Taxonomy of *Brucella*

Menachem Banai[*,1] and Michael Corbel[2]

[1]*Department of Bacteriology, Kimron Veterinary Institute, Bet Dagan 50250, Israel*

[2]*Division of Bacteriology, National Institute for Biological Standards & Control, Potters Bar, EN6 3QG, UK*

Abstract: Brucellosis is named after Dr. David Bruce who first isolated the bacterium that caused Malta fever from four fatal cases amongst the British forces on the island. The genus *Brucella* was subsequently proposed after similar bacteria were isolated from cattle and swine and the zoonotic connection recognized. The close similarities between these isolates were acknowledged but nomen species were subsequently designated on the basis of their specific host preference, phage susceptibility and oxidative metabolism pattern with specific carbohydrate and amino acid substrates. The later isolation of *B. suis* strains of divergent host preference and of strains of low human pathogenicity, such as *B. neotomae* and *B. ovis,* has inspired a debate regarding *Brucella* taxonomy. On the one hand, the DNA homologies are strikingly similar, justifying inclusion of all members of the genus in a single species with sub-divisions. On the other hand, whole genome analyses such as MLVA, MLST, microarray studies, and SNP have confirmed subtle differences between the species. As a result, a return to a multi-nomen species taxonomy has recently been proposed and accepted by the Sub-committee on Taxonomy of *Brucella*. Phylogenetic studies have shown four clades in the genus that have possibly evolved from a *Brucella – Ochrobactrum*-like common soil ancestor. These are: *B. melitensis-B. abortus*; *B. suis-B. canis*; *B. neotomae*; and *B. ceti-B. pinnipedialis* (*B. microti* not yet established), with *B. ovis* standing as a basal lineage of the tree. *B. inopinata* is a recently identified isolate that slightly diverges from classical *Brucella* according to the 16S-rRNA sequence and other molecular studies. This review elaborates on the classification of the genus *Brucella* according to insights that have emerged since it was first described.

Keywords: *Brucella*, genus, species, taxonomy, phylogeny, genome, phage typing.

INTRODUCTION

The history of the genus *Brucella* began with the recognition by Evans (1918) [1] of the similarity of the agent of Malta fever reported by Bruce (1887) [2] and later described as '*Micrococcus melitensis'*, to *Bacterium abortus* the agent of contagious abortion of cattle described by Bang (1897) [3] and the *abortus*-like bacteria isolated from swine abortions by Traum (1914) [4]. On this basis Meyer and Shaw (1920) [5] were able to define the genus *Brucella,* comprising the species *Brucella melitensis* and *Brucella abortus*. Later the organisms from swine were differentiated as a third species, *Brucella suis* [6]. Subsequently, numerous isolates which did not correspond exactly to the descriptions of the original species were identified and designated by a variety of colloquial and semi-official names e.g. 'British melitensis','Rhodesian abortus','dye-sensitive abortus', '*Brucella intermedia,*' 'American suis', *inter alia.* This chaotic situation was resolved by the application of oxidative metabolism and phage lysis tests which allowed the atypical strains to be aligned with one or other of the three original species in patterns that broadly corresponded to the preferred natural host. Within each species the different variants were assigned as biotypes or biovars.

*Address correspondence to this author at the Department of Bacteriology, Kimron Veterinary Institute, Bet Dagan 50250, Israel; Tel: 972-3-9681698; Fax: 972-3-9681753; E-mail: menachemba@moag.gov.il

The International Committee on Bacteriological Nomenclature (superseded by the International Committee on Systematic Bacteriology) formed a Sub-committee on Taxonomy of the genus *Brucella* which developed the framework of the current classification [7]. Although strains not conforming to the 'classical' species continued to be isolated and were sometimes designated as new species, e.g. *B. rangiferi tarandi* from reindeer [8], on application of the approved methods, these were assigned to the existing species, sometimes as new biovars. The first entirely new species to be added was *B. ovis* described in 1956 [9], followed by *B. neotomae* [10] and *B. canis* [11]. Other species were also proposed, eg. '*B. murium'* from rodents [12] but not accepted by the Subcommittee on Taxonomy of the genus *Brucella*.

It should be noted that the criteria used for classification of the genus were based on phenotypic characteristics, with weighting given to the preferred natural host. This was inevitable given the lack of workable genotyping methods at that time. Perhaps surprisingly, this system has proved broadly consistent with contemporary molecular genetic analysis although some inconsistencies remain. The latter raise issues that need to be resolved. In particular, there is a need to update the specifications for minimal standards for genus, species and biovar definition. These were first established 35 years ago using the information available at that time [13,14]. They were based on the framework proposed by Stableforth and Jones (1963) [7], supplemented by the genetic and biochemical data that had emerged in the interim. Some updating has been noted in the later reports of

1874-3188/10

the Sub-committee on Taxonomy [15-22] and in other publications, including the definitive Bergey's Manual of Systematic Bacteriology [23,24].

The current taxonomic structure of the genus *Brucella* includes the originally proposed *Brucella* species and recently added species as illustrated in Tables **1-3**. Emphasis is still placed on phenotypic characteristics and *Brucella* species are still assigned according to their preferred natural host affiliation with the implication that heterologous hosts are rarely involved. E.g. *B. melitensis* is mainly associated with small ruminants, *B. abortus* with cattle and other bovidae, and *B. suis* with swine. It is also assumed that the members of the genus have zoonotic potential although it is acknowledged that pathogenicity for humans is quite variable.

The host specificity has long been recognized as relative rather than absolute [7] although the extent to which some species can be transmitted outside the usually preferred host has now become more widely recognized. Examples include cross transmission of *B. abortus* to small ruminants, and of *B. melitensis* to cattle in shared common pastures. Apparent inconsistency with host preference is most evident in *B. suis*. Thus, although *B. suis* biovars 1, 2 and 3 have been shown to be infective to swine, *B. suis* biovar 2 also infects hares, *B. suis* biovar 4 is confined in nature to caribou and reindeer and *B. suis* biovar 5 apparently is found naturally only in rodents. There has been some dispute about the position of the latter biovar and it seems to be the most divergent of the *B. suis* group.

Verger *et al*. (1985) [25] challenged the taxonomical division of the genus into nomen species following DNA hybridization studies that showed *Brucella* DNA to be highly homogeneous. However, the identification, for the first time, of *Brucella* like organisms adapted to the marine environment has again fired a debate on the true taxonomy of the genus. *Brucella* strains were isolated from carcasses of seals, dolphins, porpoises and whales [26-29], shown to be associated with abortions and meningoencephalitis in several

Table 1. An Updated List of *Brucella* Type Strains, Biovars and Reference Strains

Species	Biovar[a]	Type/reference	Strain	ATCC[b] No.	NCTC[c] No.	BCCN[d]
B. melitensis	1	Type	16M	23456	10094	
	2	Reference	63/9	23457	10508	
	3	Reference	Ether	23458	10509	
B. abortus	1	Type	544	23448	10093	
	2	Reference	86/8/59	23449	10501	
	3	Reference	Tulya	23450	10502	
	4	Reference	292	23451	10503	
	5	Reference	B3196	23452	10504	
	6	Reference	870	23453	10505	
	9	Reference	C68	23455	10507	
B. suis	1	Type	1330	23444	10316	
	2	Reference	Thomsen	23445	10510	
	3	Reference	686	23446	10511	
	4	Reference	40	23447	11364	
	5	Reference	513	-	-	
B. neotomae		Type	5K33	23459	10084	
B. ovis		Type	63/290	25840	10512	
B. canis		Type	RM6/66	23365	10854	
B. ceti[e]		Type			12891^T	$94\text{-}74^T$
B. pinnipedialis[e]		Type			12890^T	$94\text{-}73^T$
B. microti[f]		Type	$CCM4915^T$			$07\text{-}01^T$
B. inopinata[g]		Type	$BO1^T$			$09\text{-}01^T$

[a] Biovars have been characterized according to agglutination in monospecific anti- A, anti – M and anti – R sera, CO_2 requirement on first isolation, production of H_2S and Urease and growth in presence of Fuchsin and Thionin, as shown in Tables **2** and **3**, respectively.
[b] ATCC stands for American Type Culture Collection
[c] NCTC stands for National Collection of Type Cultures – Great Britain
[d] BCCN stands for Brucella Culture Collection, Nouzilly
[e] Foster *et al*., 2007; Bricker *et al*., 2000
[f] Scholz *et al*., 2008 [39]
[g] Scholz *et al*., 2010 [42] (The strain was also submitted as $CPAM\ 6436^T$ (Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic)).

Table 2. Differential Characteristics of Species of the Genus *Brucella*

			Lysis by Phages[a]							
			Tb		Wb	Iz1	R/C			
Species	**Colony Morphology[b]**	**Serum Requirement**	**RTD[c]**	**104RTD**	**RTD**	**RTD**	**RTD**	**Oxidase**	**Urease Activity**	**Preferred Host**
B. abortus	S	–d	+	+	+	+	–	+e	+f	Cattle and other Bovidae
B. suis	S	–	–	+	+g	+g	–	+	+h	Biovar 1: swine Biovar 2: swine, hare Biovar 3: swine Biovar 4: reindeer Biovar 5: wild rodents
B. melitensis	S	–	–	–	–i	+	–	+	+j	Sheep and goats
B. neotomae	S	–	–k	+	+	+	–	–	+h	Desert wood rat[l]
B. ovis	R	+	–	–	–	–	+	–	–	Rams
B. canis	R	–	–	–	–	–	+	+	+h	Dogs
B. ceti	S		+m		+n	+o	–	+	+	Cetaceans
B. pinnipedialis	S		+m		+n	+o	–	+	+	Pinnipeds
B. microti	S	–	–	+	+			+	+	Vole (Microtus arvalis)
B. inopinata	S	–	P					+	+ (fast)	?

[a]Phages: Tbilisi (Tb), Weybridge (Wb), Izatnagar1(Iz1) and R/C.
[b]Normally occurring phase: S: smooth, R: rough.
[c]RTD: routine test dilution.
[d]*Brucella abortus* biovar 2 generally requires serum for growth on primary isolation.
[e]Some African isolates of *B. abortus* biovar 3 are negative.
[f]Intermediate rate, except strain 544 and some field strains that are negative.
[g]Some isolates of *B. suis* biovar 2 are not or partially lysed by phage Wb or Iz1.
[h]Rapid rate.
[i]Some isolates are lysed by phage Wb.
[j]Slow rate, except some strains that are rapid.
[k]Minute plaques.
[l]*Neotoma lepida.*
[m]Some isolates are lysed by Tb.
[n]Most isolates are lysed by Wb.
[o]Most isolates are lysed by Iz.
[P]Incomplete lysis with phages Tb, F1 and F25 at 10^4xRTD (Scholz *et al*., 2010) or not susceptible to Tb (De *et al*., 2008).

sea mammal species [30] and with pathological impacts in striped dolphins (*Stenella coeruleoalba*) [31], respectively. It was not long before these new species were also implicated in a limited number of human cases [32-34]. As with previous *Brucella* species, cross infection of hosts was also notified [35].

Because of limited information, all marine isolates were initially proposed to be included temporarily in a single new species, *B. maris*. Later studies have, however, shown categorically that the strains as a group belonged to the genus *Brucella* according to the 16S-rRNA homology although they possessed a distinctive IS*711* marker that grouped them separately from the classical terrestrial strains [26]. Within the group, the strains diverged in CO_2 requirement on primary isolation and metabolic activity on galactose, as well as in IS*711* fingerprints correlating with their predominant isolation from seals or cetaceans, respectively. Similarly, their *omp2* polymorphism justified the establishment of two distinct species, named in time *B. cetaceae* and *B. pinnipediae* [36], based on their host origin from porpoises, dolphins and Minke whales, or seals, respectively [26,29]. On grammatical grounds, the names were later modified to *B. ceti* and *B. pinnipedialis*. MLVA results have characterized marine mammal *Brucella* isolates as a distinct group but their sequence types (ST) diverged significantly according to their host origin, justifying the establishment of three rather than just two species (Fig. **1**) [37].

Soon after, an organism designated *Brucella microti* was isolated from a common field vole, *Microtus* sp. [38], from soil [39] and from red foxes [40]. *B. microti*, however, has not yet been associated with human cases.

Finally, very recently, a distinctive species, *B. inopinata*, was recovered from a breast implant in a seventy one year old woman presenting with clinical signs of brucellosis [41]. So far only a single isolation has been reported and an animal host has yet to be identified. This isolate was shown to have a divergent *omp2* sequence, and a unique 16S-rRNA sequence in 5 nucleotides in comparison to the 16S-rRNA gene consensus sequence of *Brucella* spp. In addition, the strain was grouped separately by MLST analysis in comparison to other *Brucella* spp. The MLST profile of the strain was nevertheless closer to the other *Brucella* spp. than

Table 3. Classification of *Brucella* Species and Biovars

Species	Biovar	CO_2 Requirement	H_2S Production	Growth on Dyes[a]		Agglutination with Monospecific Sera		
				Thionin	Basic Fuchsin	A	M	R
B. melitensis	1	–	–	+d	+	–	+	–
	2	–	–	+	+	+	–	–
	3	–	–	+	+	+	+	–
B. abortus	1	+b	+	–	+	+	–	–
	2	+b	+	–	–	+	–	–
	3	+b	+	+	+	+	–	–
	4	+b	+	–	+c	–	+	–
	5	–	–	+	+	–	+	–
	6	–	–	+	+	+	–	–
	9	+ or –	+	+	+	–	+	–
B. suis	1	–	+	+	–e	+	–	–
	2	–	–	+	–	+	–	–
	3	–	–	+	+	+	–	–
	4	–	–	+	–f	+	+	–
	5	–	–		–	–	+	–
B. neotomae	–	–	+	–g	–	+	–	–
B. ovis	–	+	–	+	–f	–	–	+
B. canis	–	–	–	+	–f	–	–	+
B. ceti	–	–	–	+d	+	+	–f	–
B. pinnipedialis	–	+	–	+	+	+	–f	–
B. microti	–	–	–	+	+	–	+	–
B. inopinata	-	-	+	+	+	-	+	-

[a]Dye concentration in serum dextrose medium: 20 µg/ml.
[b]Usually positive on primary isolation.
[c]Some basic fuchsin-sensitive strains have been isolated.
[d]Some strains are inhibited by dyes.
[e]Some basic fuchsin-resistant strains have been isolated.
[f]Negative for most strains.
[g]Growth at a concentration of 10 µg/ml thionin.

to *Ochrobactrum* spp. at eight distinct housekeeping genes, justifying its inclusion in genus *Brucella*. It was therefore named *Brucella inopinata* to indicate its unexpected isolation [42].

This expansion of the genus has drawn attention to the need to review and update the minimal standards for the genus *Brucella*.

PRESENT CLASSIFICATION OF *BRUCELLA* SPP. IN VIEW OF PAST AND CONTEMPORARY NEW STRAINS

In Bergey's Manual of Systematic Bacteriology [24] *Brucella* spp. have been included in Class I. Alphaproteobacteria *class. nov*., order VI Rhizobiales ord. nov., based on phylogenetic analysis of their 16S-rRNA sequences. The taxonomic structure and rules are presented in the manual, as follows:

Family III. Brucellaceae Breed, Murray and smith 1957, 394[AL]

George M. Garrity, Julia A. Bell and Timothy Lilburn

Bru.cel.la.ce.ae. M.L. fem. N. *Brucella* type genus of the family; *-aceae* ending to denote family; M.L. fem. Pl. n. *Brucellaceae* the *Brucella* family*.

*Genus I***. Brucella Mayer and Shaw 1920, *173*[AL]***

Michael Corbel and Menachem Banai

Bru.cel'la. L. dim. Ending *–ella*; M.L. fem. n. *Brucella* named after Sir David Bruce, who first recognized the organism causing undulant (Malta) fever.

**Brucella* is a type genus of the family

**Based on phylogenetic analysis of 16S-rRNA gene sequences, two other genera are included in this family: *Mycoplana*, and *Ochrobactrum*, respectively (The reader is advised that additional genera have been included in the family in the List of Prokaryotic names with Standing in Nomenclature (LNSPN), i.e., *Pseudochrobactrum*, *Daeguia* and *Crabtreella*. Most recently, genus *Paenochrobactrum* has also been validly published (Kämpfer P, Martin E, Lodders N, Jäckel U, Huber BE, Schumann P, Langer S, Busse H-J, Scholz H. *Paenochrobactrum gallinarii* gen. nov., sp. nov., isolated from the air of a duck barn and reclassification and emendation of *Pseudochrobactrum glaciei* as

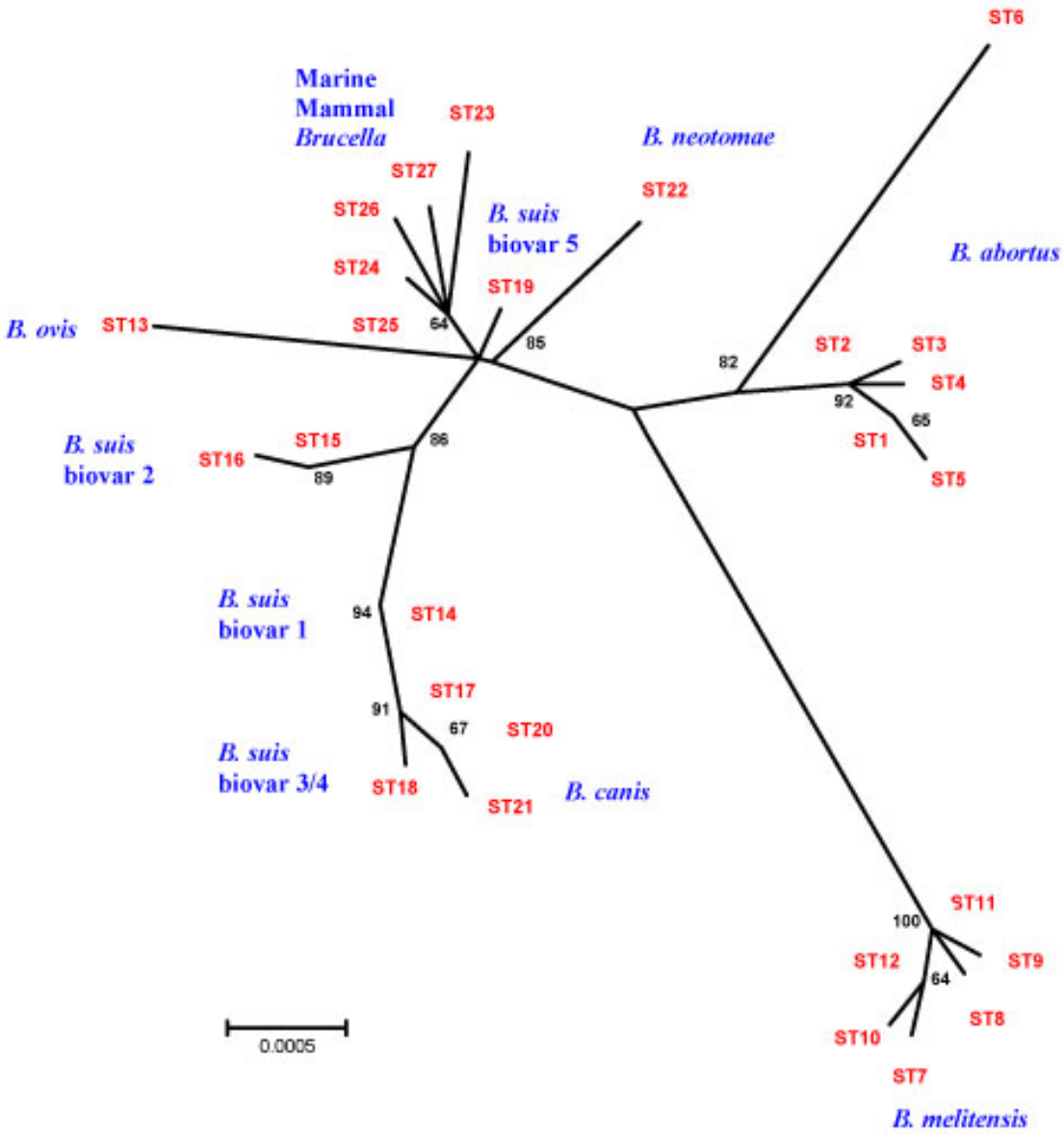

Fig. (1). Unrooted phylogenetic reconstruction of the relationships between STs. This tree was constructed with the concatenated sequence data of the nine loci (4,396 bp) using the neighbour joining approach. The Jukes-Cantor model, which is based on the assumption that all nucleotide substitutions are equally likely, was used to determine genetic distances The percentage bootstrap confidence levels of internal branches were calculated from 1,000 resamplings of the original data. BMC Microbiol. 2007; 7: 34. Published online 2007 April 20. doi: 10.1186/1471-2180-7-34. Copyright © 2007 Whatmore *et al*; licensee BioMed Central Ltd.

Paenochrobactrum glaciei comb. nov. Int J Syst Evol Microbiol 2010; 60 (Pt 7): 1493-1498).

***Page number in the original publication is shown and inclusion of the strain in the Approved Lists, first amended in 1980, is indicated.

The nomenclature used refers to the original description of the isolate, date of publication, and the use of the name by different authors for different organisms, as well as page number(s) in the main text of the scientific work in which the name was proposed (AL stands for Approved Lists of Bacterial Names (Skerman VBD, McGowan V, Sneth PHA (Eds). Approved Lists of Bacterial Names (Amended Edition): Washington, DC: American Society for Microbiology; 1989.).

By adhering to the taxonomic rules that incidentally also favored the multi-species concept, Corbel and Banai (2005) [24] have in fact envisioned the inevitable, that the *Brucella* taxonomy is based on *Brucella* species affiliation to their preferred natural hosts. This represents a return to the pre – 1986 taxonomic opinion on genus *Brucella* as later decided unanimously by the Subcommittee on *Brucella* Taxonomy in view of the zoonotic importance of the multi-species concept and the epidemiological risks associated with the different *Brucella* species, although it should be noted that the Sub-Committee had never abandoned the nomen species concept for everyday use. Based on this decision, the six *Brucella* nomen species that were validly published [43] in the Approved Lists of Bacterial Names of 1980 [22] have been officially re-endorsed. This has facilitated the inclusion of the new marine species in genus *Brucella* [36] following submission of reference strains to two collection centers and their valid publication [44-46]. *B. microti* [39] and *B. inopinata* [42] were later validly published, as well. The

current nomenclature and classification of *Brucella* species is therefore represented in Tables **1-3**.

TAXONOMICAL VIEWS ON *GENUS BRUCELLA*

Bacteriological Properties

Brucella spp. are the etiological agents of animal and human brucellosis. Brucellae are Gram-negative bacteria, stained red using the modified Ziehl Neelsen technique [47] and appearing as coccoid or short rod shaped cells from 0.5-0.7x 0.6-1.5 microns in size.

Brucella spp. live as, non-motile facultative intracellular pathogens of the reticuloendothelial cells of terrestrial and marine mammal hosts. The mechanisms of their virulence and survival inside professional phagocytes partially remains an enigma as *Brucella* has not been shown to produce the virulence factors, such as cytolysins, capsules, exotoxins, secreted proteases, pili and /or fimbriae, flagella, phage encoded toxins and virulence plasmids, implicated in such processes in other bacteria [48,49]. Nevertheless, *Brucella* has a conserved *virB* encoded type IV secretion system that may be involved in its substantiated virulence. In addition, *Brucella* has a flagellar type III secretion system that is based on a cluster of 44 genes distributed in three loci on ChrII, and *motB* and *flgJ* located at different regions on ChrI amongst the three classical species. The fact that *Brucella* is non-motile is explained, however, by inactivation of some of the flagellar genes and absence of the chemotactic systems. Despite lack of motility the presence of flagellar genes in the chromosomes has been shown to be important in *Brucella* persistence in a murine model but not in cell culture infection [50]. It has been proposed that the differences in the potential expression of the flagellum may explain *Brucella* adaptation to different hosts.

Genome sequencing of three classical *Brucella* species has provided information on their respiration and metabolic functions. The chromosomal information has shown that *Brucella* may have adapted to the intracellular habitat by selecting for a high-affinity respiratory mechanism and simultaneously losing functional nucleotide synthesis, sugar modification, polysaccharide synthesis as well as the synthesis of biotin and choline and the energy and carbon storage compounds glycogen and polyhydroxybutyrate [48,51-54].

Brucella respiration is carried out using oxygen as terminal electron acceptor with hydrogenases as donors. *B. abortus* cytochromes b, c, and o have been associated with mid-log growth whereas cytochromes a+ a_3 were identified as additional factors during late-log growth. A possible role for nitrate respiration under lowered redox potential has been proposed. Interestingly, D-erythritol 1-phosphate, an intermediate of the erythritol pathway, has been found to act as an electron donor to the respiratory chain [55]. Given that erythritol is a major product of trophoblasts that line the placenta in the late stages of pregnancy, the attraction of *Brucella* to the gravid uterus and replication in the trophoblasts has been rationalized on this basis [56]. Plommet (1991) [57] used minimal synthetic medium for possible typing of *B. suis* vaccine strain S2 [58] and its corresponding *B. suis* strain 1330 biovar 1 reference wild strain by comparing their nutritional growth requirements. Ammonium sulphate and thiosulphate sufficed to provide nitrogen and sulphur and glucose sufficed to provide carbon and energy production. An intriguing finding was the fact that *B. melitensis* strains could use glutamic acid as a sole source of nitrogen and energy requirements provided that they were stimulated by CO_2, or alternatively, that glucose was utilized for additional energy. From this result the author hypothesized that CO_2 was required for activating the flow of carbon to the tricarboxylic cycle. Interestingly, the latter may link to observations regarding some *Brucella* strains that require an atmosphere containing between 5 to 10% CO_2 for initial culture whereas on further sub-culture they may become CO_2 independent. It is not clear, however, if this results from enzyme induction or sub-population selection.

Brucella Lipopolysaccharide (LPS)

Lipid-As from members of the α-2 *Proteobacteria*, but not the β or γ subdivisions, have been shown to include n-2-hydroxylated long chain fatty acids, such as (27-OH) $C_{28:0}$ and (29-OH) $C_{30:0}$ [59]. The core structure of the lipid-A contains mono-or bisphosphorylated diaminoglucose disaccharides allowing only amide linkages to the 3-OH acyl groups. In contrast, classical lipid-A of most common Gram-negative pathogenic bacteria are formed by a bisphosphorylated disaccharide with two amide and two ester-linked 3-OH-hydroxymyristate; (3-OH) $C_{14:0}$. In *B. abortus*, the major hydroxyl fatty acids in the lipid-A moiety have been shown to lack β-OH myristic acid on the one hand but with seven major fatty acids that account for 85% of the fatty acids having equivalent chain length of 16 or greater carbon atoms, on the other [60,61]. The longer chain fatty acids were found as (27-OH) $C_{28:0}$, (3-OH) $C_{18:0}$ and (3-OH) $C_{14:0}$, respectively [62]. The nature of the long chain fatty acids (C28) has recently been inferred as conferring stealth pathogenesis on *Brucella* spp. by minimizing the host TLR4 mediated innate immune response compared with enterobacterial LPS [63,64].

Degraded oligosaccharide and native hapten extracted by phenol-water from smooth *B. abortus* LPS share structures present in smooth *Brucella* LPS core region including mannose, glucose, 2-amino-2,6-dideoxy-D-glucose (quino-vosamine), 2-amino-2-deoxy-D-glucose (glucosamine), 3-deoxy-D-manno-2-octulosonic acid (KDO) and unidentified sugars. Poly B (polysaccharide hapten extracted with trichloracetic acid from *B. melitensis* rough strain B115), on the other hand, includes traces of quinovosamine and KDO but lacks mannose, glucose and glucosamine [65].

The hydrophilic part of the LPS contains the O- chain antigen (present only in smooth *Brucella*). In *Brucella* cells bearing the A epitope (such as *B. abortus* biovar 1) it is an unbranched homopolymer of α1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranose (perosamine) of a length normally ranging between 96 to 100 glycosyl subunits. In *Brucella* cells bearing the M epitope (such as *B. melitensis* biovar 1) the O-antigen includes a linear pentasaccharide repeating unit composed of four α1,2 and one α1,3-linked perosamine residues [66]. The distinctive LPS structure, including the perosamine homopolymer O-chain in smooth strains, is a defining feature of *Brucella*.

The *gmd*, *per* and *wbkC* gene products are predicted to be involved in 4-formamido-4,6,dideoxymannose synthesis.

The *wbkA* gene product is similar to several mannosyltransferases and is probably involved in the polymerisation of the *B. melitensis* O-side-chain [67]. It is important to note that *Yersinia enterocolitica* 0:9 cross reacts with *B. abortus* A antigen in serological tests because of almost identical O-chain structure [68-71]. This cross reaction complicates serological diagnosis and can hamper eradication campaigns [72-75]. Less extensive cross reactions occur between smooth *Brucella* LPS and those of *Escherichia coli* O:157, *Francisella tularensis*, *Salmonella* O:30, *Stenotrophomonas maltophilia* and *Vibrio cholerae* [72]. These are also due to the presence of perosamine-containing structures in the LPS. This is fortuitous and the lipid A and core polysaccharide structures in these bacteria are quite different from those of *Brucella*.

Rough *Brucella* spp. lack O-chain antigen. *B. canis* was identified by Carmichael and Bruner (1968) [11] as a cause of canine abortion. It was found to grow as mucoid colonies but closely resembled *B. suis* according to its biochemical characteristics. Despite lacking O- smooth polysaccharide antigen, *B. canis* is fully virulent in dogs as well as causing zoonotic infections in humans, albeit to a lesser degree than smooth *Brucella* species. *B. ovis* is also a naturally rough organism causing epididymitis in rams and associated with abortions in ewes [9]. *B. ovis* lacks part of the chromosome [76,77] including a 15.1 kb fragment associated with a genomic island 2 that has been attributed to LPS synthesis [78] (Table **4**). This specific fragment was found to include *wboA* and two other genes involved in biosynthesis of LPS thereby justifying the rough morphology of *B. ovis* [79].

Brucella Outer Membrane Proteins (OMPS)

Certain features of the OMPs are distinctive and play an important role in determining the properties of *Brucella*. *Brucella* OMPs are grouped according to their sizes, proteins with molecular mass of 36-38 kDa are classified as group 2 porin proteins and the 31-34 kDa and 25-27 kDa OMPs as group 3 proteins. The 25-, 31-, and 36 kDa proteins are major OMPs whereas the 10-, 16-, 19-, and 89 kDa molecules are minor components. OMP 36 kDa is a porin [80] and Omp25 kDa is inferred to be important for the maintenance of *B. melitensis* infection [81]. It is worthy of note that the two component system BvrR/BvrS has been shown essential in controlling *Brucella* invasion of cells and intracellular survival in the host [82]. Specifically, the BvrR/BvrS system regulates the expression of two of the *Brucella* group 3 outer membrane proteins. Null *BvrR/BvrS* mutants lacking Omp25 showed changes in cell envelope hydrophobicity, permeability and sensitivity to surface targeted bactericidal peptides thereby leading to the attenuation of the mutants (Guzman-Verri *et al*., 2002)[83].

OMP 16 kDa shows significant similarity to the peptidoglycan-associated lipoproteins Pal of Gram-negative bacteria and like OMP 10-, 16-, and 19-, are surface exposed lipoproteins covalently bound to fatty acids. These proteins have been found in the six original *Brucella* species and biovars within species [84,85]. Omp10 and Omp19 share antigenic determinants with bacteria of the family *Rhizobiaceae* [86]. Gene deletions of *omp10* and *omp19* have led to mutant attenuation ; *omp10* mutants showing decreased survival in mice and defective growth in minimal medium whereas an *omp19* mutant exhibited increased sensitivity to polymyxin B and to sodium deoxycholate as well as yielding significantly fewer bacterial colonies in spleens of mice 4 and 8 weeks post infection [87]. A critical review has been published on these proteins [88].

Several of the *Brucella omp* genes have been cloned and sequenced [89-91]. *omp3a* encodes the formerly recognized Omp 25kDa and *omp3b* is referable to *B. melitensis* Omp31. Knowing the *omp31* gene sequence, Vizcaíno *et al*., (1997) [92] have PCR amplified the DNA fragment showing DNA polymorphism at the *omp31* locus of *Brucella* spp. They then

Table 4. Genomic Islands Identified in Genus *Brucella* as Compared to the *B. melitensis* Chromosomes, Sizes and Important ORFs (Rajashekara *et al*., 2004)

Genomic Island	Relevant to *Brucella* spp.	Size and Location	Number of ORFs	Important Genes
GI 1	*B. ovis*	~8.1 kb (ChrI)	9	Phage related genes
GI 2	*B. ovis*	~15 kb (ChrI)	20	Transposases and phage family integrase and LPS synthesis
GI 3[a]	*B. canis* *B. suis*	~21 kb (ChrI)	30	
GI 4	*B. abortus*	~3.8 kb (ChrI)	5	Butanoate metabolism
GI 5	*B. ovis*	~44 kb (ChrII)	42	Peptide ABC transporters, transcriptional regulators and two ORFs similar to cephalosporin acylases
GI 6[b]	*B. neotomae*	~7.5 kb (ChrII)	10	Transposases with significant similarity to ORFs on plasmid pNGR234a from Rhizobium species
GI 7	*B. ovis*	~4.4 kb (ChrII)	5	
GI 8	*B. abortus*	~25.1 (ChrII)	25	Proteins involved in sugar metabolism and LPS biosynthesis
GI 9	*B. ovis*	~4.9 kb (ChrII)	4	

[a] *B. canis* shares 30 ORFs with similar deletions with *B. suis*, indicating common phylogenetic origin of the two species. This is consistent with other publications [155,156]. Three additional ORFs deletions are unique to *B. canis* including a polysaccharide deacetylase that is similar to chitooligosaccharide deacetylase *NodB* of *Rhizobium* species. This factor is involved in establishing a symbiotic interaction between bacteria and host [157].

[b] Besides GI-6 deletion in *B. neotomae* genomic DNA the strain is closely homologous to *B. melitensis*, suggesting a similar phylogenetic origin of the two species [156].

used this polymorphism to distinguish between *Brucella* species by PCR-RFLP and DNA-DNA hybridization. Using this approach, the gene was absent from *B. abortus* due to a 10 kb deletion whereas *B. neotomae* could not be differentiated from *B. suis* biovars 1, 3, 4 and 5.

Analysis of the 36 kDa porin protein has revealed two genes, *omp2a* and *omp2b*, that appear in the chromosome as inverted repeats of a gene copy with identity of about 85% [91].This polymorphism may be related to differential dye sensitivity, used as one of the traditional biotyping tests (Table **3**). RFLP markers within these genes have been characterized allowing differentiation between *Brucella* species and additional classification of most biovars within species [93,94]. A further useful RFLP classification of *Brucella* spp based on *Brucella omp25* and *omp36* genes has been proposed by Cloeckaert *et al*. (1995) [95].

Brucella Genome

The properties of the genome are of primary importance in defining the genus. Thus the association of *Brucella* with the α-2 *Proteobacteria* group is supported by its possession of two chromosomes [96] like other members of the group.

The genome of *B. melitensis* biovar 1 type strain 16M was first characterised by DelVecchio *et al*. (2002) [48] as a prototype candidate of *Brucella* spp. This was followed by the genome of *B. suis* reference strain 1330 [53] and soon after, by the genome of *B. abortus* field strain 9-941 [52], and more recently the *B ovis* type strain ATCC25840 [54]. Additional genomes have subsequently been sequenced, including the *B. abortus* strain 2308 that is widely used as a challenge strain in experimental brucellosis [51] and the *B. abortus* vaccine strain S19 [97], culminating to date in 13 known *Brucella* genomes (not all published) [98,99]. This enormous progress in rapidly determining whole genome sequences of representatively widespread *Brucella* species and biovars has deepened our insight into *Brucella* taxonomy, delineating their possible phylogenetic relationships possibly down to their ancestors [98].

The DNA composition of *Brucella* reviewed by Corbel (1985) [76] showed that members of genus *Brucella* all share a homogenous base content of 55-58 % mol Guanine + Cytosine inferring a close relatedness among the six species although *B. ovis* was reported to lack a small portion of the polynucleotide sequence present in the DNA of the other species. It has been noted that *Brucella* do not have extrachromosmal replicating entities such as plasmids or phages as evidenced by lack of exotoxins, and transferrable resistance to antibiotics and the failure of attempts to purify plasmid DNA [76]. In contrast, lytic phages have been characterized as shown in Table **2**. Molecular engineering has led to development of four derivatives of the broad-host-range cloning vector pBBR1MCS [100] that replicate in *Brucella* spp. and are compatible with IncP, IncQ, and IncW group plasmids, as well as with ColE1- and P15a-based replicons [101].

An early study using restriction fragment pulsed field electrophoresis has identified the presence of two independent chromosomes in *B. melitensis* type strain 16M, estimated to be of 2.12 and 1.15 megabase size respectively [96].

Genome sequencing has confirmed existence of two chromosomes, chromosome I being larger than chromosome II with median length amongst nine established genomes of 2.1 and 1.2 Mb, respectively. *B. suis* biovar 3 was unique in having a single chromosome of size 3.1 Mb [102]. Both chromosomes have been found to have similar G+C content averaging 57.1% for chromosome I and 57.3% for chromosome II, respectively [103]. It was noted that *Brucella* chromosome I encodes the majority of the core metabolic machinery for processes such as transcription, translation, and protein synthesis, as well as phage-related proteins. In contrast, chromosome II encodes genes involved in processes such as membrane transport, regulation and energy metabolism. Although no plasmid replication origin was found [48] *B. suis* strain 1330 ChrII poses a cluster of plasmid-like replication genes including a replication initiation protein RepC (BRA001) and partitioning proteins RepA and repB (BRA1202 and BRA1203) similar to plasmid replication genes from *Agrobacterium* Ti plasmids, and plasmids from other organisms including *Rhizobium* spp. [53].

Genomic Differences Amongst *Brucella* spp.

Comparative microarray DNA-DNA genomic hybridization was conducted using *B. melitensis* genome as a substrate against the five other nomen species. In general, all six *Brucella* species showed a close identity of their genomes supporting their inclusion in a single species, as previously suggested by Verger *et al*. (1985) [25]. There were nine regions, however, containing at least three contiguous ORFs that were absent from the five *Brucella* species in comparison with *B. melitensis* strain 16M genome. As shown in Table **4**, these regions, designated genomic islands (GIs) were numbered 1 through 9 based on the gene order in 16M. Because *Brucella* live as intracellular pathogens they are not easily available to lateral DNA exchanges that could facilitate *Brucella* divergence by building different DNA composites. Contrary to this assumption, the genomic microarray analysis by Rajashekara *et al*. (2004) [78] has revealed a possible lateral DNA acquisition.

A different approach of comparative genomics has led to identification of a laterally acquired 18.3 kb cluster DNA in *B. suis* that is located on ChrII. The DNA fragment was shown to include 18 genes (BRA0362-BRA0379) with hypothetical proteins, a putative transcriptional regulator and a type IV conjugal transfer cluster of genes. It was assumed that phage integrase, found to be located in the flanking end of a 15 bp direct repeat, was involved [51]. This research also identified a gene cluster commonly found in *B. melitensis* and *B. abortus* on ChrI that *B. suis* lacks [48]. In addition, two large deletions have been found in *B. abortus*, in sizes of 25.1 and 2.8 kb, respectively [51]. The former deletion includes in *B. melitensis* 22 hypothetical genes as well as *omp31* that *B. abortus* lacks. The synthesis of a polysaccharide not yet identified in *Brucella* spp. but present in some bacteria belonging to the alpha-2-*Proteobacteria* group has also been inferred [104]. Smaller gene deletions in size of 1.2 and 0.9 kb, respectively, were correlated with a prokaryotic signaling, diguanylate cyclase/phosphodiesterase domain (GGDEF) protein and a ThiJ/PfpI family protease [78]. In *B. melitensis*, 16 complete genes, four 5' or 3' gene

segments and a tRNA-Glu are missing as the result of seven deletions, five in ChrI and two in ChrII. In addition, a 2.7-kb *B. suis*-specific region located on ChrI is missing from both *B. abortus* and *B. melitensis* [51]. Conversely, a 9-kb gene encoding Cgs, the cyclic ß1-2-glucan synthetase (BAB1V0108) known to be involved in virulence [105,106] is strictly conserved in the three species. Cgs is also conserved in the symbiont *Rhizobium* and is required for effective nodule colonization and symbiosis. Interestingly, *Brucella* Cgs successfully complemented *Rhizobium meliloti ndvB* and *Agrobacterium tumefaciens chvB* mutants [106].

In line with the close relationship between *Brucella* and plant pathogens, an intact β-ketoadipate pathway responsible for utilization of plant derived compounds as energy source was identified on ChrII of *B. suis* strain 1330, in two separate operons on opposite strands [53]. *B. suis* strain 1330 has been shown to be unique amongst the classical *Brucella* species in maintaining the intact pathway whereas in all other *Brucella* species at least 1 of 12 genes involved in this pathway has become a pseudogene and in *B. suis* strain ATCC 23445 (biovar 2) all 12 genes are missing [98].

IS*711*

The presence of a repetitive element was first established in *B. ovis* [107] and further identified as an insertion sequence, IS*711*. It is an 842 bp element having 20 bp imperfect inverted repeats at its ends. The element has been shown to be associated with possible hot-spot sites in the target molecules which are repeated elements Bru-RS1 and Bru-RS2 [108] and it may function *via* duplication of the AT-dinucleotide of the consensus target, YTAR [109]. IS*711*, also identified as IS*6501*, is not found in other bacterial genera but is closely related to IS*427* from *Agrobacterium tumefaciens*, strongly arguing in favor of the phylogenetic relatedness between the two bacteria [110]. The element has been sequenced and identified in six *Brucella* species ranging in different copy numbers between 5 to 10 in *B. melitensis, B. abortus* and *B. suis* but elevated in *B. ovis* to around 30 copies [109,110] suggesting an active mechanism of IS*711* insertions into the genome [109].

Recently, this element was identified in marine isolates at an even higher number of gene copies [26]. In a recent article, active transposition of the element in *Brucella* species that harbor high copy numbers of this element in their chromosomes, e.g., *B. ovis* and *B. pinnipedialis*, has been demonstrated [111]. In contrast, each species has a unique locus of one IS*711* copy in the chromosome, in *B. suis* on ChrI compared to *B. melitensis* and *B. abortus* both of which have this copy on ChrII. Based on polymorphism of IS*711* sequence interruptions in the *Brucella* chromosome AMOS PCR has been established, identifying *B. abortus* (biovar 1, 2 and 4), *B. melitensis* (all three biovars), *B. ovis* and *B. suis* biovar 1 [112-115].

MLVA

Multiple-Locus Variable Number Tandem Repeats (VNTR) analysis (MLVA) is an emerging molecular tool that analyzes DNA fingerprints. The method exploits the presence of a small elementary DNA repeat unit of several nucleotides in the chromosome as perfect and imperfect tandem repetitive copies. This enables identification of MLVA fingerprints of a strain by comparative DNA analysis of minisatellites (a minisatellite is defined as a repeat unit varying between 9 to 80 bp in an allele size of hundreds of bp) and microsatellites (a microsatellite is defined as a repeat unit varying between 1 to 10 bp in an allele size of tens of bp) relative to other organisms. The genome sequencing projects that span the classical *Brucella* species have defined an eight base pair tandem repeat sequence that was hypervariable in eight genomic loci amongst the three *Brucella* species. PCR was then used to characterize the number of repeat units by determining the amplicon (allele) size at each locus. The method apparently successfully identified *Brucella* type strains and biovars and it was named *Brucella* HOOF-Prints standing for Hypervariable Octameric Oligonucleotide Finger-Prints [116]. Using a similar approach, and based on the HOOF Prints it was noted that the selected loci in the early study were not sufficiently stable to assign isolates to the classical *Brucella* species. As a result, additional discriminatory markers have been studied establishing altogether a 21-locus MLVA scheme. This analysis successfully differentiated 105 out of 121 strains of the classical *Brucella* species and biovars, as well as enabling the confirmation of epidemiological linkages amongst strains originating in the same geographical location or source of infection [117]. An additional study was aimed at analyzing 21 strains of the *Brucella* genus, including 18 representative reference and type strains from the classical species and 3 marine strains. This was aimed at identifying most of the tandem repeats identified in the then three available sequenced *Brucella* genomes that showed presence of a repeat unit of 5bp with a minimum set of two alleles. From 71 confirmed loci, 15 markers were selected to be used as effective fingerprints in a *Brucella* MLVA assay. Two complementary panels were selected, panel A that included 8 markers with moderately variable minisatellite structures and panel B with 7 markers, representing highly discriminatory microsatellite structures, respectively [118]. This method was then applied in evaluating MLVA as a diagnostic method linked to epidemiological studies of human cases. Panel 1 was reliable in typing *Brucella* species by depicting some alleles that are fixed in species and biovars. Amongst *B. melitensis* human isolates, however, Panel 1 identified 20 different genotypes that were delineated further to 110 different genotypes by Panel B. These studies demonstrate that despite DNA conservation and homology amongst *Brucella* species unique DNA units may have varied throughout evolution, possibly reflecting, amongst other factors, specific host selective pressures. One can conclude, therefore, that genomic changes reflected by MLVA analysis correlate with *Brucella* evolution [119]. A recent web-site has been established presenting contemporary MLVA analyses of *Brucella* strains that were identified in different geographic sites around the world: http://mlva.u-psud.fr/, Brucella2007 MLVA database.

MLVA has recently been used in a clinical study, targeting human isolates, and extending Panel 2 by the addition of one more tandem repeat locus, on one hand, and dividing panel 2 to two sub-sets of 3 loci (Panel 2A, showing average diversity index lower than 0.75 and 5 loci (Panel 2B, showing average diversity index higher than 0.8), on the other. In all, three of the five octamers in Panel 2B have been

already included by Bricker *et al*. (2003) [116]. In this study, the MLVA–16 assay was highly discriminatory amongst unrelated human *Brucella* isolates from different geographic locations but the authors have noted that additional isolates from different sources and places need to be included in order to fine-tune the method. Interestingly, the study has revealed some *B. melitensis* strains that were identified exclusively in goats compared to others that have been associated with human cases. This specific observation is possibly suggestive of the fact that pathogenic markers are important in the zoonotic aspect of the disease [119,120].

It can be concluded that the three MLVA methods are high throughput state of art technologies that correctly identify *Brucella* species in addition to being highly discriminatory between strains from different geographical and epidemiological linkages. Nonetheless, the fact that nuances occurred between the marker composition of each method and that the typing results markedly depended on the markers (Panel A markers were species specific whereas panel B markers showing a higher diverse index) indicates that a standard method has not yet been achieved. Moreover, the validity of the MLVA typing was assessed using known classification of the test strains regarding their species, biovars and variants of the classical *Brucella* strains. Taken that genus *Brucella* is currently being widened by the inclusion of controversial isolates such as *B. inopinata*, it is yet to be proven that MLVA would be a reliable method in correctly classifying new isolates without prior identification of the strain according to its biochemical and bacteriological traits.

A major difficulty in constructing correct lineage diversifications amongst *Brucella* strains emerges from the fact that the genus *Brucella* includes monomorphic species according to the DNA composition. This is similar to other important human pathogens such as *Yersinia pestis*, *Salmonella enterica* serovar typhi, *Bacillus anthracis* and pathogenic *Mycobacteria* (*M. leprae*, *M. ulcerans* and *M. tuberculosis* complex - MTBC), respectively. Working with MTBC, Comas *et al*. (2009) [121] have found that VNTR loci exhibited different discriminatory power in different bacterial strain lineages. Their conclusion, therefore, has addressed the limited power of the method urging that a large and globally representative strain collection be analyzed in order to choose the most appropriate VNTR markers that offer the highest discriminatory power within a particular strain lineage. Herewith we amend this requirement suggesting that the application of VNTR as a standard routine in diagnostic laboratories or in establishing phylogenic relationships between *Brucella* species and their ancestors must await further validation until a larger strain collection from different sources and clinical cases have been studied.

MLST

Multi-locus sequence typing (MLST) is a contemporary supporting method in tracking epidemiological events based on specific gene sequencing and identification of single nucleotide polymorphism (SNP) in conserved genes, mostly associated with house keeping genes [122]. This method has been recently applied to *Brucella* by selecting 9 distinct genomic fragments from which seven were housekeeping genes. Because the selected genes were scattered in the *Brucella* genome no genetic linkages were expected. The analysis has justified separation of *B. abortus*, *B. melitensis*, *B. neotomae* and *B. ovis* into distinct clusters supporting the classical taxonomical view of genus *Brucella*. *B. suis* biovars 1 to 4 also separated into a distinct cluster but diversification of the cluster was prominent with *B. suis* biovar 5 protruding from the cluster. Despite establishing a distinct cluster, *B. canis* was closely related to *B. suis* biovars 3 and 4. A robust multiplex assay aimed at rapid identification of *Brucella* species based on single nucleotide polymorphism has thus been developed [123].

Molecular Insights on *B. melitensis*

MLST could not identify a clear relationship between *Brucella* biovars and sequence types (ST) [37]. Similarly, multiple VNTR typing approaches [117-118] were insufficient in correlating between a genotype and a biovar. As an example, Al Dahouk *et al*. (2007) [119], using the 16 loci MLVA typing for human brucellosis has strikingly found the collection of human *B. melitensis* isolates to be very heterogeneous and a biovar specific clustering of the *B. melitensis* strains was not achieved.

The distinction between biovars within *B. melitensis* is somewhat subjective as the three biovars present identical phenotypes when grown on agar plates with fuchsin and thionin. They differ only by their agglutination in anti-A or anti-M monospecific sera (Table **3**). Further, Banai *et al*. (1990) [124] identified in Israel an atypical *B. melitensis* biovar 1 phenotype that resembled *B. melitensis* vaccine strain Rev. 1 by its susceptibility to these dyes. As Rev. 1 vaccination is common in Israel and because adverse Rev. 1 like strains have been frequently isolated [125] a concern was raised regarding reversion of the vaccine strain to a virulent phenotype. This question was resolved by comparative analysis of the *omp2* gene polymorphism amongst local *B. melitensis* biovar 1 isolates, atypical strains and type strain 16M in comparison to Rev. 1 commercial vaccine strain and Rev. 1 like isolates. Results have shown that all biovar 1 field isolates in Israel, including the atypical ones, and irrespective of their origin whether from sheep, cows or humans have shared identical *omp2a Pst*I digestion profile that resembled that of biovars 2 and 3, differing from that of the *B. melitensis* type strain 16M. In contrast, all Rev. 1-like isolates and the commercial strain shared an identical pattern with strain 16M. Thus, it has been conclusively confirmed that an *omp2* distinct *B. melitensis* biovar 1 lineage has evolved in Israel and the region and that the atypical strains were derivatives of this lineage. Obviously, this feature has enabled the distinction of Rev. 1 like isolates from local *B. melitensis* biovar 1 strains [126].

Recently, the mutational stability of MLVA markers was analyzed using several Rev. 1 strains from different geographical and commercial sources. This study confirmed that the Rev. 1 isolates were genetically very homogeneous [127] indicating that despite showing correlation of fuchsin sensitivity with the VNTR code [119] MLVA may not be useful in analyzing biological properties of *B. melitensis* biovars. This, however, may be crucial information regarding the protective value of the Rev. 1 vaccine when encountering endemic lineages of *B. melitensis* biovars (and

very likely pathovars not yet shown to exist) that are biologically remote from the genome content of vaccine strain Rev. 1.

Brucella Phylogeny

DNA-DNA hybridization studies have placed *Brucella* strains in the ribosomal ribonucleic acid superfamily IV, which contains *Agrobacterium*, *Rhizobium*, *Mycoplana*, *Phyllobacterium* and Centers for Disease Control Group Vd [128]. The establishment of class *Proteobacteria* with the inclusion of 4 sub-division groups, alpha to delta, has been proposed by Stackebrandt, *et al*. (1988) [129], in order to sustain grouping of various genera that are not related according to current taxonomic criteria, such as phototrophic, nitrifying, nodulating, and plant and animal intracellular pathogens, in the same class.

The fact that *B. abortus* belongs to the alpha-2 subdivision of the class *Proteobacteria* has been inferred by Dorsche *et al*. (1989) [130] due to having 16S-rRNA and other DNA sequences homologous with other members of this class [130,131]. Based on the 16S-rRNA homology studies *Ochrobactrum* has been shown to belong in the alpha 2-*Proteobacteria* with *Brucella* and *Phylobacterium* being the closest genera [132]. This conclusion had been further supported by the identification and characterization of *O. intermedium* as an intermediate species between *Ochrobactrum* and *Brucella* [133].

By alignment of 2,246 protein families within the genomes of 10 *Brucella* strains, representative of *B. abortus*, *B. melitensis*, *B. suis*, *B. canis* and *B. ceti* (partial genome) and using closest relatives in the order *Rhizobiales*, *Ochrobactrum* (*O. anthropi* and *O. intermedium*), *Bartonella quintana,* and *Mesorhizobium loti* as an out group, Wattam *et al*. (2009) [103] have identified in genus *Brucella* six intra-genus phylogenetic clades, *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. ceti*. *Ochrobactrum* was shown to be the closest genus from the out group members. Shared anomalous region (SAR) 2-5 identified as *incP* island, seemed to have entered *Brucella* after breaching from *Ochrobactrum*. This SAR contains the Tra proteins associated with Type IV secretion system [134]. Because it was found in the same phylogenetic clade of *B. suis* and *B. canis*, that are closely related to *B. neotomae* and *B. ceti* (Fig. **1**), it was assumed to have been acquired laterally by a common ancestor.

SNP comparison of 13 *Brucella* genomes has identified *B. ovis* as a basal species and *B. melitensis* and *B. abortus* as forming a distant branch. This suggested that *Brucella* antecedents were first infecting sheep from which they were later transmitted to pigs, cattle and goats. As the SNP study has shown the genus to be exceptionally monomorphic, the time period that statistically justifies these minimal changes has been estimated to be a few thousand years [98]. This has been disputed on considering the evolutionary clock according to which the family *Suidae* has evolved in the past 50 to 20 million years ago preceding the sub-order *Ruminantiae* which includes sub-families *Bovinae* and *Caprinae* [51]. Nevertheless, the antiquity of the host does not seem relevant unless it can be shown that *Brucella* existed before the ruminants.

The taxonomic distance between *Brucella* and *Ochrobactrum* has been studied by comparing the *recA* and *rrs* (16S-rRNA) gene sequences of 7 out of 9 *Ochrobactrum* species and three type strains of *Pseudochrobactrum* species as well as 8 *Brucella* species (*B. inopinata* was not yet described), respectively [135]. By comparing *rrs* sequences from several *Ochrobactrum* strains as the target sequence and using *O. anthropi* type strain as an index strain, distant sub-species clades have been identified in the genus compared to *recA* analysis that formed a more homogeneous group. *Brucella* specie, on the other hand, were indistinguishable from *O. intermedium* by the *rrs* (98.6%) and *recA* (85.5%) sequences, respectively. The demonstration that *Brucella* species closely relate to genus *Ochrobactrum*, that taxonomically involves several species and diverse sub-species, has led the authors to conclude that further criteria are required for clear demarcation between the two genera [135]. With the inclusion of *B. inopinata* in genus *Brucella* the development of a correct taxonomic structure has been further complicated as according to traditional markers, such as 16S rRNA, *omp2* RFLP and MLST, this species lies on the border line between *Brucella* and *Ochrobactrum*.

One recently published taxonomic approach finds the ecological context an important factor in the demarcation of genera and species [136]. An essential feature of *Brucella* is its pathogenicity manifested as disease in the natural host and dissemination to unrelated species including humans. The zoonotic potential is a marker of unusual pathogenicity as manifested by the three classical *Brucella* species; *B. melitensis*, *B. abortus* and *B. suis*, and to a lesser extent *B. canis,* that predominate in human cases worldwide [137]. Other species are less frequently implicated in zoonotic disease although there is evidence that they have the potential. In contrast, the genus *Ochrobactrum* represents free living non-pathogenic environmental bacteria of which only *O. anthropi* and *O. intermedium* have been associated with human disease, usually in patients suffering from additional pathological problems [138]. Inherent pathogenicity and absence of a free-living state are thus distinguishing features of the genus *Brucella*.

The Monophyletic *vs* Paraphyletic Approach

Foster *et al*. (2009) [98] aimed at elucidating *Brucella* phylogeny by comparing single nucleotide polymorphism (SNP) within whole genome orthologs (homologous DNA sequences that were separated by speciation of the genus). The study included 13 genomes of five of the *Brucella* species, *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and *B. canis*. *B. ovis* was found to be a basal lineage distant from two other established nodes, *B. melitensis*-*B. abortus* and *B. suis*-*B. canis*. Within *B. melitensis*, limiting the study to only a single reference strain per biovar, the three biovars of *B. melitensis* were not clearly differentiated. Foster *et al*. (2009) [98] have thus concluded that the three biovars of *B. melitensis* have radiated simultaneously and were since undergoing additional evolution. In the *B. suis* node, strain 23445 (biovar 2) was distant from the other 3 biovars (1, 3, and 4). *B. canis* showed a closer relatedness to *B. suis* 686 (biovar 3) and *B. suis* 40 (biovar 4), suggesting it had arisen from a *B. suis* ancestor. This observation sets *B. suis* as a

paraphyletic species, i.e., a group of organisms that shares a common ancestor but does not include all decedents [98].

Does Host Adaptation Play a Key Role in *Brucella* Evolution?

Herewith we want to propose that host adaptation could be best perceived as the capacity of *Brucella* organisms to undergo biochemical adaptation to a chronic intracellular survival in the reticulo-endothelial cells of their hosts. However, *Brucella* have ensured their persistence in nature due to their capacity to establish an acute disease as free replicating organisms in the gravid uterus of the host and induction of abortion that spreads the organisms in the environment. Specifically, recent data have confirmed that chronic brucellosis is accomplished *via* establishment of sustainable replicative niches of *Brucella* Containing Vacuoles (BCV) in macrophages [139]. In contrast, *Brucella* proliferate to enormous numbers of organisms in the trophoblasts of the gravid uterus [140] resulting in necrosis of the cells and development of placentitis and abortion [141]. The latter is a profound mechanism that facilitates spread of the disease to additional hosts suggesting a linkage between *Brucella* pathogenicity and their restriction to a natural host in which they cause abortion. This has recently been associated with blue-light activation of a two-component histidine kinase sensor in *B. abortus* that triggers virulence of the organism following abortion [142]. It is very likely, therefore, that by infecting secondary hosts *Brucella* are entrapped in dead end vectors incapable of inducing abortion. This stops the abortion cycle thereby ceasing their successful propagation and transmission in nature. Thus, statistical opportunities for the organisms to find a successful replicative niche for the expansion of the population, on one hand, and human interventions such as eradication campaigns, on the other, may have affected *Brucella* evolution. *B. ovis* would be an example of a slowly perishing species following control and eradication campaigns in major endemic areas [143].

In contrast, our data in Israel have shown that *B. melitensis* may have undergone host adaptation to dairy cattle. Despite *B. abortus* S19 vaccine coverage of the female population at 3 to 6 months of age several dairy farms have been infected by *B. melitensis* biovar 1 strain(s) but none, so far, involved the atypical strain. While most cases did not commence as abortion storms on the farms, at least in a single case the infection was accompanied with this problem. This second host adaptation could be explained by possible clonal developments within species [144-146] that may have undergone pheno-genotypic changes.

Other *Brucella* types may correlate less definitely with this hypothesis. *B. abortus* biovar 8 is an extinct species [99] or may have never existed (as no-one could produce a type strain) whereas *B. abortus* biovar 7 is conceived to be a mixed culture of *B. abortus* biovars 3 and 5 [22,146] and *B. suis* biovar 5 is phylogenetically distinct from the other four species [99]. Development of new species, such as *B. microti* and *B. inopinata*, on the other hand, possibly indicate the presence of unknown forces in nature that may sustain or modify *Brucella* populations and their genomic content. Whether the strains existed in the past and were only recently identified due to careful application of improved methodologies does not change this conclusion.

CONCLUSIONS

Genus Definition

According to the 1975 minimal standards, the genus is defined in terms of morphological, biochemical, cultural, serological and pathogenic properties, with very limited genetic criteria (DNA G+C ratio, DNA-DNA hybridization homology) as other data had not emerged at that time. These criteria would not exclude any of the current members of the genus but are no longer adequate for decision-making in relation to new isolates. Indeed it was envisaged at the time that they were published that they would be updated periodically in the light of new knowledge. This is clearly long overdue. The phylogenetic position of the genus has changed dramatically since these standards were laid down. At that time *Brucella* was a distinct genus with no known close affiliation to other genera. Since then it has become clear that the genus is closely related to a number of genera within the α–2 sub division of the *Proteobacteria* and in particular to *Ochrobactrum.* This similarity is so close that it has even been suggested that these genera should be merged. It is possible that other genera will be discovered that also show a close relationship to *Brucella*. It is now assumed that the genus and its close relations such as *Ochrobactrum* and *Mycoplana*, evolved from a common environmental precursor. This may no longer exist and direct fossil evidence is unlikely! However, virtual fossil evidence may be deduced from the genome structures and virtual precursors and intermediate forms may be inferred. They may help to explain the relationships between the current genera. However, from a practical point of view a fundamental difference exists between *Brucella* and *Ochrobactrum* in that the former is a primary pathogen and associated with specific infections in a range of hosts. All known *Ochrobactrum* species are essentially saprophytic environmental bacteria with some occasionally associated with opportunistic infections in humans, usually with immune impairment. The features defining the basis of pathogenicity in *Brucella* are still only partially characterized but will eventually be defined in terms of molecular genetics. It is likely that these will form the basis for the differentiation of the genera. It is also quite conceivable that intermediate forms possessing some but not all of the pathogenicity determinants of *Brucella* against an *Ochrobactrum* genetic background will be found. Perhaps *B. inopinata* represents a step in that direction. Under such circumstances the basis of differentiation of the genera will need to be reconsidered. At present no useful purpose would be served by lumping together a group of specific pathogens with a group of essentially non-pathogenic bacteria of wide distribution. This would present legislative and practical problems for medical and veterinary authorities on a global scale.

A new genus definition should emphasize genetic features. A DNA G+C ratio of 56-59% and >95% homology with DNA of reference strains are essential features, together with typical chromosomal structure and full genome sequence. However, the latter is not convenient to determine on a routine basis with current technology and alternative

sources of information have to be considered. Key in differentiating the genus from closely related organisms is the nucleotide sequence of the 16S-rRNA. Recent near full length sequencing (1,412 bp from a full length of 1,485 bp) of the six long-established *Brucella* species and a marine isolate (ATCC M2357/93) showed all to be 100% identical. In contrast, the 16S-rRNA sequence of the closely related *Ochrobactrum anthropi* was 99.8% identical, with a difference of 16bp as well as a deletion and an insertion which were sufficient to distinguish between *Brucella* species and this closest known relative. *B. inopinata* 16S-rRNA showed only a 5 bp difference and was thus acceptable for the genus. Thus, 16S-rRNA sequencing allows determinative identification of new isolates as members of the genus [147].

It is also essential to retain fundamental phenotypic characteristics such as Gram negative morphology and absence of flagella and motility (although *Brucella* retains flagella genes, these are modified, do not confer motility and may play a role in pathogenesis). Most biochemical and cultural characteristics should remain unchanged but with more latitude for metabolic activities. The serological specificity of smooth strains is a defining feature and the presence of a lipopolysaccharide of distinctive core lipid composition and with an O- chain comprising an N-formyl 4 amino 4,6 dideoxymannose homopolymer differentiates the genus. Pathogenicity in appropriate animal species and/or the presence of functional genes encoding virulence determinants such as *virB* and the Type IV secretion system are also defining features although the possibility of deletion or disruption in individual isolates should be kept in mind.

Species Definition

It has long been maintained by some that *Brucella* comprises a single species with numerous variations. This has been reflected in the nomenclature proposed at various times eg *Brucella melitensis* var. *abortus* or var. *melitensis* [1]*; Brucella brucei* var. *abortus* &c. [148]; *Brucella melitensis melitensis* &c. [25]. While from a purely academic viewpoint there is some validity in this approach, it confers little practical assistance in differentiating the isolates from various sources and in monitoring transmission and control. In current taxonomy species are differentiated on the basis of DNA homology, with the arbitrary figure of 70% similarity being taken as the cut-off point. For many genera this level fails to differentiate many organisms that display substantial biological differences. It is also recognized that determination of homology by crude hybridization techniques fails to disclose important genomic differences. In the case of *Brucella* this has been confirmed by more subtle molecular genetic analysis. Nevertheless, it can be argued that the differences and groupings observed do not amount to true species differences. For that reason, taxa differentiated at that level are best referred to by the term nomen species which better reflects the situation.

The use of features such as CO_2 requirement, H_2S production, urease activity, dye sensitivity and antigenic type, enabled strains conforming to the original three species to be identified but rapidly fell apart once a wider range of strains was studied. The observation that oxidative metabolic pattern with carbohydrate, amino acid and urea cycle substrates allowed a correlation with preferred natural host constituted a major advance [149,150]. This was supported by the more user-friendly phage typing procedure [151,152]. This enabled differentiation of the three original species *B. abortus, B. melitensis* and *B. suis* into biovars by application of the traditional biochemical, serological and dye sensitivity tests. This system has worked reasonably well over the years although differences between isolates are not always clear-cut and the various biovars are not distinguished by characteristics of equal weight e.g. *B. melitensis* biovars 1, 2 and 3 are really only serovars; *B. abortus* biovars 3 and 6 hinge on dye sensitivity. The system can also be criticized for inconsistent application to subsequently identified species, e.g. heterogeneity exists in the properties of *B. ovis* and *B. canis* strains but is not recognized by the classification. It is clear that a more objective system for sub- division of the genus is required. Multiplex PCR has been shown, however, successful in identifying *Brucella* species and vaccine strains [153,154].

Recently, by means of whole genome single nucleotide polymorphism (SNP), *omp 2* gene RFLP analysis and multi locus sequence typing (MLST), a consensus has been obtained on the position of *B. ovis* as a basal species of the genus from which a *B. melitensis*, *B. abortus* clade and a separate *B. suis, B. canis* clade have emerged [40,41,98,103]. The other recognized species are sporadic and unorientated. Interestingly, *B. inopinata* adheres closely to the 16S-rRNA *Brucella* category but shows a divergent *omp2* sequence. Its placing as a separate species in the genus *Brucella* was based on MLST of 8 conserved housekeeping genes that showed it to be closer to *Brucella* than to *Ochrobactrum*.

In order to prepare for the unknown we therefore suggest a two step approach. Firstly, the *Brucella* taxon as presented in Tables **1-3** would be recognized as a valid structure due to conforming with the rules and codes of bacterial taxonomy. Classification of newly identified *Brucella* isolates would be achieved by the implementation of established techniques such as 16S-rRNA sequencing, phage typing, identification of oxidative metabolism patterns (gallery methods such as the API system might offer a less hazardous alternative approach although with limitations [135]) and association of the strain into an ecological niche [136]. Secondly, sub-typing to a biovar level and identification of epidemiological linkages would be accomplished by alternative molecular typing; MLVA, MLST and SNP in showing phylogenetic relationships and VNTR in establishing epidemiological linkages, respectively. Nonetheless, we propose that a pathovar nomenclature be established by recording important intra-species infection events that may impinge on possible cloning developments amongst genus members. This approach could open new horizons regarding our means of studying *Brucella* virulence and pathogenicity in different hosts.

For example, in *B. melitensis* we identified isolates that differed in their host restriction, such as the atypical biovar 1 strains that have been found associated with small ruminant and human brucellosis cases in Israel but never so far were identified in cattle (Banai, unpublished data). Al Dahouk *et al.* (2007) [119] have found a similar trend amongst *B. melitensis* reported lineages that were restricted to goats. The isolates from marine mammals also show genetic diversity

that could be reflected in differences in pathogenicity. However, there are obvious difficulties in studying this in the natural environment.

We recognize that the full rational development of such an approach is contingent on the availability of additional data on the basis of the pathogenicity and host specificity of *Brucella* and are under no illusions that this is a beginning rather than a conclusion.

REFERENCES

[1] Evans AC. Further studies on *Bacterium abortus* and related bacteria. A comparison of *Bacterium abortus* with *Bacterium bronchisepticus* and with the organism which causes Malta fever. J Infect Dis 1918; 22: 580-93.

[2] Bruce D. Note on the discovery of a microorganism in Malta fever. Practitioner 1887; 39: 161-70.

[3] Bang B. *Die Aetiologie des seuchenhaften ("infectiösen") Verwerfens*. Zeitschrift Thiermedicin 1897; 1: 241-78.

[4] Traum J. Report of the Chief of the Bureau of Animal Industry, U.S. Department of Agriculture. U.S. Department of agriculture, Washington DC. 1920; p. 30.

[5] Meyer KF, Shaw EB. A comparison of the morphologic, cultural, and biochemical characteristics of *B. abortus* and *B. melitensis*. J Infect Dis 1920; 27: 173-84.

[6] Huddleson IF. Differentiation of the species of the genus *Brucella*. Am J Pub Health Nations Health 1931; 21: 491-8.

[7] Stableforth AW, Jones LM. International Committee on Bacteriological Nomenclature subcommittee on taxonomy of *Brucella*. Report of the meeting, 18 August 1962, Montreal, Canada. Int Bull Bacteriol Nomencl 1963; 13: 145-58.

[8] Davydov NN. (Properties of *Brucella* isolated from reindeer). Trudy Vsye Inst Exp Vet 1961; 27: 24-31.

[9] Buddle MB. Studies on *Brucella ovis* (n.sp.), a cause of genital disease of sheep in New Zealand and Australia. J Hyg (Lond) 1956; 54: 351-64.

[10] Stoenner HG, Lackman DB, Bell EJ. A new species of *Brucella* isolated from the desert wood rat, Neotoma lepida Thomas. Am J Vet Res 1957; 18(69): 942-51.

[11] Carmichael LE, Bruner DW. Characteristics of a newly recognized species of *Brucella* responsible for infectious canine abortions. Cornell Vet 1968; 58: 579-92.

[12] Korol AG, Parnas J. Ein neuer serobiotyp von *Brucella-Brucella murium* (Korol). Z Gesamte Hyg1967; 13: 799-800.

[13] Corbel MJ, Morgan WJB. Proposal for minimal standards for description of new species and biotypes of the genus *Brucella*. Int J Syst Bacteriol 1975; 25: 83-9.

[14] Meyer ME. Inter-and intra-strain variants in the genus *Brucella*. Dev Biol Stand 1984; 56: 73-83.

[15] Jones LM. Report to the International Committee on Nomenclature of Bacteria by the subcommittee on taxonomy of 'Brucellae'. Minutes of meeting 22-23 July 1966, Moscow, USSR. Int J Syst Bacteriol 1967; 17: 371-5.

[16] Jones LM, Wundt W. International Committee on Nomenclature of Bacteria Subcommittee on the taxonomy of *Brucella.* Minutes of meeting, 7 August 1970, Mexico City, Mexico. Int J Syst Bacteriol 1971; 21: 126.

[17] Wundt W, Morgan WJB. Internationl Committee on Systematic Bacteriology Subcommittee on taxonomy of *Brucella.* Minutes of meeting, 3 September 1974, Tokyo, Japan. Int J Syst Bacteriol 1975; 25: 235-6.

[18] Corbel MJ. International Committee on Nomenclature of Bacteria Subcommittee on taxonomy of *Brucella.* Minutes of meeting, 4 and 5 September, 1978, Munich, Germany. Int J Syst Bacteriol 1982; 32: 260-1.

[19] Corbel MJ. International Committee on Systematic Bacteriology Subcommitteee on taxonomy of *Brucella.* Minutes of meeting, September 1982, Boston, USA. Int J Syst Bacteriol 1984; 34: 366-7.

[20] Corbel MJ. International Committee on Systematic Bacteriology Subcommittee on taxonomy of *Brucella.* Report of the meeting, 5 September 1986, Manchester, England. Int J Syst Bacteriol 1988; 38: 450-2.

[21] Corbel MJ, Moriyon I. International Committee on Systematic Bacteriology Subcommittee on Taxonomy of *Brucella.* Minutes of the meeting, 5 and 7th July 1994, Prague, Czech Republic. Int J Syst Evol Bacteriol 2006; 66:1169-70.

[22] Osterman B, Moriyón I. Minutes: International Committee on Systematics of Prokaryotes. Subcommittee on the Taxonomy of *Brucella*. Minutes of the meeting, 17 September 2003, Pamplona, Spain. Int J Sys Evol Microbiol 2006; 56: 1173-5.

[23] Corbel MJ, Morgan WJB. Genus *Brucella Meyer and Shaw 1920,173*^{AL}. In Krieg NR, Holt JG Eds. Bergey's Manual of Systematic Bacteriology, 1st ed. The Williams and Wikins Co.: Baltimore 1984; vol. 1: pp. 377-88.

[24] Corbel MJ, Banai M. *Genus I. Brucella Meyer and Shaw 1920, 173*^{AL}. In: Brenner DJ, Krieg NR, Staley JT. Eds. Bergey's Manual of Systematic Bacteriology. 2nd ed. Springer 2005; vol. 2: pp. 370-86.

[25] Verger J-M, Grimont F, Grimont PAD, *et al*. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int J Syst Bacteriol 1985; 35: 292-5.

[26] Bricker BJ, Ewalt DR, MacMillan AP, *et al*. Molecular characterization of *Brucella* strains isolated from marine mammals. J Clin Microbiol 2000; 38: 1258-62.

[27] Clavareau C, Wellemans V, Walravens K, *et al*. Phenotypic and molecular characterization of a Brucella strain isolated from a mink whale (*Balaenoptera acutorostrata*). Microbiology 1998; 144: 3267-73.

[28] Dawson CE, Stubberfield EJ, Perrett LL, *et al*. Phenotypic and molecular characterization of *Brucella* isolates from marine mammals. BMC Microbiology 2008; 8: 224.

[29] Foster G, MacMillan AP, Godfroid J, *et al*. A review of *Brucella* sp. infection of sea mammals with particular emphasis on isolates from Scotland. Vet Microbiol 2002; 90: 563-80.

[30] Bourg G, O'Callaghan D, Boschiroli ML. The genomic structure of *Brucella* strains isolated from marine mammals gives clues to evolutionary history within the genus. Vet Microbiol 2007; 125: 375-80.

[31] González-Barrientos R, Morales JA, Hernández-Mora G, *et al*. Pathology of Striped Dolphins (*Stenella coeruleoalba*) Infected with *Brucella ceti*. J Comp Pathol 2010; 142: 347-52.

[32] Sohn AH, Probert WS, Glaser CA, *et al*. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. Emerg Infect Dis 2003; 9: 485-8.

[33] McDonald WL, Jamaludin R, Mackereth G, *et al*. Characterization of a *Brucella* sp. strain as a marine-mammal type despite isolation from a patient with spinal osteomyelitis in New Zealand. J Clin Microbiol 2006; 44: 4363-70.

[34] Whatmore AM, Dawson CE, Groussaud P, *et al*. Marine mammal *Brucella* genotype associated with zoonotic infection. Emerg Infect Dis 2008; 14: 517-8.

[35] Groussaud P, Shankster SJ, Koylass MS, *et al*. Molecular typing divides marine mammal strains of *Brucella* into at least three groups with distinct host preferences. J Med Microbiol 2007; 56: 1512-8.

[36] Cloeckaert A, Verger J-M, Grayon M, *et al*. Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the *omp2* locus. Microbes Infect 2001; 3: 729-38.

[37] Whatmore AM, Perrett LL, MacMillan AP. Characterization of the genetic diversity of *Brucella* by multilocus sequencing. BMC Microbiology 2007; 7: 34.

[38] Scholz HC, Hubalek Z, Sedláček I, *et al*. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. Int J Syst Evol Microbiol 2008; 58: 375-82.

[39] Scholz HC, Hubalek Z, Nesvadbova J, *et al*. Isolation of *Brucella microti* from soil. Emerg Infect Dis 2008; 14: 1316-7.

[40] Scholz HC, Hofer E, Vergnaud G, *et al*. Isolation of *Brucella microti* from mandibular lymph nodes of red foxes, *Vulpes vulpes*, in Lower Austria. Vector Borne Zoo Dis 2009; 9: 153-5.

[41] De BK, Stauffer L, Koylass MS, *et al*. Novel *Brucella* strain (BO1) associated with a prosthetic breast implant infection. J Clin Microbiol 2008; 46: 43-9.

[42] Scholz HC, Nöckler K, Göllner C, *et al*. *Brucella inopinata* sp. nov., isolated from a breast implant infection. Int J Syst Evol Microbiol 2010; 60(4): 801-8.

[43] Meyer ME, Morgan WJB. Designation of neotype strains and of biotype reference strains for species of the genus *Brucella* Meyer and Shaw. Int J Syst Bacteriol 1972; 23: 135-41.

[44] Foster G, Osterman BJ, Godfroid J, *et al*. *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 2007; 57: 2688-93.

[45] Tindall BJ, Kämpfer P, Euzéby JP, *et al*. Valid publication of names of prokaryotes according to the rules of nomenclature: past history and current practice. Int J Syst Evol Microbiol 2006; 56: 2715-20.

[46] Tindall BJ, Rosselló-Móra R, Busse H-J, *et al*. Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Sys Evol Microbiol 2010; 60: 249-66.

[47] Stamp JT, McEwen AD, Watt IAA, *et al*. Enzootic abortion in ewes. Transmission of the disease. Vet Rec 1950; 62: 251-4.

[48] DelVecchio VG, Kapatral V, Redkar RJ, *et al*. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc Natl Acad Sci USA 2002; 99: 443-8.

[49] Seleem MN, Boyle SM, Sriranganathan N. *Brucella*: a pathogen without classic virulence genes. Vet Microbiol 2008; 129: 1-14.

[50] Fretin D, Fauconnier A, Köhler S, *et al*. The sheathed flagellum of *Brucella melitensis* is involved in persistence in a murine model of infection. Cell Microbiol 2005; 7: 687-98.

[51] Chain PSG, Comerci DJ, Tolmasky ME, *et al*. Whole-genome analyses of speciation events in pathogenic *Brucellae*. Infect Immun 2005; 73: 8353-61.

[52] Halling SM, Peterson-Burch BD, Bricker BJ, *et al*. Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. J Bacteriol 2005; 187: 2715-26.

[53] Paulsen IT, Seshadri R, Nelson KE, *et al*. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc Natl Acad Sci USA 2002; 99: 13148-53.

[54] Tsolis RM, Seshadri R, Santos RL, *et al*. Genome degradation in *Brucella ovis* corresponds with narrowing of its host range and tissue tropism. Plos ONE 2009; 4: e5519.

[55] Rest RF, Robertson DC. Characterization of the electron transport system in *Brucella abortus*. J Bacteriol 1975; 122: 139-44.

[56] Smith HA, Williams E, Pearce JH, *et al*. Foetal erythritol: a cause of the localization of *Brucella abortus* in bovine contagious abortion. Nature 1962; 193: 47-9.

[57] Plommet M. Minimal requirements for growth of *Brucella suis* and other *Brucella* species. Zbl Bakt 1991; 275: 436-50.

[58] Xie X. Orally administrable brucellosis vaccine: *Brucella suis* strain 2 vaccine. Vaccine 1986; 4: 212-6.

[59] Bhat UR, Carlson RW, Busch M, *et al*. Distribution and phylogenetic significance of 27-hydroxy-octacosanoic acid in lipopolysaccharides from bacteria belonging to the alpha-2 subgroup of Proteobacteria. Int J Syst Bacteriol 1991; 41: 213-7.

[60] Moreno E, Pitt MW, Jones LM, *et al*. Purification and characterization of smooth and rough lipopolysaccharides from *Brucella abortus*. J Bacteriol 1979; 138: 361-9.

[61] Moreno E, Cloeckaert A, Moriyón I. *Brucella* evolution and taxonomy. Vet Microbiol 2002; 90: 209-27.

[62] Ferguson GP, Datta A, Baumgarther J, *et al*. Similarity to peroxisomal-membrane protein family reveals that *Sinorhizobium* and *Brucella* BacA affect lipid-A fatty acids. Proc Natl Acad Sci USA 2004; 101: 5012-7.

[63] Lapaque N, Takeuchi O, Corrales F, *et al*. Differential inductions of TNF-α and IGTP, IIGP by structurally diverse classic and non-classic lipopolysaccharides. Cell Microbiol 2006; 8: 401-13.

[64] Tsolis RM, Young GM, Solnic JV, *et al*. From bench to bedside: stealth of enteroinvasive pathogens. Nat Rev 2008; 6: 883-92.

[65] Moreno E, Speth SL, Jones LM, *et al*. Immunochemical characterization of *Brucella* lipopolysaccharides and polysaccharides. Infect Immun 1981; 31: 214-22.

[66] Meikle PJ, Perry MB, Cherwonogrodzky JW, *et al*. Fine structure of A and M antigens from *Brucella* biovars. Infect Immun 1989; 57: 2820-8.

[67] Godfroid F, Cloeckaert A, Taminiau B, *et al*. Genetic organization of the lipopolysaccharide O-antigen biosynthesis region of *Brucella melitensis* 16M (wbk). Res Microbiol 2000; 151: 655-68.

[68] Greiser-Wilke I, Moenning V, Thon D, *et al*. Characterization of monoclonal antibodies against *Brucella melitensis*. Zentrabl Vet Med B 1985; 32: 616-27.

[69] Greiser-Wilke I, Moenning V. Monoclonal antibodies and characterization of epitopes of smooth *Brucella* lipopolysaccharides. Ann Inst Pasteur (Paris) 1987; 138: 549-60.

[70] Kittelberger R, Bundesen PG, Cloeckaert A, *et al*. Serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* 0 : 9: IV. Evaluation of the M- and C-epitope antibody response for the specific detection of *B. abortus* infections. Vet Microbiol 1998; 60: 45-57.

[71] Muñoz PMC, Marín M, Monreal D, *et al*. Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. Clin Diagn Lab Immunol 2005; 12: 141-51.

[72] Corbel MJ. Recent advances in the study of *Brucella* antigens and their serological cross reactions. Vet Bull 1985; 54: 927-42.

[73] Hilbink F, Fenwick SG, Thompson EJ, *et al*. Non-specific seroreactions against *Brucella abortus* in ruminants in New Zealand and presence of *Yersinia enterocolitica* 0:9. N Z Vet J 1995; 43: 175-8.

[74] Gerbier G, Garin-Bastuji B, Pouillot R, *et al*. False positive serological reactions in bovine brucellosis: evidence of the role of *Yersinia enterocolitica* serotype O:9 in a field trial. Vet Res 1997; 28: 375-83.

[75] Godfroid J, Saegerman C, Wellemans V, *et al*. How to substantiate eradication of bovine brucellosis when aspecific serological reactions occur in the course of brucellosis testing. Vet Microbiol 2002; 90: 461-77.

[76] Corbel MJ. *Brucella melitensis*; DNA analysis of *Brucella*: present and future. In: Verger JM, Plommet M, Eds. Martinus Nijhoff Pub., The Hague 1985; pp. 21-7.

[77] Hoyer BH, McCullough NB. Homologies of deoxyribonucleic acids from *Brucella ovis*, canine abortion organisms, and other *Brucella* species. J Bacteriol 1968; 96:1783-90.

[78] Rajashekara G, Glasner JD. Glover DA, *et al.* Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. J Bacteriol 2004: 186: 5040-51.

[79] Cardoso PG, Macedo GC, Azevedo V, *et al*. *Brucella* spp. noncanonical LPS: structure, biosynthesis, and interaction with host immune system. Microbial Cell Fact 2006; 5: 4-16.

[80] Marquis H, Ficht TA. The *omp2* gene locus of *Brucella abortus* encodes for two homologous outer membrane proteins with properties characteristic of bacterial porins. Infect Immun 1993; 61: 3785-90.

[81] Edmonds M, Cloeckaert A, Elzer P. *Brucella* species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis*. Vet Microbiol 2002; 88: 205-21.

[82] Sola-Landa A, Pizaro-Cerdá J, Grilló M-J, *et al*. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol Microbiol 1998; 29: 125-38.

[83] Guzmán-Verri C, Manterola L, Sola-Landa A, *et al*. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the *Rhizobiaceae*. Proc Natl Acad Sci USA 2002; 99: 12375-80.

[84] Tibor A, Saman E, Wergifosse P-de, *et al*. Molecular characterization, occurrence, and immunogenicity in infected sheep and cattle of two minor outer membrane proteins of *Brucella abortus*. Infect Immun 1996; 64: 100-7.

[85] Tibor A, Decelle B, Letesson J.-J. Outer membrane proteins Omp10, Omp16, and Omp19 of *Brucella* spp. are lipoproteins. Infect Immun 1999; 67: 4960-2.

[86] Cloeckaert A, Tibor A, Zygmunt M. *Brucella* outer membrane lipoproteins share antigenic determinants with bacteria of the family *Rhizobiaceae*. Clin Diag Lab Immunol 1999; 6: 627-9.

[87] Tibor A, Wansard V, Bielartz V, *et al*. Effect of *omp10* or *omp19* deletion on *Brucella abortus* outer membrane properties and virulence in mice. Infect Immun 2002; 70: 5540-6.

[88] Cloeckaert A, Verger JM, Grayon M, *et al*. Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. FEMS Microbiol Lett 1996; 145: 1-8.

[89] Vizcaino N, Cloeckaert A, Zygmunt MS, *et al*. Cloning, nucleotide sequence, and expression of the *Brucella melitensis omp31* gene coding for an immunogenic major outer membrane protein. Infect Immun 1996; 64: 3744-51.

[90] Kovach ME, Elzer PH, Robertson GT, *et al*. Cloning and nucleotide sequence analysis of a *Brucella abortus* gene encoding an 18 kDa immunoreactive protein. Microb Pathog 1997; 22: 241-6.

[91] Ficht TA, Bearden SW, Sowa BA, *et al*. DNA sequence and expression of the 36-kilodalton outer membrane protein gene of *Brucella abortus*. Infect Immun 1989; 57: 3281-91.

[92] Vizcaíno N, Verger J-M, Grayon M, *et al*. DNA polymorphism at the omp-31 locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology 1997; 143: 2913-21.

[93] Ficht TA, Husseinen HS, Derr J, *et al*. Species-specific sequences at the *omp2* locus of *Brucella* type strains. Int J Syst Bacteriol 1996; 46: 329-31.

[94] Ficht TA, Bearden SW, Sowa BA, *et al*. Genetic variation at the *omp2* porin locus of the brucellae: species-specific markers. Mol Microbiol 1990; 4: 1135-42.

[95] Cloeckaert A, Verger J-M, Grayon M, *et al*. Restriction site polymorphism of the genes encoding the major 25kDa and 36kDa outer membrane proteins of *Brucella*. Microbiology 1995; 141: 2111-21.

[96] Michaux S, Paillisson J, Carles-Nurit MJ, *et al*. Presence of two independent chromosomes in the *Brucella melitensis* 16M genome. J Bacteriol 1993; 175: 701-5.

[97] Crasta OR, Folkerts O, Fei Z, *et al*. Genome sequence of *Brucella abortus* vaccine strain S19 compared to virulent strains yield candidate virulence genes. PLoS ONE 2008; 3(5): e2193.

[98] Foster JT, Beckstrom-Sternberg SM, Pearson T, *et al*. Whole-genome-based phylogeny and divergence of the genus *Brucella*. J Bacteriol 2009; 191: 2864-70.

[99] Whatmore AM. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. Infect Genet Evol 2009; 9: 1168-84.

[100] Kovach ME, Phillips RW, Elzer PH, *et al*. pBBR1MCS: a broad-host-range cloning vector. BioTechniques 1994; 16: 800-2.

[101] Kovach ME, Elzer PH, Hill DS, *et al*. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 1995; 166: 175-6.

[102] Jumas-Bilak E, Charachon MS, Bourg G, *et al*. Differences in chromosome number and genome rearrangements in the genus *Brucella*. Mol Microbiol 1998; 27: 99-106.

[103] Wattam AR, Williams KP, Snyder EE, *et al*. Analysis of ten *Brucella* genomes reveals evidence for horizontal gene transfer despite a preferred intracellular lifestyle. J Bacteriol 2009; 191: 3569-79.

[104] Vizcaino N, Cloeckaert A, Zygmunt MS, *et al*. Characterization of a *Brucella* species 25-kilobase DNA fragment deleted from *Brucella abortus* reveals a large gene cluster related to the synthesis of a polysaccharide. Infect Immun 2001; 69: 6738-48.

[105] Briones G, de Iannino IN, Roset M, *et al*. *Brucella abortus* cyclic ß-1,2-glucan mutants have reduced virulence in mice and are defective in intracellular replication in HeLa cells. Infect Immun 2001; 69: 4528-35.

[106] de Iannino IN, Briones G, Tolmasky M, *et al*. Molecular cloning and characterization of *cgs*, the *Brucella abortus* cyclic ß(1-2) glucan synthetase gene: genetic complementation of *Rhizobium meliloti* ndvB and *Agrobacterium tumefaciens chvB* mutants. J Bacteriol 1998; 180: 4392-400.

[107] Halling SM, Zehr ES. Polymorphism in *Brucella* spp. due to highly repeated DNA. J Bacteriol 1990; 172: 6637-40.

[108] Halling SM, Bricker BJ. Characterization and occurrence of two repeated palindromic DNA elements of *Brucella* spp.: Bru-RS1 and Bru-RS2. Mol Microbiol 1994; 14: 681-9.

[109] Halling SM, Tatum FM, Bricker BJ. Sequence and characterization of an insertion sequence, IS*711*, from *Brucella ovis*. Gene 1993; 133: 123-7.

[110] Ouahrani S, Michaux S, Widada JS, *et al*. 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 1993; 139: 3265-73.

[111] Ocampo-Sosa A, García-Lobo JM. Demonstration of IS711 transposition in *Brucella ovis* and *Brucella pinnipedialis*. BMC Microbiology 2008; 8: 17.

[112] Bricker BJ, Halling SM. Differentiation of *B. abortus* bv. 1, 2, and 4, *Brucella melitensis, Brucella ovis,* and *Brucella suis* bv. 1 by PCR. J Clin Microbiol 1994; 32: 2660-6.

[113] Bricker BJ, Halling SM. Enhancement of the *Brucella* AMOS PCR assay for the differentiation of *Brucella abortus* vaccine strains S19 and RB51. J Clin Microbiol 1995; 33: 1640-2.

[114] Ewalt DR, Bricker BJ. Validation of the abbreviated *Brucella* AMOS PCR as a rapid screening method for differentiation of *Brucella abortus* field strain isolates and the vaccine strains, 19 and RB51. J Clin Microbiol 2000; 38: 3085-6.

[115] Bricker BJ, Ewalt DR, Olsen SC, *et al*. 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 Diag Invest 2003; 15: 374-8.

[116] Bricker BJ, Ewalt ER, Halling SM. *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). BMC Microbiology 2003; 3:15.

[117] Whatmore AM, Shankster SJ, Perrett LL, *et al*. Identification and characterization of variable-number tandem repeat markers for typing of *Brucella* spp. J Clin Microbiol 2006; 44: 1982-93.

[118] Le Flèche P, Jacques I, Grayon M, *et al*. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiology 2006; 6: 9.

[119] Al Dahouk S, Le Flèche P, Nöckler K, *et al*. Evaluation of *Brucella* MVLA typing for human brucellosis. J Microbiol Methods 2007; 69: 137-45.

[120] Kattar MM, Jaafar RF, Araj GF, *et al*. Evaluation of multilocus variable-number tandem-repeat analysis scheme for typing human *Brucella* isolates in a region of brucellosis endemicity. J Clin Microbiol 2008; 46: 3935-40.

[121] Comas I, Homolka S, Niemann S, *et al*. Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies. PLoS One 2009; 4: e7815.

[122] Urwin R, Maiden MC. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol 2003; 11: 479-87.

[123] Scott JC, Koylass MS, Stubberfield R, *et al*. Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. Appl Environ Microbiol 2007; 73: 7331-7.

[124] Banai M, Mayer I, Cohen A. Isolation, identification, and characterization in Israel of *Brucella melitensis* biovar 1 atypical strains susceptible to dyes and penicillin, indicating the evolution of a new variant. J Clin Microbiol 1990; 28: 1057-9.

[125] Banai, M. Control of small ruminant brucellosis by use of *Brucella melitensis* Rev.1 vaccine: laboratory aspects and field applications. Vet Micobiol 2002; 90: 497-519.

[126] Bardenstein S, Mandelboim M, Ficht TA, *et al*. Identification of the *Brucella melitensis* vaccine strain Rev.1 in animals and humans in Israel by PCR analysis of the *Pst*I site polymorphism of its *omp2* gene. J Clin Microbiol 2002; 40: 1475-80.

[127] García-Yoldi D, Le Fleche P, Marín CM, *et al*. Assessment of genetic stability of *Brucella melitensis* Rev 1 vaccine strain by multiple-locus variable-number tandem repeat analysis. Vaccine 2007; 25: 2858-62.

[128] De Ley J, Mannheim W, Segers P, *et al*. Ribosomal ribonucleic acid cistron similarities and taxonomic neighborhood of *Brucella* and CDC Group Vd. Int J Syst Bacteriol 1987; 37: 35-42.

[129] Stackebrandt E, Murray RG, Trüper HG. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives". Int J Syst Bacteriol 1988; 38: 321-5.

[130] Dorsch M, Moreno E, Stackebrandt E. Nucleotide sequence of the 16S rRNA from *Brucella abortus*. Nucleic Acids Res 1989; 17: 1765.

[131] Moreno E, Stackebrandt E, Dorsch M, *et al*. *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class *Proteobacteria*. J Bacteriol 1990; 172: 3569-76.

[132] Holmes B, Poppoff M, Kiredjina M, *et al*. *Ochrobactrum anthropi* gen. nov. sp. nov. from human clinical specimens and previously known as group Vd. Int J Syst Bacteriol 1988; 38: 406-16.

[133] Velasco J, Romero C, López-Goñi I, *et al*. Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov. a new species with a closer relationship to *Brucella* spp. Int J Syst Bacteriol 1998; 48: 759-68.

[134] Siera R, Comerci DJ, Sánchez DO, *et al*. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J Bacteriol 2000; 182: 4849-55.

[135] Scholz HC, AL Dahouk S, Tomaso H, *et al*. Genetic diversity and phylogenetic relationships of bacteria belonging to the

Ochrobactrum-Brucella group by *recA* and 16S rRNA gene-based comparative sequence analysis. Syst Appl Microbiol 2008; 31:1-16.

[136] Gevers D, Cohan FM, Lawrence JG, *et al*. Opinion: re-evaluating prokaryotic species. Nat Rev 2005; 3: 733-9.

[137] Pappas G, Papadimitriou P, Akriditis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infect Dis 2006; 6: 91-9.

[138] Barquero-Calvo E, Conde-Alvarez R, Chacón-Diaz C*, et al*. The differential interaction of *Brucella* and *Ochrobactrum* with innate immunity reveals traits related to the evolution of stealthy pathogens. PLoS ONE 2009; 4(6): e5893.

[139] Roop II RM, Gains JM, Anderson ES, *et al*. Survival of the fittest: how *Brucella* strains adapt to their intracellular niche in the host. Med Microbiol Immunol 2009; 198: 221-38.

[140] Alexander B, Schnurrenberger PR, Brown RR. Numbers of *Brucella abortus* in the placenta, umbilicus and fetal fluid of two naturally infected cows. Vet Rec 1981; 108: 500.

[141] Meador VP, Deyoe BL. Intracellular localization of *Brucella abortus* in bovine placenta. Vet Pathol 1989; 26: 513-5.

[142] Swartz TE, Tseng T-S, Frederickson MA, *et al*. Blue-light-activated histidine kinases: two-component sensors in bacteria. Science 2007; 317: 1090-3.

[143] Davidson RM. Control and eradication of animal diseases in New Zealand. N Z Vet J 2002; 50(Suppl): 6-12.

[144] Farrell ID, Robertson L. The sensitivity of the biotypes of *Brucella abortus* to three antibiotics used in selective media, and the description of a new biotype. J Hyg Camb 1967; 65: 165-72.

[145] Robertson L, Farrell ID. *Brucella abortus* (Biotype 5) infection in a milk tanker driver. A study of 23 farms. Vet Rec 1967; 80: 36-9.

[146] Valdezate S, Navarro A, Rubio V, *et al*. Emergence of a clonal lineage of *Brucella abortus* biovar 3 in clinical cases in Spain. J Clin Microbiol 2009; 47: 2687-8.

[147] Gee JE, De BK, Levett PN, *et al*. Use of 16S rRNA gene sequencing for rapid confirmatory identification of *Brucella* isolates. J Clin Microbiol 2004; 42: 3649-54.

[148] Renoux G. La notion d'espece dans le genre *Brucella*. Ann Inst Pasteur (Paris) 1958; 94: 179-206.

[149] Meyer M, Cameron HS. Metabolic characterization of the genus *Brucella*. I. Statistical evaluation of oxidative rates by which type 1 of each species can be identified. J Bacteriol 1961a; 82: 387-95.

[150] Meyer ME, Cameron HS. Metabolic characterization of the genus *Brucella*. II. Oxidative metabolic patterns of the described biotypes. J Bacteriol 1961b; 82: 396-400.

[151] Meyer ME. Metabolic characterization of the genus Brucella: IV. Correlation of oxidative metabolic patterns and phage susceptibility. J Bacteriol 1961; 82: 950-3.

[152] Morgan WJB. The examination of *Brucella* cultures for lysis by phage. J Gen Microbiol 1963; 30: 437-43.

[153] López-Goñi I, García-Yoldi D, Marín CM, *et al*. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. J Clin Microbiol 2008; 46: 3484-7.

[154] Mayer-Scholl A, Dreager A, Göllner C, *et al*. Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species. J Microbiol Methods 2010; 80:112-4.

[155] Gandara B, Merino AL, Rogel MA, *et al*. Limited genetic diversity of *Brucella* spp. J Clin Microbiol 2001; 39: 235-40.

[156] Michaux-Charachon S, Bourg G, Jumas-Bilak E, *et al*. Genome structure and phylogeny in the genus *Brucella*. J Bacteriol 1997; 179: 3244-9.

[157] Roche P, Maillet F, Plazanet C, *et al*. The common *nodABC* genes of *Rhizobium meliloti* are host-range determinants. Proc Natl Acad Sci USA 1996; 93: 15305-10.

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