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Molecular Survey of *Anaplasma* Species in Small Ruminants Reveals the Presence of Novel Strains Closely Related to *A. phagocytophilum* in Tunisia

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Abstract

A survey of *Anaplasma* species in small ruminants is still lacking in North African countries. In this study, the presence of *A. phagocytophilum*, *A. phagocytophilum*–related species, and *A. ovis* was investigated in a total of 563 healthy small ruminants (303 goats and 260 sheep), from 25 randomly selected flocks sampled in Tunisia. *Anaplasma* spp. and *A. ovis* overall infection rates were 95.0% and 93.8% in sheep and 69.6% and 65.3% in goats, respectively. *A. phagocytophilum* was not detected in any of tested animals. A total of 20 sheep (7.7%) and 144 goats (47.5%) were infected by *Anaplasma* strains genetically related to *A. phagocytophilum*. Both in sheep and goats *A. ovis* prevalence was higher in adults (≥ 2 years) than in young (<2 years) subjects ($p = 0.001$ and 0.002 for goats and sheep, respectively). In sheep, *A. ovis* prevalence was higher in ewes with respect to rams ($p = 0.010$). The *A. ovis* infection rate was significantly lower in goats of the local breed ($p = 0.049$) and it was higher in goats infested by ticks than in not infested animals ($p = 0.005$). Genetic analysis of the *msp4* gene of *A. ovis* indicated the presence of strains shared by Tunisian sheep and goats. Sequence analysis and phylogenetic studies on the basis of the *16S rRNA* gene provided evidence for the circulation of at least two different potentially novel species genetically related to *A. phagocytophilum* in Tunisian small ruminants. These findings cause concern about specificity of serological tests used for detection of *A. phagocytophilum* in ruminants and provide additional information for elucidating pathogenesis and molecular epidemiology of *A. phagocytophilum* and related species.

Key Words: *Anaplasma ovis*—*A. phagocytophilum* and related species—Small ruminants—Molecular prevalence—*16S rRNA* and *msp4* genes—Tunisia.

Introduction

Bacterial species of the genus *Anaplasma* are obligate intracellular bacteria that cause animal and human anaplasmosis, a disease widely distributed in tropical, subtropical, and temperate regions (Kocan et al. 2003). The disease impact on veterinary and public health can be significant, because at least one *Anaplasma* species has a zoonotic nature and the six species of this genus cause economic losses to farmers and in general to animal production (Woldehiwet 2010, Zobba et al. 2014).

Among *Anaplasma* species, *A. ovis,* which infects sheep, goat, and deer, is an obligate intraerythrocytic bacterium and the causative agent ovine anaplasmosis (Friedhoff 1997). In small ruminants, infection is usually subclinical; occasionally, it can be severe with hemolytic anaemia, hemoglobinuria, and fever (Barry and Van Niekerk 1990, Stoltsz 1994, Hornok et al. 2007). In addition, *A. ovis* infection may predispose to other infectious and/or parasitic diseases that aggravate an animal's condition, occasionally leading to death (Kocan et al. 2004).

A. phagocytophilum infects neutrophil granulocytes of many animal species and human (Dumler et al. 2001). In ruminants, it causes tick-borne fever (TBF) (Stuen 2007, Woldehiwet 2010), with the most common symptoms including high fever, anorexia, dullness, and milk yield decrease (Tuomi 1967, Woldehiwet 2010). In Japan, strains closely related to *A. phagocytophilum,* have been detected in cattle, in sika deer, and in some ticks species infesting ruminants (*Ixodes persulcatus*, *Ixodes ovatus*, *Haemaphysalis megaspinosa*) (Ohashi

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et al. 2005, Jilintai et al. 2009, Yoshimoto et al. 2010). On the basis of multigene analyses using *16S rRNA*, *groEL*, and *gltA* genes, these strains were placed in a distinct monophyletic cluster (Ybañez et al. 2012a). The absence of clinical signs in infected animals and the different related tick vectors (respect to *A. phagocytophilum*) provide additional evidence for species designation of these potential novel *Anaplasma* strains initially found in Japan (Jilintai et al. 2009, Yoshimoto et al. 2010, Ybañez et al. 2012a). More recently in China, Kang et al. (2014) identified *Anaplasma* sp. strains in *Hyalomma asiaticum* ticks infesting sheep, which differ from the Japanese strains (*Anaplasma* sp. Japan) and all other classified *Anaplasma* species. These latter strains generated a distinct clade in phylogenetic trees on the basis of the *16S rRNA*, *gltA*, and *groEL* genes, suggesting the presence of a new *Anaplasma* species genetically related to *A. phagocytophilum* in ticks infesting ruminants in China.

To date, molecular studies in Tunisia showed the occurrence of *A. phagocytophilum* in both animal hosts (dogs and horses) and ticks (*Ixodes ricinus, Hyalomma scupense*, and *Hyalomma marginatum*) (Sarih et al. 2005, M'ghirbi et al. 2009, 2012). *A. phagocytophilum* infection has also been reported in Tunisian horses and dromedaries by serology (Ben Said et al. 2013, 2014). Recently, *A. ovis* has been detected and characterized in sheep from northern and central Tunisia (Belkahia et al. 2014). The aim of this study was to investigate *A. ovis* and *A. phagocytophilum* and related species in small ruminants from five localities belonging to two governorates in northern Tunisia.

Materials and Methods

Blood sampling, tick collection, and DNA extraction

A cross-sectional study was carried out in five localities of northern Tunisia (Fig. 1). El Alia (37°16′N; 10°03′E) and Khetmine (37°16′N; 9°99′E) fall in the subhumid bioclimatic zone with average annual rainfall of 400 mm, whereas Joumine (36°92′N; 9°38′E), Sejnane (37°15′N; 9°23′E), and Amdoun (36°76′N; 9°08′E) are characterized by humid climate with an average annual rainfall of 650 mm. Between 2011 and 2013, a total of 563 samples were collected from 303 healthy goats (233 does and 70 bucks) and 260 healthy sheep (210 ewes and 50 rams). Goat samples originated from 16 herds located in Sejnane ($n=3$), El Alia ($n=4$), and Joumine ($n=5$) belonging to the Bizerte governorate and in Amdoun ($n=4$, Beja governorate). Sheep samples were taken from nine herds located in El Alia ($n=4$) and Khetmine ($n=5$) in the governorate of Bizerte.

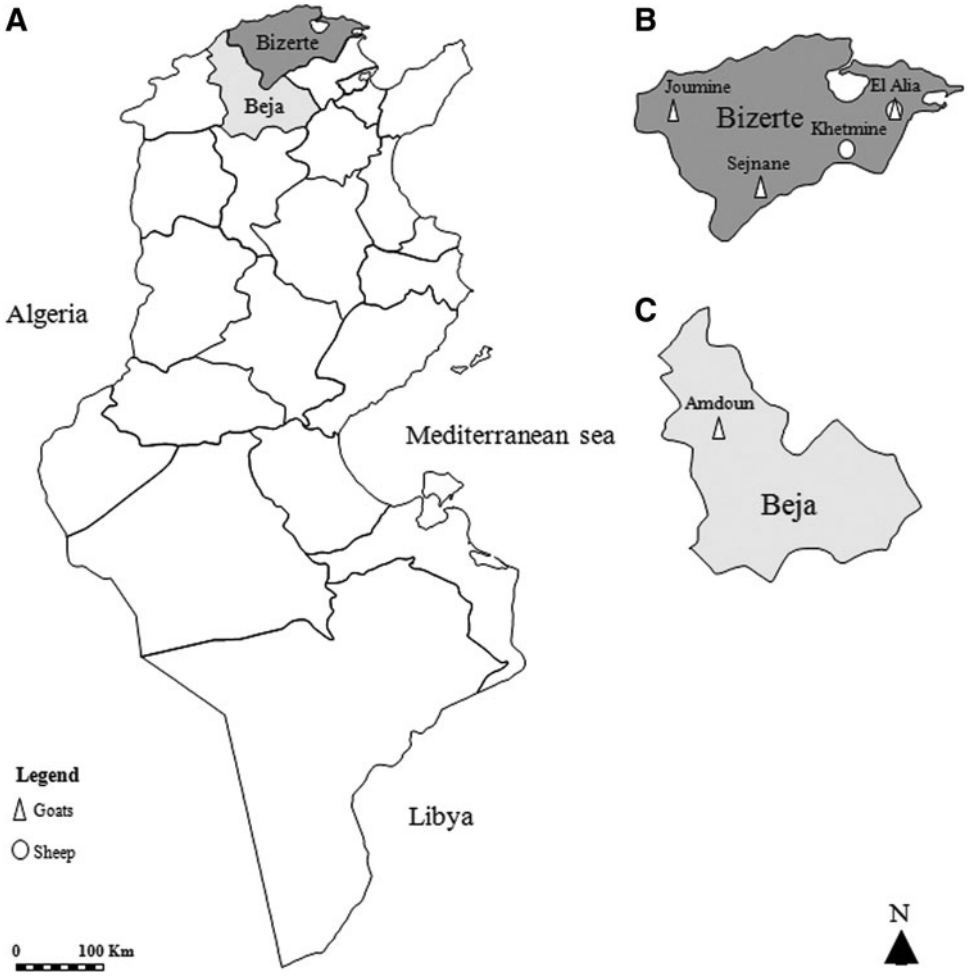

FIG. 1. Localities studied. (**A**) Geographical position of the governorates of Bizerte and Beja in Tunisia. (**B**) Position of localities sampled in the governorate of Bizerte. (**C**) Sampling area in the governorate of Beja.

For each animal, blood samples were collected in EDTA tubes from the jugular vein; gender, breed, and approximate age were noted. Goats belonged to three breeds: Local breed (275), Alpine (23), and Maltese (5). Sheep belonged to six breeds: Barbarine (118), Noire de Thibar (82), Queue Fine de l'Ouest (10), Merino (2), Sicilo-sarde (1), and crossbreeds (47). Sex ratio (male:female) was, respectively, 0.3 and 0.24 in goats and sheep, whereas mean age was 3.9 ± 1.7 years in goats and 4.9 ± 2.0 in sheep. All small ruminants were divided into three age groups for females and two age groups for males. DNA was extracted from 300 μL of each blood sample with Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. Ticks were collected manually from each sampled animal, placed in tubes containing 70% ethanol, and morphologically identified using the taxonomical key of Walker et al. (2013).

Detection of A. phagocytophilum *and related species*

Nested PCR was performed with outer primers EE1 and EE2 and inner primers SSAP2f and SSAP2r to amplify a 641-bp sequence of the *16S rRNA* gene (Liu et al. 2012). According to Ybañez et al. (2012a), inner primers allow the detection of *A. phagocytophilum* and related species (Table S1) (Supplementary Data are available at www.liebertpub/vbz/). For specific detection of *A. phagocytophilum*, positive *16S rRNA* samples were tested by hemi-nested PCR using outer primers EphplgroEL-F and EphplgroEL-R, and inner primers EphplgroEL-F and EphgroEL-R amplifying 573 bp sequence of the *groEL* gene (Alberti et al. 2005; Table S1). Each reaction was performed in a final volume of 50 μL containing 0.125 U/μL Taq DNA polymerase (Biobasic Inc, Canada), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 μL of genomic DNA, and 0.5 μM primers. Thermal cycling reactions were performed in an automated DNA thermal cycler (Techne Flexigene, Cambridge, UK). One microliter of each amplicon was used for PCR reaction with specific primers at the same conditions as for the first PCR. PCR products were electrophoresed in 1% agarose gels containing 0.5 μg/mL of ethidium bromide. Distilled water and DNA samples positive to *A. phagocytophilum* (Zobba et al. 2014) were used as negative and positive controls in each PCR experiment.

Detection of A. ovis

To detect *A. ovis* in sheep samples, loop-mediated isothermal amplification (LAMP) reactions were performed using a set of six primers targeting the *msp4* gene (Ma et al. 2011; Table S1). Reaction conditions and thermal profiles were as described by Belkahia et al. (2014). Negative and positive controls were included in all runs. After amplification, LAMP products were detected by electrophoresis in 1.5% agarose gel and either visualized under a ultraviolet (UV) light after staining with ethidium bromide or by visual inspection of tubes after adding 1 μL of 1000× SYBR Green I (Cambrex BioScience, USA). *A. ovis* infection in goat samples was detected by single PCR with the AovisMSP4Fw- and AovisMSP4Rev-specific primers designed by Torina et al. (2012). Each PCR reaction contained a mix of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.125 U/μL Taq DNA polymerase (Biobasic Inc., Canada), 2 μL (1–10 ng) of DNA, 0.5 μM primers (Table S1), and milliQ sterile water to a total volume of 50 μL. Distilled water and DNA extracted from *A. ovis* were used as negative and positive controls, respectively. Thermal cycling reactions were performed as described by Torina et al. (2012). Genotyping of the *A. ovis msp4* gene was performed by amplifying positive sheep and goat samples with *A. ovis*–specific LAMP and PCR reactions using MSP45 and MSP43 primers (de la Fuente et al. 2005, 2007; Table S1). Amplification was performed as described previously (de la Fuente et al. 2005, 2007, Belkahia et al. 2014). PCR products were electrophoresed in 1.5% agarose gel.

DNA sequencing and phylogenetic analysis

Selected positive PCR products from primers SSAP2f/SSAP2r and MSP45/MSP43 of *A. phagocytophilum* and/or related species, and *A. ovis,* respectively, were purified with the GF-1 Ambi Clean Kit (Vivantis, USA) according to manufacturer's instructions. Purified DNA fragments were sequenced in both directions, using the same primers as for the PCR amplifications (Table S1). The reactions were performed using a conventional Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems, Foster City, CA) and an ABI3730XL automated DNA sequencer by Macrogen Europe (Amsterdam, The Netherlands). The chromatograms were evaluated with Chromas Lite v. 2.01. The DNAMAN software (v. 5.2.2; Lynnon Biosoft, Quebec, Canada) was used to perform multiple sequence alignment of *16S rRNA* and *msp4* sequences and to translate nucleotide to amino acid msp4 sequences. 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 1000 reiterations.

GenBank accession numbers

The *16S rRNA* partial sequences of *Anaplasma* sp. strains related to *A. phagocytophilum* Aplike1GGo1-3 and Aplike2GGo1 isolated in goats have been deposited in GenBank under accession numbers KM285226, KM285227, KM285229, and KM285228, respectively. The *16S rRNA* partial sequences of *Anaplasma* sp. strains related to *A. phagocytophilum* AplikeGOv1-3 isolated in sheep have been deposited in GenBank under accession numbers KM285230–KM285232. The *msp4* partial sequences of *A. ovis* strains AoGOv1–5 found in sheep and the AoGGo1 genotype found in goat have been deposited in GenBank under accession numbers KM285218-22 and KM285217, respectively.

Statistical analysis

Exact confidence intervals (CI) for prevalence rates at the 95% level were calculated. Comparison of the prevalence of *Anaplasma* species in sheep and goats according to risk factors, localities, and governorates, and comparison of rates and infestation prevalence of each tick species found in goats and sheep were performed with Epi Info 6.01 (Centers for Disease Control and Prevention, Atlanta, GA), using the chi-squared test and Fisher exact test with a threshold value of 0.05. To consider any confusion factor, a chi-squared Mantel–Haenszel test was performed.

TABLE 1. MOLECULAR PREVALENCE OF *ANAPLASMA OVIS* ACCORDING TO GENDER, AGE, BREED, AND TICK INFESTATION OF GOATS AND SHEEP

	Goats			*Sheep*		
Risk factor	*Number*	*Positive (% ± CI[a])*	p *value*	*Number*	*Positive (% ± CI[a])*	p *value*
Gender						
Male	70	44 (62.9 ± 0.11)	0.617	50	43 (86.0 ± 0.10)	0.010*
Female	233	154 (66.1 ± 0.06)		210	201 (95.7 ± 0.03)	
Age						
<2 years	133	74 (55.6 ± 0.08)	0.001*	63	54 (85.7 ± 0.09)	0.002*
≥2 years	170	124 (72.9 ± 0.07)		197	190 (96.4 ± 0.02)	
Breed						
Local/Barbarine[b]	275	175 (63.6 ± 0.06)	0.049*	118	113 (95.8 ± 0.04)	0.056
Other breeds[c]	28	23 (82.1 ± 0.14)		142	127 (89.4 ± 0.05)	
Tick infestation						
Infested	113	85 (75.2 ± 0.08)	0.005*	244	229 (93.9 ± 0.03)	0.986
Not infested	190	113 (59.5 ± 0.07)		16	15 (93.8 ± 0.12)	
Total	303	198 (65.3 ± 0.05)		260	244 (93.8 ± 0.03)	

[a]CI, 95% confidence interval.
[b]Local for goats and Barbarine for sheep.
[c]Other breeds are Alpine and Maltese for goats and Noire de Thibar, Queue fine de l'Ouest, Merinos, Sicilo-sarde, and crossbred for sheep.
*Significance at the $p < 0.05$ level.

TABLE 2. NUCLEOTIDE AND AMINO ACID DIFFERENCES AMONG MSP*4* SEQUENCES (719 BP) FROM *ANAPLASMA OVIS* STRAINS

Host	*Variant*	*Sample symbol*[a]	*Country*	*GenBank*[b]	msp4 *nucleotide positions (amino acid positions)*[c]					*Reference*
					230 (77)	244 (83)	470 (157)	476 (159)	532 (178)	
Sheep	Italy 147	NI[d]	Italy	AY702924	G (R)	A (S)	C (A)	C	C (L)	de la Fuente et al. (2005)
	Italy 20	NI[d]	Italy	AY702923	*	*	T (V)	*	*	de la Fuente et al. (2005)
	GBK1	B1; B2; K2; K4	Tunisia	KC432641	*	*	*	*	*	Belkahia et al. (2014)
	GBK2	B4; K3	Tunisia	KC432642	*	*	T (V)	*	*	Belkahia et al. (2014)
	GB3	B3	Tunisia	KC432643	T (I)	*	T (V)	*	*	Belkahia et al. (2014)
	GK1	K1	Tunisia	KC432644	T (I)	*	T (V)	*	A (I)	Belkahia et al. (2014)
	AoGOv1	Kh1; Kh2	Tunisia	KM285218	*	*	T (V)	*	*	Present study
	AoGOv2	Al1	Tunisia	KM285219	G (R)	G (G)	T (V)	*	*	Present study
	AoGOv3	Al2	Tunisia	KM285220	T (I)	*	T (V)	A	*	Present study
	AoGOv4	Al3	Tunisia	KM285221	G (R)	*	T (V)	A	*	Present study
	AoGOv5	Kh3	Tunisia	KM285222	*	*	*	*	*	Present study
Goat	AoGGo1	Al1–Al5; Sj1–Sj5; Jm1–Jm5; Am1–Am5	Tunisia	KM285217	*	*	T (V)	*	*	Present study

[a]B1–B4 and K1–K4 sheep samples were collected from El Alia (Bizerte governorate) and Sbikha (Kairouan governorate), respectively, by Belkahia et al. (2014). Al1–Al3 and Kh1–Kh3 sheep samples were collected from El Alia (Bizerte governorate) and Khetmine (Bizerte governorate), respectively, in the present study. Al1–Al5, Sj1–Sj5, Jm1–Jm5, and Am1–Am5 goat samples were collected from El Alia, Sejnane, Joumine, and Amdoun, respectively.
[b]GenBank accession number of the variant.
[c]Numbers represent the nucleotide position starting at translation initiation codon Adenine. Conserved nucleotide positions with respect to the Italy 147 strain, Sicily (Italy) are indicated with asterisks (de la Fuente et al., 2005).
[d]NI, not indicated.
Amino acid changes are indicated between parentheses with single letter code.
Amino acids: R, arginine; I, isoleucine; S, serine; G, glycine; V, valine; A, alanine; L, leucine. Nucleotides: T, thymine; C, cytosine; G, guanine; A, adenine.

TABLE 3. NUCLEOTIDE DIVERSITY AMONG *16S rRNA* SEQUENCES FROM *ANAPLASMA* STRAINS CLOSELY RELATED TO *A. PHAGOCYTOPHILUM* (599 BP)

Host or vector	*Genotype*[a]	*Country*	*GenBank*[b]	*16S rRNA nucleotide positions*[c]															*Reference*
				795	802	824	827	880	983	1081	1085	1092	1120	1209	1211	1212	1223	1231	1263	
Deer	Clone 1	Japan	JN055357	C	T	G	T	A	A	G	—	C	C	T	T	C	G	G	C	Ybañez et al. (2012a)
Goat	Aplike1GGo1	Tunisia	KM285226	*	*	*	*	*	*	*	—	*	*	*	*	*	*	*	*	Present study
	Aplike1GGo2	Tunisia	KM285227	*	*	*	*	*	*	*	T	*	T	*	*	*	*	*	*	Present study
	Aplike1GGo3	Tunisia	KM285229	*	*	*	*	C	*	*	T	*	T	*	*	*	*	*	*	Present study
Sheep	Aplike1GOv1	Tunisia	KM285230	*	*	*	*	*	*	*	—	*	*	*	*	*	*	*	*	Present study
	Aplike1GOv2	Tunisia	KM285231	*	*	C	*	*	*	*	—	*	*	*	*	*	*	*	*	Present study
	Aplike1GOv3	Tunisia	KM285232	*	*	C	*	*	*	*	—	*	*	*	*	*	A	*	*	Present study
Hyalomma asiaticum	BL099-6	China	KJ410247	T	A	*	*	*	G	A	T	T	*	C	C	*	*	A	T	Kang et al. (2014)
Goat	Aplike2GGo1	Tunisia	KM285228	*	A	*	C	*	G	*	T	T	*	C	C	T	*	*	T	Present study

Conserved nucleotide positions are indicated with asterisks.

Nucleotides: T, thymine; C, cytosine; G, guanine; A, adenine.

Jm1–Jm8, Sj1–Sj4, Am1–Am3, and Al1–Al2 *Anaplasma* sp.–positive goat samples were collected from Joumine (Bizerte governorate), Sejnane (Bizerte governorate), Amdoun (Beja governorate), and El Alia (Bizerte governorate) localities, respectively.

Al1–Al4 and Kh1–Kh6 *Anaplasma* sp.–positive sheep samples were collected from El Alia (Bizerte governorate), and Khetmine (Bizerte governorate), localities, respectively.

[a]The Aplike1GGo1 genotype was isolated from Jm1, Jm2, Jm4–Jm7, Sj1, Sj3, Sj4, Am1–Am3, al1 goat samples; the Aplike1GGo2 genotype was isolated from Jm3 and Jm8 goat samples; the Aplike1GGo3 genotype was isolated from Al2 goat sample; the Aplike1GOv1 genotype was isolated from Al1-4, Kh1, and Kh4–6 sheep samples; the AplikeGOv2 genotype was isolated from Kh2 sheep sample; the AplikeGOv3 genotype was isolated from Kh3 sheep sample, and the Aplike2GGo1 genotype was isolated from Sj2 goat sample.

[b]GenBank accession number.

[c]Numbers represent the nucleotide position with respect to the HZ strain from USA for *A. phagocytophilum* (GenBank acc. no. NC_007797).

Results

Parasitological data

A total of 1223 engorged and semiengorged ticks were collected from 563 small ruminants (Table S2). Specifically, 919 ticks were found on 241 (92.69%) out of 260 sampled sheep and belonged to three *Rhipicephalus* species. *R. turanicus* (52.77%) and *R. sanguineus* (43.96%) were the dominant tick species, followed by *R. annulatus* (3.26%) ($p < 0.001$). Also, 304 adult ticks were collected from 113 (37.29%) out 303 goats and were ascribed to four species. *R. turanicus* (79.93%) and *R. bursa* (14.47%) were the dominant species, followed by *R. sanguineus* (4.93%) and a few *Hyalomma excavatum* ticks (0.65%) ($p < 0.001$).

Prevalence of Anaplasma *species*

The overall infection rates of *Anaplasma* spp. and *A. ovis* in goats were 69.6% and 65.3%, respectively. In sheep, infection rates were 95.0% (*Anaplasma* spp.) and 93.8% (*A. ovis*) (Table S1). A total of 144 goats (47.5%) and 20 sheep (7.7%) were found positive to *A. phagocytophilum* and/or related species by *16S rRNA* PCR, but specific detection of *A. phagocytophilum* conducted by targeting the *groEL* gene showed that none of these animals were positive to this zoonotic species. In goats, *A. ovis* infection rate was significantly lower in young (55.6%) than adult goats (72.9%) ($p = 0.001$) and in local breed (63.6%) compared to other breeds (82.1%) ($p = 0.049$). Goats infested by ticks (75.2%) were more infected by *A. ovis* than noninfested animals (59.5%) (p = 0.005) (Table 1). In sheep, *A. ovis* prevalence was higher in ewes (95.7%) than in rams (86.0%) ($p = 0.010$) and in adults (>2 years) (96.4%) than in young sheep (≤2 years) (55.7%) ($p = 0.002$) (Table 1).

A. ovis msp4 *genotypes*

A. ovis infection was confirmed by sequencing of 719 bp of the *msp4* gene (84.4% of the gene size) from 20 randomly selected positive goat samples (five from each sampling region) and six positive sheep samples (three from each sampling region). Alignment of these sequences revealed five different genotypes (AoGOv1–5) in sheep (GenBank acc. nos. KM285218–KM285222) and one genotype (AoGGo1) in goats (GenBank acc. no. KM285217) differed from each other in five nucleotide positions. Each nucleotide change conferred an amino acid variation, except in nucleotide position 467 (Table 2). The *msp4* gene sequences obtained in this study shared 99.6–100% and 98.7–100% nucleotides and amino acids similarity, respectively. The AoGOv1 and AoGGo1 genotypes were 100% identical to the GBK2 genotype (GenBank acc. no. KC432642) from Tunisia, and to the genotype III represented by the ''Italy20'' *A. ovis* strain from Sicilian sheep (GenBank acc. no. AY702923, Table 2). The AoGOv5 genotype revealed 100% homology with the GBK1 genotype (GenBank acc. no. KC432641) from Tunisia, and the genotype II represented by the ''Italy147'' *A. ovis* strain from Sicilian sheep (GenBank acc. no. AY702924) (Table 2). Three novel *A. ovis msp4* genotypes (AoGOv2 to AoGOv4) were identified (Table 2). Phylogenetic analysis revealed that the two new genotypes AoGOv3 and AoGOv4 clustered with the *A. ovis* Panagcy strain found in human samples from Cyprus (GenBank acc. no. FJ460443); and the new genotype AoGOv2 clustered with the genotype III

Table 4. Comparison of *16S rRNA* Sequences (599 pb) from *Anaplasma* Strains Genetically Related to *A. phagocytophilum* Isolated from Tunisian Small Ruminants and Other *Anaplasma* Species Found in GenBank

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
1	*A.* sp (Aplike1GGo1)	100															
2	*A.* sp (Aplike1GGo2)	99.8	100														
3	*A.* sp (Aplike1GGo3)	99.7	99.8	100													
4	*A.* sp (Aplike1GOv1)	100	99.8	99.7	100												
5	*A.* sp (Aplike1GOv2)	99.8	99.7	99.5	99.8	100											
6	*A.* sp (Aplike1GOv3)	99.7	99.5	99.3	99.7	99.8	100										
7	*A.* sp (Clone 1)	100	99.8	99.7	100	99.8	99.7	100									
8	*A.* sp (Aplike2GGo1)	98.7	98.5	98.3	98.7	98.5	98.3	98.7	100								
9	*A.* sp (KS8)	98.7	98.5	98.3	98.7	98.5	98.3	98.7	99.7	100							
10	*A.* sp (BL099-6)	98.5	98.3	98.2	98.5	98.3	98.2	98.5	99.2	99.5	100						
11	*A. p* (HN)	98.9	98.9	98.8	98.9	98.8	98.7	98.9	97.0	97.5	97.7	100					
12	*A. pl* (Okinawa)	98.8	98.8	98.7	98.8	98.7	98.5	98.8	97.3	97.3	97.5	99.2	100				
13	*A. o* (Jingtai)	97.7	97.8	97.7	97.7	97.5	97.3	97.7	97.3	97.3	97.5	97.5	97.7	100			
14	*A. m* (Lushi)	97.3	97.5	97.3	97.3	97.2	97.0	97.3	97.0	97.0	97.2	97.2	97.3	99.7	100		
15	*A. c* (CC)	97.0	97.2	97.0	97.0	96.8	96.7	97.0	96.7	96.7	96.8	96.8	97.0	99.3	99.0	100	
16	*A. b* (YX4)	97.0	97.2	97.0	97.0	96.8	96.7	97.0	96.5	96.5	96.3	96.8	96.3	95.7	95.7	95.7	100

The numbers represent the nucleotide identity rates found between the sequences.

A. sp, (Aplike1GGo1–3 and Aplike2GGo1), *Anaplasma* sp. isolated from Tunisian goats (AplikeGGo1–3 strains, GenBank acc. nos. KM285226, KM285227, KM285229, and KM285228, respectively); *A.* sp, (AplikeGOv1–3), *Anaplasma* sp. isolated from Tunisian sheep (AplikeGOv1–3 strains, GenBank acc. nos. KM285230–KM285232, respectively); *A.* sp (Clone 1), *Anaplasma* sp. related to *A. phagocytophilum* found on Japanese deer (Clone 1, GenBank acc. no. JN055357); *A.* sp (KS8), *Anaplasma* sp. isolated from Chinese sheep registered as *A. phagocytophilum* (KS8 isolate, GenBank acc. no. KJ782385); *A.* sp (BL099-6), *Anaplasma* sp. isolated from *Hyalomma asiaticum* tick infested Japanese ruminants (BL099-6 isolate, GenBank acc. no. KJ410247); *A. p* (HN strain), *A. phagocytophilum* strain isolated from Chinese rodent (HN strain, GenBank acc. no. KC470064); *A. pl* (Okinawa), *A. platys* isolate found on Japanese dog (Okinawa isolate, GenBank acc. no. AY077619); *A. o* (Jingtai), *A. ovis* isolate found on Chinese goat (Jingtai isolate, GenBank acc. no. AJ633049); *A. m* (Lushi), *A. marginale* isolate found on Chinese cattle (Lushi isolate, GenBank acc. no. AJ633048); *A. c* (CC), *A. centrale* strain isolated from Italian cattle (CC strain, GenBank acc. no. EF520686); *A. b* (G49), *A. bovis* isolate found on Chinese goat (G49 isolate, GenBank acc. no. JN558824).

represented by the ''Italy20'' *A. ovis* strain from Sicilian sheep (GenBank acc. no. AY702923).

Anaplasma *sp.* 16S rRNA *genotypes*

Sequencing of 599 bp (41.8%) of the *16S rRNA* gene obtained from 16 randomly selected positive goat samples (seven, four, three, and two samples from Joumine, Sejnane, Amdoun, and El Alia, respectively) and 10 positive sheep samples (six and four samples from Khetmine and El Alia, respectively) confirmed the infection by *Anaplasma* strains genetically related to *A. phagocytophilum*. Alignment of these sequences revealed four genotypes from goats (Aplike1GGo1 to Aplike1GGo3 and Aplike2GGo1; KM285226, KM285227, KM285229, and KM285228, respectively) and three from sheep (Aplike1GOv1 to Aplike1GOv3; KM 285230–KM285232, respectively). Except for the Aplike2GGo1 sequence, all *16S rRNA* sequences obtained in this study shared 99.3–100% nucleotide similarity and differed from each other in five nucleotide positions (four substitutions and one deletion; Tables 3 and 4). The Aplike1GGo1 and Aplike1GOv1 genotypes were 100% identical to the Clone 1 genotype of *Anaplasma* sp. isolated from deer in Japan (JN055357) (Tables 3 and 4). The Aplike2GGo1 genotype shared 98.3–98.7% identity with all other revealed sequences and was 99.7% and 99.2% identical to the KS8 genotype of *Anaplasma* sp. from Japanese sheep recorded as *A. phagocytophilum* (AB96720) and the BL099-6 genotype of *Anaplasma* sp. isolated from *H. asiaticum* tick infesting sheep in China (KJ410247), respectively (Table 4).

16S rRNA gene–based phylogenetic trees revealed that all *Anaplasma* sp. strains from this study clustered independently from *A. phagocytophilum* strains isolated in human, horse and rodents (NR_074113, AY527214, and DQ458805, respectively). The Aplike2GGo1 genotype clustered in *Anaplasma* sp. group 1 with *Anaplasma* sp. isolates found in Chinese sheep (KJ782381–KJ782385, registered as *A. phagocytophilum*) and *Anaplasma* sp. isolates detected in *H. asiaticum* tick infesting Chinese ruminants (JX402604, KJ410247, and KJ410249). All other genotypes clustered in *Anaplasma* sp. group 2 with *Anaplasma* sp. (Japan) strains isolated from deer (AB96720, registered as *A. phagocytophilum*, JN055357, AB588974, and AB588976), *Anaplasma* sp. strain isolated from cattle in Turkey (GU223365) and *Anaplasma* sp. strain isolated from Chinese goat (JN558817, deposited in the GenBank as *A. phagocytophilum*; Fig. 3). Nucleotide sequence identities, calculated by comparing sequences obtained in this study versus those of other *Anaplasma* species, were 97.0–99.0% with *A. phagocytophilum*, 97.3–98.8% with *A. platys*, 97.3–97.8% with *A. ovis*, 97.0–97.5% with *A. marginale*, 96.7–97.2% with *A. centrale*, and 96.5–97.2% *A. bovis*.

Discussion

Although several reports showing the presence of anaplasmosis in North Mediterranean small ruminants are available (de la Fuente et al. 2005, Torina et al. 2008a,b, Torina and Caracappa 2012, Zobba et al. 2014), similar studies are lacking in most of the South Mediterranean countries such as Tunisia, where surveys are limited to the detection of zoonotic *A. phagocytophilum* strains in horses, dogs, and ticks (Sarih et al. 2005, M'ghirbi et al. 2009, 2012).

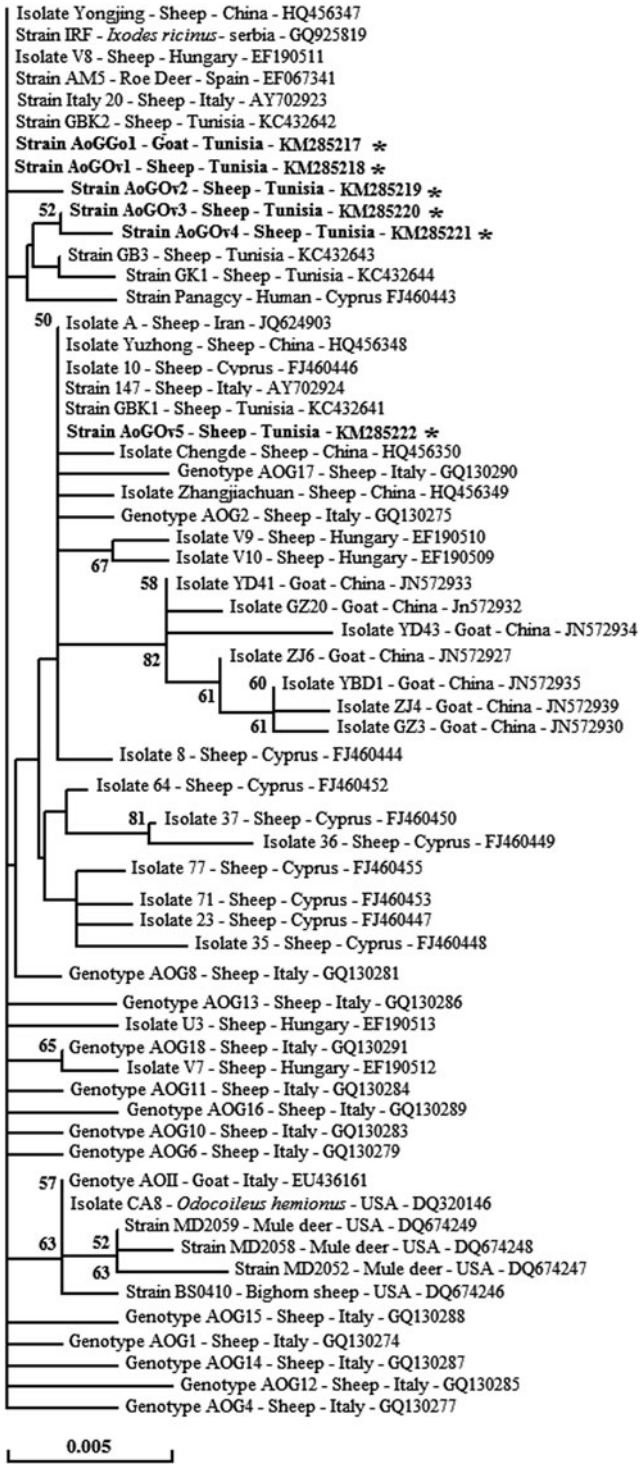

FIG. 2. Representative neighbor-joining tree based on multiple sequence alignments of the partial *msp4* nucleotide sequences (719 bp) of Tunisian strains with strains isolated in the Mediterranean area. Numbers in nodes represent the percentage of 1000 bootstrap reiterations supporting the nodes (only percentages greater than 50% are shown). The host or vector, strain or isolate identification, country of origin, and GenBank accession number are indicated in the tree for each sequence. Sequences newly obtained in this study are highlighted in bold and marked with asterisks.

In this study, we investigated the molecular epidemiology of selected *Anaplasma* species in small ruminants from northern Tunisia. Results clearly indicate evidence of *A. ovis* infection in sheep and goats sampled in the study area (Table S1). In sheep, *A. ovis* prevalence (overall 93.8%, minimum 91.4% in El Alia, maximum 96.4% in Khetmine) was similar to the prevalence reported by de la Fuente et al. (2005) in Italy (87%) and by Renneker et al. (2013) in Portugal (82.5%). It was higher than that observed in Sudan (41.7%; Renneker et al. 2013), Turkey (31.4%; Renneker et al. 2013), and Senegal (11.5%; Djiba et al. 2013). Overall prevalence in goats (65.3%; range, 44.4–78.8%) was similar to the prevalence reported by Altay et al. (2014) in Turkey (66.4%), lower than that estimated in Angola (100%; Kubelová et al. 2012), and higher than that observed in China (15.3–25.6%; Liu et al., 2012, Chi et al., 2013). The higher prevalence of *A. ovis* in sheep, also reported in Italy (Torina et al. 2008a,b, 2010, Torina and Caracappa 2012), may be the result of the different distribution of *A. ovis* tick vectors in goats and sheep sampling localities, which fall into different bioclimatic zones.

The analysis of *A. ovis msp4* sequences revealed a single genotype (AoGGO1) infecting all selected goats and five different genotypes infecting sheep (Table 2). The goat genotype is identical to the AoGOv1 genotype found in two infected sheep, whereas each one of the other sheep genotypes was found in a single sheep. In contrast, Liu et al. (2012) suggest that *A. ovis msp4* genotypes may be different among sheep and goats. Strains found in Tunisian sheep were classified in three clusters, indicating multiple introductions of genetically distinct *A. ovis* strains in the studied regions. The two new genotypes, namely AoGOv3 and AoGOv4, were classified with *A. ovis* Panagcy strain (FJ460443) in an independent cluster (Chochlakis et al. 2010; Fig. 2). In agreement with Belkahia et al. (2014), comparison of *msp4* sequences isolated from sheep from El Alia and Khetmine suggests that geographic location is not the origin of genetic diversity of the *A. ovis msp4* gene.

The *A. ovis* infection rate was significantly higher in adult goats (>2 years) compared to young goats (<2 years). This

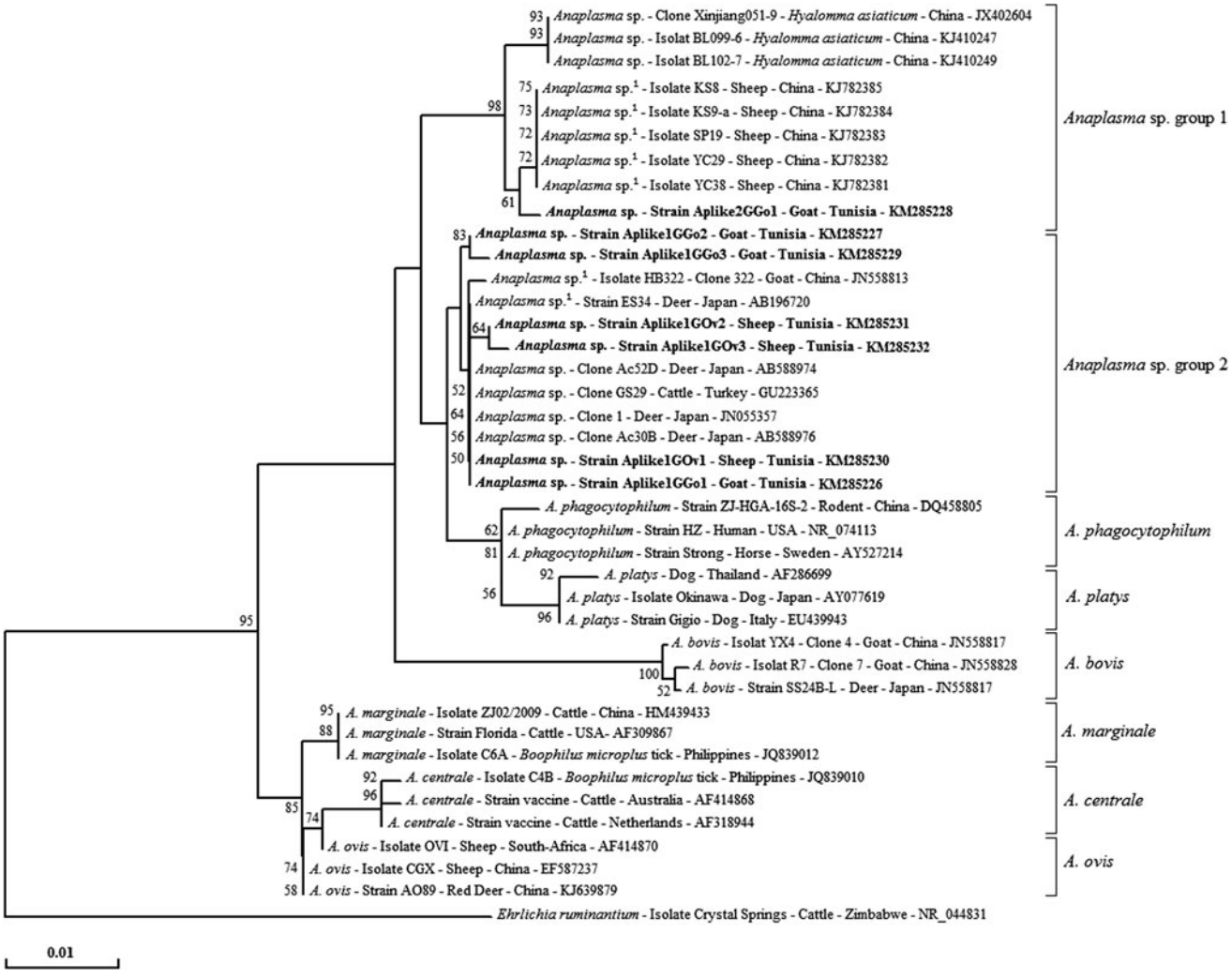

FIG. 3. Neighbor-joining tree based on the alignment of partial *A. phagocytophilum 16S rRNA* sequences (599 bp) obtained in this study with selected sequences representative of the *Anaplasma* genus. Bootstrap values (1000 replicates) are indicated in each node (only percentages greater than 50% are shown). Sequences obtained in this study are indicated in bold and marked with asterisks. The host or vector, the strain or isolate name, the country of origin and the GenBank accession number are indicated. [1]*Anaplasma* sp. deposited in the GenBank as *A. phagocytophilum.*

could be explained by the fact that adult goats were more exposed to tick infestation because they went through more tick seasons. Similarly, due to different exposures of rams and ewes to tick infestation, *A. ovis* prevalence in sheep was significantly higher in ewes than rams. Indeed, almost two-thirds of ewes were more than 4 years old and grazed on natural pastures, a trend reported by Belkahia et al. (2014). A local goat breed was less infected by *A. ovis* than other goat breeds, this difference possibly being due to genetic resistance of local breeds to hemopathogens.

Goats infested by ticks were statistically more infected by *A. ovis* than those free of ticks. Our result is consistent with that reported in sheep situated in northern Tunisia infested by *Rhipicephalus* spp. (Belkahia et al. 2014). Notably, the most dominant tick species was *R. turanicus* in both small ruminants, which is in agreement with other Tunisian surveys (Bouattour et al. 1999, Bouattour 2002, Darghouth 2004). Some of identified tick species, such as *R. turanicus*, *R. bursa*, and *R. sanguineus*, have been proposed previously as vectors of *A. ovis* in Mediterranean countries (de la Fuente et al. 2005, Torina et al. 2008a, Aktas et al. 2009). Aktas et al. (2009) reported the presence of *A. ovis* DNA in the salivary glands of *R. sanguineus* collected from Turkey. Torina et al. (2008a) and de la Fuente et al. (2005) demonstrated that *R. turanicus* is the main vector of *A. ovis* in Sicily (Italy). Until now, the vectors of *A. ovis* are still unknown in Tunisia; thus, further studies are needed to identify the main vectors of this bacterium.

PCR results based on the *groEL* gene demonstrate the absence of *A. phagocytophilum* in Tunisian small ruminants. This finding is in agreement with those reported in the North Mediterranean area by Torina et al. (2008a) and Zobba et al. (2014). On the basis of the *16S rRNA* gene, DNA fragments of *Anaplasma* species were detected in small ruminants in northern Tunisia. According to the cutoff value (99.0%) for species delineation (Adékambi et al. 2008), all genotypes obtained in this study can be potentially classified in two *Anaplasma* species different from all classified *Anaplasma* species, as sequence identities varied from 98.8% to 96.5% when comparing them to the six *Anaplasma* species officially recognized worldwide and varied from 98.7% to 98.3% between the Aplike2GGo1 genotype and all other genotypes identified in the present study.

On the one hand, genotypes from Aplike1GGo1 to Aplike1GGo 3 and from Aplike1GOv1 to Aplike1GOv 3 were 99.7–100% identical to *Anaplasma* sp. (Japan) (JN055357), considered as an unclassified novel species closely related to *A. phagocytophilum* by Ybañez et al. (2012a). Although previous reports described this potentially novel species as *A. phagocytophilum*, Ybañez et al. (2012a,b, 2013) suggested that the *A. phagocytophilum* strains initially found in Japan are distinct from those of the *A. phagocytophilum* strains in other countries. This *Anaplasma* species closely related to *A. phagocytophilum* has been detected earlier in Japanese ruminants and ticks (Ohashi et al. 2005, Jilintai et al. 2009, Yoshimoto et al. 2010, Ybañez et al. 2012a), but the presence of this species in small ruminants in Tunisia suggests that infection is not dependent on the geographical area but probably on the host. Instead, the Aplike2GGo1 genotype was 99.2% identical to *Anaplasma* sp. detected in *H. asiaticum* tick-infested Chinese ruminants (JX402604, KJ410247, and KJ410249) considered as another potentially novel species closely related to *A. phagocytophilum* by Kang et al. (2014).

In phylogenetic trees based on the *16S rRNA* gene, genotypes of *Anaplasma* species obtained in this study were consistently placed in two divergent clusters relatively distant from the *A. phagocytophilum* strains infecting humans, dogs, horses, and rodents. In addition, these novel *A. phagocytophilum*–like strains are associated to different possible tick vectors (*R. turanicus*, *R. sanguineus*, *R. annulatus*, and *H. excavatum*) with respect to *A. phagocytophilum* strains (*I. pacificus* and *I. scapularis* for American strains and *I. ricinus* for European strains; Richter et al. 1996, Telford et al. 1996, Petrovec et al. 1997, Woldehiwet 2010). Furthermore, infected small ruminants exhibited no clinical signs of active disease, as also reported for similar strains by Jilintai et al. (2009) and Yoshimoto et al. (2010). All of these findings suggest the circulation of at least two potential novel species genetically related to *A. phagocytophilum* in small ruminants in Tunisia.

In conclusion, molecular identification of potential novel *Anaplasma* species increases concerns about the specificity of serological tests routinely used in ruminants for diagnosis of anaplasmosis and provides additional information for the pathogenesis and molecular epidemiology of *A. phagocytophilum* and related species. Further studies are needed to characterize strains better by more discriminative genes and to identify the main tick vectors implicated in the transmission of these potentially novel *Anaplasma* species in Tunisia.

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Author Disclosure Statement

No competing financial interests exist.

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