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Original Article

Detection of novel strains genetically related to *Anaplasma platys* in Tunisian one-humped camels (*Camelus dromedarius*)

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Abstract

Introduction: Little information is currently available regarding the presence of *Anaplasma* species in North African dromedaries. To fill this gap in knowledge, the prevalence, risk factors, and genetic diversity of *Anaplasma* species were investigated in Tunisian dromedary camels.

Methodology: A total of 226 camels from three different bioclimatic areas were sampled and tested for the presence of *Anaplasma* species by quantitative polymerase chain reaction (qPCR) and nested polymerase chain reaction (nPCR) assays. Detected *Anaplasma* strains were characterized by 16S rRNA sequence analysis.

Results: Overall infection rate of *Anaplasma* spp. was 17.7%, and was significantly higher in females. Notably, *A. marginale*, *A. centrale*, *A. bovis*, and *A. phagocytophilum* were not detected. Animals were severely infested by three tick species belonging to the genus *Hyalomma* (*H. dromedarii*, *H. impeltatum*, and *H. excavatum*). Alignment, similarity comparison, and phylogenetic analysis of the 16S rRNA sequence variants obtained in this study suggest that Tunisian dromedaries are infected by more than one novel *Anaplasma* strain genetically related to *A. platys*.

Conclusions: This study reports the presence of novel *Anaplasma* sp. strains genetically related to *A. platys* in dromedaries from various bioclimatic areas of Tunisia. Findings raise new concerns about the specificity of the direct and indirect diagnostic tests routinely used to detect different *Anaplasma* species in ruminants and provide useful molecular information to elucidate the evolutionary history of bacterial species related to *A. platys*.

Key words: *Anaplasma* species; Dromedary (*Camelus dromedarius*); Molecular identification; 16S rRNA gene; Tunisia.

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Introduction

The genus *Anaplasma* (*Rickettsiales*: *Anaplasmataceae*) includes Gram negative obligate intracellular bacteria of significant importance in veterinary and human medicine [1]. *Anaplasma marginale*, the type species of *Anaplasma* genus, is highly pathogenic for ruminants and poses a considerable constraint to animal health in tropical and subtropical regions throughout the world [2]. It causes a variety of clinical symptoms, including fever, weight loss, abortion, lethargy, icterus, and often death of animals older than two years of age [2]. The closely related species *A. centrale* causes mild anaplasmosis in cattle [3,4]; for this reason, it has been used extensively as a live vaccine for anaplasmosis control in several countries [5]. Indeed, infection with *A. centrale* induces long-lasting protective immunity in ruminants when challenged with highly virulent *A. marginale* strains [2].

A. phagocytophilum is zoonotic and infects neutrophil granulocytes of many host species [3], including domestic ruminants, in which it causes tick-borne fever (TBF) [6,7]. The most common symptoms of TBF are high fever, anorexia, dullness, and reduced milk production [8]. *A. bovis*, a monocytotropic species, has been detected in different ruminant species from many countries [9,10]. It has been isolated from cattle and deer in Japan [11-13], cattle in Iran [14], water deer in South Korea [15], and goats in China [10]. *A. bovis* infection can cause variable clinical conditions ranging from the absence of symptoms to fever, anemia, weight loss, abortion, and death [16].

In Sicily, Italy, strains closely related to *A. platys* have been detected in neutrophils of cattle, sheep and goats [17] and in platelets of cats [18]. Based on genetic analyses using 16S rRNA and *groEL* genes, these strains revealed very high levels of nucleotide identity with canine *A. platys* strains (99% and 92%–93% identities with *A. platys* 16S rRNA and *groEL* genes, respectively) and were placed in a distinct monophyletic cluster closely related to *A. platys* sequences [17,18].

The dromedary (*Camelus dromedarius*), also known as the one-humped camel or Arabian camel, is a species of tremendous economic value in many countries, including Tunisia [19]. In central and southern regions of Tunisia, dromedary is an important source of income and is exploited for milk and meat production [19,20]. Dromedaries can be infested by a variety of tick species including *Hyalomma dromedarii*, *H. excavatum*, *H. marginatum*, *H. lusitanicum*, *H. impeltatum*, *Rhipicephalus bursa*, *R. sanguineus*, *R. pulchellus*, *R. declorotus*, *Amblyomma gemma*, and *A. variegatum* [21-25].

To date, few data on the presence of *Anaplasma* species in Tunisian domestic animals, especially in camels, are available. Molecular findings demonstrated the occurrence of *A. phagocytophilum* infections in dogs and horses [26,27], as well as *A. ovis* in sheep from the northern and central areas of the country [28]. The presence of *A. phagocytophilum* in horses and dromedaries was investigated by serology [29,30]. Indeed, surveys of anaplasmosis in camels have been focused mainly on *A. marginale* [31-35].

This study aimed to establish the presence and prevalence of *Anaplasma* species in Tunisian dromedaries by sampling three different bioclimatic areas. Molecular epidemiology of *Anaplasma* spp. strains infecting camels was also investigated by combining quantitative PCR (qPCR) with 16S rRNA sequence analyses.

Methodology

Sampling and DNA extraction

Blood samples and ticks were collected in 2009 (May to October) from 226 apparently healthy dromedaries spread throughout three localities: Bouficha (governorate of Sousse, latitude 36°18'N, longitude 10°27'E), belonging to semi-arid bioclimatic area with a mean annual rainfall of 350 mm; Sidi Bouzid (governorate of Sidi Bouzid, latitude 35°0'N, longitude 9°29'E), belonging to arid bioclimatic area with a mean annual rainfall of 237 mm, and Douz (governorate of Kebili, latitude 33°27'N, longitude 9°01'E), belonging to the Saharan bioclimatic area with a mean annual rainfall of 89 mm (Figure 1). Blood was collected from jugular veins into EDTA tubes (Becton Dickinson, Franklin lakes, USA). For each animal, the studied region, approximate age, gender, and presence/absence of ticks were noted. Ticks collected from severely infected animals were preserved in 70% ethanol and identified at genus and species levels using diagnosis keys as described by Walker *et al.* [36]. DNA was extracted from 300 µL volumes of EDTA-preserved whole blood using the Wizard Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions. DNA yields were determined with a spectrophotometer (Jenway, Genova, Italy). DNA samples were stored at -20°C until use.

Duplex real-time PCR

DNA samples were tested for the presence of *A. marginale* and *A. centrale* by using species-specific primers and TaqMan probes as described by Carelli *et al.* [37] and Decaro *et al.* [38], targeting, respectively, a fragment of the *msp1b* (77 bp) and *groEL* (95 bp)

Figure 1. Map of Tunisian studied regions

1: Bouficha region; 2: Sidi Bouzid region; 3: Douz region

genes. PCR was performed using Premix Ex Taq (Perfect Real Time) (Takara Mirus Bio, Madison, USA) in a 7500/7500 Fast Real-Time PCR System quantitative thermal cycler (Applied Biosystems, Foster City, USA). PCR amplification for *A. marginale* and *A. centrale* detection was performed in a duplex format by optimal reaction conditions using primers AM-For and AM-Rev at 600 nM each, probe AM-Pb-FAM at 200 nM, primers AC-For and AC-Rev at 900 nM each, probe AC-Pb-VIC at 200 nM, and 2 µL of template DNA (Table 1). Thermal cycling conditions included an initial activation of the Taq DNA polymerase at 95°C for 15 minutes, followed by 50 cycles of denaturation for 1 minute at 95°C followed by a 1 minute annealing-extension step at 60°C. Negative and positive controls were included in all runs.

Single and nested PCR

Primers EE1 and EE2 were used in a simple PCR run for amplifying the 16S rRNA gene of all *Anaplasma* species in dromedaries (Table 1). Reactions were performed in a final volume containing 0.125 U/µL Taq DNA polymerase (Biobasic Inc., Markham, Canada), 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 µL genomic DNA, 0.5 µM of the primers, and autoclaved MilliQ water to 50 µL. Thermal cycling reactions were performed in an automated thermal cycler (Techne Flexigene, Cambridge, UK) as described previously by Liu *et al.* [10]. Primers specific for *A. phagocytophilum* and *A. bovis* were used in two distinct nested PCRs (Table 1), in which 1 µL of the simple PCR run was used as DNA target. Thermal cycling profiles were as previously described by Kawahara *et al.* [11]. Negative (distilled water) and positive (DNA extracted from *A. phagocytophilum* and *A. bovis*) were included in each experiment. PCR products were electrophoresed on 1% agarose gel to check the size of amplified fragments by comparison with a DNA molecular weight marker (1 Kb Plus DNA Ladder, Promega, Madison, USA).

DNA sequencing and data analysis

Nine selected positive *Anaplasma* spp. PCR products (three from each sampling region) obtained with primers EE1/EE2 were purified with the GF-1 Ambi Clean Kit (Vivantis Technologies, Subang Jaya, Malaysia) according to the manufacturer's instructions. Purified DNA fragments were sequenced in both directions, using the same primers as in the PCR amplifications (Table 1). Sequencing was

Table 1. Primers and/or probes used for detection and/or characterization of *Anaplasma* spp., *A. platys*-like, *A. phagocytophilum*, *A. marginale*, *A. centrale*, and *A. bovis* in camels in the present study

Assay	Primer / probe	Sequence 5' to 3'	Target gene	Amplicon size (bp)	Reference
PCR 1[1]					
Anaplasma spp.	EE-1	TCCTGGCTCAGAACGAACGCTGGCGGC	16S rRNA	1,433	Barlough *et al*. (1996)
	EE-2	AGTCACTGACCCAACCTTAAATGGCTG			
PCR 2[2]					
A. phagocytophilum	SSAP2f[3]	GCTGAATGTGGGGATAATTTAT	16S rRNA	641	Kawahara *et al*. (2006)
	SSAP2r[3]	ATGGCTGCTTCCTTTCGGTTA			
A. bovis	AB1f[3]	CTCGTAGCTTGCTATGAGAAC	16S rRNA	551	Kawahara *et al*. (2006)
	AB1r[3]	TCTCCCGGACTCCAGTCTG			
Duplex real-time PCR					
A. marginale	AM-For	TTGGCAAGGCAGCAGCTT	*msp1b*	95	Carreli *et al*. (2007)
	AM-Rev	TTCCGCGAGCATGTGCAT			
	AM-Pb[4]	6FAM-TCGGTCTAACATCTCCAGGCTTTCAT-6TAMRA			
A. centrale	AC-For	CTATACACGCTTGCATCTC	*groEL*	77	Decaro *et al*. (2008)
	AC-Rev	CGCTTTATGATGTTGATGC			
	AC-Pb[5]	VIC-ATCATCATTCTTCCCCTTTACCTCGT-6TAMRA			

[1] Simple PCR allowing the detection of all *Anaplasma* species; [2] Second PCR, performed after the Simple PCR, allowing the specific species detection of *A. phagocytophilum* and *A. bovis;* [3] Primers used in PCR reaction for the detection of *A. phagocytophilum* and *A. bovis;* [4] The quencher dye fluorophore for the *A. marginale* probe was modified on 6-carboxyl-tetramethyl-rhodamine (6TAMRA) instead of Black Hole Quencher 1 (BHQ1) used by Carreli *et al.* [37]; [5] The reporter and quencher dye fluorophores for the *A. centrale* probe were modified on 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC) and 6-carboxyl-tetramethyl-rhodamine (6TAMRA) instead of Texas Red and Black Hole Quencher 2 (BHQ2), respectively used by Decaro *et al.* [38].

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 by Macrogen Europe (Amsterdam, the Netherlands). Chromatograms were edited with Chromas Lite version 2.01. Multiple sequence alignments were obtained with DNAMAN program (Version 5.2.2; Lynnon Biosoft, Quebec, Canada). BLAST was used to investigate homologies with *Anaplasma* sequences available in database [39]. Neighbor-joining (NJ) phylogenetic trees were constructed using the DNAMAN program based on Saitou and Nei distances [40] with bootstrap analysis of 1,000 reiterations.

Sequence accession number

The 16S rRNA partial sequences of *Anaplasma* spp. AspGDr1 to AspGDr4 variants were deposited in the GenBank under accession numbers KM401905 to KM401908, respectively.

Statistical analyses

Exact confidence intervals (CIs) for prevalence rates at the 95% level were calculated. To study the possible influence of location, gender, age and tick infestation on the molecular prevalence of *Anaplasma* spp., the Chi-square test or Fisher's exact test were performed using Epi Info version 6.01 with a cut-off value of 0.05. In order to consider any confusion factor, a Chi-square Mantel-Haenszel test was performed.

Results

Tick identification and molecular survey of Anaplasma species

Ticks collected from the camels belonged to the genus *Hyalomma* (*H. dromedarii*, *H. impeltatum*, and *H. excavatum*). The overall tick infestation prevalence was 37.6% (85/226). Overall infection rate of *Anaplasma* spp., estimated by EE1/EE2 PCR (Table 1), was 17.7% (minimum 14.8% in Sidi Bouzid and maximum 31.3% in Bouficha) (Table 2). Moreover, the infection rate of *Anaplasma* spp. was significantly higher in female (24.5%) than in male camels (11.7%, p = 0.027) (Table 2). Using qPCR tests specific for *A. marginale* and *A. centrale*, and nPCRs for *A. bovis* and *A. phagocytophilum* (Table 1), none of the classified *Anaplasma* species analyzed in this study were detected in any of the tested camels.

Molecular characterization of Anaplasma sp. 16S rRNA genotypes

Nine PCR products obtained from nine randomly selected camels (three from each sampling site) with primers EE1/EE2 targeting 1,322 bp (88.5%) of the 16S rRNA gene of *Anaplasma* spp. were successfully sequenced on both DNA strands. Based on nucleotide alignments, the sequences were grouped in four different genotypes (AspGDr1 to AspGDr4; GenBank accession numbers KM401905 to KM401908). All 16S rRNA sequences obtained in this study shared 99.8% to 99.9% nucleotide similarity and differed from each other in three nucleotide positions (Tables 3 and 4).

Table 2. Factors associated with molecular prevalence of *Anaplasma* spp. in camels from Tunisia

		***Anaplasma* spp.**	
	Number	**Positive (% ± CI[1])**	**P value**
Locality			0.086
Bouficha	32	10 (31.3 ± 0.16)	
Sidi Bouzid	155	23 (14.8 ± 0.06)	
Douz	39	7 (17.9 ± 0.12)	
Age			0.158
≤ 2 years	44	8 (18.2 ± 0.11)	
2–7 years	109	24 (22.0 ± 0.08)	
> 7 years	73	8 (11.0 ± 0.07)	
Gender			0.027*
Male	120	14 (11.7 ± 0.06)	
Female	106	26 (24.5 ± 0.08)	
Tick infestation			0.754
infested	84	14 (16.7 ± 0.08)	
Not infested	142	26 (18.3 ± 0.10)	
Total	**226**	**40 (17.7 ± 0.05)**	

[1] CI: 95% confidence interval; * Significant test.

Table 3. Nucleotide diversity among 16S rRNA sequences (1,322 bp) from *Anaplasma* sp. related to *A. platys* isolated from camels and other *Anaplasma* species found in GenBank

***Anaplasma* sp.**	**Host**	**Variant**	**Sample[1]/isolate**	**Country**	**GenBank[2]**	**16S rRNA nucleotide positions[3]**								**Reference**
						91	118	130	783	923	962	1211	1214	
Anaplasma sp.	Dromedary	AspGDr1	Sb1-Sb3	Tunisia	KM401905	G	A	A	A	C	A	T	T	Present study
		AspGDr2	Dz1-Dz3	Tunisia	KM401906	*	*	G	*	*	*	*	*	Present study
		AspGDr3	Bf1; Bf2	Tunisia	KM401907	A	*	G	*	*	*	*	*	Present study
		AspGDr4	Bf3	Tunisia	KM401908	A	G	G	*	*	*	*	*	Present study
	Goat	J3	J3	China	JN558826	A	*	G	*	T	*	*	C	Liu *et al.* (2012)
		E10	E10	China	JN558821	A	*	G	G	*	*	C	C	Liu *et al.* (2012)
A. platys	Dog	Okinawa	Okinawa	Japan	AY077619	A	*	G	*	*	G	*	C	Inokuma *et al.* (2002)

[1] Bf1-Bf3, Sb1-Sb3, and Dz1-Dz3 samples were collected from Bouficha, Sidi Bouzid, and Douz localities, respectively; [2] GenBank accession number; [3] Numbers represent the nucleotide position with respect to the clone J3 from China for *Anaplasma* sp. related to *A. platys* (GenBank accession number JN558826); Conserved nucleotide positions are indicated with asterisks. Nucleotides: T: thymine; C: cytosine; G: guanine; A: adenine.

Table 4. Comparison of 16S rRNA sequences (1,322 bp) from *Anaplasma* sp. related to *A. platys* isolated from camels and other *Anaplasma* species found in GenBank. The numbers represent the nucleotide identity rates found between the sequences.

	***A.* sp (AspGDr1)**	***A.* sp (AspGDr2)**	***A.* sp (AspGDr3)**	***A.* sp (AspGDr4)**	***A.* sp (J3)**	***A.* sp (E10)**	***A. pl* (Okinawa)**	***A. p* (China-C-Y)**	***A. b* (G49)**	***A. m* (Lushi)**	***A. c* (CC)**	***A. o* (Jingtai)**
A. sp (AspGDr1)	100											
A. sp (AspGDr2)	99.9	100										
A. sp (AspGDr3)	99.8	99.9	100									
A. sp (AspGDr4)	99.8	99.8	99.9	100								
A. sp (J3)	99.7	99.8	99.8	99.8	100							
A. sp (E10)	99.6	99.7	99.8	99.7	99.8	100						
A. pl (Okinawa)	99.7	99.8	99.8	99.8	99.8	99.8	100					
A. p (China-C-Y)	98.7	98.8	98.9	98.8	98.9	98.8	99.0	100				
A. b (G49)	97.0	97.0	97.0	97.0	96.9	97.1	96.9	97.0	100			
A. m (Lushi)	97.0	97.1	97.2	97.1	97.2	97.3	97.2	97.3	96.1	100		
A. c (CC)	97.0	96.9	97.0	96.9	97.0	97.0	97.1	97.2	96.3	99.3	100	
A. o (Jingtai)	97.0	96.9	97.0	97.0	97.0	97.0	97.1	97.1	96.1	99.2	99.5	100

A. sp (AspGDr1-4): *Anaplasma* sp. isolated from Tunisian dromedaries (AspGDr1-4 strains, GenBank accession numbers KM401905- KM401908, respectively); *A.* sp (J3, E0): *Anaplasma* sp. isolates found on Chinese goats (J3 and E10 isolates, GenBank accession numbers JN558826 and JN558821, respectively); *A. pl* (Okinawa): *A. platys* isolate found on Japanese dog (Okinawa isolate, GenBank accession number AY077619); *A. p* (China-C-Y): *A. phagocytophilum* strain isolated from Chinese sheep (China-C-Y strain, GenBank accession number GQ412338); *A. b* (G49): *A. bovis* isolate found on Chinese goat (G49 isolate, GenBank accession number JN558824); *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. o* (Jingtai): *A. ovis* isolate found on Chinese goat (Jingtai isolate, GenBank accession number AJ633049)

Based on BLASTN analyses and nucleotide alignments, the four identified genotypes were 99.6%–99.8% similar to those of J3 and E10 *Anaplasma* sp. isolates (GenBank accession numbers JN558826 and JN558821, respectively) found on Chinese goats and considered as *A. platys*-like by Liu *et al.* [10] and differed in seven and six nucleotide positions, respectively (Tables 3 and 4). Obtained sequences also shared 99.7%–99.8% similarity with an *A. platys* Okinawa isolate recovered from a dog in Japan (GenBank accession number AY077619) and differed in five nucleotide positions (Tables 3 and 4). Lower nucleotide sequence identities were obtained on comparisons with other *Anaplasma* species (98.7%–98.9% with *A. phagocytophilum*; 97.0%–97.1% with *A. marginale*; 97.0% with *A. bovis*; 96.9%–97.0% with *A. centrale*, and 96.9%–97.0% with *A. ovis*; Table 4). Similarly, comparisons based on 763 bp of the 16S rRNA gene highlighted a similarity of 99.3% with strains BovineCaprine1 and Caprine2 found on Italian cattle and goats (GenBank accession numbers KC335220–KC335222) and classified as *Anaplasma* sp. strains closely related to *A. platys* [17].

Phylogenetic analysis placed all the sequences obtained in this study in monophyletic clusters including *A. platys* (Figure 2A, 2B). In particular, all *Anaplasma* sp. Tunisian strains were closely related to *A. platys* strains isolated from Chinese goats and to Italian strains isolated from goats and cattle [10,17].

Discussion

Dromedary camels can host different pathogens, including several *Anaplasma* species [35,41]. In Tunisia, a molecular survey of *Anaplasma* species in dromedaries is still lacking [29]. In this study, molecular epidemiology of selected *Anaplasma* species was investigated in dromedary camels from different bioclimatic areas of Tunisia. Results clearly indicate evidence of *Anaplasma* infection in camels from all studied localities with an average prevalence of 17.7% (minimum 14.8% in Sidi Bouzid and maximum 31.3% in Bouficha). This is the first estimate of the molecular prevalence of *Anaplasma* spp. in Tunisian camels. Despite the important difference in bioclimatic characteristics between the three investigated areas, the difference in prevalence rates is not statistically significant ($p > 0.05$) (Table 2). This is probably due to the frequent camel movement between these areas as well as the similarity of tick populations infesting camels in sampling locations [29].

Compared to other countries, the overall prevalence rate in Tunisia remains higher than that in Spain (0%) [35], and appreciably lower than that in Saudi Arabia (95.5%) [42]. In Spain, a 3% *Anaplasma* spp. prevalence was established in camels by serology [35]. This high discrepancy between prevalence rates may result from differences in tick control programs, farm management, husbandry practices, wildlife reservoir hosts, and/or abiotic factors. In fact, several studies have reported the variability of *Anaplasma* species prevalence in ruminants according to geographic location, associated with suitable tick habitats and animal management [10,28,43]. Moreover, the infection rate of *Anaplasma* spp. was significantly higher in females compared to males ($p = 0.027$) (Table 2). This can be explained by the immunosuppression of females which may occur during pregnancy and lactation periods [41], which could last up to two years [44].

Notably, we failed to recover *A. marginale*, *A. centrale*, *A. bovis*, and *A. phagocytophilum* from investigated camels. It can be postulated that dromedaries are not relevant reservoirs for classified *Anaplasma* species in the studied regions, but alternative ruminants and other wild and domestic animal species could act as reservoir hosts in this area. The seroprevalence of *A. phagocytophilum* in the same animals was investigated in a previous study [29]. Overall, 66 out of 226 camels (29.2%) were seropositive. The discrepancy between molecular and serological tests could be explained by cross-reactivity of the antigen used in serology with anti-cytoplasmic antibodies, as well as with other autoimmune antibodies and/or with antibodies related to other *Anaplasma* species closely related to those of *A. phagocytophilum* [17,18,45]. Notably, previous studies reported a great degree of cross-reactivity in serological tests between *Anaplasma* species [46-48].

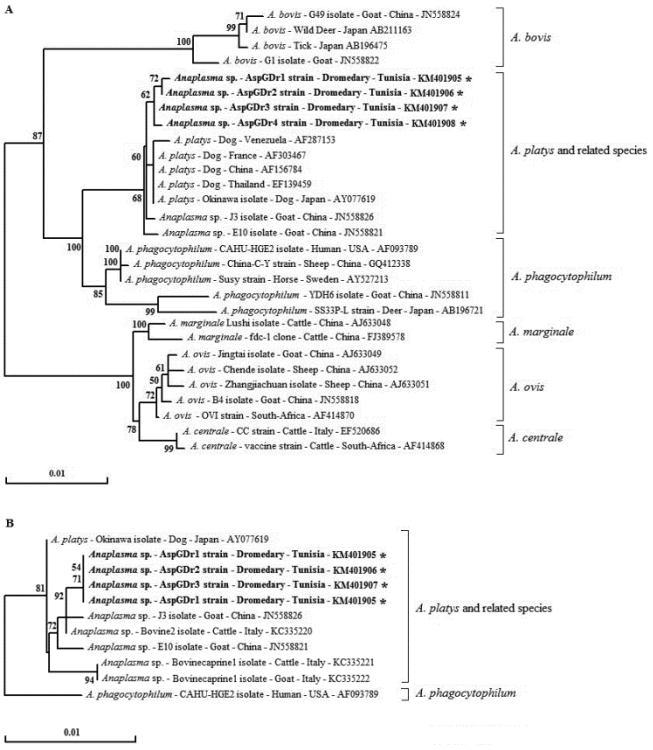
In the present study, *H. dromedarii*, *H. impeltatum*, and *H. excavatum* were collected from camels. These data are in agreement with what observed by Gharbi *et al.* [25], who reported the infestation of dromedaries by these tick species in Tunisia. All tick genera identified in investigated areas have never been reported as vectors of *A. phagocytophilum*, *A. marginale*, *A. bovis*, or *A. centrale* [36], suggesting that these tick species may be vectors of other *Anaplasma* species probably not yet classified. Further studies are needed to clarify the role of these tick species in transmission of *Anaplasma* species to camels in Tunisia.

The 16S rRNA gene is considered a sensitive molecular tool for the discrimination of *Anaplasma* species in phylogenic studies [3,49]. Sequencing of 1,322 bp of the 16S rRNA gene isolated from randomly selected *Anaplasma* spp.-positive camels revealed four different and novel *Anaplasma* sp. variants. Alignment (Table 3) and percent sequence identity comparison (Table 4) of the 16S rRNA sequence variants obtained in this study suggests that Tunisian dromedaries are infected by *Anaplasma* strains genetically related to *A. platys*. Indeed, these sequence variants shared a similarity greater than 99% with the 16S rRNA sequences of the canine *A. platys* and related strains found in Chinese and Italian ruminants [10,17] (Tables 3 and 4).

Phylogenetic analysis of 16S rRNA partial sequences performed with *Anaplasma* sp. sequences isolated from camels and selected sequences of *Anaplasma* species obtained from GenBank confirmed what was observed by percent sequence identity comparison (Figure 2). In agreement with Ooshiro *et al.* [12], Liu *et al.* [10], Ybañez *et al.* [50], and Zobba *et al.* [17], the phylogenetic tree based on 1,322 bp of the 16S rRNA gene shows two main clusters, one containing *A. phagocytophilum*, *A. platys*, *A. bovis* sequences, and another containing *A. marginale*, *A. centrale*, and *A. ovis* sequences. *Anaplasma* sp. variants isolated from Tunisian dromedaries cluster with *A. platys* and related strains (Figure 2A).

A. platys, the etiologic agent of canine infectious cyclic thrombocytopenia, has been associated with thrombocytopenia and anemia [17,18]. In this study, randomly selected dromedaries did not show any symptoms specifically referable to *A. platys* infection. Therefore the *A. platys*-like strains isolated in camels might not be pathogenic and not cause any symptoms, as previously observed in ruminants from China and Italy [10,17] and in cats from Italy [18].

Figure 2. Phylogenetic trees of *Anaplasma* species inferred with partial sequences (1,322 and 763 bp for A and B, respectively) of the 16S rRNA gene using the neighbor-joining method showing the location of the four new sequences from Tunisian camels



Sequence variants from this study represented in bold and marked with asterisks. Numbers associated with nodes represent the percentage of 1,000 bootstrap reiterations supporting the nodes (only percentages greater than 50% were represented). The host or vector, the strain or isolate name, the country of origin and the GenBank accession number are indicated

Conclusions

This paper reports the presence of novel *Anaplasma* sp. strains genetically related to *A. platys* in dromedaries from various bioclimatic areas of Tunisia. Findings open new concerns about the specificity of the direct and indirect diagnostic tests routinely used to detect different *Anaplasma* species in ruminants and provide useful molecular information to elucidate the evolutionary history of bacterial species related to *A. platys*. Further studies are needed to investigate if these *A. platys*-like strains infect other animal species in Tunisia, to better characterize these different strains by more discriminative genes, and to identify vectors implicated in the transmission of the potentially novel *Anaplasma* to which these strains could be ascribed.

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