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Examination of Taxonomic Uncertainties Surrounding *Brucella abortus* bv. 7 by Phenotypic and Molecular Approaches

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***Brucella* taxonomy is perpetually being reshuffled, at both the species and intraspecies levels. Biovar 7 of *Brucella abortus* was suspended from the *Approved Lists of Bacterial Names Brucella* classification in 1988, because of unpublished evidence that the reference strain 63/75 was a mixture of *B. abortus* biovars 3 and 5. To formally clarify the situation, all isolates previously identified as *B. abortus* bv. 7 in the AHVLA and ANSES strain collections were characterized by classical microbiological and multiple molecular approaches. Among the 14 investigated strains, including strain 63/75, only four strains, isolated in Kenya, Turkey, and Mongolia, were pure and showed a phenotypic profile in agreement with the former biovar 7, particularly agglutination with both anti-A/anti-M monospecific sera. These results were strengthened by molecular strategies. Indeed, genus- and species-specific methods allowed confirmation that the four pure strains belonged to the *B. abortus* species. The combination of most approaches excluded their affiliation with the recognized biovars (biovars 1 to 6 and 9), while some suggested that they were close to biovar 3.These assays were complemented by phylogenetic and/or epidemiological methods, such as multilocus sequence analysis (MLSA) and variable-number tandem repeat (VNTR) analysis. The results of this polyphasic investigation allow us to propose the reintroduction of biovar 7 into the *Brucella* classification, with at least three representative strains. Interestingly, the Kenyan strain, sharing the same biovar 7 phenotype, was genetically divergent from other three isolates. These discrepancies illustrate the complexity of *Brucella* taxonomy. This study suggests that worldwide collections could include strains misidentified as *B. abortus* bv. 7, and it highlights the need to verify their real taxonomic position.**

Brucellosis is a major worldwide zoonosis. This disease affects domestic and wild mammals, causing abortion and reduced fertility. The infection is transmitted to humans by animals through direct contact with contaminated animal fluids or indirectly through ingestion of unpasteurized milk products. Despite surveillance and eradication programs recommended by the World Health Organization (WHO), the Food and Agricultural Organization (FAO), and the World Organization for Animal Health (OIE), the disease remains endemic in many regions of the world (1). Accordingly, brucellosis is of serious public health importance and causes substantial losses to livestock producers and international trade for herds in areas where it is enzootic.

Brucella, the causal agent of brucellosis, is a genus of Gram-negative, nonmotile, mostly oxidase- and urease-positive, nonencapsulated, and facultative intracellular bacteria. On the basis of genetic criteria (DNA-DNA hybridization and 16S rRNA sequence comparison), the genus *Brucella* belongs to the family *Rhizobacteriaceae*, class *Alphaproteobacteria*, within the phylum *Proteobacteria* (2). Classification of these bacteria has been based primarily on phenotypic and biochemical methods and host preference (Table 1). On this basis, the *Brucella* genus currently contains 10 species, most with a preferential host: *B. abortus* infects cattle, *B. suis* is normally associated with swine, *B. melitensis* infects mainly sheep and goats, *B. ovis* seems to be responsible for a specific infection of sheep, *B. canis* is associated with dogs, *B. neotomae* is from desert wood rats, *B. ceti* and *B. pinnipedialis* infect marine mammals (3), *B. microti* has been isolated from the common vole (*Microtus arvalis*) (4, 5), and while *B. inopinata* was isolated from a human infection, its reservoir remains unknown (6). Additional novel strains, such as unnamed strains isolated from baboons (7, 8), foxes (9), frogs (10), and rodents (11), have been described recently, and ongoing updates on the *Brucella* taxonomy are expected in the near future.

Some species are subdivided into biovars, i.e., *B. melitensis* bv. 1 to 3, *B. abortus* bv. 1 to 6 and 9, and *B. suis* bv. 1 to 5. Prior to 1986, the *B. abortus* species included 8 biovars (1 to 7 and 9), biovar 8 having been deleted from *Brucella* nomenclature in 1978 by the International Committee on Systematics of Prokaryotes (ICSP) (http://www.the-icsp.org/taxa/Brucellalist.htm) because no authentic isolate of this biovar had been reported for many years and no reference strain was available (12). From 1977, strain 63/75, also designated type strain NCTC 10506 or ATCC 23454, was considered the *B. abortus* bv. 7 reference strain (13) (Table 1). Between 1986 and 1988, following successful cloning, Verger et al. (INRA, Nouzilly, France) suggested that strain 63/75 consisted of a mixture of *B. abortus* bv. 3 and bv. 5 strains (14), so biovar 7 was suspended from the *Approved Lists of Bacterial Names Brucella* classification in 1986 by the International Subcommittee on the Taxonomy of *Brucella* (ISTB) (14) until the situation could be clarified.

The genetic homogeneity of the *Brucella* genus (DNA homol-

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TABLE 1 Differential characteristics of the *Brucella* species and biovars[a]

Species	Biovar	Host preference	Morphology	CO_2	H_2S	Oxidase	Urease	Agglutination			Growth on dye[b]		Phage lysis					Reference strain
								A	M	R	Thionin	Basic fuschin	Tb RTD	10^4 Tb RTD	Wb RTD	Iz_1 RTD	R/C RTD	
B. melitensis	1		S	−	−	+	+	−	+	−	+	+	−	−	−	+	−	16M (ATCC 23456)
	2	Sheep, goats	S	−	−	+	+	+	−	−	+	+	−	−	−	+	−	63/9 (ATCC 23457)
	3		S	−	−	+	+	+	+	−	+	+	−	−	−	+	−	Ether (ATCC 23458)
B. abortus	1		S	(+)	+	+	(+)	+	−	−	−	+	+	+	+	+	−	544 (ATCC 23448)
	2		S	(+)	+	+	+	+	−	−	−	−	+	+	+	+	−	86/8/59 (ATCC 23449)
	3		S	(+)	+	(+)	+	+	−	−	+	+	+	+	+	+	−	Tulya (ATCC 23450)
	4		S	(+)	+	+	+	−	+	−	−	(+)	+	+	+	+	−	292 (ATCC 23451)
	5	Cattle	S	−	−	+	+	−	+	−	+	+	+	+	+	+	−	B3196 (ATCC 23452)
	6		S	−	−	+	+	+	−	−	+	+	+	+	+	+	−	870 (ATCC 23453)
	Ex-7[c]		**S**	−	+/−	+	+	+	+	−	+	+	+	+	+	+	−	63/75
	ND[d]		**S**	(−)	+	+	+	+	+	−	+	+	+	+	+	+	−	This study
	9		S	+/−	+	+	+	−	+	−	+	+	+	+	+	+	−	C68 (ATCC 23455)
B. suis	1	Swine, hares, rodents	S	−	+	+	+[e]	+	−	−	+	(−)	−	+	+	+	−	1330 (ATCC 23444)
	2		S	−	−	+	+[e]	+	−	−	+	−	−	+	+	+	−	Thomsen (ATCC 23445)
	3		S	−	−	+	+[e]	+	−	−	+	+	−	+	+	+	−	686 (ATCC 23446)
	4		S	−	−	+	+[e]	+	+	−	+	(−)	−	+	+	+	−	40 (ATCC 23447)
	5		S	−	−	+	+[e]	−	+	−	+	−	−	+	+	+	−	513
B. neotomae		Rodents	S	−	+	−	+[e]	+	−	−	−	−	+/−	+	+	+	−	5K33 (ATCC 23459)
B. ovis		Sheep	R	+	−	−	−	−	−	+	+	(+) (−)	−	−	−	−	+	63/290 (ATCC 25840)
B. canis		Dogs	R	−	−	+	+[e]	−	−	+	+	(−)	−	−	−	−	+	RM6/66 (ATCC 23365)
B. ceti		Dolphins, porpoises	S	(−)	−	+/−	+	+	(−)	−	(+)	(+)	−		+	+/−	−	B1/94
B. pinnipedialis		Seals	S	(+)	−	+	+[f]	(+)	(−)	−	+	(+)	−		+	+/−	−	B2/94
B. microti		Voles	S	−	−	+	+[f]	−	+	−	+	+	−	+	+	+/−	−	CCM4915
B. inopinata		Unknown	S	−	+	+	+[e]	−	+		+	+	−	+/−	−	−	−	BO1

[a] Data are from references 28 and 37 and http://www.the-icsp.org/taxa/Brucellalist.htm. R and S, rough and smooth colonial morphology, respectively; CO_2, CO_2 requirement; H_2S, H_2S production; agglutination, agglutination with monospecific A, M, and R (rough) antisera. +, growth or lysis; −, no growth or no lysis; +/−, partial lysis; (+), most strains positive; (−), most strains negative.

[b] Dye concentration of 20 $\mu g \cdot ml^{-1}$ in serum dextrose medium (1/50,000).

[c] Withdrawn (ICSP).

[d] ND, not yet designed in the *Brucella* systematics.

[e] Strong intensity.

[f] Low intensity.

ogy of >90%) initially strongly hindered the development of molecular tools for species and biovar identification. The *rrs* polymorphism is applicable for genus identification but does not allow differentiation between *Brucella* species (there is almost 100% identity in the 16S rRNA sequences) (15). To date, the most relevant genus identification technique is a real-time PCR (RT-PCR), which targets *bcsp31*, IS*711*, and *per* (16). For species identification, molecular approaches must target other loci. Polymorphism of *omp* (outer membrane protein) genes, mainly the deletion of *omp31* in *B. abortus*, is useful for identifying genetic variants (17). Other multiplex PCR assays are available, i.e., AMOS PCR (18), which discriminates *B. abortus* (biovars 1, 2, and 4), *B. melitensis* (biovars 1 to 3), *B. ovis*, and *B. suis* (only biovar 1), and Bruce-ladder (8 target genes), which differentiates between the classical, vaccine and marine *Brucella* species (19, 20). In addition, molecular typing methods with greater discriminatory power, such as multilocus variable-number tandem repeat analysis 16 (MLVA-16) (21, 22), variable-number tandem repeat 21 (VNTR-21) (23), and extended multilocus sequence analysis 21 (MLSA-21) (24; A. M. Whatmore, unpublished data) can be used to further subdivide species, giving insight into phylogenetic, taxonomic, and/or epidemiological links between different terrestrial and marine strains.

The withdrawal of *B. abortus* bv. 7 from *Brucella* systematics remains poorly understood or simply ignored within the scientific community. Indeed, the literature still abounds in typing studies that include the former biovar 7 reference (presumably mixed) strain (5, 25–27). Moreover, several laboratories still count in their strain collections some isolates probably misidentified as *B. abortus* bv. 7. Therefore, we proposed to determine if, as for the reference strain 63/75, these isolates were a mixture of various biovars or whether they constituted a new biovar with its own characteristics. The aim of our study was to clarify the situation concerning *B. abortus* bv. 7 by both conventional and molecular approaches, including RT-PCR, *omp* polymorphism, Bruce-ladder, AMOS-ERY PCR, IS*711* fingerprinting, MLSA-21, VNTR-21, and MLVA-16, and thus provide recommendations on the future taxonomic status of this biovar.

MATERIALS AND METHODS

Bacterial strains. A total of 14 strains, including strains historically labeled *B. abortus* bv. 7 from the AHVLA and ANSES *Brucella* collections and the reference strain 63/75, were analyzed. The *Brucella* strains used in this study are listed in Table 2. In order to exclude the possibility of a mixed culture of different biovars, the strains were subjected to three successive cloning isolations (probability of a mixed colony = 10^{-9}) (J.-M. Verger, personal communication).

The reference strains *B. abortus* bv. 1 strain 544, *B. abortus* bv. 2 strain 86/8/59, *B. abortus* bv. 3 strain Tulya, *B. abortus* bv. 4 strain 292, *B. abortus* bv. 5 strain B3196, *B. abortus* bv. 6 strain 870, and *B. abortus* bv. 9 strain C68 and the *B. abortus* bv. 1 vaccine strains S19, S99, and RB51, as well other reference strains, such as *B. melitensis* bv. 1 strain 16M, *B. melitensis* bv. 3 strain Ether, *B. suis* bv. 1 strain 1330, *B. suis* bv. 4 strain 40, and *B. ovis* strain 63/290, were included in this study as controls for phenotypic typing and/or for molecular analysis.

Analysis of phenotypic characteristics. Pure cloned strains were characterized using the conventional *Brucella* typing methods, as previously described, i.e., CO_2 requirement, H_2S production, urea hydrolysis, oxidase test, agglutination with monospecific sera (anti-A, anti-M, and anti-R), dye sensitivity (basic fuchsin and thionin), and phage typing (Tbilisi [Tb], 10^4 Tb, Weybridge [Wb], Izatnagar$_1$ [Iz$_1$], and R/C) (28).

TABLE 2 Strains identified as *Brucella abortus* bv. 7 in the AHVLA and ANSES collections and their phenotypic characteristics[a]

Isolate	AHVLA no.	Yr	Host	Origin	Pure strain no.	Genus	Species	Biovar	Morphology	CO_2	H_2S	Oxidase	Urease	Agglutination			Dye sensitivity[b]				Phage lysis			
														A	M	R	T1	T2	F1	F2	Tb RTD	Wb RTD	Iz RTD	R/C RTD
07-994-2401	84/35	1984	ND	Poland	07-994-2401	*Brucella*	*abortus*	4	S	+	+	+	+	−	+	−	−	−	+	+	+	+	+	−
07-994-2402	62/05	1962	ND	ND	07-994-2402	*Brucella*	*abortus*	3	S	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−
07-994-2403	84/31	1984	ND	ND	07-994-2403a	*Brucella*	*abortus*	1	S	−	+	+	+	+	−	+	−	−	+	+	+	+	+	−
					07-994-2403b	*Brucella*	*melitensis*	1	S	−	−	+	+	−	+	−	+	+	+	+	−	−	+	−
07-994-2404	84/37	1984	ND	ND	07-994-2404a	*Brucella*	*abortus*	1	S	−	+	+	+	+	−	−	−	−	−	+	+	+	+	−
					07-994-2404b	*Brucella*	*abortus*	4	S	+	+	+	+	−	+	−	−	−	+	+	+	+	+	−
07-994-2405	84/40	1984	ND	ND	07-994-2405a	*Brucella*	*abortus*	4	S	+	+	+	+	−	+	−	−	−	+	+	+	+	+	−
					07-994-2405b	*Brucella*	*abortus*	1	S	−	+	+	+	+	−	−	−	−	+	+	+	+	+	−
07-994-2406	F6/5/05	2005	Bovine	ND	07-994-2406	*Brucella*	*abortus*	3	S	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−
07-994-2407	84/32	1984	Human	ND	07-994-2407a	*Brucella*	*abortus*	1	S	−	+	+	+	+	−	−	−	−	−	+	+	+	+	−
					07-994-2407b	*Brucella*	*abortus*	4	S	+	+	+	+	−	+	−	−	−	+	+	+	+	+	−
07-994-2408	63/59	1963	ND	Poland	07-994-2408	*Brucella*	*abortus*	1	S	−	+	+	+	+	−	−	−	−	−	+	+	+	+	−
07-994-2409	63/66	1963	ND	Poland	07-994-2409	*Brucella*	*abortus*	5	S	−	−	+	+	−	+	−	−	−	+	+	+	+	+	−
07-994-2410	63/75[c]	1963	ND	Poland	07-994-2410a	*Brucella*	*abortus*	3	S	−	+	+	+	+	−	−	+	+	+	+	+	+	+	−
					07-994-2410b	*Brucella*	*abortus*	5	S	−	−	+	+	−	+	−	−	+	+	+	+	+	+	−
07-994-2411	63/294	1963	Bovine	Kenya	07-994-2411	*Brucella*	*abortus*	ND	S	−	+	+	+	+	+	−	+	+	+	+	+	+	+	−
03-4923-239-D		2003	Bovine	Turkey	03-4923-239-D	*Brucella*	*abortus*	ND	S	+/−	+	+	+	+	+	−	+	+	+	+	+	+	+	−
99-9971-135[d]		1988	Bovine	Mongolia	99-9971-135	*Brucella*	*abortus*	ND	S	−	+	+	+	+	+	−	+	+	+	+	+	+	+	−
99-9971-159		1993	Bovine	Mongolia	99-9971-159	*Brucella*	*abortus*	ND	S	−	+	+	+	+	+	−	+	+	+	+	+	+	+	−

[a] R and S, rough and smooth colonial morphology, respectively; CO_2, CO_2 requirement; H_2S, H_2S production; agglutination, agglutination with monospecific A, M, and R (rough) antisera. +, growth or lysis; −, no growth or no lysis; +/−, partial lysis. ND, not determined.

[b] T, thionin; F, basic fuchsin; T1 and F1: dye concentration of 10 μg · ml^{-1} in serum dextrose medium (1/25,000); T2 and F2, dye concentration of 20 μg · ml^{-1} in serum dextrose medium (1/50,000).

[c] Former *B. abortus* bv. 7 reference strain.

[d] Proposed reference strain for *B. abortus* bv. 7.

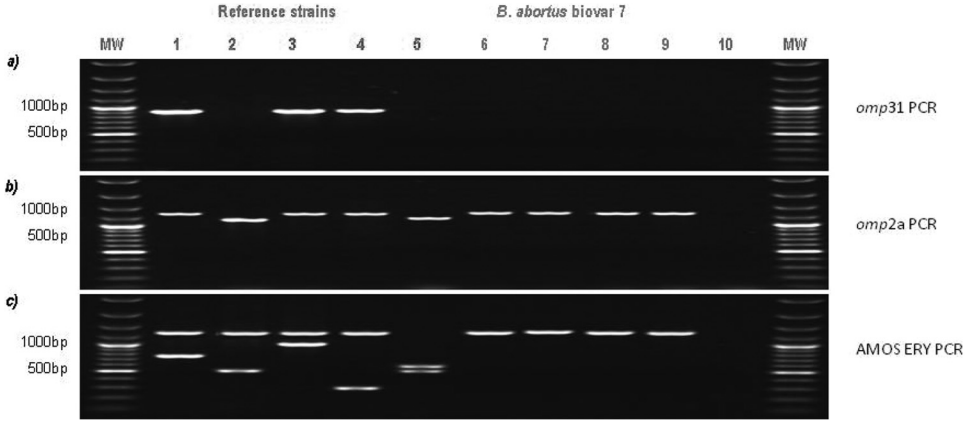

FIG 1 PCR analysis of *Brucella* reference strains and the 4 field *B. abortus* bv. 7 strains. Lanes: 1, *B. melitensis* bv. 1 strain 16M; 2, *B. abortus* bv. 1 strain 544; 3, *B. ovis* 63/290; 4, *B. suis* bv. 1 strain 1330; 5, *B. abortus* bv. 1 vaccine strain S19; 6, *B. abortus* bv. 7 field strain T; 7, *B. abortus* bv. 7 field strain M1; 8, *B. abortus* bv. 7 field strain M2; 9, *B. abortus* bv. 7 field strain K; 10, negative control. (a and b) *omp31* and *omp2a* polymorphism, respectively, for strains M1, M2, T, and K investigated in this study and the reference strains (29). (c) Discrimination between the investigated strains and the reference strains as determined by AMOS-ERY PCR assay (18).

Molecular analysis. (i) DNA preparation. Molecular tools were applied only on pure clones according to classic biotyping description of biovar 7. Genomic DNA was extracted using the High Pure PCR template preparation kit (Roche Diagnostics, France) according to the manufacturer's instructions.

(ii) PCR analysis and typing methods. *(a) Real-time PCR.* To confirm the genus *Brucella*, the RT-PCR assay which targets *bcsp31*, IS*711*, and *per* was performed on the genomic DNA as previously described (16).

(b) omp fingerprinting. Polymorphism of the *omp31*, *omp2a*, and *omp2b* genes was studied by PCR assays as previously described (29). Furthermore, restriction fragment length polymorphism (RFLP)-PCR was applied to various outer membrane protein-encoding genes (*omp31*/AvaII and/HaeIII, *omp2a*/NcoI and/StyI, and *omp2b*/KpnI and EcoRI) as previously described (30, 31).

(c) Bruce-ladder multiplex PCR. Bruce-ladder is species specific, and all the strains and biovars from the same *Brucella* species give the same profile. Pure clones obtained in our study were characterized by the Bruce-ladder multiplex PCR as previously described (19).

(d) AMOS-ERY PCR assay. For the identification and discrimination of the *B. abortus* strains of biovars 1, 2, and 4 from other *B. abortus* biovars, the S19 vaccine strain, and other species, the *B. abortus* species-specific (BaSS) PCR assay, also designated AMOS-ERY PCR, was performed following previously described approaches (18, 32).

(e) IS711 fingerprinting. Restriction profiles of the insertion sequence IS*711* (EcoRI and EcoRI plus DdeI) were investigated as previously described (33, 34).

(f) MLSA-21. Analysis by extended multilocus sequence analysis (MLSA) of the 21 distinct sequence fragments covering more than 10.2 kb of genome was performed according to previously described procedures (5, 24). Each allele at each locus gives an arbitrary numerical designation (sequence type [ST]). A representative strain of each genotype was used for phylogenetic analysis. Phylogenetic trees were constructed in MEGA (35) with the concatenated sequence data of the 21 loci using the neighbor-joining algorithm and the Jukes-Cantor model.

(g) VNTR-21 assay. The diversity of the isolates highlighted in this study was analyzed by the VNTR-21 method, based on the examination of 21 loci, including some of those described in the HOOF-Prints assay (36), as previously described (23).

(h) MLVA-16 assay. The selected pure strains, as well the *B. abortus* reference strains, were characterized by MLVA-16, using 16 genetic markers, as previously described (22). Fragment sizes converted to repeat unit numbers were imported into BioNumerics v6.6 as a character data set. The obtained MLVA patterns were compared with the *Brucella*2012 MLVA database, hosted by University Paris-Sud (Orsay, France) (http://mlva.u-psud.fr/mlvav4/genotyping/). According to the speed of molecular evolution, weights were assigned to the distinct panels (weights of 2, 1, and 0.1 per locus for panel 1, panel 2A, and panel 2B, respectively).

A minimum spanning tree (MST) was constructed to compare the pure strain genomes within the network comprising 714 *Brucella* isolates of distinct species (MLVA-16 patterns are available in the *Brucella*2010 database, http://mlva.u-psud.fr/mlvav4/genotyping/). The MST results were shown by using a logarithmic scaling. To compare the strains of interest among other *B. abortus* isolates, a cluster analysis was performed using the unweighted-pair group method (UPGMA) algorithm with categorical coefficient.

RESULTS

Cultures and biotyping. Fourteen strains, including the reference strain 63/75, previously identified as *B. abortus* bv. 7 (in particular due to their A+M+ serotype) were subjected to 3 successive cloning isolations. The characteristics of the *Brucella* cultures obtained are presented in Table 2. Five strains were found to consist of mixed cultures of different *B. abortus* biovars or a mixture of different *Brucella* species (Table 2). Three field strains, isolated in 1984, were found to represent a mixture of *B. abortus* bv. 1 (A+M−) and bv. 4 (A−M+), while, as expected, the former reference strain 63/75 was not a pure strain but was confirmed as a combination of two *B. abortus* biovars, biovar 3 (A+M−) and biovar 5 (A−M+), as described previously (14). Furthermore, one strain was characterized as a mixture of different species, *B. abortus* bv. 1 (A+M−) and *B. melitensis* bv. 1 (A−M+). In addition, five other strains that had historically been deposited in strain collections as biovar 7 were recharacterized as pure cultures of *B. abortus* bv. 1, 3, 4, or 5, with characteristics different from those expected for the former biovar 7.

Finally, only four strains, all isolated from cattle, were found to represent *B. abortus* pure clones with characteristics which did not conform to the *B. abortus* profiles of any recognized biovar (biovars 1 to 6 and 9): one strain isolated in 1963 in Kenya (07-994-2411, designated K), two strains isolated in Mongolia in 1988 and 1993 (99-9971-135 and 99-9971-159, respectively, designated M1

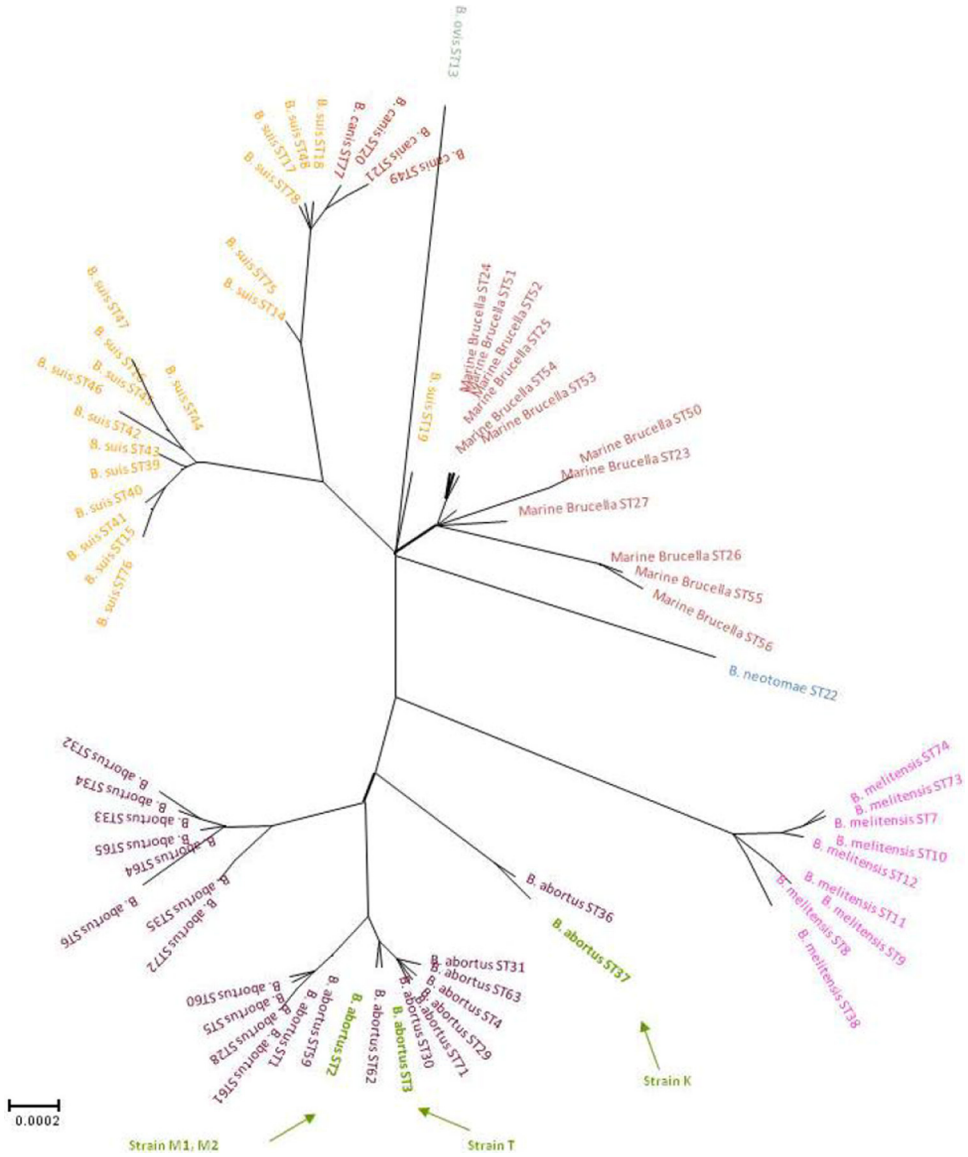

FIG 2 Unrooted phylogenetic reconstruction of relationships between 440 *Brucella* isolates representing distinct species and biovars by extended MLSA. The tree was constructed with the concatenated sequence data of the 21 loci (>10.2 kb) using the neighbor-joining algorithm with the Jukes-Cantor model. *Brucella* species are distinguished by different colors. Strains M1, M2, T, and K investigated in this study are designated by arrows (24; Whatmore, unpublished data).

and M2, respectively), and one strain isolated in Turkey in 2003 (03-4923-239D, designated T). Their particular profile conformed to the differential characteristics of the former biovar 7, particularly agglutination with both anti-A and anti-M monospecific sera (Tables 1 and 2) (37).

Molecular analysis. Accurate species-specific PCR methods (RT-PCR, Bruce-ladder, AMOS-ERY PCR, *omp* polymorphism, and IS*711* fingerprinting) were performed separately to identify the species to which the four pure strains belonged and to exclude their affiliation with the recognized biovars (biovars 1 to 6 and biovar 9). These assays were complemented by phylogenetic and/or epidemiological methods, such as extended MLST, VNTR-21, and MLVA-16.

The pure strains M1, M2, T, and K, conforming to the former biovar 7, were analyzed by RT-PCR. The cycle threshold (C_T) values indicated strong positive reactions (data not shown). The RT-PCR assay confirmed that these clones belonged to the *Brucella* genus. Likewise, the obtained Bruce-ladder pattern designated these pure strains M1, M2, T, and K as *B. abortus* (data not shown). This result was confirmed by *omp31* PCR (Fig. 1a), which evidenced the deletion of this gene for the species *B. abortus*. The *omp2a* PCR polymorphism (Fig. 1b) confirmed the thionin resistance of the four strains, in agreement with the results of the biotyping (Table 2), showing that these *B. abortus* strains were not biovar 1, 2, or 4. In parallel, in AMOS-ERY PCR (Fig. 1c), the pattern for the four strains showed uniquely the *ery* bands (1.2

kb), confirming the genus *Brucella* and showing that the biovar was different from *B. abortus* bv. 1, 2, and 4, which show an additional specific 0.5-kb band (Fig. 1c).

With regard to IS*711* fingerprinting (data not shown), the isolates M1, M2, and T shared an identical profile by both EcoRI and EcoRI-plus-DdeI digestion. The EcoRI profile was identical to that seen with some biovar 5, 6, and 9 isolates, but the EcoRI-plus-DdeI profile was unique to these three isolates. The restriction profiles of strain K were distinct from the profiles of the other three isolates using both the EcoRI and EcoRI-plus-DdeI approaches. Furthermore, the *omp* RFLP showed that the same three isolates (M1, M2, and T) shared an identical profile, mirroring the relationship determined by IS*711* fingerprinting (data not shown).

The MLSA-21 scheme indicated that the four field isolates belonged to three distinct sequence types (STs) (Fig. 2). The two Mongolian isolates M1 and M2 share an identical ST (ST2), along with many non-African *B. abortus* bv. 3 strains. Strain T was a member of a distinct ST, ST3. However, genotype ST3 is very closely related to ST2, possessing only one discriminating SNP in *gpd* in over 10.2 kb of sequence. This ST has not been described in any other isolate to date. In contrast, strain K, which represented the sole member to date of ST37, is distantly related to the above isolates and indeed is rather divergent from most other *B. abortus* strains. The only other closely related isolate is the sole representative of ST36, also a field isolate from Africa.

The VNTR-21 genotypes of the M1, M2, T, and K isolates were compared with those of *B. abortus* reference and vaccine strains (Table 3). Isolates M1 and M2 from Mongolia were closely related and shared an identical profile with isolate T at the six loci used for taxonomic resolution (VNTR14, VNTR21, VNTR27, VNTR24, VNTR7, and VNTR26) (23). In contrast, strain K appeared to be distantly related to these three isolates and possessed a unique profile, not observed in any other isolate to date, at these six loci.

The MLVA patterns obtained for the M1, M2, T, and K isolates (Table 4) were compared with those of 714 *Brucella* isolates of distinct species (Fig. 3) and with those of the *B. abortus* reference and vaccine strains (Fig. 4). The MLVA-16 scheme divided the organisms into clusters corresponding to their taxonomic designations. Isolates M1, M2, and T were closely related to the *B. abortus* members. Interestingly, isolate K, the MLVA-8 profile of which did not correspond to any described pattern in the *Brucella*2012 database, seemed to be closer to the *B. melitensis* bv. 1 cluster (only a 1-U difference in bruce42 with MLVA-8 profile of BCCN87-92, a *B. melitensis* bv. 1 strain isolated in the United States in 1997). Consequently, the MLVA-16 profile of K was very distant from those for other *B. abortus* genotypes, while strains M1, M2, and T were closer to the *B. abortus* bv. 3 strains, as also observed with the MLST assay.

DISCUSSION

In the past decades *Brucella* taxonomy has been hotly debated and has undergone many reorganizations at both the species and intraspecies levels. Indeed, from 1986, on the basis of results of DNA-DNA hybridization (high similarity values up to 99%) and in agreement with *Bergey's Manual of Systematic Bacteriology*, some scientists proposed that all the *Brucella* species should be regarded as belonging to a single species, *B. melitensis* (14). In 2003, ISTB recommendations allowed a return to the pre-1986 taxonomy of the *Brucella* genus (classical *Brucella* species with

TABLE 3 VNTR-21 genotypes for the four *B. abortus* pure strains phenotypically identified with a biovar distinct from biovars 1 to 6 and 9 in comparison with the reference strains

Strain	*B. abortus* biovar or designation	Genotype[a]																			
		Hoof 1	Hoof 2	Hoof 3	Hoof 4	Hoof 5	Hoof 6	Hoof 7	Hoof 8	VNTR2	VNTR16	VNTR17	VNTR5A	VNTR5B	VNTR12A	VNTR12B	VNTR14[b]	VNTR21[b]	VNTR27[b]	VNTR24[b]	VNTR7[b]	VNTR26[b]
544	1	3	4	6	5	2	2	4	2	3	3	5	4	2	13	1	2	1	5	2	4	4
86/8/59	2	7	4	6	2	2	2	11	2	2	4	4	5	3	2	1	2	1	5	2	4	4
Tulya	3	9	4	1	5	7	5	9	2	10	3	5	4	2	9	1	2	2	5	2	4	4
292	4	7	4	5	3	2	2	7	2	2	3	4	2	3	5	1	2	1	3	2	4	4
B3196	5	6	2	8	6	6	5	10, 11	2	2	4	5	10	8	5	1	2	1	5	1	4	4
870	6	9	2	4	10	4	2	6	2	2	4	5	9	4	13	1	2	1	5	2	4	4
C68	9	7	2	8	5	2	5	3	2	2	4	5	6	4	9	1	2	1	5	1	4	4
S19	Vaccine strain	5	4	4	2	2	2	8	2	2	4	5	6	13	5	1	2	1	5	2	4	4
RB51	Vaccine strain	5	4	4	2	2	2	4	2	2	4	4	7	4	3	1	2	1	5	2	4	4
07-994-2411[c]	K	5	3	4	5	6	4	5	5	6	2	3	2	5	4	1	2	1	5	2	3	3
03-4923-T		9	2	8	4	8	5	8	5	2	5	4	7	4	2	1	2	1	5	2	4	4
239[c]																						
99-9971-135[c]	M1	15	2	1	4	6	4	10	3	2	5	4	6	7	4	5	1	2	1	1	2	4
99-9971-159[c]	M2	13	2	1	4	6	4	9	3	2	5	4	6	6	4	5	1	2	1	1	2	4

[a] Hoof data are from reference 36, and VNTR data are from reference 28.
[b] Locus useful for taxonomic resolution.
[c] Strain investigated in this study.

TABLE 4 MLVA-16 genotypes for the four *B. abortus* pure strains phenotypically identified with a biovar distinct from biovars 1 to 6 and 9 in comparison with the reference strains

Strain	*B. abortus* biovar or designation	Host	Yr	Genotype[a]														
				Panel 1							Panel 2A			Panel 2B				
				bruce06	bruce08	bruce11	bruce12	bruce42	bruce43	bruce45	bruce55	bruce18	bruce19	bruce21	bruce04	bruce07	bruce09	bruce16	bruce30
544	1	Bovine	1942	4	5	4	12	2	2	3	3	5	21	8	3	5	3	4	5
86/8/59	2	Bovine	1959	4	5	4	12	2	1	3	3	6	21	8	3	4	3	3	5
Tulya	3	Human	1958	3	5	4	11	2	2	3	3	8	20	8	6	5	3	11	5
292	4	Bovine	1961	4	5	4	12	2	2	3	2	6	21	8	3	4	3	3	5
B3196	5	Bovine	1959	3	5	3	12	2	2	2	3	7	21	8	6	7	3	3	3
870	6	Bovine	1959	3	5	3	12	2	2	3	3	7	21	8	3	6	3	3	3
C68	9	Bovine	1958	3	5	3	12	2	2	2	3	7	21	8	6	6	3	3	3
S19	1 (vaccine)	Bovine	1943	4	5	4	12	2	2	3	3	6	21	8	3	5	3	3	5
S99	1		1957	4	5	4	12	2	2	3	3	6	21	8	3	5	3	4	6
07-994-2411[b]	K	Bovine	1963	2	4	2	12	3	2	3	3	5	22	6	5	2	6	7	5
03-4923-239[b]	T	Bovine	2003	4	5	3	12	2	2	3	1	6	21	8	6	7	6	3	3
99-9971-135[b]	M1	Bovine	1988	4	5	3	12	2	2	3	1	6	21	8	5	6	4	3	3
99-9971-159[b]	M2	Bovine	1993	4	5	3	12	2	2	3	1	6	21	8	5	6	4	3	3

[a] The 16 investigated loci are organized in agreement with their molecular evolution rate in two panels; panel 1 is useful for species identification, and panel 2 shows a higher discriminatory power (22).
[b] Strain investigated in this study.

FIG 3 Minimum spanning tree of 718 *Brucella* isolates, representing the majority of known species/biovars, by MLVA-16. An MST of 718 *Brucella* isolates of distinct species and biovars was constructed, based on MLVA-16 patterns obtained in this study or available in the *Brucella*2010 database (http://mlva.u-psud.fr/mlvav4/genotyping/). The MST results are shown by using a logarithmic scaling. *Brucella* species and *B. melitensis* biovars are distinguished by different colors. Strains M1, M2, T, and K investigated in this study are designated by arrows.

their recognized biovars) (38), and a number of molecular typing and phylogenetic studies illustrating that the classical species correspond to genetically distinct, if closely related, entities (23, 24, 39) have supported this decision.

In the same way, the presence of *B. abortus* bv. 7, represented by the reference strain 63/75, in *Brucella* systematics has been the subject of controversy. Indeed, this biovar was suspended from the *Approved Lists of Bacterial Names Brucella* classification in 1988 because of unpublished evidence that the reference strain 63/75 was a mixture of *B. abortus* bv. 3 and bv. 5 (14). To formally confirm and clarify the situation with regard to *B. abortus* bv. 7, all isolates phenotypically identified in the past as *B. abortus* bv. 7 in the AHVLA and ANSES strain collections were characterized by classical microbiological and multiple molecular approaches.

Among the 14 investigated collection strains, including the reference strain 63/75, 10 conformed to the previous *B. abortus* bv. 7 characteristics. However, they either were a mixed culture (different *B. abortus* biovars or a mixture of distinct *Brucella* species), conferring a typical A+M+ serological profile or were another pure *B. abortus* biovar, possibly reflecting the loss of another strain that resulted in the originally described A+M+ serological profile. Only four cattle strains, isolated in Kenya (K) in 1963, in Turkey (T) in 2003, and in Mongolia (M1 and M2) in 1988 and 1993, showed a particular profile, previously described as *B. abortus* bv. 7, characterized mainly by an A+M+ serological pattern.

The molecular techniques (RT-PCR and IS*711* fingerprinting) identified these four field strains as members of the *Brucella* genus. Furthermore, AMOS PCR, Bruce-ladder multiplex PCR, and the polymorphism of the outer membrane genes *omp2a*, *omp2b*, and

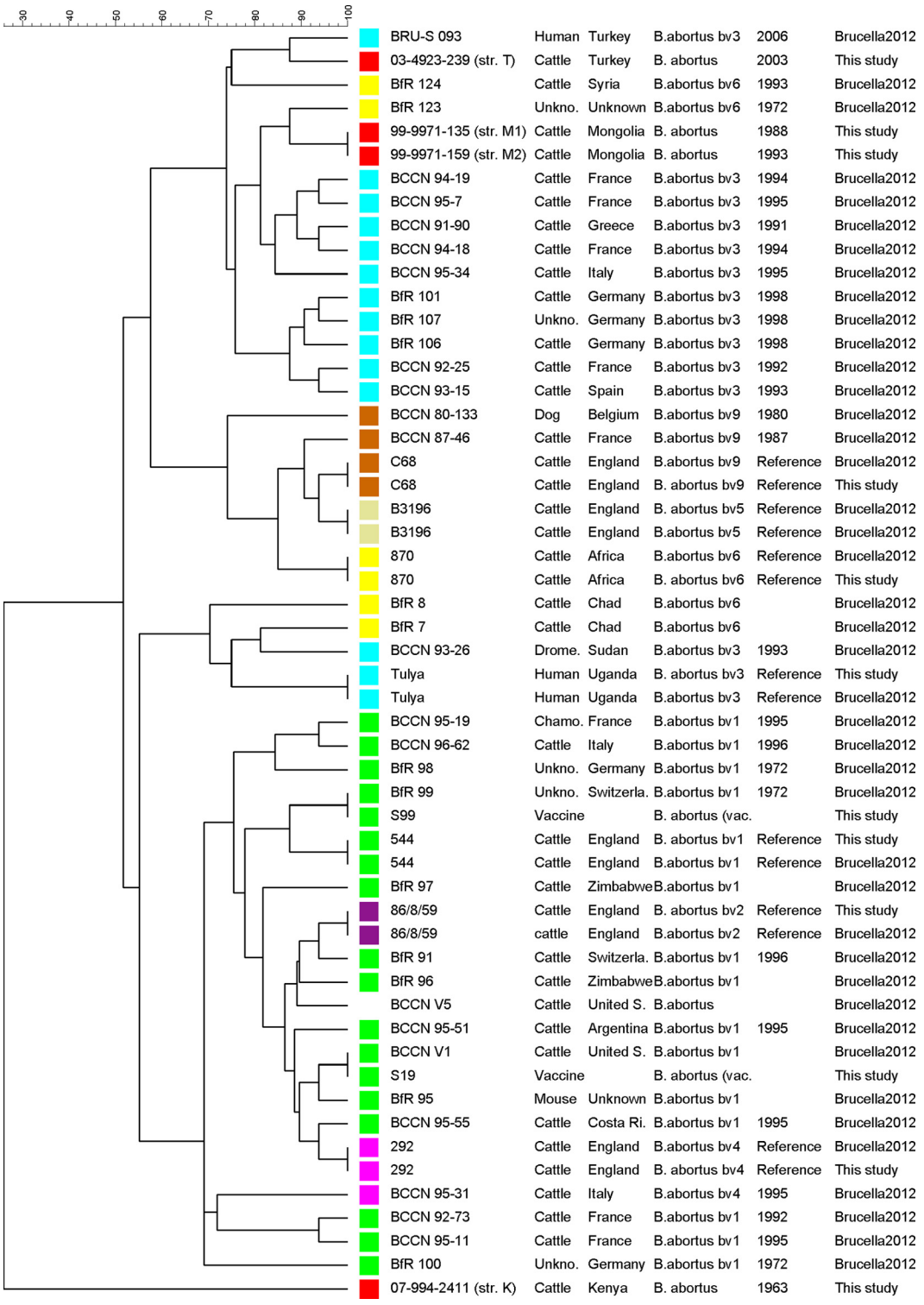

FIG 4 Analysis of relationships between 56 *B. abortus* isolates, representing all biovars, by MLVA-16. Clustering analysis was performed using the UPGMA algorithm with categorical coefficient; each data set (panel 1, panel 2A, and panel 2B) was weighted according to the molecular evolution rates (22). Reference strain genotypes were obtained both from this study and from the *Brucella*2012 database. *B. abortus* biovars are distinguished by different colors. Strains M1, M2, T, and K investigated in this study are designated in red. The strain identity, the host, the geographical origin, the biovar, and the corresponding genotype reference are listed in the dendrogram. The bars reflect the percentage of weighted divergence.

omp31 confirmed that these strains belonged to the *B. abortus* species but were distinct from the designated biovars (biovars 1 to 6 and biovar 9) and vaccine strains. The phylogenetic and/or epidemiological schemes supported these results.

In agreement with the above data, strains M1, M2, and T were found to represent a genetic cluster, with the allelic profiles, obtained by VNTR-21 and MLVA-16 assays, allowing discrimination of these strains with a characteristic MLVA-11 pattern, closely related to other *B. abortus* strains, especially with the *B. abortus* bv. 3 strains. In addition, phylogenetic multilocus sequence analysis placed the isolates as two closely related sequence types (ST2 and ST3) in the same genetically conserved group of *B. abortus* strains.

Interestingly, strain K, phenotypically identical to isolates M1, M2, and T, is divergent from them on molecular characterization grounds. Indeed, strain K possessed a distinct and novel profile using the VNTR approaches, adjacent to a *B. melitensis* bv. 1 member, and a unique MLST genotype, related to an African field isolate. While several molecular strategies (Bruce-ladder, *omp31* deletion, and AMOS PCR) allowed placement of strain K among *B. abortus* strains, this strain is unique among isolates examined to date, and genotyping data revealed that this isolate is rather distant from other *B. abortus* strains. Phenotypic and molecular discrepancies concerning strain K illustrate the complexities of *Brucella* taxonomy.

The definition of *Brucella* species is based only on characteristics of lysotyping and urease and oxidase hydrolysis (40). In agreement with these phenotypic criteria, the four field isolates M1, M2, T, and K might be classified in the species *B. abortus*. Nevertheless, their profile does not coincide with any designated *B. abortus* biovars (biovars 1 to 6 and biovar 9), but it matches the criteria of the former biovar 7. In addition, these data are partially congruent with molecular approaches. On the basis of polyphasic taxonomy results, taking into account available phenotypic and genotypic data, we propose that the *B. abortus* biovar 7 could be reintroduced into the *Brucella* classification, with as a reference strain the oldest isolate (99-9971-139), isolated in Mongolia in 1988. However, as with the existing *B. abortus* bv. 3 scenario, where a number of studies have shown that this biovar includes genetically distinct clusters (27, 41), the results here highlight the limitations of a classification system based on phenotype alone. The "clustering" of genetically unrelated strains clearly limits the value of biotyping as a typing or epidemiological tool, as equally does the finding that the ST2 type is shared by many biovar 3 isolates. Such observations strengthen the argument for moving toward a molecularly based classification, which, in the scenario described here, would readily separate the genetically distinct but phenotypically identical strain K.

Conclusion. The aim of our study was to clarify the situation concerning *B. abortus* biovar 7 by both conventional and molecular typing approaches. This consensus approach confirmed that worldwide collections could possess some strains misidentified as *B. abortus* bv. 7, a biovar suspended from the *Approved Lists of Bacterial Names Brucella* classification, and highlighted the need to verify the taxonomic position of these strains.

Our study allowed the identification of four clonal *B. abortus* strains among 14 strains investigated, with specific phenotypic criteria corresponding to the criteria defining the former biovar 7. The phenotypic data results were strengthened by molecular strategies. This polyphasic investigation allows us to propose the reintroduction of biovar 7 into the *Brucella* classification, with three representative strains (M1, M2, and T) and strain 99-9971-135 as the potential reference strain. However, the study also highlighted the existence of (i) genetically unrelated strains sharing the same biovar 7 phenotype and (ii) non-biovar 7 isolates that share the same genotype, which could compromise the reintroduction of this biovar.

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REFERENCES

1. **Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV.** 2006. The new global map of human brucellosis. Lancet Infect. Dis. **6:**91–99. http://dx.doi.org/10.1016/S1473-3099(06)70382-6.
2. **Williams KP, Sobral BW, Dickerman AW.** 2007. A robust species tree for the alphaproteobacteria. J. Bacteriol. **189:**4578–4586. http://dx.doi.org/10.1128/JB.00269-07.
3. **Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A.** 2007. *Brucella ceti* sp nov and *Brucella pinnipedialis* sp nov for *Brucella* strains with cetaceans and seals as their preferred hosts. Int. J. Syst. Evol. Microbiol. **57:**2688–2693. http://dx.doi.org/10.1099/ijs.0.65269-0.
4. **Scholz HC, Hubalek Z, Sedlacek I, Vergnaud G, Tomaso H, Al Dahouk S, Melzer F, Kämpfer P, Neubauer H, Cloeckaert A, Maquart M, Zygmunt MS, Whatmore AM, Falsen E, Bahn P, Göllner C, Pfeffer M, Huber B, Busse HJ, Nöckler K.** 2008. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. Int. J. Syst. Evol. Microbiol. **58:**375–382. http://dx.doi.org/10.1099/ijs.0.65356-0.
5. **Al Dahouk S, Hofer E, Tomaso H, Vergnaud G, Le Fleche P, Cloeckaert A, Koylass MS, Whatmore AM, Nöckler K, Scholz HC.** 2012. Intraspecies biodiversity of the genetically homologous species *Brucella microti*. Appl. Environ. Microbiol. **78:**1534–1543. http://dx.doi.org/10.1128/AEM.06351-11.
6. **Scholz HC, Nöckler K, Göllner C, Bahn P, Vergnaud G, Tomaso H, Al Dahouk S, Kämpfer P, Cloeckaert A, Maquart M, Zygmunt MS, Whatmore AM, Pfeffer M, Huber B, Busse HJ, De BK.** 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. Int. J. Syst. Evol. Microbiol. **60:**801–808. http://dx.doi.org/10.1099/ijs.0.011148-0.
7. **Schlabritz-Loutsevitch NE, Whatmore AM, Quance CR, Koylass MS, Cummins LB, Dick EJ, Jr, Snider CL, Cappelli D, Ebersole JL, Nathanielsz PW, Hubbard GB.** 2009. A novel *Brucella* isolate in association with two cases of stillbirth in non-human primates—first report. J. Med. Primatol. **38:**70–73. http://dx.doi.org/10.1111/j.1600-0684.2008.00314.x.
8. **Pappas G.** 2010. The changing *Brucella* ecology: novel reservoirs, new threats. Int. J. Antimicrob. Agents. **36:**S8–S11. http://dx.doi.org/10.1016/j.ijantimicag.2010.06.013.
9. **Hofer E, Revilla-Fernandez S, Al Dahouk S, Riehm JM, Nockler K, Zygmunt MS, Cloeckaert A, Tomaso H, Scholz HC.** 2012. A potential novel *Brucella* species isolated from mandibular lymph nodes of red foxes in Austria. Vet. Microbiol. **155:**93–99. http://dx.doi.org/10.1016/j.vetmic.2011.08.009.
10. **Eisenberg T, Hamann HP, Kaim U, Schlez K, Seeger H, Schauerte N, Melzer F, Tomaso H, Scholz HC, Koylass MS, Whatmore AM, Zschöck M.** 2012. Isolation of potentially novel *Brucella* spp. from frogs. Appl. Environ. Microbiol. **78:**3753–3755. http://dx.doi.org/10.1128/AEM.07509-11.
11. **Tiller RV, Gee JE, Frace MA, Taylor TK, Setubal JC, Hoffmaster AR, De BK.** 2010. Characterization of novel *Brucella* strains originating from wild native rodent species in North Queensland, Australia. Appl. Environ. Microbiol. **76:**5837–5845. http://dx.doi.org/10.1128/AEM.00620-10.
12. **International Committee on Systematic Bacteriology, Subcommittee on Taxonomy of *Brucella*.** 1978. Minutes of the meeting, 4 and 5 September 1978. Int. J. Syst. Bacteriol. **32:**260–261.

13. **Meyer ME, Morgan WJB.** 1973. Designation of neotype strains and of biotype reference strains for species of the genus *Brucella*—Meyer and Shaw. Int. J. Syst. Bacteriol. **23:**135. http://dx.doi.org/10.1099/00207713-23-2-135.
14. **International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Brucella*.** 1988. Report of the meeting, 5 September 1986. Int. J. Syst. Bacteriol. **38:**450–452. http://dx.doi.org/10.1099/00207713-38-4-450.
15. **Whatmore AM.** 2009. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. Infect. Genet. Evol. **9:**1168–1184. http://dx.doi.org/10.1016/j.meegid.2009.07.001.
16. **Bounaadja L, Albert D, Chenais B, Henault S, Zygmunt MS, Poliak S, Garin-Bastuji B.** 2009. Real-time PCR for identification of *Brucella* spp.: a comparative study of IS*711*, bcsp31 and *per* target genes. Vet. Microbiol. **137**(1–2)**:**156–164. http://dx.doi.org/10.1016/j.vetmic.2008.12.023.
17. **Vizcaíno N, Verger JM, Grayon M, Zygmunt MS, Cloeckaert A.** 1997. DNA polymorphism at the Omp-31 locus of *Brucella* spp—evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology **143:**2913–2921. http://dx.doi.org/10.1099/00221287-143-9-2913.
18. **Bricker BJ, Ewalt DR, Olsen SC, Jensen AE.** 2003. Evaluation of the *Brucella abortus* species-specific polymerase chain reaction assay, an improved version of the *Brucella* AMOS polymerase chain reaction assay for cattle. J. Vet. Diagn. Invest. **15:**374–378. http://dx.doi.org/10.1177/104063870301500413.
19. **Garcia-Yoldi D, Marín CM, de Miguel MJ, Munoz PM, Vizmanos JL, Lopez-Goñi I.** 2006. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. Clin. Chem. **52:**779–781. http://dx.doi.org/10.1373/clinchem.2005.062596.
20. **López-Goñi I, García-Yoldi D, Marín CM, de Miguel MJ, Barquero-Calvo E, Guzmán-Verri C, Albert D, Garin-Bastuji B.** 2011. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. Vet. Microbiol. **154**(1–2)**:**152–155. http://dx.doi.org/10.1016/j.vetmic.2011.06.035.
21. **Le Fleche P, Jacques I, Grayon M, Al Dahouk S, Bouchon P, Denoeud F, Nöckler K, Neubauer H, Guilloteau LA, Vergnaud G.** 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol. **6:**9. http://dx.doi.org/10.1186/1471-2180-6-9.
22. **Al Dahouk S, Le Fleche P, Nockler K, Jacques I, Grayon M, Scholz HC, Tomaso H, Vergnaud G, Neubauer H.** 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. J. Microbiol. Methods **69:**137–145. http://dx.doi.org/10.1016/j.mimet.2006.12.015.
23. **Whatmore AM, Shankster SJ, Perrett LL, Murphy TJ, Brew SD, Thirlwall RE, Cutler SJ, MacMillan AP.** 2006. Identification and characterization of variable-number tandem-repeat markers for typing of *Brucella* spp. J. Clin. Microbiol. **44:**1982–1993. http://dx.doi.org/10.1128/JCM.02039-05.
24. **Whatmore AM, Perrett LL, MacMillan AP.** 2007. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. BMC Microbiol. **7:**34. http://dx.doi.org/10.1186/1471-2180-7-34.
25. **Gandara B, Merino AL, Rogel MA, Martinez-Romero E.** 2001. Limited genetic diversity of *Brucella* spp. J. Clin. Microbiol. **39:**235–240. http://dx.doi.org/10.1128/JCM.39.1.235-240.2001.
26. **Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R.** 2006. Molecular characterization of the *rpoB* gene in *Brucella* species: new potential molecular markers for genotyping. Microbes Infect. **8:**860–865. http://dx.doi.org/10.1016/j.micinf.2005.10.008.
27. **Valdezate S, Navarro A, Rubio V, Garin-Bastuji B, Albert D, Hernandez P, Alonso PM, Saéz-Nieto JA.** 2009. Emergence of a clonal lineage of *Brucella abortus* biovar 3 in clinical cases in Spain. J. Clin. Microbiol. **47:**2687–2688. http://dx.doi.org/10.1128/JCM.00756-09.
28. **World Organization for Animal Health.** 2009. Bovine brucellosis. *In* Manual of diagnostic tests and vaccines for terrestrial animals. OIE, Paris, France. http://www.oie.int.
29. **Cloeckaert A, Verger JM, Grayon M, Grepinet O.** 1995. Restriction site polymorphism of the genes encoding the major 25 Kda and 36 Kda outer-membrane proteins of *Brucella*. Microbiology **141:**2111–2121. http://dx.doi.org/10.1099/13500872-141-9-2111.
30. **Al Dahouk S, Tomaso H, Prenger-Berninghoff E, Splettstoesser WA, Scholz HC, Neubauer H.** 2005. Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Crit. Rev. Microbiol. **31:**191–196. http://dx.doi.org/10.1080/10408410500304041.
31. **Garcia-Yoldi D, Marín CM, Lopez-Goñi I.** 2005. Restriction site polymorphisms in the genes encoding new members of group 3 outer membrane protein family of *Brucella* spp. FEMS Microbiol. Lett. **245:**79–84. http://dx.doi.org/10.1016/j.femsle.2005.02.026.
32. **Bricker BJ, Halling SM.** 1995. Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. J. Clin. Microbiol. **33:**1640–1642.
33. **Ouahrani S, Michaux S, Widada JS, Bourg G, Tournebize R, Ramuz M, Liautard JP.** 1993. Identification and sequence analysis of IS*6501*, an insertion sequence in *Brucella* spp. Relationship between genomic structure and the number of IS*6501* copies. J. Gen. Microbiol. **139:**3265–3273.
34. **Ouahrani-Bettache S, Soubrier MP, Liautard JP.** 1996. IS*6501*-anchored PCR for the detection and identification of *Brucella* species and strains. J. Appl. Microbiol. **81:**154–160.
35. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28:**2731–2739. http://dx.doi.org/10.1093/molbev/msr121.
36. **Bricker BJ, Ewalt DR, Halling SM.** 2003. *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). BMC Microbiol. **3:**15. http://dx.doi.org/10.1186/1471-2180-3-15.
37. **Alton GG, Jones LM, Pietz DE.** 1977. La brucellose. Techniques de laboratoire, 2nd ed, FAO/WHO, Geneva, Switzerland.
38. **International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Brucella*.** 2006. Minutes of the meeting, 17 September 2003. Int. J. Syst. Evol. Microbiol. **56:**1173–1175. http://dx.doi.org/10.1099/ijs.0.64349-0.
39. **Wattam AR, Inzana TJ, Williams KP, Mane SP, Shukla M, Almeida NF, Dickerman AW, Mason S, Moriyón I, O'Callaghan D, Whatmore AM, Sobral BW, Tiller RV, Hoffmaster AR, Frace MA, De Castro C, Molinaro A, Boyle SM, De BK, Setubal JC.** 2012. Comparative genomics of early-diverging *Brucella* strains reveals a novel lipopolysaccharide biosynthesis pathway. mBio **3**(5)**:**e00246–11. http://dx.doi.org/10.1128/mBio.00246-12.
40. **Alton GG, Jones LM, Angus RD, Verger JM.** 1988. Techniques for the brucellosis laboratory. INRA Publications, Paris, France.
41. **Ocampo-Sosa AA, Aguero-Balbin J, Garcia-Lobo JM.** 2005. Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. Vet. Microbiol. **110**(1–2)**:**41–51. http://dx.doi.org/10.1016/j.vetmic.2005.06.007.