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First molecular survey and novel genetic variants' identification of *Anaplasma marginale*, *A. centrale* and *A. bovis* in cattle from Tunisia

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ABSTRACT

Few data are available about the presence and distribution of *Anaplasma* species in cattle in North African countries. In this study prevalence, co-infections, risk factors and genetic diversity of *Anaplasma* species were evaluated in bovines from Northern Tunisia. A total of 232 cattle from 36 randomly selected farms in three Tunisian localities were investigated for the presence of *Anaplasma* species in blood by Real-time PCR and/or nested PCR. Overall infection rates of *Anaplasma* spp., *Anaplasma marginale*, *Anaplasma centrale* and *Anaplasma bovis* were 34.9%, 25.4%, 15.1%, and 3.9%, respectively. *Anaplasma phagocytophilum* was not detected in cattle. The most common co-infection pattern was an association of *A. marginale* and *A. centrale* (11.2%). Five cattle (2.1%) all reared in the sub-humid bioclimatic area, were co-infected by the three *Anaplasma* species. Molecular prevalence of *Anaplasma* infection varied significantly according to locality, bioclimatic area, tick infestation and type of breeding. Animals of the Holstein breed were less infected by *A. marginale* and *A. centrale* than other breeds. Genetic analysis of *A. marginale msp4* gene indicated a high sequence diversity of Tunisian strains, suggesting a multiple introduction of infected cattle from different origins. Phylogenetic studies based on the 16S rRNA gene showed that the most prevalent *A. centrale* strains were closely related to the *A. centrale* vaccine strain. Moreover, all *A. bovis* variants clustered with other *A. bovis* sequences obtained from domestic and wild ruminant strains. This is the first molecular investigation on *Anaplasma* species in Tunisian cattle providing pivotal background for designing epidemiological studies and to develop control strategies in the country.

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1. Introduction

Bovine anaplasmosis is a tick-borne rickettsial disease caused by *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma phagocytophilum*, and *Anaplasma bovis* (Dumler et al., 2001; Rar and Golovljova, 2011). *A. marginale*, the type species of *Anaplasma* genus, infects erythrocytes; it is highly pathogenic and widely distributed in tropical and subtropical regions (Kocan et al., 2003).

In cattle and wild ruminants, *A. marginale* causes disease, its severity increases with the age of the animal, since calves are much more resistant than adults (Kocan et al., 2003). In cattle aged of more than 2 years, *A. marginale* causes mild or severe disease characterized by persistent fever, lethargy, icterus, weight loss, abortion, milk yield decrease and death of more than 50% of untreated animals (Kocan et al., 2003). In the Mediterranean area, *A. marginale* was recorded in cattle from Morocco (Ait Hamou et al., 2012), Egypt (El-Ashker et al., 2015), Turkey (Aktas et al., 2011) and *Italy* (Carelli et al., 2007; Torina et al., 2008a,b; Zobba et al., 2014).

A. centrale, an intraerythrocytic species closely related to *A. marginale*, is often considered as a subspecies of *A. marginale* (Dumler et al., 2001). *A. centrale*, which is less pathogenic to *A. marginale*, causes mild symptoms in cattle and is considered as a naturally attenuated subspecies (Rar and Golovljova, 2011). That's why it has been used extensively as a live vaccine against

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A. marginale in several countries (Kocan et al., 2010). The infection with *A. centrale* provides a significant and long-lasting protective immunity against a challenge with highly virulent *A. marginale* strains (Kocan et al., 2003). Until now, *A. centrale* was identified only in cattle located in the North Mediterranean countries such as Italy (Georges et al., 2001; Carelli et al., 2007; Ceci et al., 2014; Zobba et al., 2014) and Turkey (Aktas et al., 2011).

A. phagocytophilum is an obligate intracellular parasite that infects granulocytes. It causes tick-borne fever (TBF) in ruminants (Rikihisa, 1991) characterized by high fever; respiratory symptoms, leucopenia, abortion and sudden milk yield decrease (Stuen, 2007). It has been reported in cattle from Czech Republic (Hulínská et al., 2004), France (Laloy et al., 2009), Switzerland (Hofmann-Lehmann et al., 2004), Italy (Torina et al., 2008a,b), Spain (de la Fuente et al., 2005b; Naranjo et al., 2006), Guatemala (Teglas et al., 2005) and Japan (Ooshiro et al., 2008; Jilintai et al., 2009; Murase et al., 2011; Ybañez et al., 2013; Zhang et al., 2012a,b).

A. bovis, a monocytotropic species, has been detected in several ruminants from many countries (Sreekumar et al., 1996; Liu et al., 2012). It has been isolated from cattle and deer in Japan (Kawahara et al., 2006; Ooshiro et al., 2008; Jilintai et al., 2009), cattle in Iran (Noaman and Shayan, 2010), water deer in South Korea (Kang et al., 2011) and goats in China (Liu et al., 2012). *A. bovis* infection has been documented as asymptomatic but it can cause fever, anaemia, weight loss, and occasionally abortion and death (Donatien and Lestoquard, 1936). Recently *Anaplasma ovis*, the causative agent of ovine anaplasmosis, has been detected and characterized in sheep from Northern and Central Tunisia (Belkahia et al., 2014).

To date, there are only few data regarding the presence of *Anaplasma* species in Tunisian livestock, especially in ruminants. Indeed, few molecular findings demonstrate the occurrence of *A. phagocytophilum* infections in dogs and horses (M'ghirbi et al., 2009, 2012). *A. phagocytophilum* was also reported in Tunisian horses and dromedaries by immunofluorescence antibody test (IFAT) (Ben Said et al., 2014a,b).

Aims of this study were to evaluate the prevalence, co-infections, risk factors, and genetic variability of *Anaplasma* species in cattle from three Tunisian localities belonging to 2 bioclimatic areas.

2. Materials and methods

2.1. Cattle population and study regions

Between July and December 2012, blood was collected from 232 cattle (199 females and 33 males) reared in 36 farms in three localities of the Bizerte governorate (Northern Tunisia): (i) Utique (37°16′N, 9°52′E) a semi-arid area with a mean annual rainfall of 400 mm, (ii) El Alia (37°16′N, 10°03′E) and Mateur (37°02′N, 9°39′E) both belonging to sub-humid bioclimatic area with a mean annual rainfall of 600 mm (Fig. 1).

2.2. Blood sampling, ticks collection and DNA extraction

Blood samples were collected from the jugular vein in EDTA tubes. For each animal, breeding type, gender, age and presence or absence of ticks were noted. DNA was extracted from 300 μl volume 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) and stored at −20 °C until use.

2.3. Duplex real time PCR

DNA samples were tested for the presence of *A. marginale* and *A. centrale* with species-specific primers and TaqMan probes as described by Carelli et al. (2007) and Decaro et al. (2008), respectively (Table 1). Briefly, 2 fragments of 95 and 77-bp, corresponding respectively to the *A. marginale msp1b* and *A. centrale groEL* genes were targeted in a real time PCR reaction performed by using Premix Ex Taq™ (Perfect Real Time, Takara Mirus Bio, Madison, WI, USA) in a 7500/7500 Fast Real-Time PCR System quantitative thermal cycler (Applied Biosystems, USA). The simultaneous amplification of *A. marginale* and *A. centrale* detection was obtained by 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), in a duplex format. Thermal cycling conditions included an initial activation of *Taq* DNA polymerase at 95 °C for 15 min followed by 45 cycles of 1 min denaturation at 95 °C and 1 min annealing-extension step at 60 °C. Distilled water and DNA bovine samples positive to *A. marginale* and *A. centrale* (Zobba et al., 2014) were included in all PCR runs.

2.4. Single and nested PCR

Primer pair EE1 and EE2 was used in a first PCR for amplifying the 16S rRNA gene of all *Anaplasma* species potentially present in cattle (Barlough et al., 1996) (Table 1). PCR 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 genomic DNA, 0.5 μM of primers. Thermal cycling profile was as described by Liu et al. (2012). *A. phagocytophilum*, *A. centrale* and *A. bovis* specific primers were used in nested PCR for strains detection and characterisation (Table 1). One microliter of the first PCR product was used as target DNA in the nested PCRs, thermally profiled as in Kawahara et al. (2006). Distilled water and DNA extracted from *A. phagocytophilum*, *A. marginale*, *A. centrale* and *A. bovis* were used as negative and positive controls, respectively. PCR products were electrophoresed in 1% agarose gel and sized with 1 Kb Plus DNA Ladder (Promega, Madison, WI, USA).

For *A. marginale* by *msp4* genotyping, bovine samples positive to *A. marginale* by real-time PCR were used in a traditional PCR with MSP45 and MSP43 primers (de la Fuente et al., 2005b, 2007a,b) (Table 1). Shortly, reactions contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.125 U/μl Taq DNA Polymerase (Biobasic Inc, Canada), 2 μl (1–10 ng) DNA, 0.5 μM of the primers, and milliQ sterile water to a total volume of 50 μl. In each experiment distilled water and DNA extracted from *A. marginale* (Zobba et al., 2014) were used as negative and positive controls, respectively. Thermal cycling reactions were performed as described by de la Fuente et al. (2005b, 2007a,b). PCR products were electrophoresed in 1.5% agarose gel.

2.5. DNA sequencing and data analysis

A selection of PCR products obtained with primers MSP45/MSP43, AC1f/AC1r and AB1f/AB1r and respectively representative of *A. marginale* (15 amplicons), *A. centrale* (18 amplicons) and *A. bovis* (5 amplicons), 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 1). The reaction was 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 (Macrogen Europe, Amsterdam, The

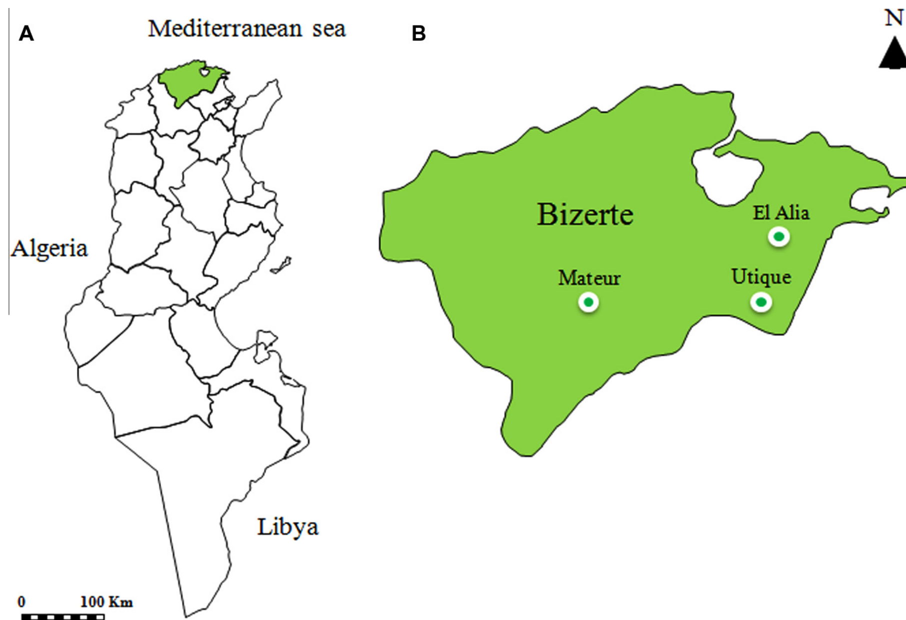

Fig. 1. Map of the Tunisian studied localities. (A) Map of Tunisia showing the governorate of Bizerte and (B) Map of Bizerte governorate showing the location of El Alia, Utique and Mateur localities.

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

Assay	Primer/probe	Sequence 5′ to 3′	Target gene	Amplicon size (bp)	References
Duplex real-time PCR					
A. marginale	AM-For	TTGGCAAGGCAGCAGCTT	*msp1b*	95	Carelli et al. 2007)
	AM-Rev	TTCCGCGAGCATGTGCAT			
	AM-Pb[c]	6FAM-TCGGTCTAACATCTCCAGGCTTTCAT-6TAMRA			
A. centrale	AC-For	CTATACACGCTTGCATCTC	*groEL*	77	Decaro et al. (2008)
	AC-Rev	CGCTTTATGATGTTGATGC			
	AC-Pb[d]	VIC-ATCATCATTCTTCCCCTTTACCTCGT-6TAMRA			
PCR 1[a]					
Anaplasma spp.	EE-1	TCCTGGCTCAGAACGAACGCTGGCGGC	16S ARNr	1433	Barlough et al. (1996)
	EE-2	AGTCACTGACCCAACCTTAAATGGCTG			
PCR 2[b]					
A. phagocytophilum	SSAP2f[e]	GCTGAATGTGGGGATAATTTAT	16S ARNr	641	Kawahara et al. (2006)
	SSAP2r[e]	ATGGCTGCTTCCTTTCGGTTA			
A. bovis	AB1f[e]	CTCGTAGCTTGCTATGAGAAC	16S ARNr	551	Kawahara et al. (2006)
	AB1r[e]	TCTCCCGGACTCCAGTCTG			
A. centrale	AC1f[e]	CTGCTTTTAATACTGCAGGACTA	16S ARNr	426	Kawahara et al. (2006)
	AC1r[e]	ATGCAGCACCTGTGTGAGGT			
PCR					
A. marginale	MSP45[f]	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	*msp4*	852	de la Fuente et al. (2005a,b)
	MSP43[f]	CCGGATCCTTAGCTGAACAGGAATCTTGC			

[a] First PCR allowing the detection of all *Anaplasma* species.
[b] Second PCR allowing the species detection of *A. phagocytophilum*, *A. bovis* and *A. centrale*.
[c] 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 Carelli et al. (2007).
[d] The reporter and quencher dye fluorophores for *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. (2008).
[e] Primers used in PCR and sequencing reactions for the detection and the characterization of *A. phagocytophilum* and *A. bovis* and the characterization of *A. centrale*.
[f] Primers used in PCR and sequencing reactions for the characterization of *A. marginale*.

Netherlands). Chromatograms were edited with Chromas Lite v 2.01. The DNAMAN program (Version 5.2.2; Lynnon Biosoft, Que., Canada) was used to perform multiple sequence alignment of 16S rRNA and *msp4* sequences and to translate nucleotide to aminoacid MSP4 sequences. Similarity searches were conducted by using BLAST (http://blast.ncbi.nlm.nih.gov, Altschul et al., 1997). DNAMAN program was also used to calculate genetic distances computed by the maximum composite likelihood method (Tamura and Nei, 1993). Neighbor-joining trees were built by using the same software (Saitou and Nei, 1987). Statistical support for internal branches was established by bootstrap analysis with 1000 reiterations.

2.6. Sequence accession numbers

The *msp4* partial sequences of *A. marginale* AmGBv1 to AmGBv9 variants have been deposited in the GenBank under accession numbers KJ512166 to KJ512174. The 16S rRNA partial sequences of *A. centrale* AcGBv1 to AmGBv6 variants have been deposited under GenBank accession numbers KM401896 to KM401901.

Finally, 16S rRNA partial sequences of *A. bovis* AbGBv1 to AbGBv3 variants have been deposited under GenBank accession numbers KM401902 to KM401905.

2.7. Statistical analyses

Exact confidence intervals (CI) for prevalence rates at the 95% level were calculated. Comparison of the prevalence of *Anaplasma* species and co-infections among different groups for each risk factor and among different localities and bioclimatic areas were performed using the χ^2 and Fisher's exact tests, with the Epi Info 6.01 software (CDC, Atlanta). Observed differences were considered to be statistically significant at a 0.05 threshold value. In order to consider any confusion factor, a chi square Mantel–Haenszel test was performed.

3. Results

3.1. Molecular survey of Anaplasma species

The overall prevalence of *Anaplasma* spp., *A. marginale*, *A. centrale* and *A. bovis* were respectively 34.9%, 25.4%, 15.1%, and 3.9%. *A. phagocytophilum* was not detected in any of the tested cattle. Cattle from El Alia and Mateur (sub-humid area) were more infected by *A. marginale* (31.5%), *A. centrale* (20.5%) and *A. bovis* (6.2%) than those from Utique (semi-arid area) (15.1; 5.8% and 0% respectively) ($p = 0.006$, $p = 0.002$ and $p = 0.018$ respectively) (Table 2). Cattle infested by ticks were statistically more infected by *A. marginale* (47.6%), *A. centrale* (33.3%) and *A. bovis* (23.8%) compared to those free of ticks (23.2; 13.3% and 1.9% respectively) ($p = 0.014$, $p = 0.014$ and $p < 0.001$ respectively). In addition, cattle reared in traditional premises were statistically more infected by *A. marginale* (55.6%), *A. centrale* (37.0%) and *A. bovis* (14.8%) compared to those living in modern premises (21.5; 12.2% and 2.4% respectively) ($p < 0.001$, $p < 0.001$ and $p = 0.001$ respectively). Furthermore, Holstein cattle were significantly less infected by *A. marginale* ($p < 0.001$) and *A. centrale* ($p < 0.001$) than other breeds (Table 2). The most common co-infection pattern was *A. marginale* and *A. centrale* (11.2%), which reached 21% in cattle from Mateur (governorate of Bizerte) (Table 3). Most of co-infected animals (96.1%) belonged to the sub-humid region. The second most common co-infection patterns were *A. marginale* and *A. bovis*, and *A. centrale* and *A. bovis*, estimated to 2.6%. Five cattle (2.2%) were co-infected by the three *Anaplasma* species and the highest co-infection rate was reported in cattle from El Alia locality (governorate of Bizerte) (5.7%) belonging to sub-humid area (Table 3).

3.2. Molecular characterization

3.2.1. A. marginale msp4 variants

A. marginale infections were validated by sequencing 805 bp (94.7%) of the *msp4* gene from 15 randomly selected positive cattle samples, five from each of the sampling regions. Sequences alignment revealed 9 distinct and novel genotypes (AmGBv1 to 9; GenBank accession numbers KJ512166 to KJ512174). Genotypes were distributed in single or multiple hosts and in one or more sampling sites (Table 4). Average nucleotide identity among genotypes was 98.5% (99.9% at the amino acid level). Fifteen SNPs were observed representing 5 non synonymous substitutions (Table 4). Genotypes were 98.1% to 99.9% homologous on comparisons with *A. marginale* genotypes published in the GenBank. When compared to the *A. centrale* reference sequence (GenBank accession number AF428090), identities ranged from 82.6 to 83.2 (nucleotides) and from 88.1% to 88.4% (amino acids).

Phylogenetic analysis based on the alignment of the 9 genotypes obtained in this study with 51 sequences obtained from the GenBank (representing the genetic variability of *A. marginale*) originated 3 main clusters (Fig. 2). The first cluster included strains from Africa (Nigeria, Zimbabwe, Kenya and South Africa), North America (represented exclusively by USA) and Southern Europe (Italy and Spain). The second cluster contained strains from Latin

Table 2
Molecular prevalence results of *Anaplasma marginale*, *A. centrale* and *A. bovis* according to bioclimatic zone, locality, gender, age, tick infestation and breed of cattle.

	Number	*A. marginale*		*A. centrale*		*A. bovis*	
		Positive (% ± C.I.[a])	*P*-value	Positive (% ± C.I.[a])	*P*-value	Positive (% ± C.I.[a])	*P*-value
Bioclimatic zone			0.006*		0.002*		0.018*
Sub-humid	146	46 (31.5 ± 0.07)		30 (20.5 ± 0.06)		9 (6.2 ± 0.03)	
Semi-arid	86	13 (15.1 ± 0.08)		5 (5.8 ± 0.05)		0 (0)	
Locality			0.003*		0.010*		0.000*
El Alia	70	17 (24.3 ± 0.10)		14 (20.0 ± 0.09)		8 (11.4 ± 0.07)	
Mateur	76	29 (38.2 ± 0.11)		16 (21.0 ± 0.09)		1 (1.3 ± 0.02)	
Utique	86	13 (15.1 ± 0.08)		5 (5.8 ± 0.05)		0 (0)	
Gender			0.865		0.288		0.785
Male	33	8 (24.2 ± 0.15)		7 (21.2 ± 0.14)		1 (3.0 ± 0.05)	
Female	199	51 (25.6 ± 0.06)		28 (14.1 ± 0.05)		8 (4.0 ± 0.02)	
Age			0.083		0.736		0.297
<1 year	30	7 (23.3 ± 0.15)		4 (13.3 ± 0.12)		0 (0)	
⩾1 and <3 years	44	16 (36.3 ± 0.14)		9 (20.4 ± 0.12)		0 (0)	
⩾3 and ⩽7 years	112	30(26.8 ± 0.08)		16(14.3 ± 0.06)		5(4.5 ± 0.03)	
>7 years	46	6(13.0 ± 0.09)		6(13.0 ± 0.09)		4(8.7 ± 0.08)	
Breed			0.004*		0.000*		0.132
Holstein	196	43 (21.9 ± 0.05)		22 (11.2 ± 0.04)		6 (3.1 ± 0.02)	
Other breeds[b]	36	16 (44.4 ± 0.16)		13 (36.1 ± 0.15)		3 (8.3 ± 0.09)	
Tick infestation			0.014*		0.014*		0.000*
Infested	21	10 (47.6 ± 0.21)		7 (33.3 ± 0.20)		5 (23.8 ± 0.18)	
Not infested	211	49 (23.2 ± 0.06)		28 (13.3 ± 0.05)		4 (1.9 ± 0.01)	
Breeding type			0.000*		0.000*		0.001*
Traditional	27	15 (55.6 ± 0.19)		10 (37.0 ± 0.18)		4 (14.8 ± 0.13)	
Modern	205	44 (21.5 ± 0.06)		25 (12.2 ± 0.05)		5 (2.4 ± 0.02)	
Total	232	59 (25.4 ± 0.06)		35 (15.1 ± 0.05)		9 (3.9 ± 0.02)	

[a] C.I.: 95% confidence interval.
[b] Charolais, Schwytz, Tarentaise and Cross-bred.
* Statistically significant test.

Table 3
Co-infection rates of *Anaplasma marginale*, *A. centrale* and *A. bovis* according to bioclimatic areas and localities.

Bioclimatic area	Locality	Number	Co-infected cattle (% ± C.I.[a])			
			A.m[b]/*A.c*[c]	*A.m*[b]/*A.b*[d]	*A.c*[c]/*A.b*[d]	*A.m*[b]/*A.c*[c]/*A.b*[d]
Sub-humid	El Alia	70	9 (12.9 ± 0.07)	5 (7.1 ± 0.06)	5 (7.1 ± 0.06)	4 (5.7 ± 0.05)
	Mateur	76	16 (21.0 ± 0.09)	1 (1.3 ± 0.02)	1 (1.3 ± 0.02)	1 (1.3 ± 0.02)
	Total	146	25 (17.1 ± 0.06)	6 (4.1 ± 0.03)	6 (4.1 ± 0.03)	5 (3.4 ± 0.02)
Semi-arid	Utique	86	1 (1.2 ± 0.02)	0 (0)	0 (0)	0 (0)
Total		232	26 (11.2 ± 0.04)	6 (2.6 ± 0.01)	6 (2.6 ± 0.01)	5 (2.2 ± 0.01)

[a] C.I.: 95% confidence interval.
[b] *A .m: Anaplama marginale.*
[c] *A.c: Anaplama centrale.*
[d] *A.b: Anaplama bovis.*

Table 4
Nucleotide and amino acid differences among *msp4* sequences from *Anaplasma marginale* strains.

Variant[a]	Genbank [b]	*msp4* nucleotide positions (amino acid positions) [c]														
		81	87	148 (50)	206 (69)	270	312	324	354	384 (128)	397 (133)	423	489	564	742 (248)	798
AmGBv1	KJ512166	G	A	G (G)	G (S)	A	A	G	G	C (S)	C (A)	A	C	G	A (I)	G
AmGBv2	KJ512167	A	*	*	*	G	G	A	A	*	*	G	*	A	*	*
AmGBv3	KJ512168	*	*	*	*	G	G	A	*	*	*	G	*	A	C (L)	A
AmGBv4	KJ512169	A	*	*	*	G	G	A	*	*	*	*	*	*	*	A
AmGBv5	KJ512170	*	*	*	*	*	*	*	*	*	*	*	T	*	*	A
AmGBv6	KJ512171	*	*	*	*	G	G	A	*	*	*	G	*	A	*	A
AmGBv7	KJ512172	A	*	*	*	G	G	A	*	*	*	*	T	*	*	*
AmGBv8	KJ512173	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*
AmGBv9	KJ512174	A	G	A (S)	A (N)	*	*	*	A	G (R)	G (G)	G	T	A	*	A

Amino acids: G, Glycine; S, Serine; A, Alanine; I, Isoleucine; L, Leucine; R, Arginine; N, Asparagine; Nucleotides: T, Thymine; C, Cytosine; G, Guanine; A, Adenine.
[a] AmGBv1 variant was isolated from Ut2 sample; AmGBv2 variant was isolated from Ut3 sample; AmGBv3 variant was isolated from Ut1; Ut4 and Ut5 samples; AmGBv4 was isolated from Mt1 sample; AmGBv5 variant was isolated from Mt2 sample; AmGBv6 variant was isolated from Mt3 sample; AmGBv7 variant was isolated from Mt4 sample; AmGBv8 variant was isolated from Mt5; Al1; Al2; Al3 and Al5 samples and AmGBv9 variant was isolated from Al4 sample. Mt1-Mt5, Ut1-Ut5 and Al1-Al5 samples were collected from Mateur, Utique and El Alia localities, respectively.
[b] GenBank accession number.
[c] Numbers represent the nucleotide position starting at translation initiation codon Adenine with respect to the Stillwater 2 strain for *Anaplasma marginale* from USA (GenBank accession number JN558825) (de la Fuente et al., 2003b). Conserved nucleotide positions relative to the first sequence are indicated with asterisks. Amino acid changes are indicated between parentheses with single letter code.

America (e.g. Mexico, Brazil, Venezuela and Argentina), Asia (like Taiwan and Australia) and Southern Europe (such as Italy). The last included two strains from Hungary. Tunisian strains were assigned to the first (8 genotypes) and to the third (1 genotype) clusters. In particular genotypes AmGBv1, AmGBv5, and AmGBv8, clustered in a sub-cluster with the Italian strain Bs19. AmGBv2 clustered in a second sub-cluster with three American strains (Saint Maries, California and Stillwater). AmGBv3 and AmGBv6 genotypes clustered in a third sub-cluster with strain Sp9 from Spain. AmGBv7 clustered in the African sub-cluster with three strains from Zimbabwe (2–4, 3–9 and 1–6 strains), one strain from South Africa (SWA strain) and one from Kenya (#661kari strain). AmGBv4 clustered in a fifth sub-cluster with the Oklahoma strain from USA. Eventually, AmGBv9 genotype clustered separately from the others in the third cluster with 2 Hungarian isolates (50(G16) and 51(G18) strains.

3.2.2. *A. centrale* 16S rRNA variants

A. centrale infections were validated by sequencing 383 bp (25.6%) of the 16S rRNA gene from 18 randomly selected positive samples (8 from El Alia, 7 form Mateur, and 3 from Utique). Alignment of these sequences allowed the identification of 5 novel 16S rRNA gene variants (AcGBv1 to AcGBv6; GenBank accession numbers KM401896 to KM401901) differing by six nucleotide substitutions (Table 5). 16S rRNA gene variants obtained in this study were 99.2% to 99.7% homologous and were 99.2% to 100% similar to the *A. centrale* genotypes published in GenBank. Nucleotide homology decreased (98.4% to 99.2%) when the novel 16S rRNA gene variants were compared to an *A. marginale* sequence isolated from Japanese cattle (Ishigaki-2007 strain).

Upon phylogenetic analyses all *A. centrale* strains clustered in a single group, relatively distant from *A. marginale* and *A. ovis*. Tunisian variants clustered into three clusters (Fig. 3). Four variants (AcGBv1, AcGBv2, AcGBv5 and AcGBv6) clustered in a first cluster with the majority of the other *A. centrale* variants included in the phylogenetic analyses. The *A. centrale* Tunisian variant AcGBv2 was the most represented in positive samples and was identical to a variant identified in cattle and buffaloes from Europe and Africa. AcGBv3 variant clustered in a second cluster with the Japanese variant (strain Aomori) while AcGBv4 clustered separately in a third cluster.

3.2.3. *A. bovis* 16S rRNA variants

Sequencing of 511 bp (34.2%) of the 16S rRNA from five randomly selected positive cattle samples validated *A. bovis* infection. Based on alignment 3 distinct variants were identified (AbGBv1 to AbGBv3; GenBank accession numbers KM401902 to KM401904). Two out of 3 showed a degree of nucleotide diversity when compared to published sequences and were considered new (Table 5). Variants differed in 2 nucleotide positions substitutions and where 99.6% to 99.8% homologous. *A. bovis* variants obtained this study were 99.6% to 100% homologous to the *A. bovis* genotypes published in the GenBank. Phylogenetic trees showed that all *A. bovis* variants clustered a monophyletic cluster distinct from other *Anaplasma* species and closely related to *Anaplasma platys* and *A. phagocytophilum* (Fig. 4). All *A. bovis* Tunisian variants were included in this cluster, albeit AbGBv1 and AbGBv2 variants clustered separately, while AbGBv3 variant clustered with two variants isolated from Japanese and South Korean deer and one variant isolated from *Haemaphysalis longicornis* in South Korea (Fig. 4).

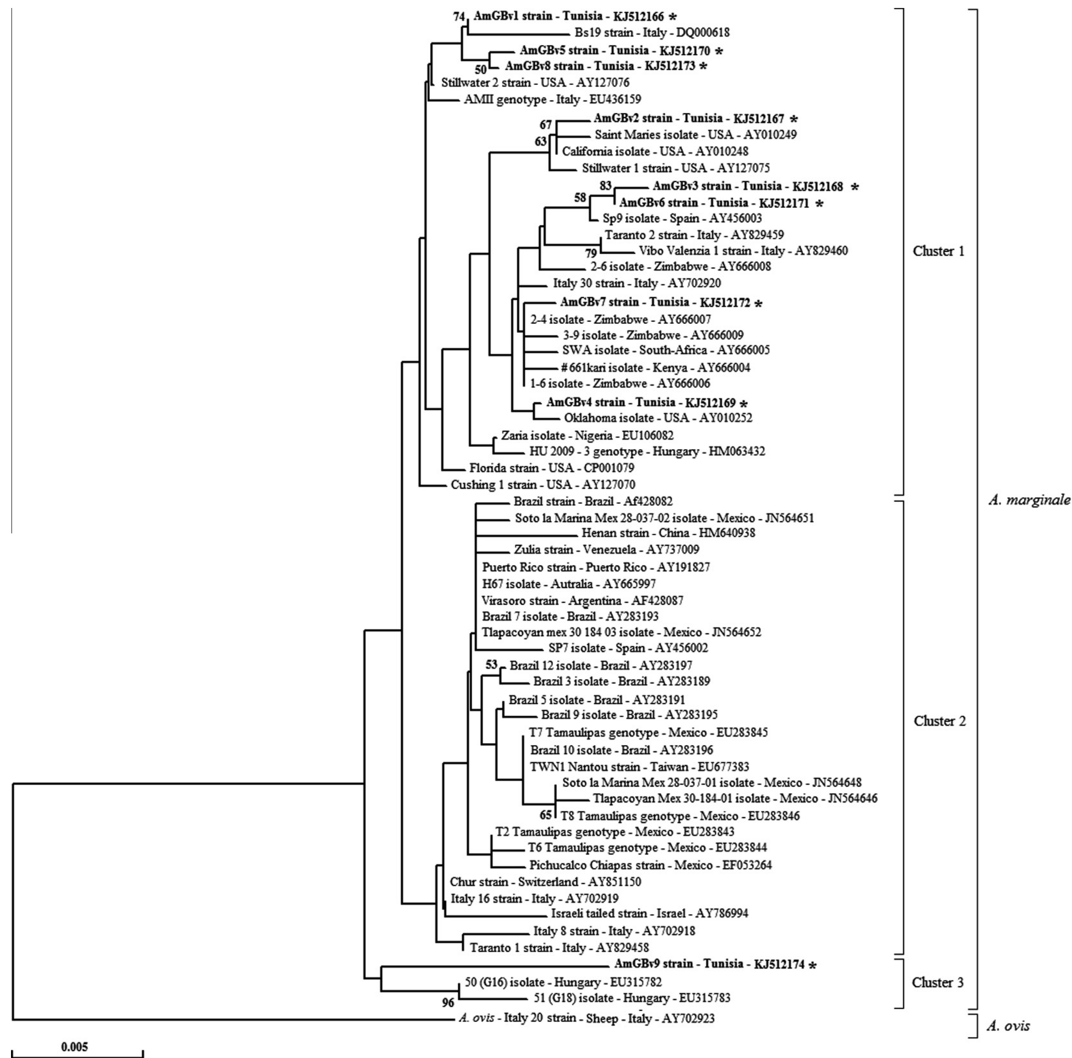

Fig. 2. Neighbor-joining tree based on the multiple alignment of partial *Anaplasma marginale msp4* sequences (805 bp). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) (only percentages greater than 50% were represented). The nine new sequences of *A. marginale* obtained in the present study are represented 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.

Table 5
Nucleotide diversity among 16S rRNA sequences from *Anaplasma centrale* (383 pb) and *Anaplasma bovis* (511 pb).

Anaplasma sp.	Variant	Sample symbol [a]	Genbank [b]	16S rRNA nucleotide positions [c]					
A. centrale				622	793	962	963	971	980
	AcGBv1	Al1; Al5; Mt1	KM401896	T	G	C	C	G	G
	AcGBv2	Al3; Al6-Al8; Mt4; Mt5; Ut2; Ut3	KM401897	*	T	*	*	*	*
	AcGBv3	Al2	KM401898	*	T	T	*	A	*
	AcGBv4	Al4	KM401899	A	T	T	*	*	*
	AcGBv5	Mt6	KM401900	*	T	*	T	*	*
	AcGBv6	Mt7	KM401901	*	T	*	*	*	C
A. bovis				82	166				
	AbGBv1	Al1; Al3; Al4	KM401902	G	T				
	AbGBv2	Al2	KM401903	A	*				
	AbGBv3	Al5	KM401904	A	A				

Nucleotides: T, Thymine; C, Cytosine; G, Guanine; A, Adenine.
[a] Al1-Al8, Mt1-Mt7 and Ut1-Ut3 *A. centrale* positive cattle samples were collected from El Alia, Mateur and Utique localities, respectively. Al1-Al5 *A. bovis* positive cattle samples were collected from El Alia locality.
[b] GenBank accession number.
[c] Numbers represent the nucleotide position with respect to *A. centrale* South-African vaccine strain (GenBank accession number AF414868) and the isolate G55 (clone 55) from China for *A. bovis* (GenBank accession number JN558825). Conserved nucleotide positions relative to the first sequence are indicated with asterisks.

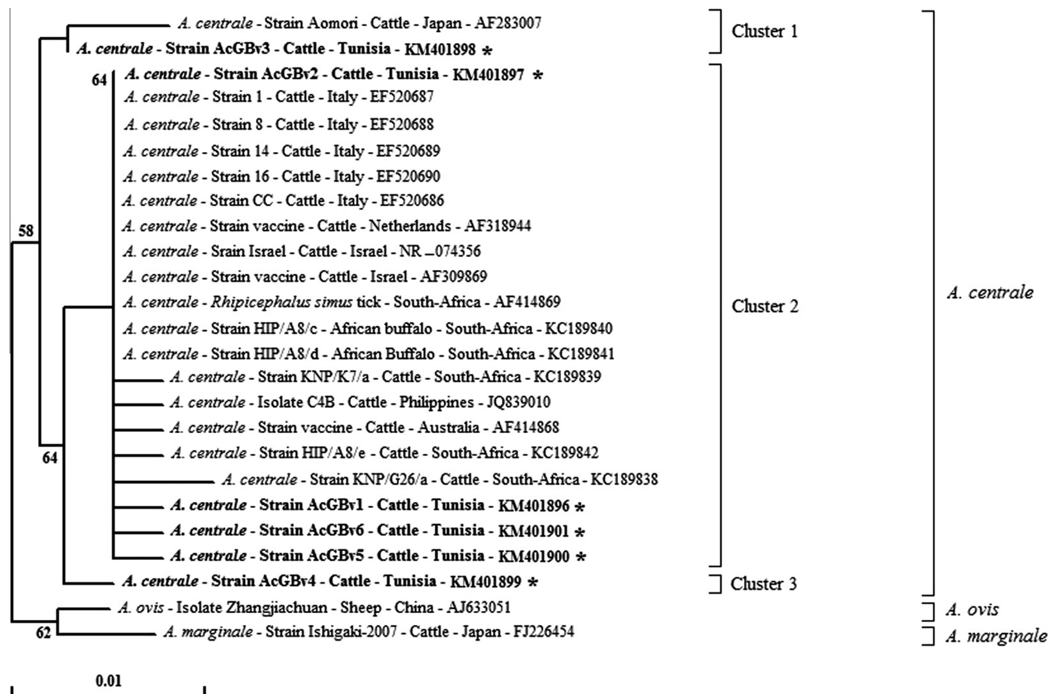

Fig. 3. Phylogenetic tree of 383 bp sequences of *Anaplasma centrale* 16S rRNA gene using the neighbor-joining method. Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting the nodes (only percentages greater than 50% were represented). *A. centrale* sequences obtained in the present study are represented 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.

4. Discussion

Despite numerous reports of anaplasmosis outbreaks in Tunisia (Sarih et al., 2005; M'ghirbi et al., 2009, 2012; Ben Said et al., 2014a,b; Belkahia et al., 2014), no epidemiological studies have been carried out to investigate the molecular evidence and spread of *Anaplasma* species in Tunisian cattle. In this study, we investigated the molecular epidemiology of *Anaplasma* infections in cattle from three localities belonging to two different bioclimatic areas.

We report for the first time the evidence of *A. marginale* infection in Tunisian cattle. *A. marginale* prevalence (overall 25.4%, minimum 15.1% in Utique, maximum 38.2% in Mateur) was similar to that reported from Morocco (21.9%) (Ait Hamou et al., 2012) and Egypt (20.1%) (El-Ashker et al., 2015), and higher than that reported in Sudan (6.1%) (Awad et al., 2011), Turkey (2.3%) (Aktas et al., 2011) and Mongolia (8.7%) (Ybañez et al., 2012). The highest *A. marginale* prevalence level observed in cattle were in Angola (38%) (Kubelová et al., 2012); Zambia (47.9%) (Yamada et al., 2009); South Africa (49% and 65% to 100%) (Mtshali et al., 2013; Mutshembele et al., 2014); *Italy* with 39% and 40% reported by Carelli et al. (2007) and Torina et al. (2008a); India with 48.7% and 51% published by Ashuma et al. (2013) and Singh et al. (2012); Philippines (54.7%) (Ybañez et al., 2013); Costa Rica (56.9%) (Shebish et al., 2012); and Brazil (60.6%) (Canever et al., 2014).

The detection of heterogenic *A. marginale* strains in this study clustering in the first and third clusters suggests that phylo-geographical resolution may be obtained at the regional level as reported by de la Fuente et al. (2003b, 2004), but not when the analysis is conducted worldwide. This heterogeneity could be explained, in part, by the importation of live cattle and/or the dissemination of *Anaplasma* spp. infected ticks with migratory birds which are proved by several studies from different countries (Alekseev et al., 2001; Ogden et al., 2008; Hildebrandt et al., 2010; Kang et al., 2013). Results suggest multiple *A. marginale* introductions in Tunisia especially from Southern Europe and North American countries. Similar findings have been documented for *A. marginale* Mexican and Italian isolates as reported by Rodríguez et al. (2000) and de la Fuente et al. (2005a), respectively. In this study, 9 novel *A. marginale* strains were characterized. Additionally to the multiple introductions of different strains, the presence of novel strains with high diversity may be caused by a co-evolution process of *A. marginale* and its potential tick vectors found in Tunisia. Exploring the MSP4 polymorphism, de la Fuente et al. (2001c) showed the presence of a co-evolution between *A. marginale* and its tick vector, *Dermacentor variabilis*, in the USA. The evolutionary history of vector-pathogen interactions could be reflected in the sequences of MSP4 (McGarey et al., 1994; de la Fuente et al., 2001a,b, 2003a,c).

In this cross-sectional study, *A. centrale* was found in 15.1% of examined cattle (minimum 5.8% in Utique and maximum 21.0% in Mateur). To our knowledge, this is the first estimate of *A. centrale* prevalence in cattle from North Africa. *A. centrale* prevalence rate was higher than that observed in Turkey (0.8%) (Aktas et al., 2011), similar to the prevalence reported by Ceci et al. (2014) in Southern Italy (13.8%) and lower to that estimated by Carelli et al. (2007) in Southern Italy (39.2%).

The analysis of *A. centrale* 16S rRNA sequences revealed six different variants all grouped in a single cluster which is separated from *A. marginale* and *A. ovis* (Table 5 and Fig. 3). Within this cluster, Tunisian strains can be classified into 3 sub-clusters suggesting multiple introductions of different strains of *A. centrale* in studied regions. Similar results have been reported by Ceci et al. (2008) in Italy. AcGBv2 is the most frequent *A. centrale* variant; it is identical to the vaccine strain from several sub-Saharan African and European countries (Bekker et al., 2002; Lew et al., 2003). Since attenuated live *A. centrale* vaccines were never used in Tunisia, we can presume that this strain was introduced with cattle from countries where this vaccine is licensed. AcGBv3 and AcGBv4 strains were distantly related to *A. centrale* vaccine strain,

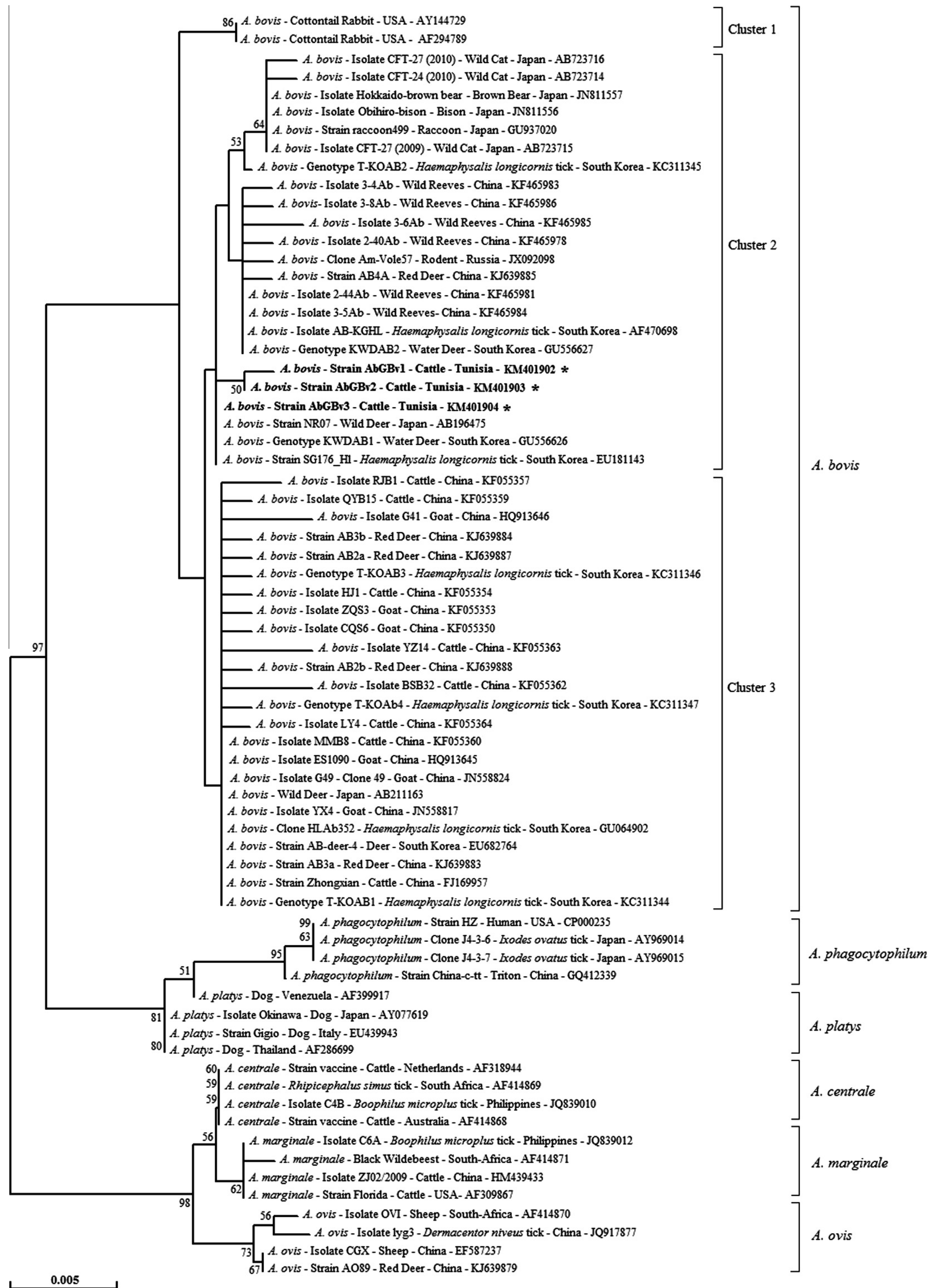

Fig. 4. Phylogenetical relationships based on the partial nucleotide sequences (511 bp) of 16S rRNA gene of new *A. bovis* variants with other variants from *A. bovis* strains and *Anaplasma* species available in GenBank. Numbers associated with nodes represent the percentage of 1000 bootstrap iterations 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. Sequences newly obtained in the present study are indicated in bold and marked with asterisks.

especially AcGBv3 strain which is closely related to Japanese strain (Inokuma et al., 2001) and which will require, according to Lew et al. (2003), further analysis for a better comprehension of its taxonomic status.

Molecular survey of *A. bovis* in cattle showed that overall prevalence was 3.9% (minimum 0% in Utique, maximum 11.2% in El Alia). This is the first *A. bovis* molecular prevalence estimate in cattle from a country located in the South Mediterranean area. Our findings were similar to those reported from Italy (4.2%) (Ceci et al., 2014) and Iran (2.7%) (Noaman and Shayan, 2010), lower to those from Japan (15–53.3%) (Ooshiro et al., 2008; Jilintai et al., 2009) and to other ruminants such as goats (49.6%) in

China (Liu et al., 2012), water deer (34.8%) in South Korea (Kang et al., 2011) and deer (23%) in Japan (Kawahara et al., 2006). *A. bovis* infection in investigated cattle was validated by 16S rRNA gene sequencing. In agreement with several other authors (Ooshiro et al., 2008; Doan et al., 2013; Ybañez et al. 2013), the phylogenetic analysis showed two main groups, one consisting of *A. phagocytophilum*, *A. platys* and *A. bovis*, and a second consisting of *A. marginale*, *A. centrale* and *A. ovis*. Indicating a low geographic segregation, only three *A. bovis* 16S rRNA sequences were isolated from cattle in different Tunisian areas.

Although 16S rRNA and *msp4* genes are considered as molecular markers with low variability compared to other genes like *groEL*, genetic analysis of these genes isolated from the 3 analyzed *Anaplasma* species showed a high diversity especially within *A. marginale* and *A. centrale*. This finding implies that livestock populations of Tunisia were most probably affected by an intense cattle movement from different regions. Further analyses using 16S rRNA, MSP4 and other more discriminating genes are needed investigate and clarify the complex origin of livestock populations in Tunisia.

In this study, *A. marginale* rather than *A. centrale* or *A. bovis* was found to be the dominant species in cattle. As reported in Southern Italy by Ceci et al. (2014), the most common co-infection pattern was *A. marginale* and *A. centrale.* In the sub-humid bioclimatic area, 5 cattle were co-infected by the three *Anaplasma* species. Also reported by Ceci et al. (2014) in Italy, the co-infections by three *Anaplasma* species in several geographic areas require further studies to better understand relations between these species and their impact on the host immune system especially in the presence of poor health management in the Mediterranean region. Furthermore, the absence of *A. phagocytophilum* in studied cattle could suggest that in these geographic regions belonging to the sub-humid and semi-arid areas, cattle are not relevant reservoirs of granulocytic anaplasmosis, as observed in other Mediterranean countries (Zobba et al., 2014). This finding is probably related to the absence of *Ixodes ricinus*, the main vector of *A. phagocytophilum* in Tunisia (Sarih et al., 2005), which is more represented in humid area (Bouattour, 2002).

A significant difference in *Anaplasma* species infection prevalence was recorded among bioclimatic zones; cattle from sub-humid area were more infected than those from semi-arid area. This difference, which was reported in Morocco for *A. marginale* by Ait Hamou et al. (2012), is probably due to the effect of bioclimatic conditions on the distribution and the phenology of arthropod vectors. In addition, overall prevalence of *A. marginale*, *A. centrale* and *A. bovis* differed statistically among geographic location. This discrepancy may be due to differences in tick control programmes, farm management, husbandry practices, wildlife reservoir hosts, and/or abiotic factors as reported by others (Torina et al., 2008a; Kocan et al., 2010; Ait Hamou et al., 2012). Cattle infested by ticks were statistically more infected by the three *Anaplasma* species than those free of ticks. Our results are consistent with those reported in cattle infested by *Rhipicephalus microplus* (Krantz and Walter, 2009; Ashuma et al., 2013). In addition, significantly higher prevalence of *A. marginale*, *A. centrale* and *A. bovis* was recorded in traditionally managed farms when compared to modern ones. Poor management, lack of tick control practices and inadequate economic sustainability of poor resource smallholders may contribute to the higher prevalence of *Anaplasma* (Atif et al., 2012, 2013). The present study showed that Holstein breed was less infected by *A. marginale* and *A. centrale* compared to other breeds (Schwyz and crossbreeds). This result may be due to a genetic resistance of this breed to hemopathogens (Bouattour et al., 1994; Darghouth et al., 1996). In Pakistan, similar trends were reported in cattle infected by *A. marginale* (Atif et al., 2012). In contrast, Ait Hamou et al. (2012) found no significant difference of *A. marginale* prevalence rates according to breed.

In conclusion, investigation on prevalence, co-infections, risk factors and genetic variability of *Anaplasma* species in cattle reported in this study is important for the implementation of control programmes. Further studies are needed to determine the vectors and reservoirs of *Anaplasma* species in Tunisian cattle and to clarify the pathogenicity of different strains especially *A. marginale* and *A. bovis*.

Competing interests

The authors declare that they have no competing interests.

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References

Ait Hamou, S., Rahali, T., Sahibi, H., Belghyti, D., Losson, B., Goff, W., Rhalem, A., 2012. Molecular and serological prevalence of *Anaplasma marginale* in cattle of North Central Morocco. Res. Vet. Sci. 93, 1318–1323.

Aktas, M., Altay, K., Dumanli, N., 2011. Molecular detection and identification of *Anaplasma* and *Ehrlichia* species in cattle from Turkey. Ticks Tick Borne Dis. 2, 62–65.

Alekseev, A.N., Dubinina, H.V., Semenov, A.V., Bolshakov, C.V., 2001. Evidence of ehrlichiosis agents found in ticks (Acari: Ixodidae) collected from migratory birds. J. Med. Entomol. 38, 471–474.

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

Ashuma, Sharma, A., Singla, L.D., Kaur, P., Bal, M.S., Kaur, B.K., Juyal, P.D., 2013. Prevalence and haemato-biochemical profile of *Anaplasma marginale* infection in dairy animals of Punjab (India). Asian Pac. J. Trop. Med. 6, 139–144.

Atif, F.A., Khan, M.S., Iqbal, H.J., Roheen, T., 2012. Prevalence of tick-borne diseases in Punjab (Pakistan) and hematological profile of *Anaplasma marginale* infection in indigenous and crossbred cattle. Pakistan J. Sci. 64, 11–15.

Atif, F.A., Khan, M.S., Muhammad, F., Ahmad, B., 2013. Sero-epidemiological study of *Anaplasma marginale* among cattle. J. Anim. Plant Sci. 23, 740–744.

Awad, H., Antunes, S., Galindo, R.C., do Rosário, V.E., de la Fuente, J., Domingos, A., El Hussein, A.M., 2011. Prevalence and genetic diversity of *Babesia* and *Anaplasma* species in cattle in Sudan. Vet. Parasitol. 181, 146–152.

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

Bekker, C.P., de Vos, A., Taoufik, A., Sparagano, O.A., Jongejan, F., 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridisation. Vet. Microbiol. 89, 223–238.

Belkahia, H., Ben Said, M., El Hamdi, S., Yahiaoui, M., Gharbi, M., Daaloul-Jedidi, M., Mhadhbi, M., Jedidi, M., Darghouth, M.A., Klabi, I., Zribi, L., Messadi, L., 2014. First molecular identification and genetic characterization of *Anaplasma ovis* in sheep from Tunisia. Small Ruminant Res. 121, 404–410.

Ben Said, M., Belkahia, H., Héni, M.M., Bouattour, A., Ghorbel, A., Gharbi, M., Zouari, A., Darghouth, M.A., Messadi, L., 2014a. Seroprevalence of *Anaplasma phagocytophilum* in well-maintained horses from Northern Tunisia. Trop. Biomed. 31, 432–440.

Ben Said, M., Belkahia, H., Sayahi, L., Aloui, M., Jemli, M.H., Hadj Mohamed, B., Sassi, L., Darghouth, M.A., Djaïem, A.A., Bayoudh, M., Messadi, L., 2014b. Première étude sérologique de la prévalence d'*Anaplasma phagocytophilum* chez le dromadaire (*Camelus dromedarius*) en Tunisie. Bull. Soc. Pathol. Exot. 107, 1–6.

Bouattour, A., Darghouth, M.A., Ben Miled, L., 1994. Epidémiologie de la theilériose bovine en Tunisie. Arch. Inst. Pasteur Tunis 71, 459–462.

Bouattour, A., 2002. Clé dichotomique et identification des tiques (Acari : Ixodidae), parasites du bétail au Maghreb. Arch. Inst. Pasteur Tunis 79, 43–50.

Canever, M.F., Vieira, L.L., Reck, C., Richter, L., Miletti, L.C., 2014. First evaluation of an outbreak of bovine babesiosis and anaplasmosis in Southern Brazil using multiplex PCR. Korean J. Parasitol. 52, 507–511.

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L., Buonavoglia, C., 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. Vet. Microbiol. 124, 107–114.

Ceci, L., Decaro, N., Lorusso, E., Paradies, P., Elia, G., Martella, V., Buonavoglia, C., Carelli, G., 2008. First report of bovine anaplasmosis by *Anaplasma centrale* in Europe, molecular identification and phylogenetic analysis. Vet. Res. Commun. 32, S263–S266.

Ceci, L., Iarussi, F., Greco, B., Lacinio, R., Fornelli, S., Carelli, G., 2014. Retrospective study of hemoparasites in cattle in southern Italy by reverse line blot hybridization. J. Vet. Med. Sci. 76, 869–875.

Darghouth, M.A., Bouattour, A., Ben Miled, L., Kilani, M., Brown, C.G., 1996. Epidemiology of tropical theileriosis (*Theileria annulata* infection of cattle) in an endemic region of Tunisia: characterization of endemicity states. Vet. Parasitol. 65, 199–211.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Kocan, K.M., 2001a. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int. J. Parasitol. 31, 145–153.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., McEwen, B.R., Clawson, D., Kocan, K.M., 2001b. Major surface protein 1a effects tick infection and transmission of the ehrlichial pathogen *Anaplasma marginale*. Int. J. Parasitol. 31, 1705–1714.

de la Fuente, J., Van Den Bussche, R.A., Kocan, K.M., 2001c. Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae). Vet. Parasitol. 97, 65–76.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Kocan, K.M., 2003a. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. Vet. Microbiol. 91, 265–283.

de la Fuente, J., Golsteyn Thomas, E.J., Van Den Bussche, R.A., Hamilton, R.G., Tanaka, E.E., Druhan, S.E., Kocan, K.M., 2003b. Characterization of *Anaplasma marginale* isolated from North American bison. Appl. Environ. Microbiol. 69, 5001–5005.

de la Fuente, J., Van Den Bussche, R.A., Prado, T.M., Kocan, K.M., 2003c. *Anaplasma marginale* msp1alpha genotypes evolved under positive selection pressure but are not markers for geographic isolates. J. Clin. Microbiol. 41, 1609–1616.

de la Fuente, J., Vicente, J., Höfle, U., Ruiz-Fons, F., Fernández De Mera, I.G., Van Den Bussche, R.A., Kocan, K.M., Gortázar, C., 2004. *Anaplasma* infection in free-ranging Iberian red deer in the region of Castilla–La Mancha, Spain. Vet. Microbiol. 100, 163–173.

de la Fuente, J., Torina, A., Caracappa, S., Tumino, G., Furlá, R., Almazán, C., Kocan, K.M., 2005a. Serologic and molecular characterization of *Anaplasma* species infection in farm animals and ticks from Sicily. Vet. Parasitol. 133, 357–362.

de la Fuente, J., Naranjo, V., Ruiz-Fons, F., Höfle, U., Fernández De Mera, I.G., Villanúa, D., Almazán, C., Torina, A., Caracappa, S., Kocan, K.M., Gortázar, C., 2005b. Potential vertebrate reservoir hosts and invertebrate vectors of *Anaplasma marginale* and *A. phagocytophilum* in central Spain. Vector Borne Zoonotic Dis. 5, 390–401.

de la Fuente, J., Atkinson, M.W., Naranjo, V., Fernández de Mera, I.G., Mangold, A.J., Keating, K.A., Kocan, K.M., 2007a. Sequence analysis of the *msp4* gene of *Anaplasma ovis* strains. Vet. Microbiol. 119, 375–381.

de la Fuente, J., Ruybal, P., Mtshali, M.S., Naranjo, V., Shuqing, L., Mangold, A.J., Rodríguez, S.D., Jiménez, R., Vicente, J., Moretta, R., Torina, A., Almazán, C., Mbati, P.M., de Echaide, S.T., Farber, M., Rosario-Cruz, R., Gortazar, C., Kocan, K.M., 2007b. Analysis of world strains of *Anaplasma marginale* using major surface protein 1a repeat sequences. Vet. Microbiol. 119, 382–390.

El-Ashker, M., Hotzel, H., Gwida, M., El-Beskawy, M., Silaghi, C., Tomaso, H., 2015. Molecular biological identification of *Babesia*, *Theileria*, and *Anaplasma* species in cattle in Egypt using PCR assays, gene sequence analysis and a novel DNA microarray. Vet. Parasitol. 207, 329–334.

Decaro, N., Carelli, G., Lorusso, E., Lucente, M.S., Greco, G., Lorusso, A., Radogna, A., Ceci, L., Buonavoglia, C., 2008. Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*. J. Vet. Diagn. Invest. 20, 606–611.

Doan, H.T.T., Noh, J.H., Choe, S.E., Yoo, M.S., Kim, Y.H., Reddy, K.E., Quyen, D.V., Nguyen, L.T., Nguyen, T.T., Kweon, C.H., Jung, S.C., Chang, K.Y., Kang, S.W., 2013. Molecular detection and phylogenetic analysis of *Anaplasma bovis* from *Haemaphysalis longicornis* feeding on grazing cattle in Korea. Vet. Parasitol. 196, 478–481.

Donatien, A., Lestoquard, F., 1936. *Rickettsia bovis*, nouvelle espèce pathogène pour le boeuf. Bull. Soc. Pathol. Exot. 29, 1057–1061.

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

Georges, K., Loria, G.R., Riili, S., Greco, A., Caracappa, S., Jongejan, F., Sparagano, O., 2001. Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. Vet. Parasitol. 99, 273–286.

Hildebrandt, A., Franke, J., Meier, F., Sachse, S., Dorn, W., Straube, E., 2010. The potential role of migratory birds in transmission cycles of *Babesia* spp., *Anaplasma phagocytophilum*, and *Rickettsia* spp. Ticks Tick Borne Dis. 1, 105–107.

Hofmann-Lehmann, R., Meli, M.L., Dreher, U.M., Gonczi, E., Deplazes, P., Braun, U., Engels, M., Schüpbach, J., Jörger, K., Thoma, R., Griot, C., Stärk, K.D., Willi, B., Schmidt, J., Kocan, K.M., Lutz, H., 2004. Concurrent infections with vector-borne pathogens associated with fatal haemolytic anemia in a cattle herd in Switzerland. J. Clin. Microbiol. 42, 3775–3780.

Hulínská, D., Langøová, K., Pejèoch, M., Pavlásek, I., 2004. Detection of *Anaplasma phagocytophilum* in animals by real-time polymerase chain reaction. APMIS 112, 239–247.

Inokuma, H., Terada, Y., Kamio, T., Raoult, D., Brouqui, P., 2001. Analysis of the *16S rRNA* gene sequence of *Anaplasma centrale* and its phylogenetic relatedness to other Ehrlichiae. Clin. Diag. Lab. Immunol. 8, 241–244.

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

Kang, J.G., Ko, S., Kim, Y.J., Yang, H.J., Lee, H., Shin, N.S., Choi, K.S., Chae, J.S., 2011. New genetic variants of *Anaplasma phagocytophilum* and *Anaplasma bovis* from Korean water deer (*Hydropotes inermis argyropus*). Vector Borne Zoonotic Dis. 11, 929–938.

Kang, J.G., Kim, H.C., Choi, C.Y., Nam, H.Y., Chae, H.Y., Chong, S.T., Klein, T.A., Ko, S., Chae, J.S., 2013. Molecular detection of *Anaplasma*, *Bartonella*, and *Borrelia* species in ticks collected from migratory birds from Hong-do Island, Republic of Korea. Vector Borne Zoonotic Dis. 13, 215–225.

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

Kocan, K.M., de la Fuente, J., Guglielmone, A.A., Meléndez, R.D., 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin. Microbiol. Rev. 16, 698–712.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., Ewing, S.A., 2010. The natural history of *Anaplasma marginale*. Vet. Parasitol. 167, 95–107.

Krantz, G.W., Walter, D.E., 2009. A Manual of Acarology. University Press, Lubbock, TX, Texas Tech, p. 807.

Kubelová, M., Mazancová, J., Siroký, P., 2012. *Theileria*, *Babesia*, and *Anaplasma* detected by PCR in ruminant herds at Bié Province, Angola. Parasite 19, 417–422.

Laloy, E., Petit, E., Boulouis, H.J., Gandoin, C., Bouillin, C., Gounot, G., Bonnet, S., Maillard, R., 2009. Dynamics of natural infection by *Anaplasma phagocytophilum* in a dairy cattle herd in Brittany, France. Clin. Microbiol. Infect. 15, 24–25.

Lew, A.E., Gale, K.R., Minchin, C.M., Shkap, V., de Waal, D.T., 2003. Phylogenetic analysis of the erythrocytic *Anaplasma* species based on 16S rDNA and GroEL (HSP60) sequences of *A. marginale*, *A. centrale*, and *A. ovis* and the specific detection of *A. centrale* vaccine strain. Vet. Microbiol. 92, 145–160.

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

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

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

McGarey, D.J., Barbet, A.F., Palmer, G.H., McGuire, T.C., Allred, D.R., 1994. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect. Immunol. 62, 4594–4601.

Mtshali, M.S., de la Fuente, J., Ruybal, P., Kocan, K.M., Vicente, J., Mbati, P.A., Shkap, V., Blouin, E.F., Mohale, N.E., Moloi, T.P., Spickett, A.M., Latif, A.A., 2013. Prevalence and genetic diversity of *Anaplasma marginale* strains in cattle in South Africa. Zoonoses Public Health 54, 23–30.

Murase, Y., Konnai, S., Hidano, A., Githaka, N.W., Ito, T., Takano, A., Kawabata, H., Ato, M., Tajima, T., Tajima, M., Onuma, M., Murata, S., Ohashi, K., 2011. Molecular detection of *Anaplasma phagocytophilum* in cattle and *Ixodes persulcatus* ticks. Vet. Microbiol. 149, 504–507.

Mutshembele, A.M., Cabezas-Cruz, A., Mtshali, M.S., Thekisoe, O.M., Galindo, R.C., de la Fuente, J., 2014. Epidemiology and evolution of the genetic variability of *Anaplasma marginale* in South Africa. Ticks Tick Borne Dis. 5, 624–631.

Naranjo, V., Ruiz-Fons, F., Hofle, U., Fernandez De Mera, I.G., Villanua, D., Almazan, C., Torina, A., Caracappa, S., Kocan, K.M., Gortázar, C., de La Fuente, J., 2006. Molecular epidemiology of human and bovine anaplasmosis in southern Europe. Ann. N. Y. Acad. Sci. 1078, 95–99.

Noaman, V., Shayan, P., 2010. Molecular detection of *Anaplasma bovis* in cattle from central part of Iran. Vet. Res. Forum 1, 117–122.

Ogden, N.H., Lindsay, L.R., Hanincová, K., Barker, I.K., Bigras-Poulin, M., Charron, D.F., Heagy, A., Francis, C.M., O'Callaghan, C.J., Schwartz, I., Thompson, R.A., 2008. Role of migratory birds in introduction and range expansion of *Ixodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada. Appl. Environ. Microbiol. 74, 1780–1790.

Ooshiro, M., Zakimi, S., Matsukawa, Y., Katagiri, Y., Inokuma, H., 2008. Detection of *Anaplasma bovis* and *Anaplasma phagocytophilum* from cattle on Yonaguni island, Okinawa. Japan. Vet. Parasitol. 154, 360–364.
Rar, V., Golovljova, I., 2011. *Anaplasma*, *Ehrlichia*, and "*Candidatus Neoehrlichia*" bacteria: Pathogenicity, biodiversity, and molecular genetic characteristics, a review. Infect. Genet. Evol. 11, 1842–1861.
Rikihisa, Y., 1991. The tribe Ehrlichieae and ehrlichial diseases. Clin. Microbiol. Rev. 4, 286–308.
Rodríguez, S.D., Garcîa Ortiz, M.A., Hernández Salgado, G., Santos Cerda, N.A., Aboytes Torre, R., Cantó Alarcón, G.J., 2000. *Anaplasma marginale* inactivated vaccine: dose titration against a homologous challenge. Comp. Immunol. Microbiol. Infect. Dis. 23, 239–252.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Sarih, M., M'ghirbi, Y., Bouattour, A., Gern, L., Baranton, G., Postic, D., 2005. Detection and identification of *Ehrlichia* spp. in ticks collected in Tunisia and Morocco. J. Clin. Microbiol. 43, 1127–1132.
Shebish, E., Vemulapalli, R., Oseto, C., 2012. Prevalence and molecular detection of *Anaplasma marginale, Babesia bovis* and *Babesia bigemina* in cattle from Puntarenas Province, Costa Rica. Vet. Parasitol. 188, 164–167.
Singh, H., Jyoti, I., Haque, M., Singh, N.K., Rath, S.S., 2012. Molecular detection of *Anaplasma marginale* infection in carrier cattle. Ticks Tick Borne Dis. 3, 55–58.
Sreekumar, C., Anandan, R., Balasundaram, S., Rajavelu, G., 1996. Morphology and staining characteristics of *Ehrlichia bovis*. Comp. Immunol. Microbiol. Infect. Dis. 19, 79–83.
Stuen, S., 2007. *Anaplasma phagocytophilum* – the most widespread tick-borne infection in animals in Europe. Vet. Res. Commun. 31, 79–84.
Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512.
Teglas, M., Matern, E., Lein, S., Foley, P., Mahan, S.M., Foley, J., 2005. Ticks and tick-borne disease in Guatemalan cattle and horses. Vet. Parasitol. 131, 119–127.
Torina, A., Alongi, A., Naranjo, V., Estrada-Peña, A., Vicente, J., Scimeca, S., Marino, A.M., Salina, F., Caracappa, S., de la Fuente, J., 2008a. Prevalence and genotypes of *Anaplasma* species and habitat suitability for ticks in a Mediterranean ecosystem. Appl. Environ. Microbiol. 74, 7578–7584.
Torina, A., Alongi, A., Naranjo, V., Scimeca, S., Nicosia, S., Di Marco, V., Caracappa, S., Kocan, K.M., de la Fuente, J., 2008b. Characterization of *Anaplasma* infections in Sicily, Italy. Ann. N.Y. Acad. Sci. 1149, 90–93.
Yamada, S., Konnai, S., Imamura, S., Simuunza, M., Chembensofu, M., Chota, A., Nambota, A., Onuma, M., Ohashi, K., 2009. PCR-based detection of blood parasites in cattle and adult *Rhipicephalus appendiculatus* ticks. Vet J. 182, 352–355.
Ybañez, A.P., Sivakumar, T., Battsetseg, B., Battur, B., Altangerel, K., Matsumoto, K., Yokoyama, N., Inokuma, H., 2012. Specific molecular detection and characterization of *Anaplasma marginale* in Mongolian cattle. J. Vet. Med. Sci. 75, 399–406.
Ybañez, A.P., Tagawa, M., Matsumoto, K., Kishimoto, T., Yokoyama, N., Inokuma, H., 2013. Specific molecular detection of *Anaplasma* sp. closely related to *Anaplasma phagocytophilum* in Ixodid ticks and cattle in a pastureland in Hokkaido, Japan. Vector-Borne Zoonotic. Dis. 13, 6–11.
Zhang, L., Liu, H., Xu, B., Lu, Q., Li, L., Chang, L., Yu, X.J., 2012a. *Anaplasma phagocytophilum* infection in domestic animals in ten provinces/cities of China. Am. J. Trop. Med. Hyg. 87, 185–189.
Zhang, X.C., Zhang, L.X., Li, W.H., Wang, S.W., Sun, Y.L., Wang, Y.Y., Zhang, L.J., 2012b. Ehrlichiosis and zoonotic anaplasmosis in suburban areas of Beijingm, China. Vector-Borne Zoonotic Dis. 12, 932–937.
Zobba, R., Anfossi, A.G., Pinna Parpaglia, M.L., Dore, G.M., Chessa, B., Spezzigu, A., Rocca, S., Visco, S., Pittau, M., Alberti, A., 2014. Molecular investigation and phylogeny of *Anaplasma* spp. in Mediterranean ruminants reveal the presence of neutrophil-tropic strains closely related to *A. platys*. Appl. Environ. Microbiol. 80, 271–280.