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Original article

Genetic characterization of *Anaplasma marginale* strains from Tunisia using single and multiple gene typing reveals novel variants with an extensive genetic diversity

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ABSTRACT

Anaplasma marginale, which is responsible for bovine anaplasmosis in tropical and subtropical regions, is a tick-borne obligatory intraerythrocytic bacterium of cattle and wild ruminants. In Tunisia, information about the genetic diversity and the phylogeny of *A. marginale* strains are limited to the *msp4* gene analysis. The purpose of this study is to investigate *A. marginale* isolates infecting 16 cattle located in different bioclimatic areas of northern Tunisia with single gene analysis and multilocus sequence typing methods on the basis of seven partial genes (*dnaA*, *ftsZ*, *groEL*, *lipA*, *secY*, *recA* and *sucB*). The single gene analysis confirmed the presence of different and novel heterogenic *A. marginale* strains infecting cattle from the north of Tunisia. The concatenated sequence analysis showed a phylogeographical resolution at the global level and that most of the Tunisian sequence types (STs) formed a separate cluster from a South African isolate and from all New World isolates and strains. By combining the characteristics of each single locus with those of the multi-loci scheme, these results provide a more detailed understanding on the diversity and the evolution of Tunisian *A. marginale* strains.

1. Introduction

Tunisian ecosystems represent a favorable ecology for several tick species which can transmit numerous bacterial agents (Ben Said et al., 2018). Among these, *Anaplasma marginale* is a tick-borne rickettsial endemic worldwide especially in tropical and subtropical areas (Ben Said et al., 2018; Kocan et al., 2003). This bacterium causes a variety of clinical signs, including fever, weight loss, abortion, lethargy, icterus, and often death of the animals which are older than 2 years (Kocan et al., 2003). In Tunisia, the infection by this *Anaplasma* species has been investigated only in cattle and dromedaries. Belkahia et al. (2015b) failed to detect *A. marginale* in Tunisian camels, but in cattle, the infection has been confirmed by several cross-sectional and longitudinal investigations (Belkahia et al., 2015a, 2017; M'ghirbi et al., 2016).

The analysis of some risk factors was carried out in cattle in Tunisia. Indeed, *A. marginale* infection prevalence varied according to geographic region, bioclimatic area, tick infestation, animal age and breed, and type of breeding (Belkahia et al., 2015a, 2017; M'ghirbi et al., 2016). However, the molecular characterization of Tunisian strains is essential for evaluating the impact of anaplasmosis and their clinical signification. But until now, information about the genetic diversity and the phylogeny of Tunisian *A. marginale* strains are limited to the *msp4* gene analysis. In particular, genetic variation has been found in Tunisian *A. marginale* strains infecting cattle located in different localities and bioclimatic zones in Tunisia; however it was not possible to attribute a certain geographic origin to genetic variants (Belkahia et al., 2015a, 2017; M'ghirbi et al., 2016). These investigations showed the existence of different and novel *msp4* genetic variants of *A. marginale* proving an extensive genetic heterogeneity of Tunisian strains compared to those found in other countries.

Recently, multilocus sequence typing (MLST) was developed and applied by Guillemi et al. (2015) for the characterization of *A. marginale* isolates using seven different loci (*dnaA*, *ftsZ*, *groEL*, *lipA*, *secY*, *recA* and *sucB*). High nucleotide diversity, a large proportion of synonymous substitutions and recombination events were detected in almost all genes. However, the authors failed to find any evident association between geographic locations and sequence types obtained from different isolates.

In the present study, for further characterization of Tunisian *A. marginale* strains, two approaches were used on *A. marginale* strains infecting naturally 16 cattle selected from different geographic locations from northern Tunisia. Firstly, by using earlier cited housekeeping genes initially employed in a MLST scheme, each locus was examined

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Table 1
Designation and information about origins and sequence types of the 16 *Anaplasma marginale* Tunisian isolates characterized in this study.

Isolate name	ST[a]	District (governorate, bioclimatic area)[b]	Reference	Genotype (GenBank accession number)						
				dnaA	*ftsZ*	*groEL*	*lipA*	*recA*	*secY*	*sucB*
TunBv10	1	Omaden (Bz, LH)	Belkahia et al. (2017)	dnaAGV1 (MG807922)	ftsZGv1 (MG807938)	groELGv1 (MG807954)	lipAGv1 (MG807970)	recAGv1 (MG807986)	secYGv1 (MG808002)	sucBGv1 (MG808018)
TunBv25	2	Oued Abid (Nb, SH)	Belkahia et al. (2017)	dnaAGV1 (MG807923)	ftsZGv2 (MG807939)	groELGv1 (MG807955)	lipAGv2 (MG807971)	recAGv2 (MG807987)	secYGv2 (MG808003)	sucBGv2 (MG808019)
TunBv11	3	Omaden (Bz, LH)	Belkahia et al. (2017)	dnaAGV2 (MG807924)	ftsZGv3 (MG807940)	groELGv1 (MG807956)	lipAGv3 (MG807972)	recAGv3 (MG807988)	secYGv3 (MG808004)	sucBGv1 (MG808020)
TunBv49	4	Chott Solimen (Nb, HSA)	Belkahia et al. (2017)	dnaAGV1 (MG807925)	ftsZGv2 (MG807941)	groELGv2 (MG807957)	lipAGv1 (MG807973)	recAGv4 (MG807989)	secYGv1 (MG808005)	sucBGv1 (MG808021)
TunBv31	5	Oued Abid (Nb, SH)	Belkahia et al. (2017)	dnaAGV1 (MG807926)	ftsZGv2 (MG807942)	groELGv3 (MG807958)	lipAGv2 (MG807974)	recAGv2 (MG807990)	secYGv3 (MG808006)	sucBGv2 (MG808022)
TunBv55/1	6	El Mabtouh (Bz, HSA)	Belkahia et al. (2015a)	dnaAGV3 (MG807927)	ftsZGv4 (MG807943)	groELGv4 (MG807959)	lipAGv1 (MG807975)	recAGv2 (MG807991)	secYGv1 (MG808007)	sucBGv3 (MG808023)
TunBv53/1	7	Bech Hamba (Bz, HSA)	Belkahia et al. (2015a)	dnaAGV4 (MG807928)	ftsZGv2 (MG807944)	groELGv2 (MG807960)	lipAGv1 (MG807976)	recAGv5 (MG807992)	secYGv1 (MG808008)	sucBGv4 (MG808024)
TunBv56/1	8	El Mabtouh (Bz, HSA)	Belkahia et al. (2015a)	dnaAGV3 (MG807929)	ftsZGv5 (MG807945)	groELGv4 (MG807961)	lipAGv1 (MG807977)	recAGv2 (MG807993)	secYGv1 (MG808009)	sucBGv3 (MG808025)
TunBv59/1	9	Bech Hamba (Bz, HSA)	Belkahia et al. (2015a)	dnaAGV3 (MG807930)	ftsZGv4 (MG807946)	groELGv4 (MG807962)	lipAGv1 (MG807978)	recAGv2 (MG807994)	secYGv1 (MG808010)	sucBGv3 (MG808026)
TunBv60/1	10	Ezzogb (Bz, SH)	Belkahia et al. (2015a)	dnaAGV3 (MG807931)	ftsZGv4 (MG807947)	groELGv4 (MG807963)	lipAGv1 (MG807979)	recAGv2 (MG807995)	secYGv1 (MG808011)	sucBGv3 (MG808027)
TunBv61/1	11	Utique (Bz, HSA)	Belkahia et al. (2015a)	dnaAGV3 (MG807932)	ftsZGv4 (MG807948)	groELGv4 (MG807964)	lipAGv1 (MG807980)	recAGv2 (MG807996)	secYGv1 (MG808012)	sucBGv3 (MG808028)
TunBv69/4	12	Sidi Ali Chebeb (Bz, SH)	Belkahia et al. (2015a)	dnaAGV5 (MG807933)	ftsZGv2 (MG807949)	groELGv4 (MG807965)	lipAGv4 (MG807981)	recAGv6 (MG807997)	secYGv1 (MG808013)	sucBGv1 (MG808029)
TunBv68/4	13	Sidi Ali Chebeb (Bz, SH)	Belkahia et al. (2015a)	dnaAGV3 (MG807934)	ftsZGv3 (MG807950)	groELGv4 (MG807966)	lipAGv5 (MG807982)	recAGv6 (MG807998)	secYGv2 (MG808014)	sucBGv1 (MG808030)
TunBv76/7	14	Techga (Bz, SH)	Belkahia et al. (2015a)	dnaAGV6 (MG807935)	ftsZGv6 (MG807951)	groELGv4 (MG807967)	lipAGv1 (MG807983)	recAGv7 (MG807999)	secYGv1 (MG808015)	sucBGv1 (MG808031)
TunBv74/1	15	Techga (Bz, SH)	Belkahia et al. (2015a)	dnaAGV1 (MG807936)	ftsZGv7 (MG807952)	groELGv5 (MG807968)	lipAGv4 (MG807984)	recAGv6 (MG808000)	secYGv1 (MG808016)	sucBGv1 (MG808032)
TunBv82/4	16	Utique (Bz, HSA)	Belkahia et al. (2015a)	dnaAGV7 (MG807937)	ftsZGv3 (MG807953)	groELGv4 (MG807969)	lipAGv1 (MG807985)	recAGv6 (MG808001)	secYGv2 (MG808017)	sucBGv1 (MG808033)

[a] ST, Sequence type.
[b] Governorate: Bz, Bizerte; Nb, Nabeul; Bioclimatic area: LH, Low humid; SH, Sub-humid; HSA, Higher semi-arid.

independently according to the single gene analysis (SGA) method, which was performed to search for possible phylogeographic resolution at least for one in each of the seven analyzed genes. To deepen the analysis, we performed multigenic analysis based on concatenated sequences in order to acquire additional phylogeographical information necessary for the worldwide classification of our Tunisian isolates.

2. Materials and methods

2.1. Infected cattle and Anaplasma marginale selected strains

Sixteen cattle samples infected by *A. marginale* and not infected by other *Anaplasma* spp. (such as *A. centrale*, *A. phagocytophilum* and related strains, *A. bovis*, *A. ovis,* and *A. platys* and related strains) were selected from positive samples revealed earlier by Belkahia et al. (2015a, 2017) (Table 1). Selected animals were from seven districts belonging to governorate of Bizerte: Omaden (n = 2), El Mabtouh (n = 2), Bach Hamba (n = 2), Utique (n = 2), Techga (n = 2), Sidi Ali Chebeb (n = 2) and Ezzogb (n = 1) and from two districts belonging to governorate of Nabeul: Oued Abid (n = 2) and Chott Soliman (n = 1) (Fig. 1). Bioclimatic areas of studied districts are presented in Table 1.

2.2. DNA extraction and polymerase chain reaction

DNA was re-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.

Single PCRs were performed on seven genes namely *dnaA*, *ftsZ*, *groEL*, *lipA*, *secY*, *recA* and *sucB* used in MLST scheme recently developed by Guillemi et al. (2015). However, following a failure of the amplification of the *groEL* partial sequence using the reverse primer of Guillemi et al. (2015), another inverse primer has been designed that allows the amplification of a larger sequence (1025 bp) and which covers the sequence amplified by Guillemi et al. (2015). Studied genes and their corresponding sizes are presented in Table 2. PCR reaction was performed in a final volume of 50 µl containing 0,125 U/µl Taq DNA polymerase (Biobasic Inc, Canada), 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 µl genomic DNA and 0.5 µM of primers. Thermal cycling profile was as described by Guillemi et al. (2015). In this experiment, distilled water (and DNA from bovine blood not infected with *Anaplasma* spp.) and DNA extracted from *A. marginale* (Belkahia et al., 2015a) were used as negative and positive controls, respectively. PCR products were electrophoresed in 1.5% agarose gel.

2.3. DNA sequencing, molecular typing and phylogenetic analysis

All PCR products obtained with primers used for the amplification of the seven loci *dnaA*, *ftsZ*, *groEL*, *lipA*, *secY*, *recA and sucB* 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 2). 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. 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 single and concatenated sequences and to translate nucleotide to amino-acid