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Short communication

First molecular survey of *Anaplasma bovis* in small ruminants from Tunisia



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ABSTRACT

To date, no information is available regarding the presence of *Anaplasma bovis* in the South Mediterranean area. In this study, prevalence, risk factors, and genetic diversity of *A. bovis* were assessed in small ruminants. A total of 563 healthy small ruminants (260 sheep and 303 goats), from 25 randomly selected flocks located in 5 localities from two bioclimatic areas in Tunisia, were investigated for the detection of *A. bovis* in blood by nested polymerase chain reaction (nPCR) assay. The overall infection rates of *A. bovis* were 42.7 and 23.8% in sheep and goats, respectively. Goats located in a sub-humid area were statistically more infected than those located in a humid area. *A. bovis* prevalence rate varied significantly according to sheep and goat flocks, and to the sheep breed. Infection with *A. bovis* was validated by sequencing. Sequence analysis based on the 16S rRNA gene showed that *A. bovis* from Tunisian goats and sheep clustered with other strain sequences detected from wild and domestic animals and published in GenBank. This study gives the first insight of presence of *A. bovis* DNA in small ruminants in Tunisia and suggests that these animal species may be playing an important role in the bovine anaplasmosis natural cycle caused by *A. bovis* in the South Mediterranean ecosystem.

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1. Introduction

In small ruminants, the diseases due to the tick-borne pathogens belonging to the genera *Anaplasma*, *Babesia*, *Theileria* and *Toxoplasma* are widespread in Tunisia (Gharbi et al., 2013; M'ghirbi et al., 2013; Belkahia et al., 2014).

Within the genus *Anaplasma*, *Anaplasma bovis*, a monocytotropic bacterial species, has been detected in several animals from many countries (Liu et al., 2012). It has been commonly reported in ruminants such as cattle, goats and wild reeves (Liu et al., 2012; Ceci et al., 2014; Yang et al., 2014), and in other animal species like dogs (Sakamoto et al., 2010), rabbits (Goethert and Telford, 2003), cats (Sasaki et al., 2012) and small wild mammals (Masuzawa et al., 2014). In cattle, *A. bovis* infection has been reported as usually asymptomatic, but it can cause a variety of clinical symptoms, including reduced body weight, fever, anemia, depression,

lymphadenopathy and rarely abortion, and death in some cases (Noaman and Shayan, 2010).

Recently, Ybañez et al. (2014) clarified the phylogenetic position of *A. bovis* by single gene and multi-loci trees and established the existence of two subclades within *Anaplasma* genus where *A. bovis* consistently formed a cluster with *A. phagocytophilum*, *A. platys* and *Anaplasma* sp. Japan (a potentially novel *Anaplasma* species related to *A. phagocytophilum* identified in Japan).

Until now, molecular findings demonstrated the occurrence of *A. phagocytophilum* infections in dogs, horses, *Ixodes ricinus*, *Hyalomma scupense*, and *H. marginale* ticks in Tunisia (Sarih et al., 2005; M'ghirbi et al., 2009, 2012). *A. phagocytophilum* was also reported in Tunisian horses and dromedaries by serological method (Ben Said et al., 2014a,b). Recently, *A. ovis* has been detected and characterized in sheep from Northern and Central Tunisia (Belkahia et al., 2014).

To our best knowledge, there have been no reports of small ruminants infected with *A. bovis* in countries of South Mediterranean area like Tunisia. Therefore, the main issue of this study was to investigate *A. bovis* in small ruminants from this country.

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2. Materials and methods

2.1. Blood sampling and tick collection

Five localities situated in Northern Tunisia have been investigated in this cross-sectional study. The two localities of El Alia and Khetmine, both situated in Bizerte governorate, belong to sub-humid bioclimatic area with a mean annual rainfall of 400 mm. The three localities of Joumine, Sejnane, both situated in Bizerte governorate, and Amdoun, situated in Beja governorate, belong to humid bioclimatic area with a mean annual rainfall of 650 mm.

Sheep ($n=260$), from 9 herds, situated in two localities from Bizerte governorate, and goats ($n=303$), from 16 herds, situated in four localities from Bizerte and Beja governorates (Table 1), were sampled in May 2011 and from May to September 2013, respectively. For each animal, blood sample was collected from the jugular vein and placed in an EDTA tube. The approximate age, gender, and breed were noted (Table 2). Goats belonged to three breeds: local breed (275), Alpine (23) and Maltese (5); while sheep belonged to six breeds: Barbarine (118), Noire de Thibar (82), Queue Fine de l'Ouest (10), Merinos (2), Sicilo-sarde (1) and crossbred (47). All ticks found on the animals were manually collected and placed in tubes with 70% ethanol, and later identified using the morphological identification keys of Walker et al. (2013).

2.2. Anaplasma bovis specific detection by nested PCR

DNA was extracted from 300 μ l of each blood sample using Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions. The primer pair EE1 and EE2 was used in the first PCR to amplify the 16S rRNA gene

Table 1

Prevalence rates of *Anaplasma bovis* according to governorates, localities and farms in goats and sheep from Tunisia.

Host	Governorate	Locality	Farms	Number	Positive (% \pm C.I. ^a)
Goats	Bizerte	El Alia	1	30	0 (0)
			2	09	0 (0)
			3	15	12 (80.0 \pm 0.20)
			4	16	13 (81.3 \pm 0.19)
			Total	70	25 (35.7 \pm 0.11)
		Sejnane	9	23	6 (26.1 \pm 0.18)
			10	25	18 (72.0 \pm 0.18)
			11	32	12 (37.5 \pm 0.17)
			Total	80	36 (45.0 \pm 0.11)
			Joumine	12	19
		13		07	1 (14.3 \pm 0.26)
		14		20	0 (0)
		15		20	0 (0)
		16		15	0 (0)
		Total	81	8 (9.9 \pm 0.06)	
	Total	231	69 (29.9 \pm 0.06)		
	Beja	Amdoun	5	16	0 (0)
			6	24	0 (0)
			7	15	2 (13.3 \pm 0.17)
			8	17	1 (5.9 \pm 0.11)
			Total	72	3 (4.2 \pm 0.05)
Total		303	72 (23.8 \pm 0.05)		
Sheep	Bizerte	El Alia	1	30	2 (6.7 \pm 0.09)
			2	20	9 (45 \pm 0.21)
			3	30	5 (16.7 \pm 0.13)
			8	30	5 (16.7 \pm 0.13)
			9	30	6 (20 \pm 0.14)
			Total	140	27 (9.3 \pm 0.06)
			Khetmine	4	30
		5		30	13 (43.3 \pm 0.17)
		6		30	23 (76.7 \pm 0.15)
		7		30	25 (83.3 \pm 0.13)
		Total		120	84 (70 \pm 0.08)
		Total		260	111 (42.7 \pm 0.06)

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

Table 2

Molecular prevalence of *Anaplasma bovis* according to gender, age, breed and tick infestation of goats and sheep.

Risk factor	Goats			Sheep		
	Number	Positive (% \pm C.I. ^a)	P-value	Number	Positive (% \pm C.I. ^a)	P-value
Gender			0.601			0.455
Male	70	15 (21.4 \pm 0.10)		50	19 (38.0 \pm 0.13)	
Female	233	57 (24.5 \pm 0.05)		210	92 (43.8 \pm 0.06)	
Age			0.127			0.538
<2 years	133	26 (19.5 \pm 0.07)		63	29 (40.0 \pm 0.12)	
\geq 2 years	170	46 (27.1 \pm 0.07)		197	82 (41.6 \pm 0.07)	
Breed			0.872			0.020 ^d
Local/Bar ^b	275	65 (23.6 \pm 0.05)		118	59 (50 \pm 0.09)	
Other breeds ^c	28	7 (25.0 \pm 0.16)		142	52 (21.1 \pm 0.08)	
Tick ^e			0.192			0.929
Infested	113	33 (27.7 \pm 0.08)		244	104 (42.6 \pm 0.06)	
Not infested	190	39 (21.2 \pm 0.06)		16	7 (43.7 \pm 0.24)	
Total	303	72 (23.8 \pm 0.05)		260	111 (42.7 \pm 0.06)	

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

^b Local for goats and Barbarine for sheep.

^c Other breeds are Alpine and Maltese for goats and Noire de Thibar, Queue fine de l'Ouest, Merinos, Sicilo-sarde and crossbred for sheep.

^d Indicates significance at the $P < 0.05$ level.

^e Tick infestation.

of any *Anaplasma* species (Barlough et al., 1996) (Supplementary file 1). The reactions were performed in a final volume of 50 μ l containing 0.125 U/ μ l Taq DNA polymerase (Biobasic Inc Canada), 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 μ l (50–150 ng) genomic DNA, 0.5 μ M of primers. Thermal cycling reactions were performed in an automated DNA thermal cycler (Techne Flexigene, Cambridge, UK). *A. bovis* specific primers were used for the second PCR (Supplementary file 1). One microliter of the first PCR amplicon was used in the second PCR utilizing the same reaction conditions as the first PCR (Kawahara et al., 2006). Distilled water and DNA extracted from *A. bovis* South African strain isolated from a rodent were used as negative and positive controls, respectively. PCR products were electrophoresed on 1% agarose gels to check the size of amplicons.

2.3. DNA sequencing and data analysis

Twenty six randomly selected positive PCR products from primers AB1f/AB1r of *A. bovis* were purified using 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 (Supplementary file 1). The reactions were performed using a conventional Big Dye Terminator cycle sequencing ready reaction kit (PerkinElmer, Applied Biosystems, Foster City, USA) and an ABI3730XL automated DNA sequencer (MacroGen Europe, Amsterdam, The Netherlands). The chromatograms were evaluated with Chromas Lite (Version 2.01; Technelysium, Helensvale, Australia). The DNAMAN program (Version 5.2.2; Lynnon Biosoft, Que. Canada) was used to perform multiple sequence alignment of 16S rRNA sequences. Sequence homology search was made by BLASTn analysis of GenBank (<http://blast.ncbi.nlm.nih.gov/>) (Altschul et al., 1997). A phylogenetic tree was constructed using 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.

2.4. GenBank accession numbers

The 16S rRNA partial sequences of *A. bovis* of AbGGo1 genotype found in goat and of AbGOv1 and AbGOv2 genotypes found in sheep have been deposited under GenBank accession numbers from KM285223 to KM285225, respectively.

2.5. Statistical analysis

Comparison of the prevalence of *A. bovis* in sheep and goats according to risk factors, farms, localities and governorates, and comparison of rates of each tick species found in goats and sheep were performed with Epi Info 6.01 (CDC, Atlanta), using the χ^2 test and Fisher's exact test. Observed differences were considered to be statistical significant when the *p* value was less or equal than 0.05. In order to consider any confusion factor, a chi square Mantel-Haenszel test was performed.

3. Results

3.1. Parasitological data

A total of 304 adult ticks (126 males and 178 females) were collected from 113 infested goats (*n* = 303). The ticks belonged to 2 genera and 4 species. *Rhipicephalus turanicus* (79.9%) and *R. bursa* (14.5%) were the dominant species, followed by *R. sanguineus* (4.9%) and there were few specimens of *Hyalomma excavatum* (0.7%) (*p* < 0.001). From infested sheep (244/260), 919 adult ticks (473 males and 446 females) were collected. These ticks belong to 3 species of *Rhipicephalus* genus. *R. turanicus* (52.8%) and *R. sanguineus* (44.0%) were the dominant species, followed by *R. annulatus* (3.3%) (*p* < 0.001).

3.2. Molecular prevalence and risk factor analysis

In goats, the overall infection rate of *A. bovis* was 23.8% (72/303). A significant difference in infection rates was recorded among governorates (*p* < 0.001), localities (*p* < 0.001), farms (*p* < 0.001)

and bioclimatic zones (*p* = 0.007). The highest prevalence was reported in goats of the sub-humid area represented by El Alia locality (35.7%; 25/70) compared to the humid area (20.2%; 47/233) represented by Sejnane, Joumine and Amdoun localities (Table 1). For goats, no risk factors had any effect on the probability for an individual to be infected with *A. bovis* (Table 2). Among sheep, the overall infection rate of *A. bovis* was 42.7% (111/260). Prevalence rates differed statistically among localities (*p* < 0.001) and among farms (*p* < 0.001). *A. bovis* prevalence was significantly higher in Barbarine (50%; 59/118) compared to other breeds (21.1%; 52/142) (*p* = 0.020) (Table 2).

3.3. Genetic characterization using 16S rRNA sequences

A. bovis infections were validated by sequencing of 511 bp 16S rRNA fragment (34.2% of the gene size) from randomly selected sixteen goat samples (four, five, two and five samples from Joumine, Sejnane, Amdoun and El Alia, respectively) and ten sheep samples (seven and three samples from Khetmine and El Alia, respectively). Alignment of these sequences revealed one genotype from goats (AbGGo1; GenBank accession number KM285223) and two genotypes from sheep (AbGOv1 and AbGOv2; GenBank accession numbers KM285224 and KM285225, respectively). AbGGo1 and AbGOv1 genotypes were 100% identical to G55 variant isolated from goat in China (GenBank accession number JN558825) and different from AbGOv2 genotype by two substitutions with an identity rate estimated at 99.6% (Table 3).

Phylogenetic analysis of the partial 16S rRNA gene was performed with *A. bovis* sequences from this study and selected sequences of *A. bovis* isolated from ruminants and other *Anaplasma* species found in GenBank (Fig. 1). All *A. bovis* Tunisian strains were classified in *A. bovis* cluster that was closely related to *A. platys*, *A. phagocytophilum* and *Anaplasma* sp. (Japan) (Fig. 1). However, AbGGo1 and AbGOv1 variants were identical to that of G55 isolate isolated from Chinese goat, while AbGOv2 variant was closely related to that of AB4A strain isolated from Chinese red deer (Fig. 1). Nucleotide sequence identities were evaluated between all revealed sequences and those of other *Anaplasma* species that were *A. platys* (97.7–98% sequence identity), *Anaplasma* sp. (Japan) (97.7–98%), *A. phagocytophilum* (96.9–97.3%), *A. centrale* (97.3%), *A.*

Table 3
Nucleotide diversity among 16S rRNA sequences (511 bp) from *Anaplasma bovis* Tunisian strains and from other strains or isolates infected wild and domestic ruminants published in GenBank.

Host	Country	Strain/isolate	GenBank ^d	16S rRNA nucleotide positions ^e																
				82	98	144	146	151	166	200	226	357	375	380	441	448	451	465	523	540
Goat	Tunisia	AbGGo1 ^a (This study)	KM285223	A	T	A	G	G	A	G	T	T	T	G	A	C	G	A	C	A
	China	G55	JN558825	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		G41	HQ913646	*	*	*	A	*	*	A	*	*	*	*	G	*	*	C	*	*
Sheep	Tunisia	AbGOv1 ^b (This study)	KM285224	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		AbGOv2 ^c (This study)	KM285225	G	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*
Cattle	China	YZ14	KF055363	*	C	*	A	A	*	*	*	*	*	*	*	*	A	*	*	*
		BSB32	KF055362	*	*	*	A	*	*	*	*	*	*	A	*	T	*	*	T	*
		RJB1	KF055357	*	*	G	A	*	*	*	*	*	C	*	*	*	*	*	*	*
		KWDAB2	GU556627	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Deer	China	Ab4a	KJ639885	G	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T
		NR07	AB196475	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
		Ab2a	KJ639887	*	*	*	A	*	*	*	*	*	C	*	*	*	*	*	*	*
		Obihiro-bison	JN811556	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*	T

^a AbGGo1 strain was isolated from Jm1-4, Sj1-5, Am1, 2 and Al1-5 *A. bovis* positive goat samples. Jm1-4, Sj1-5, Am1, 2 and Al1-5 *A. bovis* positive goat samples were collected from Joumine, Sejnane, Amdoun and El Alia localities, respectively.

^b AbGOv1 strain was isolated from Kh1-7; Al1; Al3 *A. bovis* positive sheep samples.

^c AbGOv2 strain was isolated from Al2 *A. bovis* positive sheep sample. Al1-3 and Kh1-7 *A. bovis* positive sheep samples were collected from El Alia and Khetmine localities, respectively.

^d GenBank accession number.

^e Numbers represent the nucleotide position with respect to the isolate G55 (clone 55) from China for *A. bovis* (GenBank accession number JN558825). Conserved nucleotide positions are indicated with asterisks. Nucleotides: T, Thymine; C, Cytosine; G, Guanine; A, Adenine).

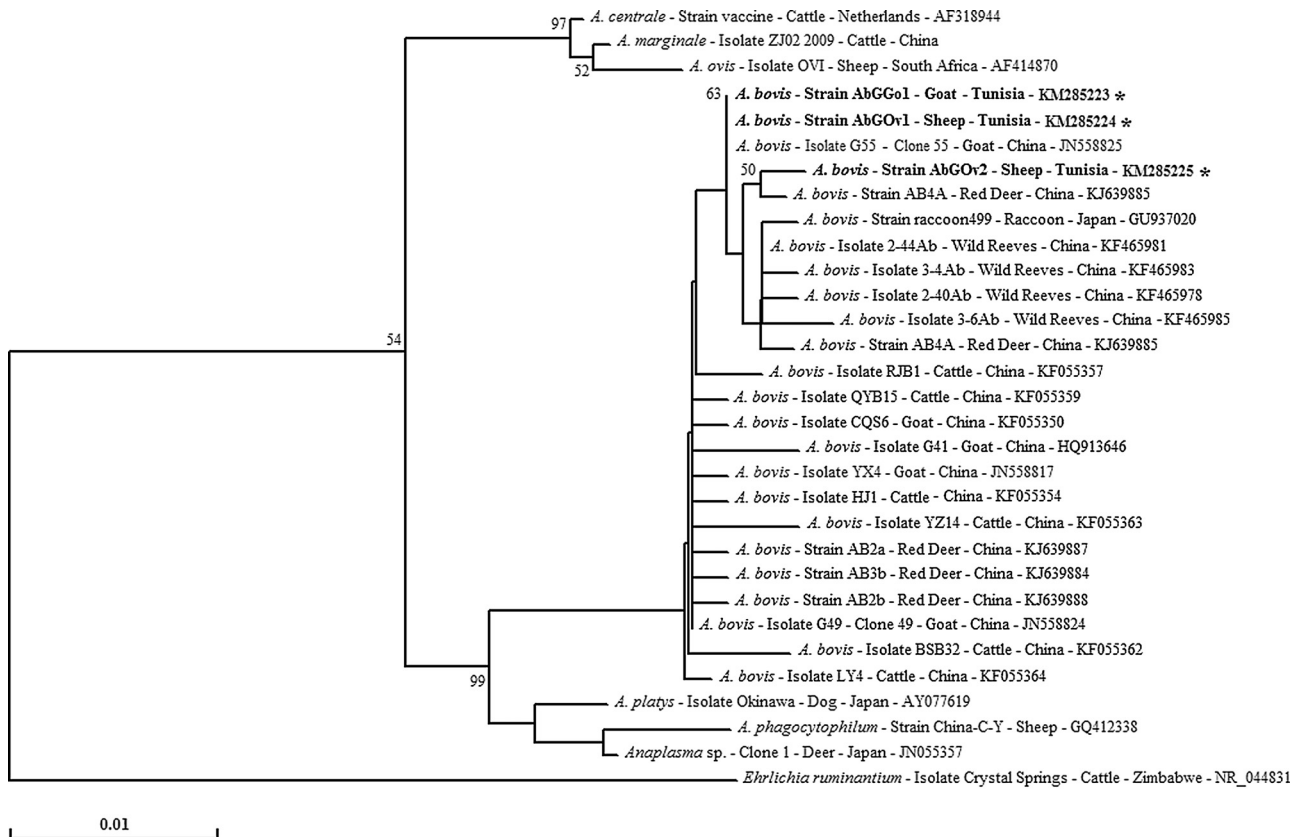


Fig. 1. Phylogenetic tree inferred with partial sequences (511 bp) of the 16S rRNA gene of *Anaplasma bovis* isolated from ruminants and other *Anaplasma* species found in GenBank using the neighbor-joining method. Numbers associated with nodes represent the percentage of 1000 bootstrap iterations supporting the nodes (only percentages greater than 50% were presented). The host or vector, the strain or isolate name, the country of origin and the GenBank accession number are indicated. The new sequences of *A. bovis* obtained in the present study are represented in bold and marked with asterisks.

marginale (97.1%), and *A. ovis* (96.7%). The outgroup, *Ehrlichia ruminantium*, had 92% sequence homology.

4. Discussion

The results clearly indicated the presence of *A. bovis* infection for the first time in sheep and goats from Tunisia. In sheep, *A. bovis* overall prevalence rate was 42.7%. *A. bovis* has been detected in several domesticated ruminants from many countries. It has been isolated from cattle in Iran (2.7%) (Noaman and Shayan, 2010), India (3.3%) (Nair et al., 2013), Italy (4.2%) (Ceci et al., 2014) and Japan (15–53.3%) (Ooshiro et al., 2008; Jilintai et al., 2009), and from goats (49.6%) in China (Liu et al., 2012).

In goats, *A. bovis* prevalence (overall 23.8%) was lower than that found in the investigated sheep (42.7%), and in goats from China (49.6%) (Liu et al., 2012) but similar to that found in deer (23%) from Japan (Kawahara et al., 2006). It can be suggested that this difference could be related to the susceptibility of each animal species to *A. bovis* infection and to differences in rates of infestation by its tick vectors as it was observed in the present study where the tick infestation rate in sheep (93.8%) was statistically more important than in goats (33.6%) ($p < 0.001$).

The presence of *A. bovis* in investigated small ruminants was validated by sequencing of a 16S rRNA partial sequence. In agreement with Ybañez et al. (2014), the phylogenetic analysis shows the presence of two subclades within *Anaplasma* genus, where *A. bovis* formed a subclade with *A. phagocytophilum*, *A. platys* and *Anaplasma* sp. Japan. Indicating low geographic segregation, only two different 16S rRNA sequences isolated from *A. bovis* strains were revealed in sheep and goats from different Tunisian

locations. Since none of the investigated small ruminants showed any apparent clinical sign, these strains seem to have a limited pathogenicity in sheep and goats. Actually, this finding is in agreement with those reported by Liu et al. (2012).

In goats, a significant difference in average infection rates was recorded among bioclimatic areas. Thus, animals located in sub-humid zone were statistically more infected than those located in a humid zone. This difference, which has been reported in other studies on blood parasites in general, is probably related to the effect of bioclimatic conditions on the distribution of tick vectors (M'ghirbi et al., 2013). In addition, overall prevalence rate differed statistically among sheep and goats farms. 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 *Anaplasma* species prevalence according to geographic location, associated with suitable tick habitats and animal management (Liu et al., 2012; Belkahlia et al., 2014).

In sheep, Barbarine breed is the most infected by *A. bovis*, probably explained by the fact that sheep of this breed are not in their natural environment, which is the steppe of dry land in pre-Saharan system mainly represented by the Center of Tunisia. This event matches with results reported by Rjeibi et al. (2014) during an investigation on *Babesia ovis* in Tunisian sheep and goats.

In the present study, the parasitological data are in agreement with several studies which reported that the major tick species infesting small ruminants in Tunisia is *R. turanicus* (Bouattour, 2002). All of identified tick species have a vernal activity (Walker et al., 2013), which corresponds to our sampling period. This finding suggests that one or several of these tick species could be

vectors of *A. bovis* in the investigated regions. However, *Hyalomma* species have been proposed previously as vectors of *A. bovis* in Africa (Donatien and Lestoquard, 1936). More recently, *A. bovis* has been isolated from *R. turanicus* and *R. sanguineus* in Israel (Harrus et al., 2011). Until now, the vectors of *A. bovis* are still unknown in Tunisia; thus, further studies are needed to identify the main vectors of this bacterium.

In conclusion, this study gives the first insight of presence of *A. bovis* in small ruminants in Tunisia and demonstrates that these animal species may be playing an important role in the bovine anaplasmosis natural cycle caused by *A. bovis* in this country. Further studies are needed to better characterize the different strains of this species by more discriminative genes and to identify the main vectors implicated in the transmission of this *Anaplasma* species in South Mediterranean ecosystem.

Competing interests

The authors declare that they have no competing interests.

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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.vetmic.2015.05.022>.

References

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.

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.

Belkahlia, 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., Belkahlia, 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., Belkahlia, H., Sayahi, L., Aloui, M., Jemli, M.H., Hadj Mohamed, B., Sassi, L., Darghouth, M.A., Djaïem, A.A., Bayouh, 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., 2002. Clé dichotomique et identification des tiques (Acari: Ixodidae) parasites du bétail au Maghreb. *Arch. Inst. Pasteur Tunis* 79, 43–50.

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.

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

Gharbi, M., Zribi, L., Jedidi, M., Chakkhari, H., Hamdi, S., R'hayem, S., Zribi, N., Souli, M., Darghouth, M.A., 2013. Prévalence d'infection des ovins par *Toxoplasma gondii* en Tunisie. *Bull. Soc. Pathol. Exot.* 106, 184–187.

Goethert, H.K., Telford, S.R., 2003. Enzootic transmission of *Anaplasma bovis* in Nantucket cottontail rabbits. *J. Clin. Microbiol.* 41, 3744–3747.

Harrus, S., Perlman-Avrahami, A., Mumcuoglu, K.Y., Morick, D., Eyal, O., Baneth, G., 2011. Molecular detection of *Ehrlichia canis*, *Anaplasma bovis*, *Anaplasma platys*, *Candidatus Midichloria mitochondrii* and *Babesia canis vogeli* in ticks from Israel. *Clin. Microbiol. Infect.* 17, 459–463.

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.

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.

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.

Masuzawa, T., Uchishima, Y., Fukui, T., Okamoto, Y., Pan, M.J., Kadosaka, T., Takada, N., 2014. Detection of *Anaplasma phagocytophilum* and *Anaplasma bovis* in small wild mammals from Taichung and Kinmen Island, Taiwan. *Jpn. J. Infect. Dis.* 67, 111–114.

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. Vec.* 180, 30.

M'ghirbi, Y., Ros-García, A., Iribar, P., Rhaim, A., Hurtado, A., Bouattour, A., 2013. A molecular study of tick-borne haemoprotozoan parasites (*Theileria* and *Babesia*) in small ruminants in Northern Tunisia. *Vet. Parasitol.* 198, 72–77.

Nair, A.S., Ravindran, R., Lakshmanan, B., Sreekumar, C., Kumar, S.S., Raju, R., Tresamol, P.V., Vimalkumar, M.B., Saseendranath, M.R., 2013. Bovine carriers of *Anaplasma marginale* and *Anaplasma bovis* in South India. *Trop. Biomed.* 30, 105–112.

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

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.

Rjeibi, M.R., Gharbi, M., Mhadhbi, M., Mabrouk, W., Ayari, B., Nasfi, I., Jedidi, M., Sassi, L., Rekik, M., Darghouth, M.A., 2014. Prevalence of piroplasms in small ruminants in North-West Tunisia and the first genetic characterization of *Babesia ovis* in Africa. *Parasite* 21, 23.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Bio. Evol.* 4, 406–425.

Sakamoto, L., Ichikawa, Y., Sakata, Y., Matsumoto, K., Inokuma, H., 2010. Detection of *Anaplasma bovis* DNA in the peripheral blood of domestic dogs in Japan. *Jpn. J. Infect. Dis.* 63, 349–352.

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.

Sasaki, H., Ichikawa, Y., Sakata, Y., Endo, Y., Nishigaki, K., Matsumoto, K., Inokuma, H., 2012. Molecular survey of *Rickettsia*, *Ehrlichia*, and *Anaplasma* infection of domestic cats in Japan. *Ticks Tick Borne Dis.* 3, 308–311.

Walker, A.R., Bouattour, A., Camicas, J.-L., Estrada-Peña, A., Horak, I.G., Latif, A.A., Pegram, R.G., Prestom, P.M., 2013. Ticks of domestic animals in Africa: a guide to identification of species. *Biosci. Reports. Part 4: Species Ticks* pp. 45–221.

Yang, J., Li, Y., Liu, Z., Guan, G., Chen, Z., Luo, J., Wang, X., Yin, H., 2014. Molecular evidence for *Anaplasma bovis* infection in wild Reeves' muntjac (*Muntiacus reevesi*) Southwest China. *J. Wildl. Dis.* 50, 982–985.

Ybañez, A.P., Sashika, M., Inokuma, H., 2014. The phylogenetic position of *Anaplasma bovis* and inferences on the phylogeny of the genus *Anaplasma*. *J. Vet. Med. Sci.* 76, 307–312.