Brucella ceti sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts

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Small Gram-negative coccobacilli resembling *Brucella* strains have been reported from marine mammals since the mid-1990s. Their placement in the genus *Brucella* has been supported by the following characteristics: they are aerobic, non-motile and catalase-positive, do not produce acid from carbohydrates and have a DNA–DNA relatedness value of >77% with the six established members of the genus. Twenty-eight European isolates of the genus *Brucella* from marine mammals were distinguished from the six recognized species by their pattern of utilization of eleven substrates in oxidative metabolism tests and phage lysis. The 28 strains could be further separated into two groups with cetaceans and seals as their respective preferred hosts on the basis of molecular methods and on differences in the metabolism of L-arabinose, D-galactose and D-xylose. The names *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. are proposed for the isolates from cetaceans and seals, respectively. The type strain of *Brucella ceti* sp. nov. is NCTC 12891^T (=BCCN 94-74^T) and the type strain of *Brucella pinnipedialis* sp. nov. is NCTC 12890^T (=BCCN 94-73^T).

The genus *Brucella* contains aerobic, intracellular bacteria that can infect many species of animals and man. Corbel & Brinley-Morgan (1984) listed six species in the genus; however, subsequent DNA–DNA hybridization studies demonstrated that the genus *Brucella* is a highly homogeneous group (>90% DNA–DNA relatedness) (Verger *et al.*, 1985, 1987). A proposal that this genus should comprise only one genomic species (Verger *et al.*, 1985, 1987) was accepted by the *Brucella* Taxonomy Subcommittee with some guardedness (Manchester, UK, 1986; Corbel, 1988). This proposal did not gain acceptance amongst brucellosis researchers, however, and classification of *Brucella* strains into six species, with particular emphasis on the preferential host (Corbel & Brinley-Morgan, 1984), was still favoured. Thus, the outcome of the Manchester meeting-sanctioned taxonomic opinion that the genus *Brucella* was monospecific was the use of two systems of nomenclature, one for taxonomic purposes and one, using vernacular names, for non-taxonomic purposes. The following, poorly attended, *Brucella* Taxonomy

Subcommittee meeting in Osaka, Japan, in 1990 was not reported. A Subcommittee Correspondence Report 1991–1993 (Gargani & López-Merino, 2006) was followed by a meeting in Prague, Czech Republic, in 1994 (Corbel & Moriyón, 2006). After a period of inactivity, a convened *Brucella* Subcommittee meeting was held in Nîmes, France, in 2000 (MacMillan, 2006), with the intention of relaunching the *Brucella* Taxonomy Subcommittee; a feat that was not finally achieved for another three years.

The six previously recognized species of the genus *Brucella* can be distinguished on the basis of preferred host, phenotype and genotype. In addition, the seriousness of human *Brucella* infections, the political significance of the disease for agriculture and the potential role for *Brucella* as an agent of bioterrorism are strong supportive reasons for the widely held view that the original six species format should prevail. When the International Committee on the Systematics of Prokaryotes Subcommittee on the Taxonomy of *Brucella* met in 2003 (in Pamplona, Spain),

there was a unanimous verdict to return to the six species taxonomy for the genus *Brucella*. This led to a recommendation from the *Brucella* Subcommittee that there should be a return to the pre-1986 taxonomy of the genus *Brucella* (Osterman & Moriyón, 2006). This Subcommittee recommendation implies the reapproval of the six classical *Brucella* nomenspecies with their recognized biovars; the classical names related to these six species having been validly published in the Approved List of Bacterial Names (Skerman *et al.*, 1980, 1989). In this context, reference should also be made to the *Bacteriological Code* (1990 Revision) (Lapage *et al.*, 1992) and an updated Taxonomic Note (Tindall *et al.*, 2006).

Brucella infections of terrestrial mammals have long been recognized and have been researched extensively; however, it was only during the last few years of the twentieth century that the first reports of *Brucella* species from animals living in the marine environment were made. The first marine mammal isolations of *Brucella* strains came from common seals (*Phoca vitulina*), a porpoise (*Phocoena phocoena*) and a common dolphin (*Delphinus delphis*) in Scotland (Ross *et al.*, 1994) and a captive bottlenose dolphin (*Tursiops truncatus*) in the USA (Ewalt *et al.*, 1994). Since these first reports, there have been many more isolations and the range of hosts has expanded significantly (Foster *et al.*, 2002). Additional species from which bacteriological cultures have proved positive include the Atlantic white-sided dolphin (*Lagenorhynchus acutus*), striped dolphin (*Stenella coeruleoalba*) (Foster *et al.*, 1996), minke whale (*Balaenoptera acutorostrata*) (Clavareau *et al.*, 1998; Foster *et al.*, 2002), hooded seal (*Cystophora cristata*), grey seal (*Halichoerus grypus*) (Foster *et al.*, 1996), Pacific harbour seal (*Phoca vitulina richardsi*) (Garner *et al.*, 1997), ringed seal (*Phoca hispida*), harp seal (*Phoca groenlandica*) (Forbes *et al.*, 2000; Maratea *et al.*, 2003) and a European otter (*Lutra lutra*) (Foster *et al.*, 1996). In addition, there have also been reports of human infections with strains from marine mammals (Brew *et al.*, 1999; Sohn *et al.*, 2003; McDonald *et al.*, 2006).

Since their discovery, *Brucella* strains from marine mammals have been subjected to a range of characterization tests which have compared them with each other and also with the terrestrial *Brucella* species. Representative *Brucella* strains of marine mammals, which include the designated type strains of *B. ceti* sp. nov. and *B. pinnipedialis* sp. nov., have been shown by DNA–DNA hybridization to be related to the six classical *Brucella* nomenspecies at a level of >77 % DNA–DNA relatedness. The type strains of all these species form a homogeneous DNA–DNA relatedness group (Verger *et al.*, 2000; Wayne *et al.*, 1987). These results may be interpreted as evidence for the two proposed novel marine mammal *Brucella* species being affiliated to the genus *Brucella*. The appearance of these novel *Brucella* strains fits with previous descriptions of members of the genus *Brucella* made by Corbel & Brinley-Morgan (1975, 1984) and by Corbel & Banai (2005). The *Brucella* strains from marine mammals

are small Gram-negative cocci, coccobacilli or short rods, 0.5–0.7 µm in diameter and 0.6–1.5 µm in length, non-motile, aerobic, catalase-positive, oxidase-positive, able to reduce nitrates, urease-positive, indole-negative, negative for gelatin liquefaction, do not produce acid from carbohydrates in conventional media and possess *Brucella* antigens, as demonstrated by reactions with *Brucella*-specific antisera.

Jahans *et al.* (1997) managed to distinguish the marine mammal strains from the terrestrial species of the genus *Brucella* on the basis of a substrate-specific oxidative metabolism test and suggested that they may belong to a separate species for which the name '*Brucella maris*' was suggested but not formally proposed. Jahans further demonstrated that galactose metabolism served as a useful test to separate cetacean-derived species from those derived from seals. Ribotyping further suggested that marine strains of the genus *Brucella* may represent a separate group on the basis of their rRNA gene *Hind*III restriction pattern (Verger *et al.*, 2000). Other distinctive characteristics at the molecular and genomic level have been provided by IS711 DNA fingerprinting which showed that a higher number of IS711 copies occur in the genomes of *Brucella* isolates from marine mammals than in strains isolated from land mammals (Clavareau *et al.*, 1998; Bricker *et al.*, 2000). A specific marker of marine mammal isolates has been identified, consisting of an IS711 element downstream of the *bp26* gene (Cloeckaert *et al.*, 2000). Most recently, a study of DNA polymorphism at the *omp2* locus showed that the isolates from marine mammals can be classified into two major groups, one comprising isolates from seals that carry one copy of both the *omp2a* and *omp2b* genes and another group comprising the cetacean isolates carrying two copies of the *omp2b* gene (Cloeckaert *et al.*, 2001). Both copies of the *omp2* gene were shown to contain motifs typical of marine mammal isolates. 16S rRNA gene sequence data, as well as *recA* gene sequences, identify the strains from marine mammals as belonging to the genus *Brucella* (Scholz *et al.*, 2006).

With respect to the classification of species of the genus *Brucella* according to their preferential host, two new species names have been suggested, but not validly published: '*B. pinnipediae*' (for isolates from seals) and '*B. cetaceae*' (for isolates from cetaceans) (Cloeckaert *et al.*, 2001). Furthermore, development of an infrequent restriction site-PCR (IRS-PCR) method, taking into account the higher number of IS711 elements in the genome of isolates from marine mammals compared with *Brucella* species from terrestrial mammals, allowed the classification of the marine mammal isolates into two distinct clusters of strains. These two clusters were also distinct from the clusters representing the recognized *Brucella* species from terrestrial mammals (Cloeckaert *et al.*, 2003). These two clusters of strains correlated well with the hosts from which they had been isolated, i.e. a cluster of isolates from cetaceans and a cluster of isolates from pinnipeds. Therefore, IRS-PCR confirmed the previous classification

of isolates from marine mammals, based on DNA polymorphism at the *omp2* locus and their preferential hosts, into two novel species of the genus *Brucella*: '*Brucella cetaceae*' for cetacean isolates and '*Brucella pinnipediae*' for pinniped isolates (Cloeckaert *et al.*, 2001).

Studies by Vizcaino *et al.* identified an additional specific marker for '*B. cetaceae*' found in most cetacean isolates consisting of a 1.7 kb inversion including a gene coding for one of the Omp25/Omp31 family of proteins (Vizcaino *et al.*, 2004). More recently, Jacques *et al.* (2007) used oxidative metabolism to confirm the findings of Jahans and provide further support for these two novel species of the genus *Brucella*. The approval of the taxonomic rank of species for each of these two groups of *Brucella* strains from marine mammals is in line with the recommendations made by the ad hoc Committees on approaches to taxonomy (Wayne *et al.*, 1987; Murray *et al.*, 1990; Stackebrandt *et al.*, 2002) and with our current understanding of the evolution of the genus and of the relevant criteria for inclusion in this group (Moreno *et al.*, 2002).

In view of the data presented above and in the descriptions of the novel isolates from marine mammals (given below), the characteristics of the two novel species meet the criteria for inclusion in the genus *Brucella* and conform to the minimal standards for the description of novel *Brucella* species as laid down by the *Brucella* Taxonomy Subcommittee (Corbel & Brinley-Morgan, 1975). The recommended investigation procedures have been used and have provided results that concur with the requirements for members of the genus *Brucella* as regards particular morphological features observable by electron microscopy, cultural characteristics and colony morphologies. Strains of the two novel species are obligate aerobes with an oxidative metabolism and have oxidative metabolic profiles that enable inter-species differentiation. The *Brucella* antigenic profiles and results from *Brucella* phage sensitivity tests, as well as results from classical *Brucella* biochemical tests (Table 1), also confirm their assignment to the genus *Brucella*. The facultatively intracellular behaviour of the proposed novel species is also in congruence with that of recognized members of the genus

Table 1. Differential characteristics of *Brucella ceti* sp. nov., *Brucella pinnipedialis* sp. nov. and other members of the genus *Brucella*

Data from Alton *et al.* (1975, 1988); Corbel & Banai (2005); Jacques *et al.* (2007); Meyer & Cameron (1961); Verger & Grayon (1977). Results are expressed as QO₂(N) values corresponding to microlitres of oxygen uptake per milligram of bacterial nitrogen per hour. +, QO₂(N) over 100 for all strains; –, QO₂(N) below 100 for all strains; D, doubtful, some strains showing QO₂(N) over 100, some strains showing QO₂(N) below 100; NL, no lysis; L, lysis; RTD, routine test dilution.

Characteristic	<i>B. ceti</i> (17 strains)	<i>B. pinnipedialis</i> (11 strains)	<i>B. melitensis</i>	<i>B. abortus</i>	<i>B. suis</i> biovars					<i>B. neotomae</i>	<i>B. ovis</i>	<i>B. canis</i>
					1	2	3	4	5			
Urease	+	+	+	+	+	+	+	+	+	+	–	+
Lysis by phage at RTD:												
Tb	NL	NL†	NL	L	NL	NL	NL	NL	NL	D	NL	NL
Wb	L	L	NL	L	L	L	L	L	L	L	NL	NL
Iz	L	L	L	L	L	L	L	L	L	L	NL	NL
R/C	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	L	L
Oxidation of:												
L-Alanine	–	–	+	+	D	–	D	–	–	–	D	–
L-Arabinose	+	–	–	+	+	+	–	–	–	+	–	–
L-Arginine	–	–	–	–	+	+	+	+	+	–	–	+
L-Asparagine	–	–	+	+	–	D	–	–	D	+	+	–
meso-Erythritol	D	+	+	+	+	+	+	+	+	+	–	D
D-Galactose	+	–	–	+	+	+	–	–	–	+	–	–
L-Glutamic acid	+	+	+	+	D	+	+	+	+	+	+	+
L-Lysine	–	–	–	–	+	D	D	D	D	–	–	D
DL-Ornithine	–	–	–	–	+	+	+	+	+	–	–	+
D-Ribose	+	+	–	+	+	+	+	+	+	D	–	+
D-Xylose	+	–	–	–	+	D	D	D	D	–	–	–
Preferred host	Cetaceans	Seals	Sheep, goats	Cattle	Swine	Swine, hares	Swine	Reindeer	Rodents	Desert wood rat	Sheep	Dogs

*Except for reference strain 544 and occasional field strains which are negative.

†Lysis occurs with a few strains.

Brucella, as are the special characteristics of specific host preferences and virulence-pathogenicity.

The proposals of these two groups of marine mammal *Brucella* strains as being of the taxonomic rank of species of the genus *Brucella* are based on investigations according to the Subcommittee recommendation (Corbel & Brinley-Morgan, 1975) 'that strains should be classified into species on the basis of (i) preferred natural host, (ii) sensitivity to *Brucella* phages, and (iii) oxidative metabolic profiles'. The results of molecular investigations are also included. The proposal is in line with decisions (focusing on new methods of pheno-genotypic analysis) taken by the Subcommittee at the 2003 Pamplona meeting (Osterman & Moriyón, 2006), where the earlier acknowledged importance of host-preference and virulence-pathogenicity for the delineation of species of the genus *Brucella* was re-emphasized. The results are based on phenotypic and molecular tests on over 30 isolates collected from marine mammals in several European countries, thereby providing clear evidence of host preference for cetacean- and pinniped-derived strains, respectively.

Following the return to the six-species taxonomy based on the unanimous verdict of the International Subcommittee on the Taxonomy of *Brucella* (Osterman & Moriyón, 2006) and the reasons presented above, we formally propose two novel species of the genus *Brucella* for strains from marine mammals (with corrected etymology). The name *Brucella ceti* sp. nov. is proposed for *Brucella* strains with cetaceans as their preferred host and the name *Brucella pinnipedialis* sp. nov. is proposed for *Brucella* strains with pinnipeds as their preferred host.

Description of *Brucella ceti* sp. nov.

Brucella ceti [ce.ti. L. n. *cetus* large sea animal (whale, porpoise, dolphin); L. gen. n. *ceti* of a large sea animal].

Cocci, coccobacilli or short rods, 0.5–0.7 µm in diameter and 0.6–1.5 µm in length. Arranged singly and, less frequently, in pairs, short chains or small groups. Gram-negative. Non-motile; does not produce flagella. Aerobic. Colonies on Columbia sheep blood agar and Farrell's medium are normally visible after 3–4 days and are raised, convex, circular and entire with a diameter of 0.5–1.0 mm. Non-haemolytic. Nitrate reductase is produced. Most strains do not require supplementary CO₂ for growth. Growth is improved by the addition of serum or blood. Colonies on serum-glucose agar are transparent, raised, convex, with an entire edge and a smooth shiny surface and appear as a pale honey colour by transmitted light. Optimum temperature is 37 °C. Growth occurs between 20 and 40 °C. Optimum pH is between 6.6 and 7.4. Catalase-, oxidase- and urease-positive. H₂S negative. L-glutamic acid, L-arabinose, D-galactose, D-ribose and D-xylose are oxidized, but L-alanine, L-asparagine, L-arginine, DL-ornithine and L-lysine are not oxidized. *meso*-Erythritol may or may not be oxidized. Cultures are lysed by Iz and Wb phages, but no lysis occurs with Tb and R/C phages. A

antigen dominant. The preferred hosts are porpoises, dolphins and whales in which a range of pathologies occur.

The type strain, NCTC 12891^T (=BCCN 94-74^T), was isolated from a skin lesion on a harbour porpoise.

Description of *Brucella pinnipedialis* sp. nov.

Brucella pinnipedialis (pin.ni.ped.i.a'lis. N.L. fem. adj. *pinnipedialis* pertaining to pinnipeds).

Cocci, coccobacilli or short rods, 0.5–0.7 µm in diameter and 0.6–1.5 µm in length. Arranged singly and, less frequently, in pairs, short chains or small groups. Gram-negative. Non-motile; does not produce flagella. Aerobic. Colonies on Columbia sheep blood agar are normally visible after 3–4 days and are raised, convex, circular, entire and non-haemolytic with a diameter of 0.5–1.0 mm. Growth on Farrell's medium is typically slower (7–10 days) or absent. Nitrate reductase is produced. Most strains require supplementary CO₂ for growth. Growth is improved by the addition of serum or blood. Colonies on serum-glucose agar are transparent, raised, convex, with an entire edge and a smooth shiny surface and appear as a pale honey colour by transmitted light. Optimum temperature is 37 °C. Growth occurs between 20 and 40 °C. Optimum pH is between 6.6 and 7.4. Catalase-, oxidase- and urease-positive. H₂S negative. In oxidative metabolism tests, L-glutamic acid, D-ribose and *meso*-erythritol are oxidized, but L-alanine, L-arginine, L-asparagine, DL-ornithine, L-lysine, L-arabinose, D-galactose and D-xylose are not oxidized. Cultures are lysed by Iz and Wb phages, but no lysis occurs with R/C. A small number of strains exhibit lysis with the Tb phage. A antigen dominant. The preferred hosts are seals, in which the strains cause a range of pathologies.

The type strain, NCTC 12890^T (=BCCN 94-73^T), was isolated from the spleen of a common seal (*Phoca vitulina*).

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