



Research paper

Anaplasma platys-like strains in ruminants from Tunisia

Mourad Ben Said^a, Hanène Belkahia^a, Narjese El Mabrouk^a, Mariem Saidani^a, Alberto Alberti^b, Rosanna Zobba^b, Amal Cherif^a, Tarek Mahjoub^a, Ali Bouattour^c, Lilia Messadi^{a,*}

^a Laboratoire de Microbiologie, Ecole Nationale de Médecine Vétérinaire, Institution de la Recherche et de l'Enseignement Supérieur Agricoles, Université de La Manouba, Sidi Thabet, Tunisia

^b Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Sassari, Italy

^c Laboratoire d'Entomologie, Institut Pasteur de Tunis, Tunisia

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ABSTRACT

Molecular diagnosis of *Anaplasma platys* and related strains (*A. platys*-like) in carnivores and ruminants is challenging due to co-infections with cross-reacting strains, and require post-amplification sequencing of the hemi-nested PCR products traditionally generated by targeting the *groEL* gene. In this study, a Restriction Enzyme Fragment Length Polymorphism (RFLP) assay coupled to hemi-nested *groEL* PCR was developed to discriminate among *A. platys* and genetically related strains. This novel approach was used for investigating *A. platys*-like infection in 963 domesticated ruminants (241 goats, 355 sheep, and 367 cattle) from 22 delegations located in North Tunisia. Overall prevalence rates of *A. platys*-like were 22.8, 11, and 3.5% in goats, sheep, and cattle, respectively. Alignment, identity comparison, and phylogenetic analysis of the *groEL* sequence variants obtained in this study confirmed RFLP data suggesting that Tunisian ruminants are infected by novel unclassified *Anaplasma* strains genetically related to *A. platys*. Compared to sequencing, RFLP assay allows fast detection of *A. platys* and *A. platys*-like pathogens in the same sample and has a potential value especially when screening ticks, cats and ruminants, which can be a common host for these two bacteria. This newly developed molecular technique would provide valuable molecular tool for epidemiological studies related to *A. platys* as well as remove concern over specificity of serological and molecular methods routinely used to identify diverse *Anaplasma* strains and species in wild and domestic ruminants.

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1. Introduction

Anaplasma spp. are obligate intracellular bacteria that replicate within a mammalian eukaryotic host cell and tick cell membrane-derived vacuole (Rikihisa, 2007). Eight species are currently assigned to the genus *Anaplasma* including *Anaplasma marginale*, *A. centrale*, *A. bovis*, *A. phagocytophilum*, *A. ovis*, *A. platys*, “*A. capra*” and *Aegyptianella pullorum* (Kocan et al., 2015; Li et al., 2015a; Atif, 2016).

Anaplasma platys, the agent of cyclic thrombocytopenia in dogs, is the only classified rickettsial species known to infect platelets (Dumler et al., 2001). Apart from dogs, this pathogen has been found infecting cats (Lima et al., 2010) and some ruminants like cattle, goats, camels, buffalo and red deer (Chochlakis et al., 2009; Dahmani et al., 2015; Li et al., 2015b, 2016; Lorusso et al., 2016a, 2016b; Machado et al., 2016).

Phylogenetic comparisons based on the 16S rRNA gene sequences of *A. platys* strains isolated worldwide from dogs, some other animals and ticks like *Rhipicephalus sanguineus* sensu lato group with *Anaplasma* spp.

species show that *A. platys* sequences form a monophyletic cluster, in which identity among different strains is always >99.3% (Pinyoowong et al., 2008; Li et al., 2015b). However, in Sardinia (Italy), *A. platys*-like strains have been identified in neutrophils of cattle, sheep and goats (Zobba et al., 2014) and in cat platelets (Zobba et al., 2015). Genetic analyses based on 16S rRNA and *groEL* genes, these strains reveal considerable levels of nucleotide identity (99% and 92–93%, respectively) with the canine *A. platys* strains but form a distinct monophyletic cluster closely related to *A. platys* sequences (Zobba et al., 2014, 2015). In addition, *A. platys*-like strains have been isolated from camels (Bastos et al., 2015; Belkahia et al., 2015b).

In Tunisia, several *Anaplasma* spp. were identified in ruminants such as *A. marginale*, *A. bovis* and *A. centrale* in cattle (Belkahia et al., 2015a), and *A. ovis*, *A. bovis*, and strains closely related to *A. phagocytophilum* in small ruminants (Belkahia et al., 2014; Ben Said et al., 2015a, 2015b). However, to date, *A. platys*-like strains have been detected in dromedaries only (Belkahia et al., 2015b). These strains genetically related to *A. platys* were amplified by using a 16S rRNA PCR specific for *Anaplasma* spp. (Liu et al., 2012; Belkahia et al., 2015b; Yang et al., 2015) or alternatively a *groEL* hemi-nested PCR designed for simultaneous identification of *A. platys* and related strains complex (Zobba et al., 2014, 2015). Both methods required post-amplification sequencing to confirm results.

* Corresponding author at: Laboratoire de Microbiologie, Ecole Nationale de Médecine Vétérinaire, 2020 Sidi Thabet, Tunisie.

E-mail address: lilia_messadi@yahoo.fr (L. Messadi).

In this study, we identified a restriction enzyme site that allows discrimination of *A. platys* and *A. platys*-like strains upon amplification of the *groEL* gene by hemi-nested PCR (Alberti et al., 2005). We applied this novel PCR-RFLP approach to investigate for the first time presence and prevalence of *A. platys*-like strains in goats, sheep and calves located in several delegations (intermediate administrative districts between the governorates and the sectors) belonging to three different Tunisian bioclimatic areas as well as genetic characterization and phylogeny of the genetic variants recorded during this study.

2. Materials and methods

2.1. Blood sampling and DNA extraction

A cross-sectional study was carried out in twenty-two delegations of Northern Tunisia belonging to five governorates (Tunis, Ariana, Bizerte, Beja and Nabeul) and three bioclimatic areas (lower humid, sub-humid and higher semi-arid) (Fig. 1, Table 1 and Supplementary file 1). A total of 963 blood samples were collected from apparently healthy cattle ($n = 367$), sheep ($n = 355$) and goats ($n = 241$) during May to June 2015 (Table 1 and Supplementary file 1). Blood samples were collected in EDTA tubes from the jugular vein. The DNA was extracted from 300 μ l of each blood sample with the Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to manufacturer's instructions.

2.2. Hemi-nested PCR

For the detection of *A. platys* and related strains, all DNA samples were tested by hemi-nested PCR using outer primers EphplgroEL-F and EphplgroEL-R, and inner primer EplgroEL-R, amplifying 515 bp of the *groEL* gene (Alberti et al., 2005; Alberti and Sparagano, 2006; Zobba et al., 2014, 2015; Fig. 2). Each reaction was performed in a final volume of 50 μ l containing 0.125 U/ μ l Taq DNA polymerase (Biobasic Inc., Canada), $1 \times$ PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 μ l genomic DNA and 0.5 μ M of primers. Thermal cycling reactions were performed by using an automated DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler, Foster City, California, USA). One microliter of each amplicon was used for PCR reaction with specific primers under the same conditions as for the first PCR. The PCR products were electrophoresed in 1% agarose gels containing 0.5 μ g/ml of

ethidium bromide. Distilled water and DNA samples positive to *A. platys* (Zobba et al., 2014, 2015) were used as negative and positive controls in each PCR experiment.

2.3. RFLP analysis of the amplified products

In silico analyses of the *A. platys* and *A. platys*-like *groEL* gene were performed in order to identify restriction sites useful to differentiate *A. platys* from *A. platys*-like strains. Briefly, 53 *groEL* partial sequences (476 bp, excluding the two primers regions) derived respectively from 19 and 34 *A. platys* and *A. platys*-like strains (20 ruminants and 14 felines), were aligned (Supplementary file 2). A *Stu*I site (AGG/CCT), at position 713 referring to the full *groEL* sequence, was identified in all analyzed *A. platys* sequences and was absent in all analyzed *A. platys*-like sequences caused by the substitution of "G" by "A" and of "T" by "G" in positions 713 and 716, respectively (Fig. 2; Supplementary file 2). All hemi-nested PCR products obtained in this study were digested by *Stu*I to discriminate among specific *A. platys* and *A. platys*-like strains. Restriction reaction was performed in a final volume of 20 μ l containing 10 μ l PCR product, 2 μ l buffer (10 \times), 1 μ l *Stu*I enzyme (10,000 U/ml) (Biolabs, New England, UK) and 7 μ l distilled water. Digestions were incubated 1 h at 37 °C and run in 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 subsequently visualized at UV light after with ethidium bromide staining.

2.4. DNA sequencing and phylogenetic analysis

Twenty-seven *A. platys*-like PCR products (originated from 22 goats, 3 sheep and 2 cattle) obtained with primers EphplgroEL-F/EplgroEL-R were purified with GF-1 Ambi Clean kit (Vivantis technologies, Malaysia) according to manufacturer's instructions. Purified DNA fragments were directly sequenced in both directions by using hemi-nested primers. Sequencing was performed by using Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Applied Biosystems, Foster City, California, USA) and an ABI3730XL automated DNA sequencer. Chromatograms were edited with Chromas Lite v 2.01. Multiple nucleotide and amino-acid sequence alignments were obtained with the DNAMAN software Version 5.2.2. (Lynnon Biosoft, Que., Canada). The BLAST was used to investigate identities with *Anaplasma*

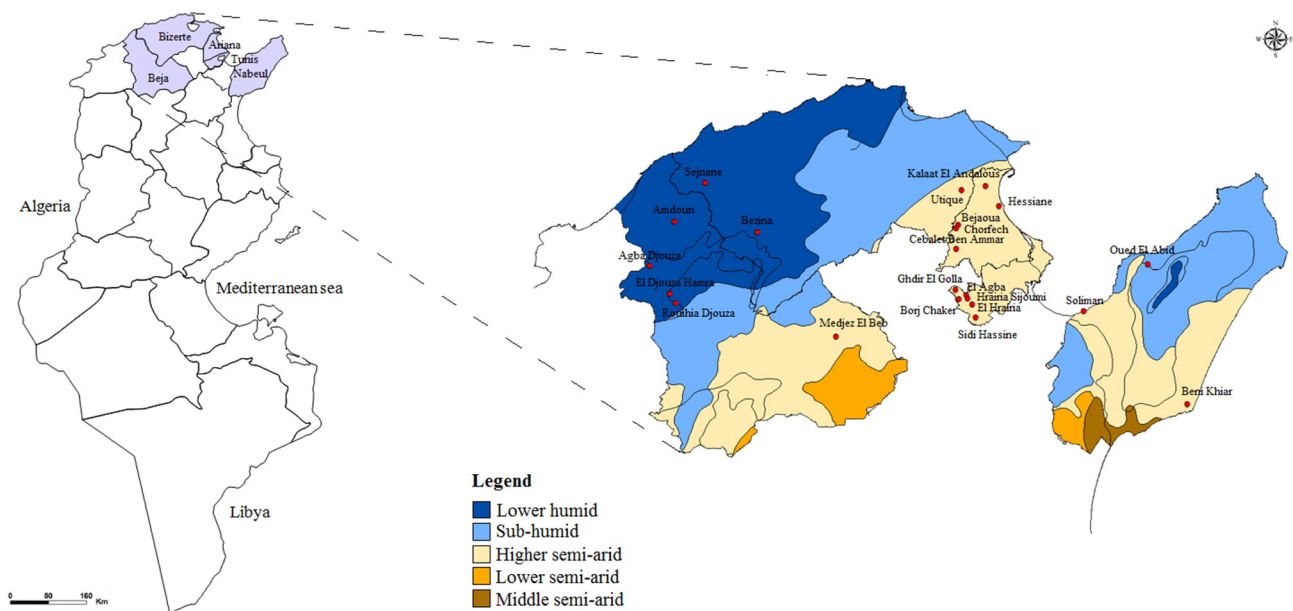


Fig. 1. Map of the Tunisian studied regions. (A) Map of Tunisia showing investigated governorates and (B) Map of the five governorates showing the twenty-two studied delegations belonging to three bioclimatic areas.

Table 1
Prevalence rates of *A. platys*-like strains in goats, sheep and cattle according to governorates and delegations.

Governorates	Delegations	Goats			Sheep			Cattle		
		Number	Positive (% ± C.I. ^a)	p-Value ¹	Number	Positive (% ± C.I. ^a)	p-Value ¹	Number	Positive (% ± C.I. ^a)	p-Value ¹
Bizerte		61	34 (55.7 ± 0.13)	0.000*	85	9 (10.6 ± 0.07)	0.009*	103	8 (7.8 ± 0.05)	0.029*
	Sejnane	49	41 (83.7 ± 0.10)	0.430	48	9 (18.8 ± 0.11)	0.020*	59	8 (13.6 ± 0.09)	0.000*
	Utique	12	11 (91.7 ± 0.16)		15	0 (0)		NA	NA	
	Bezina	NA	NA		22	0 (0)		44	0 (0)	
Tunis		76	0 (0)		107	3 (2.8 ± 0.03)		60	0 (0)	
	Agba	NA	NA		20	1 (5 ± 0.09)	0.465	10	0 (0)	–
	Hrairia	20	0 (0)	–	87	2 (2.3 ± 0.03)		29	0 (0)	
	Ghdir El Golla	10	0 (0)		NA	NA		NA	NA	
	Sidi Hassine	20	0 (0)		NA	NA		10	0 (0)	
	Hrairia Sijoumi	26	0 (0)		NA	NA		NA	NA	
	Borj Chaker	NA	NA		NA	NA		11	0 (0)	
		48	4 (8.3 ± 0.08)		66	10 (15.2 ± 0.67)		82	0 (0)	
Beja	El Djouza Hamra	22	0 (0)	0.000*	22	0 (0)	0.003*	20	0 (0)	–
	Amdoun	16	0 (0)		34	10 (29.4 ± 0.15)		21	0 (0)	
	Medjez El Beb	10	4 (40 ± 0.30)		10	0 (0)		10	0 (0)	
	Rouihia Djouza	NA	NA		NA	NA		10	0 (0)	
	Agba Djouza	NA	NA		NA	NA		21	0 (0)	
		37	17 (45.9 ± 0.16)		56	11 (19.6 ± 0.10)		66	3 (4.5 ± 0.05)	
Nabeul	Oued El Abid	11	3 (27.3 ± 0.26)	0.003*	32	1 (3.1 ± 0.06)	0.000*	35	1 (2.9 ± 0.05)	0.426
	Soliman	20	14 (70 ± 0.20)		20	10 (50 ± 0.22)		22	2 (9.1 ± 0.12)	
	Beni Khair	06	0 (0)		04	0 (0)		9	0 (0)	
Ariana		19	0 (0)		41	6 (14.6 ± 0.05)		56	2 (3.6 ± 0.05)	
	Cebalet Ben Ammar	05	0 (0)	–	30	1 (3.3 ± 0.03)	0.003*	NA	NA	0.022*
	Hessiane	10	0 (0)		11	5 (45.5 ± 0.27)		10	2 (20 ± 0.25)	
	Chorfech	04	0 (0)		NA	NA		20	0 (0)	
	Kalaat El Andalous	NA	NA		NA	NA		16	0 (0)	
	Bejaoua	NA	NA		NA	NA		10	0 (0)	
Total		241	55 (22.8 ± 0.05)		355	39 (11 ± 0.03)		367	13 (3.5 ± 0.02)	

NA: Not analyzed.

^a C.I.: 95% confidence interval.

¹ p-Value represented in the first line is calculated according to governorates and p-values represented in the other lines are calculated according to delegation of each appropriated governorate.

* Statistically significant test.

sequences available in the GenBank database (Altschul et al., 1997). Neighbor-Joining (NJ) phylogenetic trees based on nucleotide and amino-acid sequences were constructed using the DNAMAN program (Saitou and Nei, 1987), based on the maximum likelihood evolutionary distance method (Tamura, 1992). Statistical evaluation of tree branches was conducted by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

2.5. Sequence accession numbers

The *groEL* nucleotide partial sequences of *Anaplasma* sp. strains genetically related to *A. platys* were deposited in the GenBank under accession numbers KX650588 to KX650613.

2.6. Statistical analyses

Exact confidence intervals (CI) for prevalence rates at the 95% level were calculated. To study the possible influence of geographic location, bioclimatic area and host on the molecular prevalence of *A. platys*-like, chi square test or Fisher's exact test were performed using Epi Info 6.01 (CDC, Atlanta, USA) with a cut-off value of 0.05.

3. Results

3.1. Specific detection of *Anaplasma platys*-like strains

The DNA from *A. platys* and related strains was amplified from 107 ruminant positive samples resulted from analyzing the 983 samples and 2 canine positive controls and produced a clear single band on agarose electrophoresis at 515 bp. Amplicons digestion with *StuI* allowed differentiation of specific *A. platys* (from 2 canine positive controls)

from *A. platys*-like strains (from 107 ruminant samples). *Anaplasma platys* DNA always produced fragments of 371 and 144 bp when digested with *StuI*, whereas *A. platys*-like PCR products were always unaffected by *StuI* treatment (Fig. 2).

3.2. Molecular prevalence of *Anaplasma platys*-like strains

Overall prevalence rates of *A. platys*-like were 22.8 (55/241); 11 (39/355) and 3.5% (13/367) in goats, sheep, and cattle, respectively (Table 1; Supplementary file 1). Goats from Bizerte and Nabeul showed *A. platys*-like infection rates of 55.7 (34/61) and 45.9% (17/37), respectively. Infection rates in these locations were greater than those observed in other governorates ($p < 0.001$; Table 1). Within the governorate of Bizerte, the two delegations Utique and Sejnane showed very high infection rates estimated at 91.7 (11/12) and 81.7% (41/49), respectively (Table 1). Within Nabeul governorate, the higher infection rate was observed in the delegation of Soliman (70%; 14/20, $p = 0.003$), followed by delegation of Oued Abid (27.3%; 3/11) (Table 1). In sheep, Nabeul, Beja, Ariana and Bizerte governorates showed similar prevalence rates estimated at 19.6 (11/56); 15.2 (10/66); 14.6 (6/41) and 10.6% (9/85), while infection rate of sheep located in the governorate of Tunis was statistically lower (2.8%; 3/107, $p = 0.009$). Within Ariana governorate, animals from Hessiane were the most infected by *A. platys*-like with an infection rate of 45.5% (5/11) (Table 1). Only cattle from Bizerte, Nabeul and Ariana governorates were infected with prevalence rates of 7.8 (8/103), 4.5 (3/66), and 3.6% (2/56) ($p = 0.029$). As for Sheep, cattle from Hessiane delegation were the most infected with a prevalence rate of 20% (2/10) (Table 1). For all analyzed host species, no statistically significant differences were found among *A. platys*-like prevalence rates according to bioclimatic areas (Supplementary file 1).

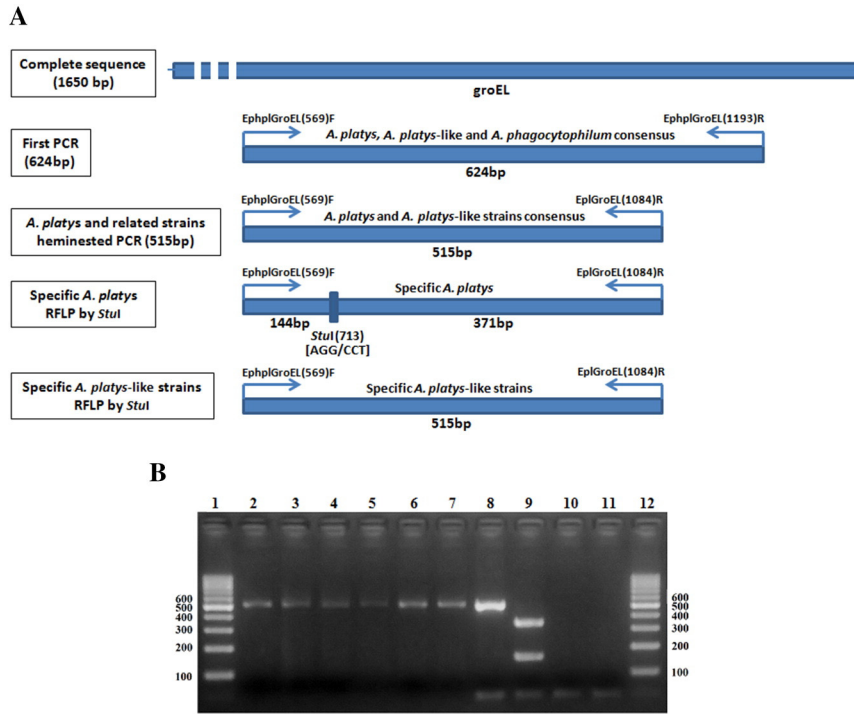


Fig. 2. RFLP strategy and results of restriction analysis. (A) RFLP strategy developed and validated during this study for the detection and differentiation of canine *A. platys* and *A. platys*-like strains. Numbers in parentheses indicate positions of primers and *Stul* restriction sites calculated using the sequence of NCH-1 strain of *A. phagocytophilum* as a reference; ¹: A *Stul* site (AGG/CCT), at position 713 referring to the full *groEL* sequence, was identified in all analyzed *A. platys* sequences and was absent in all analyzed *A. platys*-like sequences caused by the substitution of “G” by “A” and of “T” by “C” in positions 713 and 716, respectively. (B) Results of restriction analysis using *Stul* enzyme on DNA analyzed samples. Lines 1 and 12: 1 Kb ladder; lines 2, 4, 6 and 8: *A. platys*-like PCR (515 bp) products before RFLP assay isolated from goat, sheep and cattle, and of canine *A. platys* (515 bp) isolated from dog, respectively; lines 3, 5, 7 and 9: PCR products after RFLP assay of *A. platys*-like strains (515 bp) isolated from goat, sheep and cattle, and of canine *A. platys* (144 and 371 bp) isolated from dog, respectively and Lines 10 and 11: PCR results of negative control before and after RFLP assay, respectively.

Table 2
Designation and information about sequencing and RFLP data of the nine *groEL* genetic variants of *Anaplasma* sp. closely related to *A. platys* identified in this study.

<i>Anaplasma</i> spp.	Genetic variant	Host (isolate)	Geographical location	GenBank accession no.	BLAST analysis	RS of <i>Stul</i> ^a	RFLP by <i>Stul</i> ^b
<i>A. platys</i>	AplDog Lara	<i>Canis canis</i> Lara	Sardinia, Italy	AY848753	100% <i>A. platys</i>	+	+
<i>A. platys</i> -like	ApllikeCpOv1	<i>Capra hircus</i> Bz1Cp9	Bizerte, Tunisia	KX650597	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp12	Bizerte, Tunisia	KX650600	93% <i>A. platys</i>	–	–
		<i>Ovis aries</i> Bz1Ov11	Bizerte, Tunisia	KX650603	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp7	Bizerte, Tunisia	KX650595	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp8	Bizerte, Tunisia	KX650596	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp10	Bizerte, Tunisia	KX650598	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz2Cp12	Bizerte, Tunisia	KX650609	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> N1Cp1	Nabeul, Tunisia	KX650610	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp1	Bizerte, Tunisia	KX650590	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp3	Bizerte, Tunisia	KX650591	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp4	Bizerte, Tunisia	KX650592	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz1Cp5	Bizerte, Tunisia	KX650593	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz2Cp8	Bizerte, Tunisia	KX650605	93% <i>A. platys</i>	–	–
	<i>Capra hircus</i> Bz2Cp9	Bizerte, Tunisia	KX650606	93% <i>A. platys</i>	–	–	
	<i>Capra hircus</i> Bz2Cp10	Bizerte, Tunisia	KX650608	93% <i>A. platys</i>	–	–	
	<i>Capra hircus</i> Bz1Cp11	Bizerte, Tunisia	KX650599	93% <i>A. platys</i>	–	–	
	<i>Capra hircus</i> Bz1Cp13	Bizerte, Tunisia	KX650601	93% <i>A. platys</i>	–	–	
	ApllikeCpOv2	<i>Ovis aries</i> Bz1Ov9	Bizerte, Tunisia	KX650602	93% <i>A. platys</i>	–	–
		<i>Capra hircus</i> N1Cp8	Nabeul, Tunisia	KX650612	93% <i>A. platys</i>	–	–
	ApllikeCp1	<i>Capra hircus</i> Bz1Cp6	Bizerte, Tunisia	KX650594	92% <i>A. platys</i>	–	–
		<i>Capra hircus</i> N1Cp6	Nabeul, Tunisia	KX650611	92% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz3Cp9	Bizerte, Tunisia	KX650607	92% <i>A. platys</i>	–	–
		<i>Capra hircus</i> Bz2Cp6	Bizerte, Tunisia	KX650604	93% <i>A. platys</i>	–	–
<i>Ovis aries</i> N1Ov30		Nabeul, Tunisia	KX650613	92% <i>A. platys</i>	–	–	
<i>Bos taurus</i> A1Bv6		Ariana, Tunisia	KX650588	85% <i>A. platys</i>	–	–	
<i>Bos taurus</i> A1Bv10		Ariana, Tunisia	KX650589	86% <i>A. platys</i>	–	–	

^a Presence (+) or absence (–) of the restriction site of *Stul* enzyme during *in silico* analysis.
^b Restriction (+) or not (–) of the PCR product with *Stul* enzyme during RFLP assay.

3.3. Molecular characterization of *Anaplasma platys*-like *groEL* variants

After specific detection by PCR-RFLP assay, *A. platys*-like infections were validated by sequencing 476 bp of the *groEL* gene from 27 randomly selected positive ruminant samples (22, 3 and 2 from goats, sheep and cattle, respectively; Table 2). Sequences alignment revealed 9 distinct genotypes out of these 7 were declared as novel (ApllikeCpOv1, ApllikeCpOv2, ApllikeCp1–4, ApllikeOv1, ApllikeBv1 and ApllikeBv2; GenBank accession numbers KX650588 to KX650613) (Table 2). Genotypes were distributed in single or double hosts (goat and sheep) and in one or more sampling sites (Table 2). Nucleotide identity among genotypes ranged from 95.9 to 99.8% (from 96.2 to 100% at the amino acid level). Revealed genotypes were 95.9 to 100% identical on comparison with 18 *A. platys*-like genotypes (from 96.2 to 100% at the amino acid level) isolated from Italian ruminants and cats published in the GenBank. In fact, 94 SNPs were observed representing 19 non synonymous substitutions (Supplementary files 3 and 4). When compared to the *A. platys* reference sequence (GenBank accession number AY848753), identities ranged from 85.7 to 93.3% (nucleotides) and from 98.7 to 100% (amino-acids) (Table 3).

Phylogenetic analysis based on the alignment of the nine genetic variants obtained in this study was compared with all sequence types of *A. platys*-like detected in Italian ruminants and cats, and other *Anaplasma* species found in GenBank (Fig. 3). All revealed Tunisian strains were classified in *A. platys*-like cluster that was closely related to *A. platys* (Fig. 3). The ApllikeBv1 and ApllikeBv2 variants clustered separately from the others in a first separate sub-cluster. Eventually, the remaining Tunisian variants were assigned to the second sub-cluster with all *A. platys*-like Italian variants isolated from ruminants and cats. In particular, variant ApllikeCpOv1 was identical to Caprinecervus1 sequence type isolated from a goat and a red deer (GenBank accession numbers KC335247) and variant ApllikeCp2 was identical to Caprine6 and Feline8 sequence types isolated from a goat and a cat; respectively (GenBank accession numbers KC335252 and KP090285 respectively). All the other Tunisian variants were novel but closely related to Italian variants isolated from ruminants and cats (Zobba et al., 2014, 2015; Fig. 3).

Nucleotide and amino-acid sequence identities were evaluated among all sequences obtained and those of other *Anaplasma* species, namely *A. phagocytophilum* (78.6–80.3% nucleotide sequence identity and 91.8–93.7% amino-acid sequence identity), *A. centrale* (77.5–78.8% and 88.6–92.4%), *A. marginale* (76.7–77.7% and 88.6–90.5%), *A. ovis* (75.6–78.8% and 88.6–90.5%) and *A. bovis* (75.2–77.3% and 88.0–89.9%). The outgroup, *Rickettsia rickettsii*, had 56.3–58.9 and 61.7–62.3% nucleotide and amino-acid sequence identity, respectively (Table 3).

4. Discussion

Despite detection of *A. platys*, the etiologic agent of canine infectious cyclic thrombocytopenia, has been previously performed by hemi-nested PCR based on the *groEL* gene developed by Alberti et al. (2005). In ruminants and cats, this method fails as it detects strains genetically related to, but distinct from *A. platys*. Discrimination of these *A. platys*-like strains from the typical canine pathogen depends therefore on sequencing of hemi-nested PCR products. To date, this is the only method for confirmation and validation of host and vector infections by these strains. In this study, EphplgroEL-F/EplgroEL-R primers along with restriction enzyme digestion proved specific and sensitive for the detection of *A. platys* and related strains complex. By this way, confirmation with post amplification sequencing step to discriminate among *A. platys* and *A. platys*-like strains was eliminated and replaced with *StuI* restriction enzyme digestion that allows discrimination by RFLP assay (Fig. 2). Furthermore, allowing faster and reliable diagnostic method for the estimation of *A. platys* and related strains prevalence. This method has the advantage of potentially allowing co-infecting *A. platys* and *A. platys*-like pathogens in the same sample particularly useful when dealing with ticks, which can be vectors of these two bacteria, if they infest ruminants and dogs sharing in the same area. The use of this direct, specific, and less expensive tool has potential application when performing field epidemiological investigations in resource-poor countries.

Results obtained in this study clearly indicated for the first time, the presence of *A. platys*-like infection in goats, sheep and cattle from Tunisia (Table 1). In goats, overall prevalence rate was 22.8% which is lower

Table 3
Comparison of *groEL* sequences (476 bp) from *Anaplasma* sp. related to *A. platys* isolated from ruminants and other *Anaplasma* species found in GenBank. The numbers represent the nucleotide (shaded dark gray) and amino-acid (shaded clear gray) identity rates found between the sequences.

<i>Anaplasma</i> sp. (Variant)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 <i>A. sp.</i> (ApllikeCpOv1)	100	100	97.5	100	98.7	99.4	99.4	98.7	100	100	93.7	90.5	90.5	90.5	89.9	62.3
2 <i>A. sp.</i> (ApllikeCpOv2)	99.8	100	97.5	100	98.7	99.4	99.4	98.7	100	100	93.7	90.5	90.5	90.5	89.9	62.3
3 <i>A. sp.</i> (ApllikeCp1)	98.9	98.7	100	97.5	96.8	96.8	96.8	96.2	97.5	97.5	91.8	88.6	88.6	88.6	88.0	61.7
4 <i>A. sp.</i> (ApllikeCp2)	99.6	99.4	98.5	100	98.7	99.4	99.4	98.7	100	100	93.7	90.5	90.5	90.5	89.9	62.3
5 <i>A. sp.</i> (ApllikeCp3)	99.6	99.4	98.7	99.2	100	98.1	98.1	97.5	98.7	98.7	98.7	92.4	89.2	89.2	88.6	61.7
6 <i>A. sp.</i> (ApllikeCp4)	99.8	99.6	98.7	99.4	99.4	100	98.7	98.1	99.4	99.4	93.0	89.9	89.9	89.9	89.2	61.7
7 <i>A. sp.</i> (ApllikeOv1)	99.6	99.8	98.5	99.2	99.2	99.4	100	98.1	99.4	99.4	93.0	89.9	89.9	89.9	89.9	61.7
8 <i>A. sp.</i> (ApllikeBv1)	87.0	87.0	85.9	86.6	86.6	86.8	86.8	100	98.7	98.7	93.0	90.5	90.5	90.5	89.9	62.3
9 <i>A. sp.</i> (ApllikeBv2)	88.0	88.0	87.0	88.0	87.6	87.8	87.8	95.2	100	100	93.7	90.5	90.5	90.5	89.9	62.3
10 <i>A. pl</i> (Santiago 17)	93.3	93.1	92.2	92.9	92.9	93.1	92.9	85.7	86.3	100	93.7	90.5	90.5	90.5	89.9	62.3
11 <i>A. p</i> (Horse Sogno)	80.3	80.3	79.8	80.3	79.8	80.3	80.0	78.6	79.0	79.0	100	90.5	90.5	89.9	91.1	61.0
12 <i>A. c</i> (LT)	78.8	78.8	78.2	78.4	78.4	78.6	78.6	78.6	77.5	79.4	77.3	100	100	98.7	89.2	63.0
13 <i>A. m</i> (C7D)	77.5	77.5	76.9	77.1	77.1	77.5	77.3	77.7	76.7	78.2	76.7	98.1	100	98.7	98.2	63.0
14 <i>A. o</i> (OVI)	76.3	76.3	75.6	75.8	75.8	76.3	76.1	78.2	78.8	76.9	77.3	89.7	90.1	100	88.6	63.0
15 <i>A. b</i> (Kh-Hc215)	75.8	75.8	75.2	75.4	75.4	75.6	75.6	76.3	77.3	77.3	76.9	73.3	73.3	71.6	100	61.7
16 <i>R. r</i> (R)	58.7	58.7	58.9	58.7	58.7	58.7	58.5	56.6	56.3	56.8	57.2	54.2	53.8	53.8	56.8	100

A. sp. (ApllikeCpOv1, ApllikeCpOv2, ApllikeCp1–4, ApllikeOv1, ApllikeBv1 and ApllikeBv2): *Anaplasma sp.* (*A. platys*-like strains) isolated from Tunisian ruminants (ApllikeCpOv1, ApllikeCpOv2, ApllikeCp1–4, ApllikeOv1, ApllikeBv1 and ApllikeBv2, GenBank accession numbers KX650588 to KX650613). *A. pl* (Santiago 17): *A. platys* isolate found on dog from Chile (Santiago 17 isolate, GenBank accession number EF201806); *A. p* (Horse Sogno): *A. phagocytophilum* isolate found on Italian horse (Horse Sogno isolate, GenBank accession number AY848749); *A. c* (LT): *A. centrale* strain isolated on *Rhipicephalus simus* tick from South Africa (LT strain, GenBank accession number AF414866); *A. m* (C7D): *A. marginale* isolate found on *Rhipicephalus microplus* tick from Philippines (C7D isolate, GenBank accession number JQ839003); *A. o* (OVI): *A. ovis* strain isolated from South African sheep (OVI strain, GenBank accession number AF441131); *A. b* (Kh-Hc215): *A. bovis* clone found on *Haemaphysalis concinna* tick from Russia (Kh-Hc215 clone, GenBank accession number JX092095) and *R. r* (R): *R. rickettsii* strain isolated from Human in USA (R strain, GenBank accession number U96733).

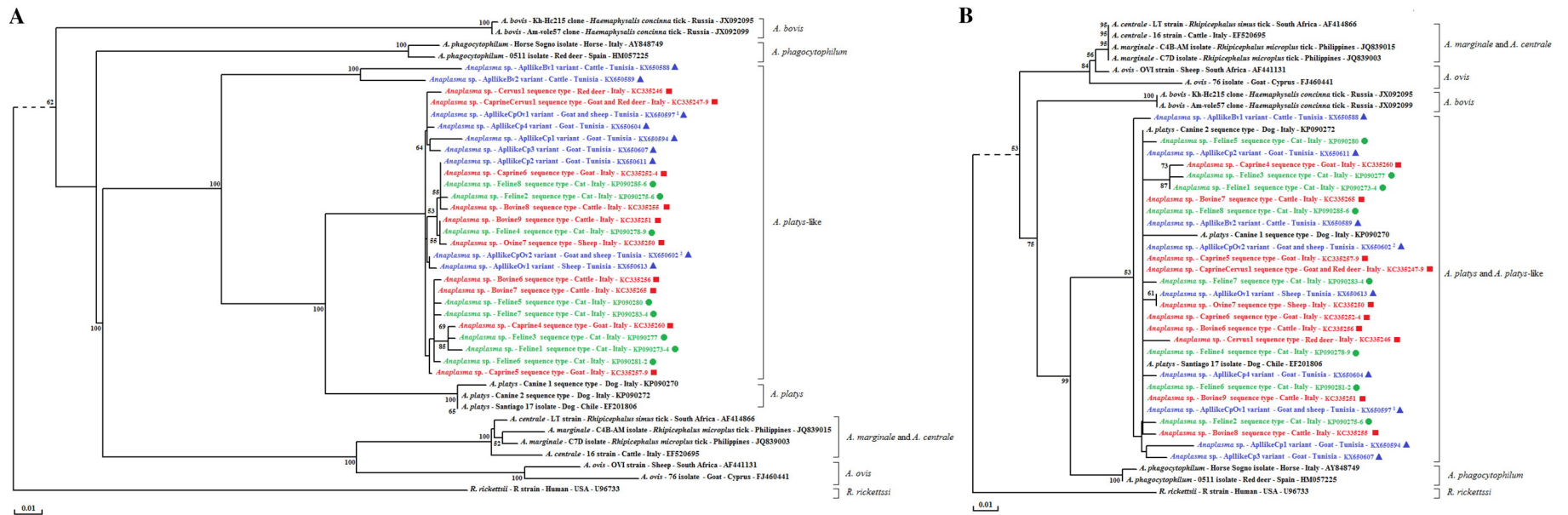


Fig. 3. Neighbor-joining tree based on nucleotide (A) and amino-acid (B) multiple alignments of partial *Anaplasma* spp. groEL sequences (476 bp). Numbers over the branches indicate the percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (1000 replicates, only percentages >50% were represented). The 9 *A. platys*-like sequences obtained in this study are represented in blue and marked with triangles. Ruminant related sequence types of *A. platys* Italian strains published by [Zobba et al. \(2014\)](#) are represented in red and marked with rectangles. Feline related sequence types of *A. platys* Italian strains published by [Zobba et al. \(2015\)](#) are represented in green and marked with circles. The host or vector, the variant, sequence type, strain or isolate name, the country of origin and GenBank accession number are indicated. ¹ApllikeCpOv1 sequence was deposited in the GenBank (KX650590 to KX650593, KX650595 to KX650601, KX650603, KX650605, KX650606 and KX650608 to KX650610). ²ApllikeCpOv2 variant was also deposited under accession number KX650612. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than that found in Italian goats (79.4%) (Zobba et al., 2014). In sheep, *A. platys*-like prevalence (overall 11%) was lower than that found in the investigated goats, and in goats from Italy (Zobba et al., 2014) but higher to that found in studied cattle (3.5%), and in cattle from Algeria (4.8%) (Dahmani et al., 2015). It can be postulated that this difference could be related to the susceptibility of each animal species to *A. platys*-like infection and differences in infestation rates by their tick vectors. This discrepancy in infection rates has been reported in other studies on *A. ovis* and *A. bovis* between Tunisian sheep and goats (Ben Said et al., 2015a, 2015b). By combining PCR to 16S rRNA or groEL sequence analyses, *A. platys*-like has been detected in several domesticated ruminants from many countries. It has been isolated from goats in China (Liu et al., 2012), from sheep in Italy (Zobba et al., 2014), from cattle in Italy (Zobba et al., 2014) and China (Yang et al., 2015), and from dromedaries in Tunisia (Belkahlia et al., 2015b) and South Africa (Bastos et al., 2015).

For each ruminant species, overall prevalence rate differed statistically among governorates and also between delegations (Table 1). This discrepancy may essentially result from differences in tick control programs, husbandry practices, farm organization, wildlife reservoir hosts, and/or abiotic factors. In fact, several studies reported the variability of *Anaplasma* species prevalences according to geographic position, associated with appropriate tick environment and animal management (Liu et al., 2012; Belkahlia et al., 2014, 2015a; Ben Said et al., 2015a, 2015b).

Although the dissimilarity in bioclimatic characteristics among the three investigated areas, the difference in the prevalence rates is not statistically significant (Supplementary file 1, $p > 0.05$). This finding is probably due to the frequent ruminant movement between these areas as well as the similarity of tick populations, especially belonging to *Rhipicephalus* (Bouattour, 2002), infesting each ruminant species in sampling locations. Some studies suggest that one or several tick species of this genus could be vectors of *A. platys*-like strains. Notably, *Rhipicephalus* spp. infesting camels have been previously proposed as vectors of *A. platys*-like in China (Li et al., 2015b). Earlier, *A. platys* and/or related strains (reported as *A. platys*) has been isolated from *R. bursa* feeding on goats from Turkey (Aktas et al., 2009) and *Rhipicephalus* spp. infesting cattle in Malaysia (Tay et al., 2014). Until now, the vectors of *A. platys*-like are unknown in Tunisia; accordingly, additional studies are required to recognize the main vectors of these unclassified *Anaplasma* strains.

Sequencing of 476 bp of the *groEL* gene identified from randomly selected *Anaplasma* sp. positive ruminants revealed nine different *Anaplasma* sp. variants, seven of which are novel. RFLP data (Table 2), nucleotide and amino-acid alignments (Supplementary files 3 and 4), and percent sequence identity comparison (Table 3) of the *groEL* sequence variants obtained in this study confirm that Tunisian ruminants are infected by *A. platys*-like strains, with extensive nucleotide diversity observed between ApllikeBv1 and ApllikeBv2 variants, and other *A. platys*-like variants revealed in this study, or published in the GenBank. All Tunisian sequence variants shared higher similarity with the *groEL* sequences of *A. platys*-like found in Italian ruminants and cats (Zobba et al., 2014, 2015) (Tables 2 and 3).

Phylogenetic analysis of *groEL* based on alignments of *Anaplasma* sp. partial sequences isolated from Tunisian ruminants, and selected sequences of *Anaplasma* species obtained from the GenBank confirmed the observations obtained with BLASTN analysis (Table 2), and percent sequence identity comparison (Table 3). In agreement with Zobba et al. (2015), phylogenetic trees based on nucleotide and amino acid *groEL* sequences show that all the sequences isolated from Tunisian ruminants group in a monophyletic clade including Italian ruminants and cats (Fig. 3), despite the fact that ruminant strains show tropism for neutrophil granulocytes (Zobba et al., 2014), and feline strains preferentially infect platelets (Zobba et al., 2015). Since none of the investigated animals showed any apparent clinical sign, these strains seem to have a limited pathogenicity in ruminants and in other domesticated animals. Actually, this finding is in agreement with those reported by Zobba et

al. (2014) in sheep, goats and cattle, Belkahlia et al. (2015b), Bastos et al. (2015) and Li et al. (2015b) in camels, and Zobba et al. (2015) in cats.

In conclusion, this study indicates, for the first time, the occurrence of *A. platys*-like strains in goats, sheep and cattle from various Tunisian bioclimatic areas. Present data open new concerns about the specificity of serological and molecular tools traditionally employed to identify diverse *Anaplasma* species in wild and domesticated ruminants, and offer helpful molecular information to clarify the evolutionary history of bacterial strains genetically related to *A. platys*. Further studies are required to improve molecular characterization of these different strains by more discriminative genes, and to recognize the main vectors involved in the transmission of this unclassified *Anaplasma* species in Mediterranean areas.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meegid.2017.01.023>.

Competing interests

The authors declare that they have no competing interests.

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