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First molecular identification and genetic characterization of *Anaplasma ovis* in sheep from Tunisia

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ABSTRACT

The aims of this study consist of first estimating the molecular prevalence of *Anaplasma ovis* with specific species loop-mediated isothermal amplification (LAMP) method in sheep ($n = 204$) from Northern and Central Tunisia. This study also characterizes the genotypes of this *Anaplasma* species by partial *msp4* gene sequence. The molecular prevalence of *A. ovis* used to record 70.1%. Prevalence rate was significantly higher in El Alia locality (governorate of Bizerte, Northern Tunisia), where it indicated 91.1%, than in Sbikha locality (governorate of Kairouan, Central Tunisia), where it indicated 49.5%. In Sbikha, *A. ovis* prevalence varied significantly according to two factors, i.e., gender and age. In fact, ewes were significantly more infected than ram lambs. Ticks collected on animals allowed recovering 271 ixodid ticks from El Alia. Ticks belonged to 3 species of *Rhipicephalus* genus: first, *R. turanicus* (59.78%) which was the dominant species and which was followed by *R. sanguineus* (36.16%) and *R. annulatus* (4.06%). In Sbikha, an absence of ticks was noted in all of the examined sheep. The analysis of *A. ovis msp4* sequences revealed four different genotypes; two of them were previously described in Italy and two new other genotypes were classified in an independent cluster. The present work is the first published report of *A. ovis* infection in sheep in Tunisia. The results indicate the presence of *A. ovis* at a high rate; hence, the need for a survey into the medical and economic impact of the disease, and the identification of vectors of this bacterium in Tunisia must be made.

1. Introduction

Anaplasma ovis is a tick-borne obligatory intraerythrocytic bacterium of sheep, goats and wild ruminants (Friedhoff, 1997; Yabsley et al., 2005; de la Fuente et al., 2006, 2007). The infection is frequently subclinical but can also cause disease which is more severe among goats than sheep, which is particularly proved in stressed or debilitated animals (Friedhoff, 1997). The acute phase of the disease is characterized by fever, progressive anemia, icterus, weight loss, milk yield decrease, and sometimes death (Splitter et al., 1956; Yasini et al., 2012). In addition, the infection with *A. ovis* may predispose animals to other infectious or parasitic diseases that aggravate the condition of the animal and can lead to its death (Kocan et al., 2004).

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Recently, Ciani et al. (2013) observed the possible influence of the breed of lambs experimentally infected by *A. ovis* on the severity of infection.

In Europe, the majority of *A. ovis* infection cases in sheep and goats were reported in several Mediterranean countries, including France (Cuille and Chelle, 1936), Italy (de la Fuente et al., 2005b), Turkey (Sayin et al., 1997; Renneker et al., 2013), Greece (Papadopoulos, 1999), and Portugal (Renneker et al., 2013). In the same way, the infection was also reported in Northern Hungary (Hornok et al., 2007), Slovakia and Czech Republic (Derdáková et al., 2011). In North Africa, *A. ovis* has been detected in sheep from Algeria (Lestoquard, 1924). In Tunisia, to the best of our knowledge, there has been no published data about *A. ovis* infection in sheep.

Accordingly, nucleic acid-based molecular tools, such as PCR amplifying the 16S *rRNA* gene (Liu et al., 2005) and major surface protein 4 (*msp4*) gene (de la Fuente et al., 2007; Torina et al., 2012), and the reverse line blotting method (Bekker et al., 2002) proved to be very useful for the *A. ovis* molecular screening. Recently, a loop-mediated isothermal amplification (LAMP) was developed for *A. ovis* screening by Ma et al. (2011), who reported higher sensitivity than conventional PCR.

The interaction between *Anaplasma* spp. and their hosts is mainly mediated by the major surface proteins (MSPs). Rapid evolution of the genes encoding these proteins is likely to be due to the immune pressure (Kocan et al., 2004; de la Fuente et al., 2005a; Brayton et al., 2006; Dunning Hotopp et al., 2006; Psaroulaki et al., 2009). The genetic diversity of *A. ovis* was determined using the *msp4* gene in sheep and goats strains from different regions (de la Fuente et al., 2002, 2005b, 2006; Yabsley et al., 2005; Hornok et al., 2007; Torina et al., 2008, 2010; Psaroulaki et al., 2009; Derdáková et al., 2011; Liu et al., 2012).

In short, the aim of this study was to estimate the molecular prevalence of *A. ovis* by LAMP method in healthy sheep blood samples from Northern and Central Tunisia. The *msp4* gene of *A. ovis* strains collected from sheep was sequenced to investigate the genetic diversity of this bacterium in Tunisia.

2. Materials and methods

2.1. Study regions

The present study was carried out in five flocks from two Tunisian governorates: (i) four flocks from El Alia (Bizerte governorate, Northern Tunisia), characterized by a subhumid climate and a mean altitude of 33 m above sea level (latitude 37°16′ N, longitude 9°52′ E) and (ii) one flock from Sbikha (Kairouan governorate, Central Tunisia), characterized by an arid climate and a mean altitude of 60 m above sea level (latitude 35°40′ N, longitude 10°06′ E) (Fig. 1). In El Alia, investigated flocks are traditionally managed, animals are housed in traditional shelters and they graze on natural pastures. The sheep are irregularly treated with external acaricides. Sheep from Sbikha were reared in traditional shelters under semi-intensive conditions for ewes with grazing on natural grassland, and zero grazing for lambs. All the sheep were monthly treated with an external acaricide from May to November.

2.2. Blood sampling and tick collection

A total number of 204 apparently healthy sheep, 103 from Sbikha and 101 from El Alia, were sampled during the tick season from June to July 2008 and during May 2011, respectively. Blood samples were collected in EDTA tubes from the jugular vein. DNA was extracted from 300 μl of blood

Fig. 1. Map showing the two Tunisian studied regions. The maps of Bizerte and Kairouan show El Alia and Sbikha localities, respectively.

Table 1
Primers used for molecular detection and characterization of *Anaplasma ovis* in sheep in the present study.

Assay	Primer/probe	Sequence 5′ to 3′	Target gene	Amplicon size (bp)	Reference
LAMP					
A. ovis	MSP4F3	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	*A. ovis msp4*	–	Ma et al. (2011)
	MSP4B3	CCGGATCCTTAGCTGAACAGGAATCTTGC			
	MSP4FIP	GTGTTGCACACAGATTTGCC			
	MSP4BIP	AGGCTTTTGCTTCTCCGG			
	MSP4LF	GCCCCTGTAGGCTAGCTTTGTG-CCCATATGTGTGTGCCGG			
	MSP4LB	TGGTGGTAGGTGGGTTCTACCA-ATGTGCGGGTATGTCCTTG			
	MSP4F3	TGTCGACAAAGCTAGCACC			
	MSP4B3	CGGACTCTTTGACGAGTCTT			
PCR					
A. ovis/*A. marginale*	MSP45[a]	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	*A. ovis*/*A. marginale msp4*	852	de la Fuente et al. (2005b)
	MSP43[a]	CCGGATCCTTAGCTGAACAGGAATCTTGC			

[a] Primers used for the sequencing reactions.

samples with Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions. Purified DNA was stored at −20 °C until used. During the visits, the entire body of each sheep was inspected for ticks, they were collected manually and placed in 70% ethanol in labeled tubes then identified with a stereomicroscope with the key of Walker et al. (2013).

2.3. LAMP *A. ovis* detection

LAMP reactions were performed using six primers of the *A. ovis msp4* gene (Ma et al., 2011) (Table 1). Briefly, 2 μl of template DNA were mixed with 23 μl LAMP mixture that consisted of 6 μl of primer mix (40 pmol of each FIP and BIP, 20 pmol of each LF and LB, 5 pmol of each F3 and B3) (Table 1), 2.5 μl of 10× ThermoPol Reaction Buffer (20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 at 25 °C) (New England BioLabs, United Kingdom), 1.5 μl (6 mM) of supplementary $MgSO_4$ (100 mM), 4 μl (0.8 M) of Betaine (Sigma–Aldrich, USA) (5 M), 3.5 μl (1.4 mM) of each deoxynucleoside triphosphate (dNTP) (Sigma–Aldrich, USA) (10 mM), 1.0 μl (8 U) Bst DNA polymerase (New England BioLabs, United Kingdom) (8 U/μl) and 4.5 μl of distilled water. The mixtures were incubated in a heat block at 62 °C for 60 min and subsequently, at 80 °C for 5 min to stop the reaction. Negative and positive controls were included in all runs. The LAMP products were detected by electrophoresis in 1.5% agarose gel and either visualized under a UV light after staining with ethidium bromide or by visual inspection of the tubes after adding 1 μl of 1000× SYBR Green I (Cambrex BioScience, USA) to amplicons.

2.4. Amplification of partial sequences from *msp4* gene

To obtain partial sequences from *msp4* gene, a PCR was performed for LAMP positive samples with the primers msp43 and msp45 to amplify a 852 bp fragment of the *A. ovis*/*A. marginale msp4* gene (de la Fuente et al., 2005b, 2007) (Table 1). PCR amplifications were performed as follows: 1× Taq MasterMix (Vivantis, USA) (containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.125 U/μl Taq DNA polymerase), 2 μl (1–10 ng) DNA, 0.5 μM of the primers (Table 1) and milliQ sterile water to a total volume of 50 μl. To ensure the accuracy of the method, negative (distilled water) and positive controls (*A. ovis* positive DNA) were used in each experiment every 10 samples. Thermal cycling reactions were performed in an automated DNA thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, USA) using the following conditions: an initial step of 30 s at 94 °C followed by 35 cycles (a denaturing step of 30 s at 94 °C, an annealing step of 30 s at 60 °C and an extension step of 1 min at 68 °C) and a final extension step of 68 °C for 7 min. PCR products were electrophoresed in 1.5% agarose gel.

2.5. DNA sequencing and phylogenetic analysis

Eight selected PCR products, four from each sampling area, 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 with a conventional Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Applied Biosystems, Foster City, USA) and ABI3730XL automated DNA sequencer by Macrogen Europe (Amsterdam, The Netherlands). The chromatograms were evaluated 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 the amplicons and to translate nucleotides to amino acids sequences. BLAST analysis of GenBank was used to identify previously reported sequences with identity to those obtained in the present study (http://blast.ncbi.nlm.nih.gov/) (Altschul et al., 1997). A phylogenetic tree was constructed with the DNAMAN program based on the distance method using the Neighbor-Joining (NJ) algorithm of Saitou and Nei (1987) with bootstrap analysis of 1000 iterations. Four different sequences corresponding to *msp4* Tunisian strains have been submitted to GenBank and can be retrieved under accession numbers from KC432641 to KC432644.

2.6. Statistical analysis

To study the possible influence of region, gender and age on the molecular prevalence of *A. ovis* and to compare the tick infestation rates, chi square test or Fisher's exact test were performed using Epi Info 6.01 (CDC, Atlanta) with a cut-off value of 0.05.

3. Results

3.1. Molecular prevalence of *A. ovis* in Tunisian sheep

The overall *A. ovis* infection was 70.1% (143/204), with a higher rate in El Alia (Bizerte governorate, Northern Tunisia) (91.1%; 92/101) than Sbikha (Kairouan governorate, Central Tunisia) (49.5%; 51/103) ($p < 0.001$) (Table 2). In Sbikha, the infection prevalence was higher in females (92.8%) than males (33.3%) ($p < 0.001$) (Table 2). In addition, a significant difference was observed between the infection prevalence in adults (92.8%) compared to lambs (33.3%) ($p < 0.001$). In El Alia, there was no statistically significant association between prevalence and age or gender (Table 2). A total of 271 ticks were collected from 101 sheep located in El Alia with tick infestation burdens ranged from one to 8 ticks per animal. These ticks belonged to *Rhipicephalus* genus. *Rhipicephalus turanicus* (59.78%, 162 ticks) was the dominant species followed by *R. sanguineus* (36.16%, 98 ticks) and *R. annulatus* (4.06%, 11 ticks) ($p < 0.001$). In Sbikha, no ticks were collected on all examined sheep.

Table 2
Prevalence of *Anaplasma ovis* according to gender and age of studied sheep from El Alia locality (governorate of Bizerte, Northern Tunisia) and Sbikha locality (governorate of Kairouan, Central Tunisia).

	El Alia (governorate of Bizerte)			Sbikha (governorate of Kairouan)		
	Positive/total (% ± C.I.[a])	χ^2 (df[b])	*P*-value	Positive/total (% ± C.I.[a])	χ^2 (df[b])	*P*-value
Gender		2.17 (1)	0.140		28.90 (1)	0.000*
Male	8/11 (72.7 ± 26.8)			25/75 (33.3 ± 10.9)		
Female	84/90 (93.3 ± 5.2)			26/28 (92.8 ± 9.7)		
Age		0.14 (1)	0.710		28.90 (1)	0.000*
≤1 year	7/8 (87.5 ± 23.3)			25/75 (33.3 ± 10.9)		
>1 year	85/93 (91.4 ± 5.8)			26/28 (92.8 ± 9.7)		
Total	92/101 (91.1 ± 5.6)			51/103 (49.5 ± 9.8)		

[a] C.I., 95% confidence interval.
[b] df, degree of freedom.
* Significant.

3.2. Sequence diversity of *A. ovis msp4* gene

A. ovis infection was confirmed by partial sequencing of the *msp4* gene from randomly selected eight positive samples (four from each sampling region). Alignment of these sequences revealed 4 different genotypes with 3 distinctions of nucleotide positions (GenBank accession numbers KC432641 to KC432644). Each nucleotide change conferred an amino acid variation (Table 3). GBK1 genotype (GenBank accession number KC432641) revealed 100% homology with the genotype II represented by "Italy147" *A. ovis* strain (GenBank accession number AY702924) (Table 3). GBK2 genotype (GenBank accession number KC432642) from Bizerte (B4) and Kairouan (K3) governorates showed 100% sequence identity to the genotype III represented by "Italy20" *A. ovis* strain from Sicilian sheep (GenBank accession number AY702923) (Table 3, Fig. 2). Two novel *A. ovis msp4* genotypes (GB3 and GK1, GenBank accession number KC432643 and KC432644, respectively) were identified. They differ from each other by a single nucleotide and amino acid position. They have 99% sequence homology to genotype III represented by "Italy20" *A. ovis* strain (GenBank accession number AY702923) (Table 3). Phylogenetic analysis showed that these two new variants are clustered with *A. ovis* Panagcy strain found in human samples from Cyprus (GenBank accession number FJ460443) (Chochlakis et al., 2010) (Fig. 2).

4. Discussion

A cross-sectional study was performed using LAMP reaction to investigate *A. ovis* infection in sheep from Northern (El Alia, Bizerte governorate) and Central Tunisia (Sbikha, Kairouan governorate). The results confirmed the presence of *A. ovis* infection in these regions with an overall prevalence of 70.1%. Iraq witnessed a high infection rate of 66.7% (Renneker et al., 2013). Similarly, China recorded 69.2% (Ma et al., 2011), Hungary 72.7% (Hornok et al., 2007), Portugal 82.5% (Renneker et al., 2013), and Italy 87% (de la Fuente et al., 2005b) and also lower prevalences in other countries, such as Senegal (11.5%) (Djiba et al., 2013), China (15.3%) (Liu et al., 2012), Italy (13.1–47.3%) (Torina et al., 2008, 2010; Torina and Caracappa, 2012), Slovakia (22.6%) (Derdáková et al., 2011), Sudan (41.7%) and Turkey (31.4%) (Renneker et al., 2013).

The prevalence of *A. ovis* infection was higher in El Alia (91.1%) than in Sbikha (49.5%). This discrepancy may result from differences in tick control programs, farm management, husbandry practices, wildlife reservoir hosts, and/or abiotic factors. In fact, several studies reported the variability of *A. ovis* prevalence according to localities, associated with suitable tick habitats and animal management, increasing tick-borne pathogens transmission (Torina et al., 2008; Liu et al., 2012). Regional variation of *A. ovis* infection prevalence was also reported by Torina et al. (2008) between Eastern and Western regions of Sicily, Italy.

Table 3
Nucleotide and amino acid differences among *msp4* sequences from *Anaplasma ovis* strains.

Strain or sample[a] symbol	Genbank accession number[b]	Location (country)	Genotype	*msp4* nucleotide positions (amino acid positions)[c]		
				230 (77)	470 (157)	532 (178)
Italy 147	AY702924	Sicily (Italy)	II	G (R)	C (A)	C (L)
B1; B2; K2; K4	KC432641	Bizerte and Kairouan (Tunisia)	GBK1	*	*	*
Italy 20	AY702923	Sicily (Italy)	III	*	T (V)	*
B4; K3	KC432642	Bizerte and Kairouan (Tunisia)	GBK2	*	T (V)	*
B3	KC432643	Bizerte (Tunisia)	GB3	T (I)	T (V)	*
K1	KC432644	Kairouan (Tunisia)	GK1	T (I)	T (V)	A (I)

[a] B1, B2, B3 and B4 samples were collected from Bizerte governorate and K1, K2, K3 and K4 samples were collected from Kairouan governorate.
[b] Genbank accession number of the variant.
[c] Numbers represent the nucleotide position starting at translation initiation codon Adenine.
Conserved nucleotide positions with respect to the Italy 147 strain, Sicily (Italy) are indicated with asterisks (de la Fuente et al., 2005b). Amino acid changes are indicated between parentheses with single letter code. (Amino acid: R, Arginine; I, Isoleucine; V, Valine; A, Alanine; L, Leucine; Nucleotide: T, Thymine; C, Cytosine; G, Guanine; A, Adenine).

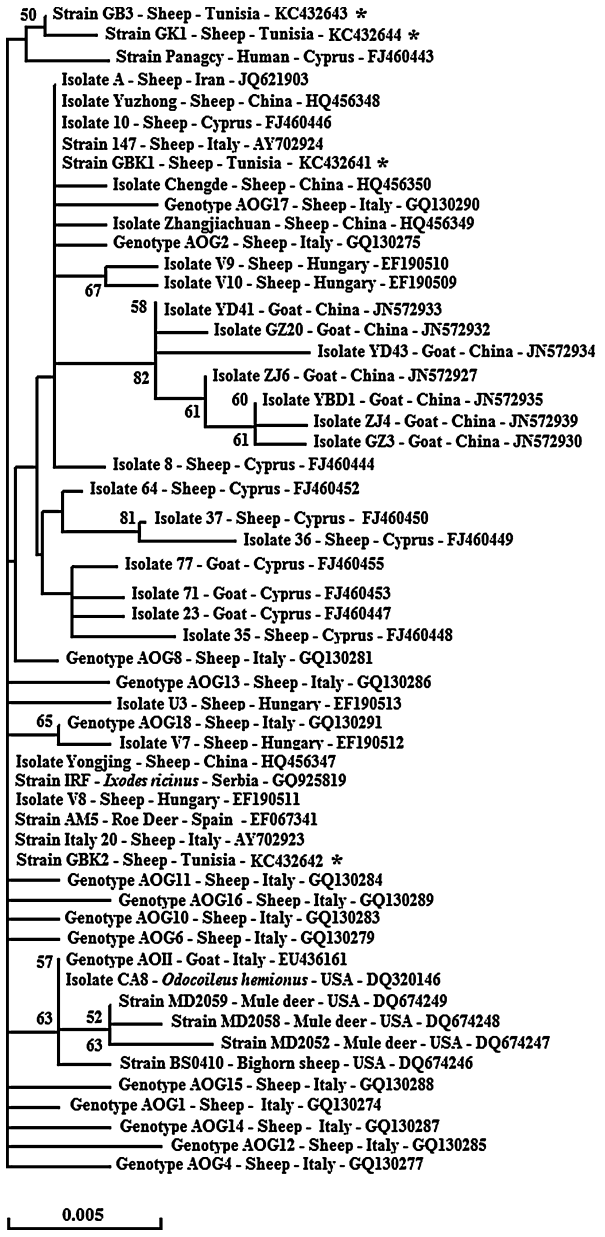

Fig. 2. Neighbor-joining tree based on the alignment of partial *msp4* sequences (719 bp) of *Anaplasma ovis*. Multiple sequence alignments were generated with DNAMAN program (Version 5.2.2; Lynnon Biosoft, Que., Canada). Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting the nodes (only percentages greater than 50% were represented). The new sequences of *A. ovis* obtained in the present study are marked with asterisks. The host or vector, the strain or isolate name, the country of origin and the GenBank accession number are indicated.

In the present study, three tick species were collected from sheep in El Alia. The most dominant tick species was *R. turanicus* (59.78%) followed by *R. sanguineus* (36.16%) and then *R. annulatus* (4.06%). The present data are in agreement with the results of Bouattour (2002) who reported that the major tick species infecting sheep in Tunisia is *R. turanicus* (Bouattour et al., 1999; Darghouth, 2004). All of these three tick species have a vernal activity (Walker et al., 2013), which corresponds to our sampling period in El Alia. This premise suggests that these tick species could be the vectors of *A. ovis* in the investigated area. Some of these tick species such as *R. turanicus* and *R. sanguineus* have been proposed previously as vectors of *A. ovis* in Mediterranean countries (Torina et al., 2008; Aktas et al., 2009). Actually, Aktas et al. (2009) reported the presence of *A. ovis* DNA in salivary glands of *R. sanguineus* collected from Turkey. Torina et al. (2008) reported that *R. turanicus* is one of the main vectors of *A. ovis* in Sicily (Italy). Several authors have identified some other vectors of *A. ovis*. Indeed, Lu et al. (1990) showed that *Dermacentor nuttalli*, *Hyalomma asiaticum* and *Rhipicephalus pumilio* are the main vectors of *A. ovis* in China. Hornok et al. (2007) reported that *Dermacentor marginatus* could be implicated in the transmission of *A. ovis*. *Dermacentor* spp. and *R. bursa* are vectors of *A. ovis* respectively in the New World as well as the Old World (Friedhoff, 1997; Stiller et al., 1999). However, the vectors of *A. ovis* are still unknown in Tunisia; thus, further studies are needed to be done to identify the main vectors of *A. ovis* in this country.

In Sbikha, the prevalence of *A. ovis* infection was significantly affected by the animal's gender and age. Since all females were adults (>1 year) and all males were young (≤1 year), there is no difference identified in *A. ovis* infection prevalence between gender and age. Actually, ewes were significantly more infected than ram lambs. This can be explained by the fact that ewes were probably exposed to several tick seasons. As a matter of fact, Palmer et al. (1998) demonstrated that persistence of *A. ovis* can reach 17–21 months after experimental infection. This led us to suggest that the higher prevalence of *A. ovis* in ewes from Sbikha, compared to lambs, could be due to their possibility to be infected with *A. ovis* during previous tick seasons. Moreover, the lower exposure to ticks due to the absence of lambs grazing and the protective effect of colostral antibodies that can last up to three months among young animals (Friedhoff, 1997) may also explain the lower *A. ovis* prevalence among lambs compared to ewes in Sbikha. In agreement with our result, Mustafa (2012) reports a lower prevalence of *A. ovis* in lambs from Iraq.

The absence of relevant clinical signs in investigated sheep suggests that the causative agent is not a virulent strain like most *A. ovis* strains; and, the predisposing factors that may aggravate its manifestation are not sufficiently present (Friedhoff, 1997). Since the high infection prevalence is association to the absence of clinical symptoms of anaplasmosis, we can confirm that these farms are in endemic stability state to *A. ovis* infection. In general, anaplasmosis usually progresses to a lifelong persistent and subclinical infection (Palmer et al., 1998; Kieser et al., 1990) simultaneously representing a reservoir for arthropod vectors (Kocan et al., 2003). However, clinical cases may occur in stressful conditions, co-infection or if the animals are heavily challenged (Manickam, 1987).

Sequence analysis showed that although *A. ovis msp4* genotypes may be less variable than *A. phagocytophilum* and *A. marginale* strains (de la Fuente et al., 2007), genetic polymorphism occurs in this locus. Indeed, four different genotypes were revealed in eight sequenced samples. This

is in agreement with the observations of Hornok et al. (2007) in Hungary and Torina et al. (2010) in Italy, which showed a high polymorphism, represented by 5 and 17 genotypes, respectively. In addition to the two novel genotypes detected in a sheep (GB3 and GK1), GBK1 and GBK2 genotypes, previously described in Italy (AOG2 and AOG3) where they seem to be predominant (de la Fuente et al., 2005b), were detected in animals from the two studied regions.

The comparison of *msp4* sequences between El Alia and Sbikha suggests that the geographic location is not the origin of genetic diversity of *A. ovis msp4*. The presence of identical *A. ovis* genotypes in both regions could be explained by sheep movements across the country. Similarly to Hornok et al. (2007), the sequences analyses show nucleotide heterogeneity between the sheep of the same region, which demonstrates that *A. ovis msp4* genotypes may vary between animals of the same geographic location.

The present strains can be classified into three different clusters, suggesting multiple introductions of genetically different strains of *A. ovis* in the two regions. The two new genotypes (GB3 and GK1) are classified with *A. ovis* Panagcy strain in an independent cluster (GenBank accession number FJ460443) (Chochlakis et al., 2010). In agreement to other observations, the strong bootstrap values in the phylogenetic tree demonstrate *A. ovis msp4* gene may vary among human, sheep, goat and deer hosts (de la Fuente et al., 2007; Torina et al., 2008). However, there is no phylogeographic information that was obtained by phylogenetic analysis (Fig. 2).

To the best of our knowledge, this is the first published molecular detection of *A. ovis* in sheep from Tunisia. Further studies are needed to investigate other co-infected *Anaplasma* species in Tunisian sheep, to determine the vectors and the wildlife reservoirs of *A. ovis* and also to improve our knowledge on the regional epidemiology of this infection and its financial, human and health impacts.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.smallrumres.2014.07.009.

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