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The characterization of *Brucella* strains isolated from cattle in Algeria reveals the existence of a *B. abortus* lineage distinct from European and Sub-Saharan Africa strains

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ABSTRACT

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella* that causes important economic losses and human suffering worldwide. Brucellosis control requires an understanding of the *Brucella* species circulating in livestock and humans and, although prevalent in African countries of the Mediterranean basin, data for this area are mostly restricted to isolates obtained from humans and small ruminants. Here, we report the characterization of twenty-four *Brucella* strains isolated from Algerian cattle. Bruce-ladder multiplex PCR and conventional biotyping showed that Algerian cattle are infected mostly by *B. abortus* biovar 3, and to less extent by *B. abortus* biovar 1 and *B. melitensis* biovar 3. Extended AMOS-ERY PCR showed that all Algerian *B. abortus* biovar 3 strains were of the subgroup 3b. Although by multi locus variable number of tandem repeats analysis (MLVA) most isolates were closer to the European counterparts, five strains displayed characteristics distinct from the European isolates and those of countries across the Sahara, including three repetitions of marker Bruce55. These five strains, plus an earlier isolate from an Algerian human patient, may represent a lineage close to clades previously described in Africa. These data provide the basis for additional molecular epidemiology studies in northern Africa and indicate that further bacteriological and molecular investigations are necessary for a complete understanding of the epidemiology of cattle brucellosis in countries north and south of the Sahara.

1. Introduction

Bacteria of the genus *Brucella* cause brucellosis, a zoonosis that affects a variety of vertebrates and causes a grave and debilitating disease in humans. Eradicated from domestic ruminants in some industrialized countries, brucellosis is endemic in livestock in many areas of the world, where it represents a substantial source of economic losses and human suffering (McDermott et al., 2013). Up to now, twelve nominal *Brucella* species have been described (http://www.bacterio.net/-allnamesac.html), among which *B. abortus* and *B. melitensis* preferentially infect cattle and small ruminants, respectively. Despite this typical host range, *B. melitensis* is also a source of cattle infections in mixed breeding systems (Godfroid et al., 2013) and less frequently some *B. suis* biovars can also infect cattle (Alton et al., 1988; Corbel, 1997; Ewalt et al., 1997; Musser et al., 2013; Szulowski et al., 2013; Tae et al., 2012), which makes necessary *Brucella* species identification for a complete understanding of the epidemiology of the disease in these animals. However, because of the existence of immunodominant epitopes common to *B. abortus*, *B. melitensis* and *B. suis*, the *Brucella* species infecting a given individual, herd or flock, cannot be identified by serological testing (Ducrotoy et al., 2016). Moreover, most laboratories cannot carry out a direct identification of species-specific DNA

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sequences in animal tissues or fluids, and the vast majority of these methods have not been validated for specificity and sensitivity. Therefore, bacteriological isolation and typing are currently necessary for epidemiological surveys under most circumstances. Phenotypic methods for species identification and further division into biovars were developed decades ago (Alton et al., 1988). However, these methodologies use a limited number of characters, pose reproducibility problems and require specialized laboratories. Thus, they have been surpassed by the analysis of DNA obtained from strains previously isolated from milk, vaginal swabs or necropsy samples. Methods like multiplex Bruce-ladder PCR for species identification (López-Goñi et al., 2008) and multi locus variable number of tandem repeat analysis (MLVA) for in depth epidemiological studies have gained wide acceptance (Al-Dahouk et al., 2007; Alton et al., 1988; Bertu et al., 2015; Corbel, 1997; Ducrotoy et al., 2017; Ewalt et al., 1997; Ferreira et al., 2012; García-Yoldi et al., 2007; Jiang et al., 2011; Le Fleche et al., 2006; Maquart et al., 2009; Mick et al., 2014; Musser et al., 2013; Szulowski et al., 2013; Tae et al., 2012; Tian et al., 2017; Whatmore et al., 2016). Not surprisingly MLVA has provided data with phylogeographic implications and revealed inconsistencies within the classical biovars (Le Fleche et al., 2006; Whatmore et al., 2016).

Mostly based on serological evidence, brucellosis has been reported throughout much of Africa (Ducrotoy et al., 2017; Hegazy et al., 2011; Lounes et al., 2014; McDermott et al., 2013). Although brucellosis is known to be endemic in Mediterranean Africa, bacteriological data are scarce (Aggad and Boukraa, 2006; Benhabyles et al., 1992; Gabli et al., 2015; Lounes et al., 2014; Verger and Grayon, 1984; Whatmore et al., 2016) and seldom supported by DNA analyses. For the Maghreb (western North Africa), Lounes et al. (2014) presented the results of a MLVA of 90 *B. melitensis* strains of human origin, but to the best of our knowledge there are no DNA molecular analyses of *B. abortus* isolates obtained in this area. Here, we present the characterization and MLVA carried out on 24 Algerian strains isolated from cattle plus one human isolate. Taken together with the molecular data of Lounes et al. (2014) on *B. melitensis*, the results provide the basis for a better understanding of the epidemiology of the brucellosis in Mediterranean Africa.

2. Methods

2.1. Study site/area

The study was conducted in 2015 in farms and slaughterhouses of the Algeria Center region (Medea and El-Azizia), an agropastoral area located in the heart of the Tellian Atlas, a transit area between the Tell and the Sahara, and between the Highlands of eastern and western Algeria. Investigated cattle include imported breeds (Montbeliarde, Flekveih, Holstein, Brune des Alpes or Frisonne) and local breeds as well as crossbred animals (Magpie Black or Red Magpie).

2.2. Animal samples

Blood (serum) and mammary and retropharyngeal lymph nodes were collected from 225 cows in Medea city and El-Azizia slaughterhouses. In addition, blood sera and milk were obtained from 295 cattle (261 females and 34 males) in dairy farms. Lymph nodes were collected in sterile plastic bags, sealed and transported to the laboratory in iceboxes. Milk and necropsy samples were preserved at −20 °C. Rose Bengal and complement fixation tests were conducted following OIE guidelines (Alton et al., 1988; OIE, 2016). In addition, positive sera of Medea and El-Azizia were confirmed by the double gel diffusion immunoprecipitation test with *B. melitensis* native hapten (NH)-rich lipopolysaccharide (LPS) preparations in hypertonic gels (OIE, 2016), as a positive result in this test has been shown to correlate with a positive culture (Jones et al., 1980).

2.3. Bacterial culture

Milk samples (n = 8) and lymph nodes (n = 30) of all seropositive cattle (n = 32) were cultured on CITA medium (de Miguel et al., 2011). Lymph nodes were degreased and surface sterilized by brief immersion on ethanol and gentle burning. Then, they were suspended in the minimal possible amount of sterile saline, homogenized, and at least 0.5 ml of each homogenate inoculated per plate. Milk was plated directly on the same media (0.5 ml/plate; one plate for each quarter), a procedure that results in the same sensitivity as the classical centrifugation method (Alton et al., 1988) and avoids the biohazards inherent to aerosols (J.M. Blasco, personal communication). All plates were incubated aerobically under a 5–10% CO_2 atmosphere at 37 °C and examined daily for suspicious colonies during the next 3–7 days. Colonies containing Gram-negative, oxidase positive coccobacilli were subcultured on Blood Agar Base N°2 supplemented with 5% sterile calf serum, and pure cultures were stored in skim milk at −80 °C.

2.4. Conventional species and biovar identification

All suspicious isolates were tested for oxidase and urease activity and CO_2 requirement, and then for phage sensitivity (Tb, Wb, Iz and R/C), acriflavine agglutination, crystal violet exclusion, agglutination with anti-A and anti-M sera and sensitivity to thionin (10 μg/ml, 20 μg/ml and 40 μg/ml), basic fuchsin (10 μg/ml and 20 μg/ml) and safranin (100 μg/ml) (Alton et al., 1988; OIE, 2016). *B. abortus* strains S19, 2308, 544 and Tulya, and *B. melitensis* strains 16 M and Ether (all from the collection kept at Centro de Investigación y Tecnología Agroalimentaria [CITA]) were used as controls.

2.5. Molecular typing

Genomic DNA was extracted using The PowerMicrobial ® Maxi DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Species identification was confirmed by Bruce-ladder multiplex PCR (8 target genes) (López-Goñi et al., 2008), and the enhanced AMOS-ERY PCR, which discriminates two different haplotypes (3a and 3b) within *B. abortus* biovar 3 was subsequently carried out (Ocampo-Sosa et al., 2005). MLVA genotyping was performed using the 16-primer-pair PCR assay described elsewhere (Al-Dahouk et al., 2007; Le Fleche et al., 2006) and the DNA fragment sizes obtained were converted to repeat unit (U) numbers using the corresponding formula for each locus. Cluster analysis of MLVA data was performed with the Bionumerics 7.6.1 software (Applied Maths, Belgium) using the UPGMA (Unweighted Pair Group Method Algorithm) algorithm or a minimum spanning tree (MST) with categorical distance matrices. The biovar 1 reference strain *B. melitensis* 16 M was included as outgroup for molecular analysis. The Algerian MLVA pattern (MLVA-11 and MLVA-16) investigated in this study, including the reference strain *B. abortus* biovar 3 Tulya, were compared with 115 MLVA genotypes from Nigeria, Kenya, Sudan, France and Spain previously published and/or available at CITA and ANSES collections and the *Brucella* 2012 database (http://mlva.u-psud.fr/mlvav4/genotyping/) hosted by Paris Sud University (Orsay, France).

3. Results

A total of 32 (6.15 ± 2%) sera were found positive when 520 cattle from Medea were analyzed by RBT and DGD-NH immunoprecipitation, and samples of these seropositive animals were cultured for brucellae. Two milk samples from two different farms (strains AB0114 and AB0115) and 22 retropharyngeal and/or mammary lymph nodes (Table 1) yielded colonies of Gram-negative coccobacilli that were oxidase and urease positive. As expected, some animals yielded bacteria in more than one organ/source (Table 1). Thus, the 24 isolates corresponded to 18 infected animals. All isolates were smooth by the crystal

Table 1
Summary of bacteriological results.

Location[a]	Sample[b]	Strain code[c]	Species and biovar (bv)	Subtype (AMOS-ERY PCR)	Bruce55 repetitions (MLVA)
Medea (f)	Milk	AB0114	*B. abortus* bv 3	3b	3
Medea (f)	Milk	AB0115	*B. abortus* bv 3	3b	3
Medea city (s)	Mammary	AB0338	*B. abortus* bv 1	–	–
Medea city (s)	Mammary	AB0339	*B. abortus* bv 1	–	–
El-Azizia (s)	Retropharyngeal	AB0122	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary	AB0123	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary	AB0124	*B. abortus* bv 3	3b	3
El-Azizia (s)	Mammary	AB0125	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary	AB0126	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary	AB0127	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary/Retropharyngeal	AB0116/AB0117	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary/Retropharyngeal	AB0129/AB0130	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary/Retropharyngeal	AB0118/AB0119	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary	AB0128	*B. abortus* bv 3	3b	1
El-Azizia (s)	Mammary/Retropharyngeal	AB0120/AB0121	*B. abortus* bv 3	3b	3
El-Azizia (s)	Mammary/Retropharyngeal	AB0336/AB0337	*B. abortus* bv 1	–	–
El-Azizia (s)	Mammary/Retropharyngeal	AZB240/AZB241	*B. melitensis* bv 3	–	–
El-Azizia (s)	Mammary	AB0131	*B. abortus* bv 3	3b	1

[a] (f) farm, (s) slaughterhouse.
[b] Collected samples for bacteriological purposes include milk (from farms) and mammary and/or retropharyngeal lymph nodes (from farms and slaughterhouses).
[c] See also Supplemental Figs. S4 and S5 .

violet dye exclusion and/or the acriflavine agglutination tests and, consistent with these results, all were resistant to the rough-specific bacteriophage R/C. Twenty-two isolates were lysed by phages Tb, Wb and Iz, and 2 were resistant to phages Tb and Wb but lysed by phage Iz, patterns that are typical of smooth *B. abortus* and *B. melitensis*, respectively. Species classification was confirmed by Bruce-ladder multiplex PCR (Supplemental Fig. S1).

As expected, the 2 *B. melitensis* isolates (AZB240 and AZB241) did not require CO_2 for growth. Both agglutinated with anti-A and anti-M sera and were resistant to thionine at 20 µg/ml, to basic fuchsin at 20 µg/ml and to safranin at 100 µg/m. Accordingly, they were classified as *B. melitensis* biovar 3. Among the *B. abortus* isolates, 4 (AB0336 to AB0339) belonged to biovar 1 (CO_2-dependent, sensitive to thionine and resistant to basic fuchsin at 20 µg/ml, and safranin at 100 µg/ml), and 18 to biovar 3 (CO_2-dependent on primary isolation and resistant to thionin and basic fuchsin at 20 µg/ml and safranin at 100 µg/ml). This phenotypic discrimination between biovar 1 and biovar 3 strains was in concordance with the profiles obtained with the enhanced AMOS-ERY PCR. All the *B. abortus* biovar 3 strains yielded an enhanced AMOS-ERY PCR profile that was different from that of the biovar 3 Tulya reference strain (biovar 3a) and similar to that of the Spanish biovar 3 (biovar 3b) strains (Table 1 and Supplemental Fig. S2).

The MLVA-16 assay (16 loci) showed that the isolates from the same animal (Table 1) were indistinguishable by this method and clustered together. As regards to the *B. abortus* biovar 3 strains, MLVA-11 allowed clustering the 89 *B. abortus* biovar 3 included in the analysis, covering the 18 Algerian strains investigated in this study, into three distinct lineages. Remarkably, 6 Algerian strains (5 from this study [AB0114, AB0115, AB0120, AB0121, AB0124] plus one *B. abortus* of human origin taken from the data base [AB0078]) were in a group distinct from the 3a non-Mediterranean Africa strains (Nigeria, Kenya, Sudan and Uganda for Tulya) and the 3b European (Spanish and French) strains (Fig. 1 and Supplemental Fig. S3), showing the existence of a different genotype within biovar 3b (see below). The existence of three biovar 3 lineages, including this third type, was confirmed by the MLVA16 (Supplemental Fig. S4), and closer examination showed that while the Algerian isolates in the larger biovar 3 cluster presented only one repeat unit (1 U) for Bruce55, the third group of Algerian strains (AB0114, AB0115, AB0120, AB0121, AB0124 and AB0078) harbored 3 U of this marker.

Concerning the 4 *B. abortus* biovar 1 isolates, they were grouped together with strains of Morocco, France, Italy, Portugal, Germany and

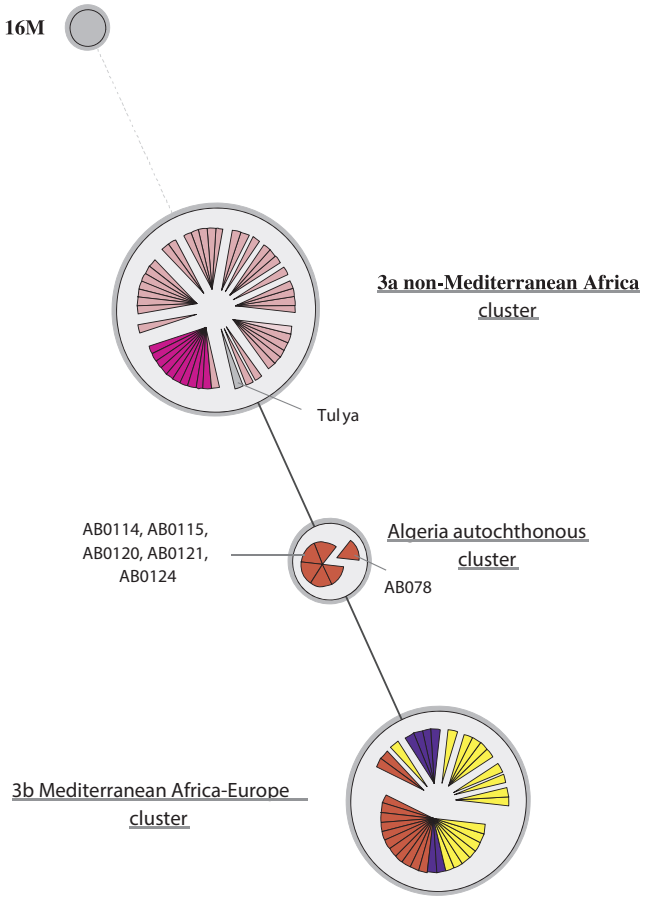

Fig. 1. MST of MLVA11 genotypes of 89 worldwide *B. abortus* biovar 3 isolates, including Tulya. The MST was constructed with a categorical coefficient using *B. melitensis* bv 1 16 M as outgroup. Size of circles reflects the number of isolates with a particular MLVA genotype. Width of the line reflects the genetic distance between the genotypes (heavy short lines connect SLVs, thin longer lines connect DLVs). Each country is assigned a different colour. Blue: France; Fuchsia: Kenya; Grey: reference strains; Light pink: Sudan; Pink: Nigeria; Red: Algeria; Yellow: Spain (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) .

Zimbabwe (Supplemental Fig. S5). Also, although isolated from cattle, the 2 *B. melitensis* biovar 3 bovine isolates (AZB240 and AZB241) clustered together but interspersed among other *B. melitensis* strains isolated from human cases in several African Mediterranean countries (Supplemental Fig. S6), all of them belonging to the autochthonous Maghreb lineage described previously (Lounes et al., 2014).

4. Discussion

Despite solid and longstanding serological and clinical evidence of the presence of brucellosis in sheep and goats and consequently in humans in Algeria and other Maghreb countries (Benhabyles et al., 1992; Lounes et al., 2014), data about bovine brucellosis in this area are rather meager and seldom supported by bacteriological evidence (Verger and Grayon, 1984). To the best of our knowledge, this is the first study conducted to clarify the *Brucella* spp. predominant in cattle in Algeria, information critically necessary when the possibility of *B. melitensis* infections acquired from the abundant small ruminant local population is considered (Corbel, 1997; Godfroid et al., 2013; Verger, 1985). Although the results indicate the dominance of the classical biovar 3 of *B. abortus* as well as the presence of *B. melitensis* biovar 3 MLVA shows a more complex picture.

Concerning *B. abortus*, several works have suggested that Mediterranean and Sub-Saharan strains may represent different lineages. Verger and Grayon (1985) investigated 273 strains of African origin isolated from 1976 to 1983 that included 12 Moroccan strains, the others being from Senegal, Togo, Guinea Bissau, Rwanda and Niger. Even though their study was based on classical biotyping, these authors pointed out that the *B. abortus* biovar 1 and 3 strains from Morocco had been isolated from “Pied Noir” cattle and were similar to those isolated in Europe but different from those of the other African countries represented in the collection. Recent molecular analyses have confirmed this insight on the heterogeneity of African *B. abortus* strains and the existence of differences between the European and Maghreb strains on one hand and those of Sub-Saharan countrieson the other (reviewed in Bertu et al., 2015; Ducrotoy et al., 2017 Ducrotoy et al., 2017; see also Mathew et al., 2015; Matope et al., 2009; Whatmore et al., 2016). Indeed, most *B. abortus* biovar 3 strains characterized in the present work also show a MLVA pattern closer to the Spanish and French strains, which bear the characteristics of the biovar 3b identified by Ocampo-Sosa and coworkers as a subgroup within biovar 3 (Ocampo-Sosa et al., 2005). However, there were exceptions. A subgroup of 6 strains, including a human isolate, represented a distinct lineage, and the repetition of Bruce55 shows that, contrary to what was suspected (Bertu et al., 2015), Bruce55 by itself is not a marker for the biovar 3a grouping. Presently, it is difficult to conclude whether these results reflect gaps in the capacity of the MLVA methods to discriminate a few local strains or a real connection of these strains with those of countries across the Sahara (Whatmore et al., 2016). However, taking into account the repeated validation of the MLVA methodology, we favor the second conclusion. Further studies will be necessary to compare these strains with the African genotypes described previously as Clade C1 or A using an extended BruMLSA21 (Whatmore et al., 2016). The connection of the *B. abortus* biovar 3b strains in the main cluster and the *B. abortus* biovar 1 strains with those of Spain and Morocco is easier to interpret taking into account the socio-economical and commercial links existing among Mediterranean countries. Similarly, the presence of *B. melitensis* in cattle is not unexpected and confirms that where cattle are kept in close association with sheep or goats, the former are at risk of being infected by *B. melitensis* (Corbel, 1997; Godfroid et al., 2013; Verger, 1985).

5. Conclusions

This study shows that Algerian cattle are infected mostly by *B. abortus* biovar 1 and 3 strains closer to the European lineages than to the Sub-Saharan strains, and that *B. melitensis* biovar 3 is also present in these animals. However, a few cattle strains display distinct characteristics and may represent a lineage close to Sub Saharan strains. While the data provide the bases for further studies, they also indicate that additional bacteriological and molecular studies are necessary for a complete understanding of the epidemiology of cattle brucellosis in Algeria.

Conflicts of interest

The authors declare not to have conflicts of interest.

Authorship

Conceived the study M.K., M.O., I.M.; isolated the bacteria, M.K.; performed classical biotyping and Bruce-ladder PCR, M.K., M.J.M; performed MLVA assays G.G., P.M.M.; performed clustering analysis: P.M.M, A.Z.-R., V.M.; supervised the laboratory work A.Z-R., R.C-A, P.M.M., V.M.; wrote the paper, A.Z.-R., I.M and P.M.M. All authors participated in the presentation and discussion of results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2017.10.008.

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