*	0 (0)	0.000*
	Hrairia	87	8 (40±0.06)		9 (45±0.06)		5 (5.8±0.05)	
	Medjez El Bab	10	4 (40±0.30)		3 (30±0.28)		3 (30±0.28)	
	Cebalet Ben Ammar	30	1 (3.3±0.03)		0 (0)		0 (0)	
	Hessiane	11	3 (27.3±0.23)		2 (18.2±0.19)		2 (18.2±0.23)	
	Soliman	20	0 (0)		0 (0)		0 (0)	
	Beni Khiar	04	0 (0)		0 (0)		0 (0)	
	Utique	15	6 (40±0.25)		5 (33.3±0.24)		4 (26.7±0.22)	
Total		355	25 (7.0±0.03)		19 (5.4±0.02)		14 (3.9±0.02)	

¹: C.I.: 95% confidence interval; ²: P-value represented in the first line is calculated according to bioclimatic areas and P-values represented in the other lines are calculated according to delegation of each appropriated bioclimatic areas; *: Statistically significant test; *A.ph-like*: *Anaplasma phagocytophilum*-like.

Table 3: Prevalence and co-infection rates of *Anaplasma* spp. closely related to *A. phagocytophilum* in cattle according to bioclimatic zones and delegations.

Bioclimatic zone	Delegation	Number	Positive (%±C.I. ¹)				Co-infected (%±C.I. ¹)		
			<i>A. ph</i> -like 1	P-value ²	<i>A. ph</i> -like 2	P-value ²	<i>A. ph</i> -like 1/ <i>A. ph</i> -like 2	P-value ²	
Lower humid		175	6 (3.4±0.03)	0.122	1 (0.6±0.01)	0.896	1 (0.6±0.01)	0.896	
	Sejnane	59	6 (10.2±0.08)	0.031	1 (1.7±0.03)	0.852	1 (1.7±0.03)	0.852	
	Bezina	44	0 (0)		0 (0)		0 (0)		
	Rouihia Jouza	10	0 (0)		0 (0)		0 (0)		
	Jouza Hamra	20	0 (0)		0 (0)		0 (0)		
	Agba Jouza	21	0 (0)		0 (0)		0 (0)		
	Amdoun	21	0 (0)		0 (0)		0 (0)		
Sub-humid		35	0 (0)		0 (0)		0 (0)		
	Oued Abid	35	0 (0)	-	0 (0)	-	0 (0)	-	
Higher semi-arid		157	1 (0.6±0.01)		1 (0.6±0.01)		1 (0.6±0.01)		
	Soliman	22	0 (0)	0.160	0 (0)	0.160	0 (0)	0.160	
	Beni Khaled	09	0 (0)		0 (0)		0 (0)		
	Bejaoua	10	0 (0)		0 (0)		0 (0)		
	Kalaat El Andalous	16	0 (0)		0 (0)		0 (0)		
	Hrairia	29	0 (0)		0 (0)		0 (0)		
	Borj Chaker	11	0 (0)		0 (0)		0 (0)		
	Agba	10	0 (0)		0 (0)		0 (0)		
	Mjez El Bab	10	0 (0)		0 (0)		0 (0)		
	Sidi Hassine	10	0 (0)		0 (0)		0 (0)		
	Hessiane	10	1 (10±0.19)		1 (10±0.19)		1 (10±0.19)		
	Chorfech	20	0 (0)		0 (0)		0 (0)		
Total		367	7 (1.9±0.01)		2 (0.5±0.01)		2 (0.5±0.01)		

¹: C.I.: 95% confidence interval; ²: P-value represented in the first line is calculated according to bioclimatic areas and P-values represented in the other lines are calculated according to delegation of each appropriated bioclimatic areas; *: Statistically significant test.

Table 4: Designation and information about sequencing and RFLP data of the two and twelve 16S rRNA genetic variants analyzed in this study of specific *A. phagocytophilum* and *Anaplasma* sp. closely related to *A. phagocytophilum*, respectively.

<i>Anaplasma</i> spp.	Sequence type	Isolate	Host	Geographical location	GenBank accession no.	BLAST analysis	RFLP by XcmI ¹	RFLP by BsaI ²
<i>A. phagocytophilum</i>	HZ	HZ	<i>Homo sapiens</i>	New York, USA	NC_007797	100% <i>A. phagocytophilum</i>	+	NA ³
	ApDg	Dog1	<i>Canis canis</i>	Ariana, Tunisia	KX702986	100% <i>A. phagocytophilum</i>	+	NA ³
<i>A. phagocytophilum</i> -like 1 (Japan)	Aplike1BvCp1	R1	<i>Bos taurus</i>	Bizerte, Tunisia	KX702974	98.9% <i>A. phagocytophilum</i>	-	-
		R2	<i>Bos taurus</i>	Bizerte, Tunisia	KX702975	98.9% <i>A. phagocytophilum</i>	-	-
		R3	<i>Capra hircus</i>	Beja, Tunisia	KX702976	98.9% <i>A. phagocytophilum</i>	-	-
		R4	<i>Capra hircus</i>	Beja, Tunisia	KX702977	98.9% <i>A. phagocytophilum</i>	-	-
		R5	<i>Ovis aries</i>	Bizerte, Tunisia	KX702978	98.9% <i>A. phagocytophilum</i>	-	-
<i>A. phagocytophilum</i> -like 2 (China)	Aplike2OvCp1	R6	<i>Ovis aries</i>	Bizerte, Tunisia	KX702979	98.9% <i>A. phagocytophilum</i>	-	-
		R7	<i>Ovis aries</i>	Tunis, Tunisia	KX702980	97.5% <i>A. phagocytophilum</i>	-	+
		R8	<i>Capra hircus</i>	Tunis, Tunisia	KX702981	97.5% <i>A. phagocytophilum</i>	-	+
		R9	<i>Capra hircus</i>	Tunis, Tunisia	KX702982	97.5% <i>A. phagocytophilum</i>	-	+
		R10	<i>Ovis aries</i>	Tunis, Tunisia	KX702983	97.5% <i>A. phagocytophilum</i>	-	+
		R11	<i>Ovis aries</i>	Tunis, Tunisia	KX702984	97.5% <i>A. phagocytophilum</i>	-	+
	Aplike2Cp1	R12	<i>Capra hircus</i>	Tunis, Tunisia	KX702985	97.7% <i>A. phagocytophilum</i>	-	+

¹ Restriction (+) or not (-) of the PCR product with XcmI enzyme during RFLP assay.

² Restriction (+) or not (-) of the PCR product with BsaI enzyme during RFLP assay.

³: Not analyzed.

Table 5: Nucleotide diversity among 16S rRNA sequences from *Anaplasma* strains closely related to *A. phagocytophilum* (598-599 bp).

Host or vector	Genetic variant ¹	Country	GenBank ²	16S rRNA nucleotide positions ³																	Reference	
				823	830	852	855	908	1011	1109	1111	1113	1120	1137	1148	1237	1239	1240	1251	1259		1291
Human	Webster	USA	NR_044762	T	T	G	T	A	A	G	T	A	C	A	T	T	T	C	G	G	C	Chen et al. (1994)
Horse	Camawi	USA	AF172167	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Unpublished
Dog	Dog2	USA	CP006618	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Unpublished
Deer	Clone 1	Japan	JN055357	C	*	*	*	*	*	*	A	-	*	G	C	*	*	*	*	*	*	Ybañez et al. (2012a)
Goat	Aplike1GGo1	Tunisia	KM285226	C	*	*	*	*	*	*	A	-	*	G	C	*	*	*	*	*	*	Ben Said et al. (2015)
	Aplike1GGo2	Tunisia	KM285227	C	*	*	*	*	*	*	A	T	*	G	*	*	*	*	*	*	*	Ben Said et al. (2015)
	Aplike1GGo3	Tunisia	KM285229	C	*	*	*	C	*	*	A	T	*	G	*	*	*	*	*	*	*	Ben Said et al. (2015)
Sheep	Aplike1GOv1	Tunisia	KM285230	C	*	*	*	*	*	*	A	-	*	G	C	*	*	*	*	*	*	Ben Said et al. (2015)
	Aplike1GOv2	Tunisia	KM285231	C	*	C	*	*	*	*	A	-	*	G	C	*	*	*	*	*	*	Ben Said et al. (2015)
	Aplike1GOv3	Tunisia	KM285232	C	*	C	*	*	*	*	A	-	*	G	C	*	*	*	A	*	*	Ben Said et al. (2015)
Cattle and goat	Aplike1BvCp1	Tunisia	KX702974	C	*	*	*	*	*	*	A	-	*	G	C	*	*	*	*	*	*	Present study
Sheep	Aplike1Ov1	Tunisia	KX702978	C	*	*	*	*	*	*	A	-	*	G	*	*	*	*	*	*	*	Present study
<i>H. asiaticum</i> ⁴	BL099-6	China	KJ410247	*	A	*	*	*	G	A	A	T	T	G	C	C	C	*	*	A	T	Kang et al. (2014)
Goat	Aplike2GGo1	Tunisia	KM285228	C	A	*	C	*	G	*	A	T	T	G	C	C	C	T	*	*	T	Ben Said et al. (2015)
Sheep and goat	Aplike2OvCp1	Tunisia	KX702980	C	A	*	*	*	G	*	A	T	T	G	C	C	C	T	*	*	T	Present study
Goat	Aplike2Cp1	Tunisia	KX702985	C	A	*	*	*	G	*	A	T	T	G	C	C	C	T	*	*	T	Present study

¹: Aplike1BvCp1 variant is represented by the GenBank accession numbers KX702974-KX702977; Aplike1Ov1 variant is represented by the GenBank accession number KX702978 and KX702979; Aplike2OvCp1 variant is represented by the GenBank accession numbers KX702980-KX702984 and Aplike2Cp1 variant is represented by the GenBank accession number KX702985.

²: GenBank accession number.

³: Numbers represent the nucleotide position with respect to the HZ strain from USA for *A. phagocytophilum* (GenBank accession number NC_007797) (Lin et al., 2011). Conserved nucleotide positions are indicated with asterisks. Nucleotides: T, Thymine; C, Cytosine; G, Guanine; A, Adenine.

Nucleotide position shaded clear gray shows the substitution of A by G permitting the discrimination between *A. phagocytophilum*-like 1 and 2 by BsaI enzyme after the first differentiation between specific *A. phagocytophilum* and *A. phagocytophilum*-like strains. Nucleotide position shaded dark gray shows the substitution of A by G permitting the differentiation between specific *A. phagocytophilum* and *A. phagocytophilum*-like strains (*A. phagocytophilum*-like 1 and 2) by XcmI enzyme.

⁴: *H. asiaticum* represents *Hyalomma asiaticum* tick.

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Table 6: Homology and genetic distance between 16S rRNA sequences (598-599 bp) from *Anaplasma phagocytophilum*-like 1 and 2 isolated from Tunisian ruminants and other *Anaplasma* species found in GenBank. The numbers represent the nucleotide identity rates found between the sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 <i>A. sp</i> (Aplike1BvCp1)	100/0	0.002	0.000	0.002	0.003	0.000	0.002	0.003	0.000	0.012	0.013	0.013	0.015	0.011	0.012	0.023	0.027	0.030	0.034	0.079
2 <i>A. sp</i> (Aplike1Ov1)	99.8	100/0	0.002	0.000	0.002	0.002	0.003	0.005	0.002	0.013	0.015	0.015	0.017	0.011	0.012	0.022	0.025	0.028	0.032	0.077
3 <i>A. sp</i> (Aplike1GGo1)	100	99.8	100/0	0.002	0.003	0.000	0.002	0.003	0.000	0.012	0.013	0.013	0.015	0.011	0.012	0.023	0.027	0.030	0.034	0.079
4 <i>A. sp</i> (Aplike1GGo2)	99.8	100	99.8	100/0	0.002	0.002	0.003	0.005	0.002	0.013	0.015	0.015	0.017	0.011	0.012	0.022	0.025	0.028	0.032	0.077
5 <i>A. sp</i> (Aplike1GGo3)	99.7	99.8	99.7	99.8	100/0	0.003	0.005	0.007	0.003	0.015	0.017	0.017	0.018	0.012	0.013	0.023	0.027	0.030	0.033	0.078
6 <i>A. sp</i> (Aplike1GOv1)	100	99.8	100	99.8	99.7	100/0	0.002	0.003	0.000	0.012	0.013	0.013	0.015	0.011	0.012	0.023	0.027	0.030	0.034	0.079
7 <i>A. sp</i> (Aplike1GOv2)	99.8	99.7	99.8	99.7	99.5	99.8	100/0	0.002	0.002	0.013	0.015	0.015	0.017	0.012	0.013	0.025	0.028	0.032	0.035	0.080
8 <i>A. sp</i> (Aplike1GOv3)	99.7	99.5	99.7	99.5	99.3	99.7	99.8	100/0	0.003	0.015	0.017	0.017	0.018	0.013	0.015	0.027	0.030	0.033	0.037	0.082
9 <i>A. sp</i> (Clone 1)	100	99.8	100	99.8	99.7	100	99.8	99.7	100/0	0.012	0.013	0.013	0.015	0.011	0.012	0.023	0.027	0.030	0.034	0.079
10 <i>A. sp</i> (Aplike2OvCp1)	98.8	98.7	98.8	98.7	98.5	98.8	98.7	98.5	98.8	100/0	0.002	0.002	0.007	0.023	0.025	0.025	0.028	0.032	0.037	0.085
11 <i>A. sp</i> (Aplike2Cp1)	98.7	98.5	98.7	98.5	98.3	98.7	98.5	98.3	98.7	99.8	100/0	0.003	0.005	0.025	0.027	0.027	0.030	0.033	0.038	0.083
12 <i>A. sp</i> (Aplike2GGo1)	98.7	98.5	98.7	98.5	98.3	98.7	98.5	98.3	98.7	99.8	99.7	100/0	0.008	0.025	0.027	0.027	0.030	0.033	0.038	0.087
13 <i>A. sp</i> (BL099-6)	98.5	98.3	98.5	98.3	98.2	98.5	98.3	98.2	98.5	99.3	99.5	99.2	100/0	0.023	0.025	0.025	0.028	0.032	0.040	0.082
14 <i>A. p</i> (HN)	98.9	98.9	98.9	98.9	98.8	98.9	98.8	98.7	98.9	97.7	97.5	97.0	97.7	100/0	0.008	0.025	0.028	0.032	0.035	0.077
15 <i>A. pl</i> (Okinawa)	98.8	98.8	98.8	98.8	98.7	98.8	98.7	98.5	98.8	97.5	97.3	97.3	97.5	99.2	100/0	0.023	0.027	0.030	0.040	0.078
16 <i>A. o</i> (Jingtai)	97.7	97.8	97.7	97.8	97.7	97.7	97.5	97.3	97.7	97.5	97.3	97.3	97.5	97.5	97.7	100/0	0.003	0.007	0.047	0.067
17 <i>A. m</i> (Lushi)	97.3	97.5	97.3	97.5	97.3	97.3	97.2	97.0	97.3	97.2	97.0	97.0	97.2	97.2	97.3	99.7	100/0	0.010	0.047	0.065
18 <i>A. c</i> (CC)	97.0	97.2	97.0	97.2	97.0	97.0	96.8	96.7	97.0	96.8	96.7	96.7	96.8	96.8	97.0	99.3	99.0	100/0	0.047	0.073
19 <i>A. b</i> (YX4)	96.6	96.8	97.0	97.2	97.0	97.0	96.8	96.7	97.0	96.3	96.2	96.5	96.3	96.8	96.3	95.7	95.7	95.7	100/0	0.094
20 <i>E. r</i> (Crystal Springs)	92.1	92.3	92.1	92.3	92.2	92.1	92.0	91.8	92.1	91.5	91.7	91.3	91.8	92.3	92.2	93.3	93.5	92.7	90.6	100/0

A. phagocytophilum-like 1 (*Anaplasma sp.*-Japan) variants were represented by *Anaplasma sp.* (Aplike1BvCp1) (R1-4 isolates; GenBank accession numbers KX702974-KX702977), *Anaplasma sp.* (Aplike1Ov1) (R5 and R6 isolates; GenBank accession numbers KX702978 and KX702979), *Anaplasma sp.* (Aplike1GGo1-3) (AplikeGGo1-3 variants, GenBank accession numbers KM285226, KM285227 and KM285229), *Anaplasma sp.* (Aplike1GOv1-3) (AplikeGOv1-3 variants, GenBank accession numbers KM285230- KM285232) and *Anaplasma sp.* (Clone 1) (Clone 1, GenBank accession number JN055357).

A. phagocytophilum-like 2 (*Anaplasma sp.*-China) variants were represented by *Anaplasma sp.* (Aplike2OvCp1) (R7-11 isolates; GenBank accession numbers KX702980-KX702984), *Anaplasma sp.* (Aplike2Cp1) (R12 isolate; GenBank accession number KX702985), *Anaplasma sp.* (Aplike2GGo1) (Aplike2GGo1 variant, GenBank accession numbers KM285228) and *Anaplasma sp.* (BL099-6) (BL099-6 isolate, GenBank accession number KJ410247).

A. p (HN strain): *A. phagocytophilum* strain isolated from Chinese rodent (HN strain, GenBank accession number KC470064); *A. pl* (Okinawa): *A. platys* isolate found on Japanese dog (Okinawa isolate, GenBank accession number AY077619); *A. o* (Jingtai): *A. ovis* isolate found on Chinese goat (Jingtai isolate, GenBank accession number AJ633049); *A. m* (Lushi): *A. marginale* isolate found on Chinese cattle (Lushi isolate, GenBank accession number AJ633048); *A. c* (CC): *A. centrale* strain isolated from Italian cattle (CC strain, GenBank accession number EF520686); *A. b* (G49): *A. bovis* isolate found on Chinese goat (G49 isolate, GenBank accession number JN558824) and *E. r* (Crystal Springs): *Ehrlichia ruminantium* isolate found on cattle from Zimbabwe (Crystal Springs isolate, GenBank accession number NR_044831).