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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Ruminants' populations and study regions

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

2.2. Sampling and DNA extraction

Blood was collected from the animals' jugular vein into EDTA tubes (Becton Dickinson). DNA was extracted from 300 µl volumes of EDTA-preserved whole blood using the Wizard® Genomic DNA 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 $MgCl_2$, 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.*

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

2.5. DNA sequencing and data analysis

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

2.6. GenBank accession numbers

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

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

2.7. Statistical analyses

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

3. Results

3.1. Strains' molecular identification

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

3.2. Anaplasma spp. prevalence and co-infection rates

Nested 16S rRNA PCR-RFLP and *groEL* hemi-nested PCR failed to detect *A. phagocytophilum* in all ruminant species, indicating that none of the analysed animals were positive to this zoonotic species.

In goats, *A. phagocytophilum*-like 1 and 2 overall prevalence rates were respectively 13.1 and 5%, with a co-infection rate estimated at 2.5% (Table 1). Goats from lower humid area were statistically more infected by *A. phagocytophilum*-like 1 (21.8%) than those from higher semi-arid and sub-humid areas (9.1 and 0% respectively; $p = 0.009$). Beja, Ariana and Bizerte governorates showed similar *A. phagocytophilum*-like 1 prevalence rates (25, 21.1, and 19.7%), while rate of infection was statistically lower or absent in goats of other governorates (2.8 and 0% in Tunis and Nabeul respectively; $p < 0.001$) (Supplementary file 3). Within the Ariana and Beja governorates, the delegations of Cebalet Ben Ammar and Jouza Hamra showed higher infection rates respect to other delegations belonging to the same governorate (80 and 40.9%, $p < 0.001$ and $p = 0.014$ respectively) (Supplementary file 3). Goats from Tunis were statistically more infected by *A. phagocytophilum*-like 2 (11.8%) than those from other governorates ($p = 0.017$), with Hrairia Sejoumi delegation showing the highest infection rates of this governorate (26.9%, $p = 0.030$) (Supplementary file 3).

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

Competing interests

The authors declare that they have no competing interests.

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References

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

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic 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.

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 X.P., Holmes, E.C., Gillespie, J.J., Dumler, S.J., Zhang, Y.Z., 2014. Extensive diversity of Rickettsiales bacteria in two species of ticks from China and the evolution of the Rickettsiales. BMC Evol. Biol. 14, 167.

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

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

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

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

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

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

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

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Torina, A., Alongi, A., Naranjo, V., Estrada-Peña, A., Vicente, J., Scimeca, S., Marino, A.M., Salina, F., Caracappa, S., de la Fuente, J., 2008. Prevalence and genotypes of *Anaplasma* species and habitat suitability for ticks in a Mediterranean ecosystem. Appl. Environ. Microbiol. 74, 7578–7584.

Tuomi, J., 1967. Experimental studies on bovine tick-borne fever .1. Clinical and haematological data, some properties of the causative agent, and homologous immunity. Acta Pathol. Microbiol. Scand. 70, 429–445.

Woldehiwet, Z., 2010. The natural history of *Anaplasma phagocytophilum*. Vet. Parasitol. 167, 108–122.

Ybañez, A.P., Matsumoto, K., Kishimoto, T., Inokuma, H., 2012. Molecular analyses of a potentially novel *Anaplasma* species closely related to *Anaplasma phagocytophilum* detected in sika deer (*Cervus nippon yesoensis*) in Japan. Vet. Microbiol. 157, 232–236.

Yoshimoto, K., Matsuyama, Y., Matsuda, H., Sakamoto, L., Matsumoto, K., Yokoyama, N., Inokuma, H., 2010. Detection of *Anaplasma bovis* and *Anaplasma phagocytophilum* DNA from *Haemaphysalis megaspinosa* in Hokkaido, Japan. Vet. Parasitol. 168, 170–172.

Sarih, M., M'ghirbi, Y., Bouattour, A., Gern, L., Baranton, G., Postic, D., 2005. Detection and identification of *Ehrlichia* spp. in ticks collected in Tunisia and Morocco. J. Clin. Microbiol. 43, 1127–1132.

Zobba, R., Anfossi, A.G., Pinna Parpaglia, M.L., Dore, G.M., Chessa, B., Spezzigu, A., Rocca, S., Visco, S., Pittau, M., Alberti, A., 2014. Molecular investigation and phylogeny of *Anaplasma* spp. in Mediterranean ruminants reveal the presence of neutrophil-tropic strains closely related to *A. platys*. Appl. Environ. Microbiol. 80, 271–280.

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**

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

Supplementary file legends

Supplementary file 1

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

Supplementary file 2

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

Supplementary file 3

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

Supplementary file 4

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

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.

MINISTERE DE L'AGRICULTURE
ET DES RESSOURCES HYDRAULIQUES

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MINISTERE DE L'ENSEIGNEMENT
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Université de la Manouba

**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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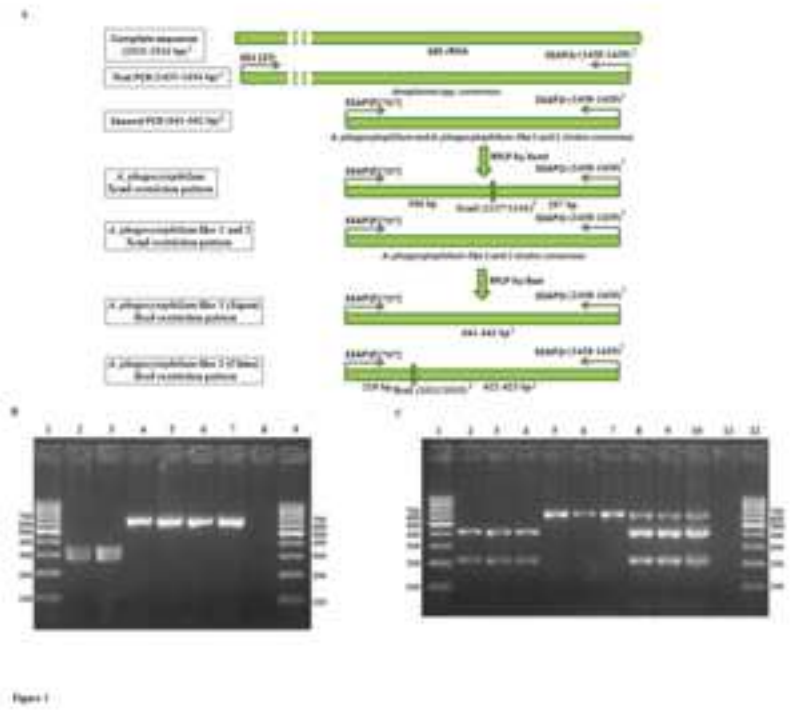

Figure 2 Ben Said et al., for TTBD

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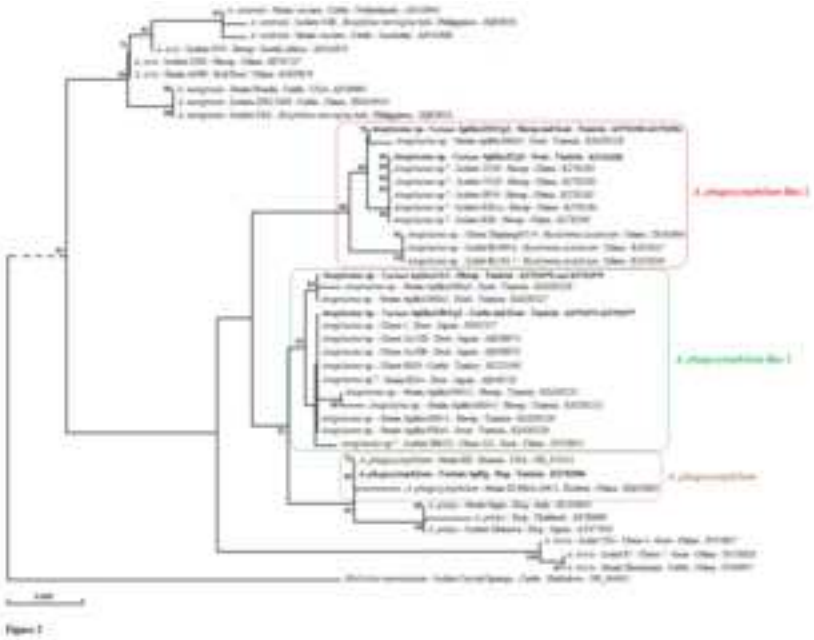

Table 1: Prevalence and co-infection rates of *Anaplasma* spp. closely related to *A. phagocytophilum* in goats according to bioclimatic zones and delegations.

Bioclimatic zone	**Delegation**	**Number**	**Positive (%±C.I.[1])**				**Co-infected (%±C.I.[1])**	
			***A. ph*-like 1**	**P-value[2]**	***A. ph*-like 2**	**P-value[2]**	***A. ph*-like 1/ *A. ph*-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])**	
			***A. ph*-like 1**	**P-value[2]**	***A. ph*-like 2**	**P-value[2]**	***A. ph*-like 1/ *A. ph*-like 2**	**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])**	
			***A. ph*-like 1**	**P-value[2]**	***A. ph*-like 2**	**P-value[2]**	***A. ph*-like 1/ *A. ph*-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.

***Anaplasma* spp.**	**Sequence type**	**Isolate**	**Host**	**Geographical location**	**GenBank accession no.**	**BLAST analysis**	**RFLP by XcmI[1]**	**RFLP by BsaI[2]**
A. phagocytophilum	HZ	HZ	*Homo sapiens*	New York, USA	NC_007797	100% *A. phagocytophilum*	+	NA[3]
	ApDg	Dog1	*Canis canis*	Ariana, Tunisia	KX702986	100% *A. phagocytophilum*	+	NA[3]
A. phagocytophilum-like 1 (Japan)	Aplike1BvCp1	R1	*Bos taurus*	Bizerte, Tunisia	KX702974	98.9% *A. phagocytophilum*	-	-
		R2	*Bos taurus*	Bizerte, Tunisia	KX702975	98.9% *A. phagocytophilum*	-	-
		R3	*Capra hircus*	Beja, Tunisia	KX702976	98.9% *A. phagocytophilum*	-	-
		R4	*Capra hircus*	Beja, Tunisia	KX702977	98.9% *A. phagocytophilum*	-	-
	Aplike1Ov1	R5	*Ovis aries*	Bizerte, Tunisia	KX702978	98.9% *A. phagocytophilum*	-	-
		R6	*Ovis aries*	Bizerte, Tunisia	KX702979	98.9% *A. phagocytophilum*	-	-
A. phagocytophilum-like 2 (China)	Aplike2OvCp1	R7	*Ovis aries*	Tunis, Tunisia	KX702980	97.5% *A. phagocytophilum*	-	+
		R8	*Capra hircus*	Tunis, Tunisia	KX702981	97.5% *A. phagocytophilum*	-	+
		R9	*Capra hircus*	Tunis, Tunisia	KX702982	97.5% *A. phagocytophilum*	-	+
		R10	*Ovis aries*	Tunis, Tunisia	KX702983	97.5% *A. phagocytophilum*	-	+
		R11	*Ovis aries*	Tunis, Tunisia	KX702984	97.5% *A. phagocytophilum*	-	+
	Aplike2Cp1	R12	*Capra hircus*	Tunis, Tunisia	KX702985	97.7% *A. phagocytophilum*	-	+

[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
H. asiaticum[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.

Table 6 Ben Said et al., for TTBD

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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A.* sp (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	*A. p* (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	*A. pl* (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	*A. o* (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	*A. m* (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	*A. c* (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	*A. b* (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	*E. r* (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) (R5and 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 *A*naplasma 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 *A*naplasma 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).