In: Brucellosis in Goats and Sheep ISBN: 978-1-53614-962-3
Editors: J. C. Caetano Simões et al.

Chapter 2

ETIOLOGY: THE GENUS *BRUCELLA*

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ABSTRACT

Brucellosis is caused by bacteria of the *Brucella* species. Twelve species have been identified in the genus. In this chapter, we address the taxonomy and phylogenetic relationships of the presently recognised *Brucella* species and biovars, as well as *Brucella* morphology and metabolism and epidemiological features of the *Brucella* species. *Brucella melitensis* and *B. ovis* are the aetiological agents of small ruminant brucellosis. *B. melitensis* may infect both, goats and sheep, producing a disease mainly characterised by abortion, retained placenta and birth of weakened offspring, while *B. ovis* is pathogenic to sheep, inducing epididymitis and decreased fertility in males as main symptoms. *B. melitensis* is the main agent of zoonotic brucellosis, while *B. ovis* has lower zoonotic significance. Other *Brucella* species have negative impact on public health. Given the recent knowledge about the genus *Brucella*, it is worth describing the most relevant characteristics of the different species. Therefore, a short description of each species is presented.

Keywords: *Brucella* spp., taxonomy, phylogenetic, morphology, metabolism

INTRODUCTION

Brucellosis is a zoonotic disease caused by faculative, intracellular bacteria of the genus *Brucella* that can survive and multiply within phagocytic cells of the host and can be sequestered within monocytes and macrophages of the mononuclear phagocytic system (MPS), such as lymph nodes, liver, spleen and bone marrow (Meyer and Shaw, 1920). These bacteria belong to the family Brucellaceae, order Rhizobiales of the α-Proteobacteria class, and are closely related to phylogenetic genera, such as *Agrobacterium, Phyllobacterium, Rhizobium* and *Ochrobactrumn.* Members of the class proteobacteria include families of organisms that establish intimate relationships with plant or animal cells, as well as possess capacity for intracellular growth such as pathogens or symbionts (Corbel, 1997; Garrity et al., 2005; Corbel, 2006; Bohlin et al., 2010; Ficht, 2010; Barbier et al., 2017).

It has been commonly believed that *Brucella* spp. are typical mammal cell pathogens. However, a *Brucella* strain was recently isolated from a Pac-Man frog (*Ceratophyrus ornate*) at a veterinary hospital in Texas (Soler-Lloréns et al., 2016), and amphibian strains were isolated and described by Al Dahouk et al. (2017) as a remarkable group of heterogeneous brucellae, characterized by some unique features as the first *Brucella*-like organism in African bullfrogs and other frogs (unique cold-blooded hosts). These strains have versatile adaptability both to cold-blooded animals and endotherms. Also, a single amphibian strain was recently shown to be motile, a characteristic that is a common feature of diverse organisms that are possible ancestral to the classical zoonotic *Brucella* and, finally, the bullfrog strains show long-term persistence in mice without causing disease.

Considering the many novel characteristics in strains belonging to an emerging group within the *Brucella* genus, accurate identification tools are necessary for such atypical *Brucella* isolates and reliable methodology for evaluation of their zoonotic potential is urgently required (Soler-Lloréns et al., 2016).

Therefore, other than the biochemical and phenotypic description of *B. melitensis* and *B. ovis*, and their distinguishing individualities from other *Brucella*, including the preferred hosts, which classify the distinct species (Garin-Bastuji et al., 2014), the present chapter also addresses the genomic approach. With the aim of better elucidating the apparent duality between the heterogeneous brucellae traits and their genetic homogeneity, with more than 90% of DNA homology (Ratushna et al., 2006; Whatmore, 2009), some features of each small ruminant *Brucella* species are also described.

TAXONOMY

The genus *Brucella* is currently classified into 12 known species (Table 2.1), according to basic differences in pathogenicity and host preference: *Brucella melitensis* (goats and sheep), *B. abortus* (cattle and

bison), *B. suis* (swine, hares, rodents, and reindeer), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (desert wood rats), *B. ceti* (cetaceans), and *B. pinnipedialis* (pinnipeds) (Corbel, 1997; Garrity et al., 2005; Pappas, 2010; Kang et al., 2015; Sankarasubramanian et al., 2017) plus the most recently identified species are *B. microti* (common vole), *B. inopinata* (a human breast implant infection), *B. papionis* (baboons) and *B. vulpis* (red foxes) (Scholz et al., 2008b, 2010; Whatmore et al., 2014; Scholz et al., 2016).

Table 2.1. Epidemiological features of *Brucella* species

Species	Natural host	Prevalent region	Reported human cases
B. melitensis	Sheep, goats	Mediterranean littoral, Arabian Peninsula, Latin America	Several cases
B. abortus	Cattle	Asian countries, Europe	Several cases
B. suis	Pigs	Latin America, Southern China, Southeast Asia, Europe	Several cases (biovar 1)
B. canis	Dogs	Argentina, Brazil, China, Czech Republic, Germany, Japan, Madagascar, Mexico, Papua New Guinea, Peru, Philippines	Rare cases
B. ovis	Sheep	Argentina, Chile, France, Germany, South Africa, USA, Spain, countries of the former Soviet Union	No human cases
B. neotomae	Rodents (desert wood rats)	United States	Two human cases
B. microti	Wild voles	North Europe	No human cases
B. ceti	Marine mammals	Mainly Northern Hemisphere	One laboratory infection
B. pinnipedialis	Marine mammals	Mainly Northern Hemisphere	No human cases
B. inopinata	Unknown	-	Prosthetic breast implant infection (one human case)
B. papionis	Baboons	-	No human cases
B. vulpis	Red foxes	Austria	No human cases

Adapted from Foster et al. (2002); Godfroid et al. (2011), Percin (2013), Smirnova et al. (2013), Olsen and Palmer (2014), Whatmore et al. (2014), ECDC (2017) and Suárez-Esquivel et al. (2017)

The three major species in terms of disease and economic impact for man, *B. melitensis*, *B. abortus* and *B. suis* are further divided into biovars based on a range of phenotypic and serological characteristics: *B. melitensis* with 3 biovars, *B. abortus* with 8 biovars, and *B. suis* with 5 biovars.

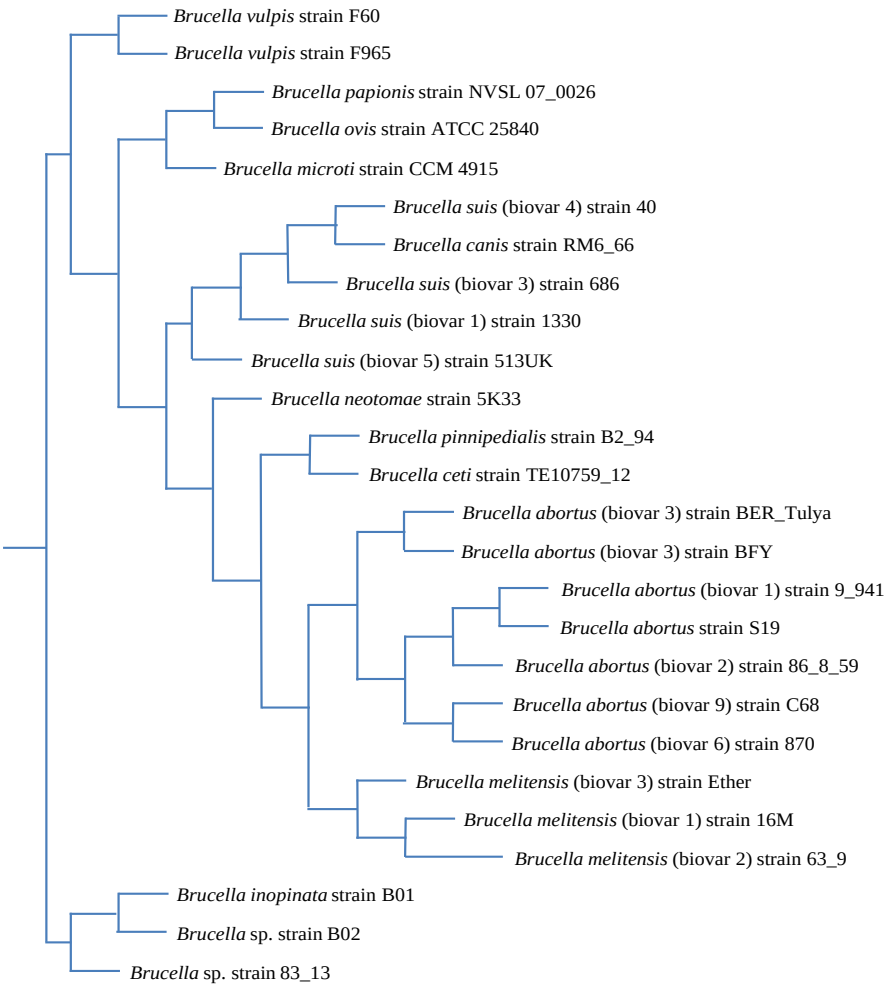

Figure 2.1. Phylogenetic relationships of the recognised *Brucella* species and biovars. Branch lengths do not reflect phylogenetic distances. Adapted from Scholz et al. (2016).

Brucella species are genetically similar to each other. However, the division into several species has been influenced by the restriction of each, so-called species virulence to one or a small number of mammalian hosts. Therefore, the evaluative lineages are restricted to a narrow niche. The main reason is that brucellae cannot actively multiply in the environment, but only in infected animals inside their tissues (Michaux-Charachon et al., 1997). For instances, *B. papionis* was first isolated from baboons (*Papio* spp.) and phylogenetic analysis confirmed that they represent a well-separated lineage related most closely to *B. ovis* (Figure 2.1). This was the first report of a *Brucella* isolate in association with two cases of stillbirth in nonhuman primates (Whatmore et al., 2014).

CHARACTERIZATION OF *BRUCELLA*

Brucellae are Gram-negative cocci, coccobacilli or short rods of 0.5-0.7 by 0.6-1.5 µm in size, usually have individual arrangement and less frequently are found in pairs, short chains, or small groups. They are non-motile and do not produce flagella. Multiplication is slow at the optimum temperature of 37°C, growth occurring between 20 and 40ºC and optimal pH of 6.6–7.4. *Brucella* spp. are fastidious bacteria that need rich culture medium to support adequate growth. The growth occurs on *Brucella* agar, MacConkey Agar, Trypticase Soy agar, Sheep Blood agar and Standard Nutrient agar at 25–42ºC. Colonies on translucid media are transparent, convex and have an entire edge. They are usually small (0.5–1.0 mm after 2–3 days of incubation of a fresh inoculum), but there are variations that depend on the medium and strain. A culture can be identified as belonging to the genus *Brucella* on the basis of colonial morphology, staining and slide agglutination with anti-*Brucella* serum, smooth or rough. (Alton et al., 1988; Garrity et al., 2005; Vicente et al. 2014).

Brucella strains are catalase positive and superoxide dismutase positive, most of them are also oxidase positive (Michaux et al., 1993; Alton and Forsyth, 1996; Zinsstag et al., 2011; Percin, 2013). The mode of metabolism is aerobic with a cytochrome-based electron transport system

using oxygen or nitrate as the terminal electron acceptor. Many strains require supplementary CO_2 for growth (Garrity et al., 2005; Percin, 2013). The brucellae metabolism is mainly oxidative and depends exclusively on low molecular weight carbon sources, such as carbohydrates and amino acids; therefore, the oxidative activity of *Brucella* strains towards different carbohydrates and amino acids (Table 2.2) is very important for identification (Percin, 2013; Barbier et al., 2017).

Table 2.2. Oxidative metabolism and urease activity of classical species of *Brucella*

		B. melitensis	***B. bortus***	***B. canis***	***B. neotomae***	***B. suis***	***B. ovis***
Amino acids	L-Alanine	+	+	V	V	V	V
	L-Asparagine	+	+	-	+	V	+
	L-Glutamate	+	+	+	+	V	+
	L-Arginine	-	-	+	-	+	-
	DL-Citrullin	-	-	+	-	+	-
	L-Lysine	-	-	+	-	V	-
	DL-Ornithine	-	-	+	-	+	-
Carbohydrates	L-Arabinose	-	+	V	+	V	-
	D-Galactose	-	+	V	+	V	-
	D-Ribose	-	+	+	V	+	-
	D-Xylose	-	V	-	-	-	-
	D-Glucose	+	+	+	+	+	-
	Isoerythritol	+	+	V	+	+	-
Urease activity		1 hour	1 hour	5 min	1 hour	5 min	7 days

+ positive; - negative; V variable. Adapted from Percin (2013) and Barbier et al. (2017).

Phenotyping identification, through biochemical profiling, could not be conclusive for all *Brucella* species due to the high similarity between them. Notwithstanding, as there is little antigenic variation among *Brucella* spp., the differentiation of species and strains is based on approximately 25 biological and physiological characteristics (phenotype) (Bricker and Halling, 1994). Classical microbiological identification depends on CO_2 requirement, H_2S production, urease activity (as in Table 2.2), dye (thionin and fuchsin) sensitivity, lysis by phages F1, F25, Tb, BK2, Iz, Wb, Fi, R/C, agglutination with monospecific sera A and M, agglutination with rough *Brucella* antiserum and dissociation tests with crystal violet and trypaflavin

(Alton et al., 1988; Garrity et al., 2005; OIE, 2012; Hamidi et al., 2016; Kang et al., 2017).

In the last decades several PCR-based methods have been developed and implemented in diagnostic laboratories to confirm pure cultures of brucellae and differentiate among *Brucella* species and biovars, as well as vaccine strains. Concerning *B. abortus* (biovars 1, 2 and 4), *B. melitensis* (biovars 1, 2 and 3), *B. ovis* and *B. suis* (biovar 1), the AMOS PCR assay was the first species-specific multiplex PCR assay which can identify and differentiate them, using five-primer cocktails targeting, the IS711 sequence (AMOS is an acronym for the *Brucella* species identified); further improvement of AMOS PCR assay allows identification of *B. abortus* vaccine strain 19 (S19) and vaccine strain RB51 (Bricker and Halling, 1994, 1995).

More recently the Suis-ladder multiplex PCR has been developed for fast and accurate identification of the five *B. suis* biovars. Bruce-ladder multiplex PCR has been used for identification of all known *Brucella* species and vaccine strains. Two molecular typing methods, MLVA (multi-locus variable-number tandem-repeat analysis) and MLST (multi locus sequence typing), are useful tools for identifying and genotyping *Brucella* spp., being first-line tools for molecular epidemiological studies within outbreak investigations; MLST is also appropriate for phylogenetic analysis applied for identification (Le Flèche et al., 2006; Al Dahouk et al., 2007; Whatmore et al., 2007; López-Goñi et al., 2011; Gyuranecz et al., 2016; Sankarasubramanian et al., 2008; Scholz et al., 2016) (see also chapter 8).

Several characteristics found in *Brucella* are common to other phylogenetically-related species of the α-Proteobacteria class and are considered ancestral (Moreno and Moriyó, 2006). Blasting the genomes of *B. melitensis, B. abortus, B. suis, B. neotomae* and *B. canis* against that of *B. ovis* reveals an overall DNA homology of 95%, indicating that they all were diverged from a common ancestor very close to *B. ovis* (Figure 2.2) and suggests that brucellosis in animals such as pigs, goats, and cattle emerged from contact with infected sheep. Furthermore, this contact was

fairly recent regarding the live evolution on Earth, occurring roughly in the past 86,000 to 296,000 years (Foster et al., 2009; Ficht, 2010).

Considering that all genomes of different *Brucella* species show a gene content similarity of more than 70%, the question whether the genomes should be divided into different strains within a species, rather than a species within the *Brucella* genus, was raised (Bohlin et al., 2010), nevertheless due to the host specificity of *Brucella* species, and the presence of specific genes related to infection and intracellular life, they were divided into different species.

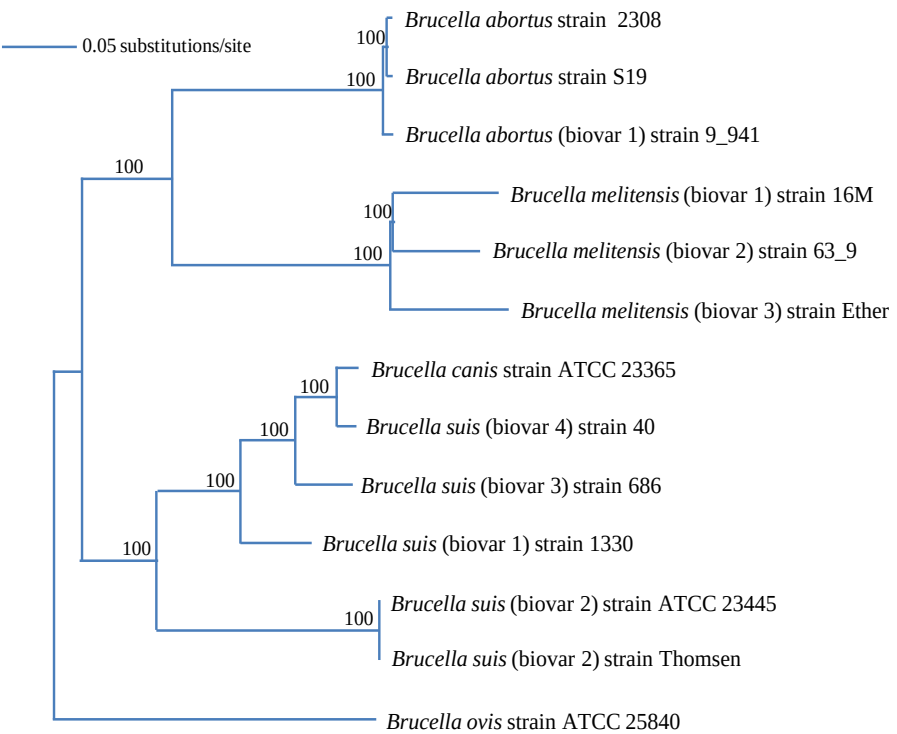

Figure 2.2. Rooted phylogeny of the genus *Brucella*, including 13 genomes of five species related to domestic animals. Tree was constructed by using neighbor joining and percent bootstrap support based on 1,000 repetitions as shown at each node. Adaptated from Foster et al. (2009).

Brucella genome is usually composed of two circular chromosomes of approximately 2.1 and 1.2 Mb in size. *Brucella* ancestor was most likely a free-living bacterium with one chromosome, which evolved into an animal parasite with two separate chromosomes, a large-sized chromosome and a smaller plasmid originating the smaller one. However, some *Brucella* species still have only one chromosome, others kept their ancestor accessory genes responsible for utilization of plant derived nutrients such as *B. suis*, which possess transport and metabolic activities similar to those of certain soil-plant associated bacteria (El-Sayed and Awad, 2018).

Brucella Affecting Small Ruminants

Brucella melitensis

The species *B. melitensis* includes three biovars (biovars 1, 2 and 3). This is primarily the causative agent of small ruminant brucellosis (sheep and goat), but this species can infect other animal hosts (*e.g.*, camels and cattle) and is the main agent of human brucellosis (Alton and Forsyth, 1996).

Although *B. melitensis* are not truly acid-fast, they are stained by the modified Ziehl-Neelsen method of Stamp. When examined with obliquely reflected light, the smooth colonies appear moist and glistening and somewhat bluish (Moreno and Moriyó, 2006). Smooth *B. melitensis* cultures tend to undergo variation during growth, especially with subcultures, and dissociate to rough (R) forms, and sometimes mucoïd (M) forms. Colonies are then much less transparent with a more granular, dull surface (R) or a sticky glutinous texture (M), and range in colour from matt white to brown in reflected or transmitted light. Intermediate (I) forms between S, R and M forms may occur in cultures undergoing dissociation to the non-smooth state. Changes in the colonial morphology are generally associated with changes in virulence, serological properties and phage sensitivity (EC, 2001).

The genome from *B. melitensis* biovar 1 strain 16M was the first to be sequenced (DelVecchio et al., 2002). Like most of the *Brucella* species, this genome consists of two circular chromosomes of 2.1 Mbp (Chr I) and 1.2 Mbp (Chr II). Genomic analysis indicates that the two chromosomes probably have distinct evolutionary origins. The origin of replication of Chr I is typical of bacterial circular chromosomes, while that of Chr II possesses a cluster of plasmid-like replication genes. Most of the essential genes for protein synthesis are located in Chr I, while those encoding enzymes for sugar metabolism, protein regulators and membrane transport proteins for sugar, dipeptides and amino acids reside on Chr II (Paulsen et al., 2002; Halling et al., 2004; El-Sayed and Awad, 2018).

All three *B. melitensis* biovars give rise to disease in sheep and goats, but their geographic distribution differs (Michaux et al., 1993; Nagati and Hassan, 2016). Biovars 1 and 3 are the most frequently isolated in Mediterranean countries (Garin-Bastuji et al., 2006). Pathologically and epidemiologically, *B. melitensis* infection is very similar to *B. abortus* infection in cattle and causes mainly clinical manifestations in the reproductive system, with abortion around the 4th month of gestation, retained placenta and birth of weakened offspring, arthritis, orchitis, and epididymitis (Garin-Bastuji et al., 2006). Orchitis and epididymitis are symptoms usually observed in sheep infected with *B. ovis*, but *B. melitensis* biovar 3 was identified after histopathology, microbiology, and PCR analysis in a ram with those clinical signs in Turkey (Büyükcangaz et al., 2013).

Brucellosis in humans is mainly caused by *B. melitensis*. The results from a study performed in Portugal between 2010 and 2013, in which 162 human patients were analyzed by real-time PCR (blood, cerebrospinal fluid, biopsies and strains isolated from blood cultures), *Brucella melitensis* was the only species identified in 12.3% (20/162) of the analyzed cases (Pelerito et al., 2014). In 2014, a brucellosis outbreak with 13 cases transmitted by goat cheese occurred in northern Portugal (ECDC, 2017). Biovar 3 has been related to many cases in Turkey, China, Italy and other countries, where brucellosis is endemic (Di Giannatale et al., 2008; Ica et al., 2012; de Massis et al., 2015; Xiao et al., 2015). In China (Shanxi

Province), between 2009 and 2011, 81 *Brucella* isolates from human patients with brucellosis symptoms were identified as *B. melitensis* biovar 3 by conventional biotyping and the identification was confirmed by AMOS-PCR (Xiao et al., 2015).

The high prevalence of infected humans by *B. melitensis* biovar 3 confirms the same trend in animal flocks. *Brucella melitensis* biovar 3 was isolated in 2011, the first time in Kosovo, from a sample of goat's milk in a herd vaccinated with Rev1, which presented abortions in approximately 40% of the pregnant sheep (Hamidi et al., 2016). Furthermore, from 59 *Brucella* isolates obtained from the Xinjiang area (China), during a six-year period (2010-2015), 50 were identified as *B. melitensis* (biovar 1of 16%; biovar 2 of 4%; biovar 3 of 80%), compared to 9 isolates identified as *B. abortus* biovar 3. In terms of host origin, the majority of *B. melitensis* strains (47) were isolated from sheep, and one from a goat and a cow (Sun et al., 2016).

Brucella ovis

B. ovis infects sheep and has been reported in sheep-raising regions, such as Australia, New Zealand (also reported infection in red deer, *Odocoileus virginianus)*, North and South America, South Africa, and many countries in Europe. In ovine livestock, *B. ovis* infection affects the genital system and the main symptoms are epididymitis and decreased fertility in males, occasional abortions, and increased lamb mortality. A frequent route of infection is venereal transmission, but direct contact (ram-to-ram transmission) is also common, principally in Europe, where rams are usually housed together (OIE, 2009; Ridler, 2008; Dorneles et al., 2014; Picard-Hagen et al., 2015, Costa et al., 2016; Cvetnić et al., 2017).

The Farrell's medium described for the culture of smooth brucellae is not appropriate for the culture of *B. ovis* as it does not grow on this medium. For good growth, supplementary serum or blood must be added to the culture media and CO_2 (5-10%) is required. Growth occurs in the presence of thionin and basic fuchsin at different concentrations but does not occur in the presence of methyl violet. *B. ovis* are positive on catalase and acriflavine tests. The strains are oxidase and urease negative, do not

produce H_2S, and do not reduce nitrate to nitrite. The cultures are not lysed by *Brucella*-phages of the Tb, Wb and Iz groups but are lysed by phage R/C (Garrity et al., 2005; Moreno and Moriyó, 2006; OIE, 2009; Soler-Lloréns et al., 2016). *B. ovis* is differentiated from the rest of the *Brucella* species, and together with *B. canis*, belongs to the group of "rough *Brucella*", characterized by their scarce amount or total absence of the polysaccharide O of the outer lipopolysaccharide (LPS) wall. For this reason, the classical serologic tests that detect smooth LPS, does not detect *B. ovis.* The presence of LPS-R (rough) in *B. ovis* and *B. canis* is the cause of fundamental differences in its pathogenesis, compared to the smooth strains (*B. abortus, B. melitensis, B. suis*) that have a greater relevance and high zoonotic potential. *B. ovis* is not associated with human infections. Other specific characteristics are listed in Table 2.2 (Garrity et al., 2005; Moreno and Moriyón, 2006; Costa et al., 2016).

B. ovis presents high genetic diversity among strains and sometimes in the same herd. This was observed in southern Brazil where 13 distinct genotypes among the 14 *B. ovis* isolates were found, as evaluated by MLVA-16 (Dorneles et al., 2014).

Clinical alterations in sheep are observed by palpation, mainly on the epididymis and testicle, and thus *B. ovis* induces genital lesions and alters the semen quality, leading to an alteration of sexual function of the ram. In a study with 218 rams in France, 60 animals presented epididymis alterations, i.e., head and tail hypertrophy, indurations and nodules, and 13 animals showed testicle asymmetry, indurations, degeneration and atrophy (Picard-Hagen et al., 2015). In addition, Cvetnić et al. (2017) pathomorphological results of the epididymis and testicle in 22 rams showed pathological changes, such as granulomas, fibrosis, and atrophy. In deer, transmission can occur between animals that are in direct contact and invade the male reproductive tract resulting in characteristic pathological changes and subsequent decreases in semen quality. In contrast, the impact on females is low (Ridler, 2001).

Other *Brucella* Species

Brucella abortus

In cattle, bison and buffalo, brucellosis is mainly caused by *Brucella abortus* (Bamaiyi et al., 2012). This organism is a facultative intracellular pathogen and has eight biovars (1-7 and 9) as has been reported in several works (Whatmore, 2009a; Çiftci et al., 2017). The authenticity of *B. abortus* biovar 7, however, has been questioned for many years because the reference strain was a mixture of *B. abortus* biovars 3 and 5 (Allix et al., 2008) and there are no cultures anymore of biovar 8 in existence, so the status of this biovar was suspended by the Subcommittee on the Taxonomy of the genus *Brucella* in 1978 (Garrity et al., 2005). *B. abortus* can infect humans, cross occupational exposure to infected animals, or from ingesting contaminated dairy products (CFSPH, 2009a).

Brucella suis

Brucellosis in pigs is a chronic disease, which is most often expressed by infertility and abortion in females and by orchitis in males. While biovars 1, 2 and 3 have affinity for porcine, biovar 4 preferentially infects reindeer and caribou, whereas biovar 5 infects wild rodents. *B. suis* biovar 4 (rangiferine brucellosis) may be transmitted in reindeer and caribou by contact with aborted fetuses and fetus membranes. This strain can also infect moose, cattle, arctic foxes and wolves. In humans, biovars 1, 3 and 4 are more pathogenic. Biovar 2 is zoonotic, but has been reported very rarely in humans (Garrity et al., 2005; Moreno and Moriyó, 2006; CFSPH, 2007; Kutlu et al., 2016; Di Sabatino et al. 2017; Ferreira et al. 2017).

B. suis biovar 2 is commonly found in Europe and, besides the usual symptoms mentioned above, this biovar in pigs can also cause miliary lesions, that often become purulent, particularly in reproductive tissues. The wild boars are considered the main wild reservoir of this infection and they are recognised as a source of biovar 2 transmission to domestic pigs in Europe. Although rare in humans, this biovar is reported in cases of immuno-compromised hunters, extensively exposed through gutting or skinning boars or hares. In addition, rare cases of *B. suis* biovar 2 infection,

without clinical signs, have been reported in Europe in cattle and sheep exposed to infected wild boars (Garrity et al., 2005; Kreizinger et al., 2014; OIE 2016; Di Sabatino et al., 2017; Franco-Paredes et al., 2017). These metabolic characteristics, as described for the genus and specific species, are indicated in Table 2.2.

Brucella canis

B. canis is the etiologic agent of canine brucellosis, which can lead to severe economic loss in infected kennels. Infection with *B. canis* is common and endemic in Central and South America, in the southern USA and East Asia, but some cases of this disease have also been reported in Canada and Europe (Holst et al., 2012; Keid et al., 2017; Tuemmers et al., 2013; Bílman et al., 2014; Krueger et al., 2014; Whatmore et al., 2017b; Morgan et al., 2017).

Its metabolism is aerobic, does not need CO_2 to grow, being catalase, oxidase and urease positive, usually reduces nitrates and it does not produce H_2S. The species grows well in enriched media with yeast, serum or blood extract. Hemolysis is not observed. *B. canis* colonies appear after 72 h of aerobic incubation at 37ºC on *Brucella* agar, SDA agar (Sabouraud-dextrose), TSA agar, Farrell agar and modified Thayer Martin agar. The colonies are initially observed small (1-5 mm), translucent with a slight bluish tone, with defined edges and mucoid in the first isolation. On incubation for more than a few days, the colonies become opaque, tenacious and viscous. *B. canis* grows in media with thionin (10μg/mL), but not with basic fuchsin and this is a differential characteristic than the other species of the genus. Cultures are not agglutinated by antisera monospecific for the A and M antigens but do agglutinate with antiserum to the R antigen of *B. ovis*. The species is not lysed by *Brucella*-phages Tb, Fi, Wb or Bk2. *B. canis* belongs to the "rough *Brucella*" group, together with *B. ovis*. Other specific characteristics are listed in table 2.2 (Alton, 1988; Garrity, et al. 2005; Percin, 2013; Vicente, 2014; Chacón-Díaz et al., 2015).

Different PCR protocols, such as qPCR (quantitative PCR), multiplex PCR and MLVA are necessary for the molecular identification of *B. canis*.

However, the interpretation of the results is difficult due to the great genetic homogeneity among the species (Figure 2.1), particularly with *B. suis* (López-Goñi et al., 2011; Kauffman et al., 2014; Lee et al., 2015; Piao et al., 2017).

The role of *B. canis* in human disease is considered limited, but bacteria are transferred to humans by contact with the secretions and extraction of aborted dogs. Although asymptomatic mild infection is usually induced in humans, in Argentina and the USA one case of endocarditis one of peritonitis were, respectively, reported (Manias et al., 2013; Bilman et al.,2014; Krueger et al., 2014; Javeri et al., 2014; Keid et al., 2017).

The few existing reports to this species may arise from inefficient classical serological diagnosis (Pappas, 2010, Keid et al., 2017). The serological tests used routinely to diagnose human infections with *B. abortus*, *B. suis* and *B. melitensis* do not detect antibodies to *B. canis*. These tests use "smooth phase" antigens, while *B. canis* is a "rough" form of *Brucella* (CFSPH, 2007; Pujol et al., 2017). Nevertheless, there is a growing number of human infections caused by *B. canis* reported in Turkey, USA, China and Argentina. This disease has been reported mainly in humans who had direct contact with infected dogs, including breeders, kennel employees, veterinary personnel, animal shelter workers, and dog show handlers and owners (Manias et al., 2013; Bilman et al.,2014; Di et al., 2014; Krueger et al., 2014; Piao et al., 2017; Viana et al., 2017).

The infection in dogs occurs by venereal transmission, or by contact with the fetus and fetus membranes, after abortions and stillbirths. Puppies can be infected in utero and through contaminated milk. Other potential sources of infection include blood transfusions and contaminated syringes (CFSPH, 2007; Reynes et al., 2012).

The main consequences of *B. canis* infection in dogs relate to genital organs, including late term abortion and fetus resorption in pregnant females and orchiepididymitis, prostatitis and sperm abnormalities in males. *B. canis* infection can persist even after antibiotic treatment. In kennels, infected dogs are often euthanized to prevent them from infecting other dogs or people (CFSPH, 2007; Holst et al., 2012).

Brucella neotomae

B. neotomae strains were isolated from *Neotoma lepida,* a desert wood rat that inhabits the western regions of the USA in 1957 (Stones and Hayward, 1968; Olsen and Palmer, 2014).

The strains are aerobic, non-spore-forming and fastidious. They also oxidase positive for L-arabinose, L-aspargine, *meso*-Erythritol, D-galactose, L-glutamic acid and D-glucose. Acid production (but not gas) occurs from carbohydrates in conventional media from D-glucose, D-galactose, L-arabinose, and D-xylose in peptone water sugar media (Cameron and Meyer, 1958; Garrity et al., 2005; Moreno and Moriyó, 2006).

Optimal temperature for growth is 37ºC, however, growth occurs between 20 and 40ºC. Colonies on serum-dextrose agar are transparent, raised, convex, with an entire edge and a smooth, shiny surface. They appear a pale honey color by transmitted light and produce perosamine synthetase and a distinctive LPS. Colonies are formed within 18 h, with a diameter of approximately 1–2 mm, smooth and opaque. Good growth does not require CO_2 and improves with supplementary serum or blood and no haemolysis is observed. Growth does not occur in the presence of thionin and basic fuchsin. Incomplete lysis occurs only with bacteriophages Wb, Fi and Bk_2. Smooth *Brucella* cultures will produce agglutination with either A and/or M sera. *B. neotomae* are catalase, urease reaction, nitrate reductase and H_2S formation positive, negative for Voges–Proskauer reaction, indol production, citrate and oxidase (Cameron and Meyer, 1958; Garrity et al., 2005; Moreno and Moriyó, 2006; Suárez-Esquivel et al., 2017).

B. neotomae is not pathogenic for domestic animals, or humans. In a laboratory experiment with white mice, the dose of bacteria found in the livers and spleens was 1/10 of the applied dose, which could not be enough to allow horizontal transfer between the animals (Gibby and Gibby, 1965; Corbel, 2006; Whatmore, 2009). However, a recent study showed that in 2008 and 2011, *B. neotomae* was isolated from cerebrospinal fluid of two men with neurobrucellosis. The species was confirmed by molecular methods. In this way, *B. neotomae,* as a cause of zoonotic disease, raises

questions about possible underrepresentation of reported cases and the nonzoonotic status of *B. neotomae* should be reassessed (Suárez-Esquivel et al., 2017). The symptoms of *B. neotomae* in its natural host, (the desert wood rat), does not apparently produce disease, and laboratory animals showed minimal pathogenicity. Guinea pigs with intraperitoneal inoculation develop slight splenomegaly, and sometimes epididymo-orchitis or testicular abscesses and small granulomatous lesions in the liver (Garrity et al., 2005).

Brucella ceti and Brucella pinnipedialis

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. Since their discovery in 1994, it was found in common seals (*Phoca vitulina*), a porpoise (*Phocoena phocoena*) and a common dolphin (*Delphinus delphis*) in Scotland (Ross et al., 1994). The name *Brucella ceti* is proposed for *Brucella* strains with cetaceans, as their preferred host, and the name *Brucella pinnipedialis* is proposed for *Brucella* strains with pinnipeds, as their preferred host. *Brucella* strains from marine mammals (*B. ceti* and *B. pinnipedialis*) have been subjected to a range of characterization tests (Foster et al. 2007; CFSPH, 2009b; Whatmore et al., 2017a).

Colonies on Columbia sheep blood agar and Farrell's medium are raised, convex, circular and entire with a diameter of 0.5–1.0 mm and non-haemolytic. Distinctive characteristics, like a transparent, smooth shiny surface and a pale honey colour transmitted by light are observed in colonies on serum-glucose agar. Growth is improved by the addition of serum or blood. Optimum temperature is 37ºC. Growth occurs between 20 and 40ºC. Optimum pH is between 6.6 and 7.4 (Foster et al., 2002; Tryland et al., 2005; Foster et al. 2007; CFSPH, 2009b).

These species have aerobic metabolism, produce nitrate reductase, are catalase, oxidase and urease positive and H_2S negative. The A antigen is dominant. Cultures are lysed by Iz and Wb phages, but no lysis occurs with Tb and R/C phages. Only a small number of *B. pinnipedialis* strains exhibit

lysis with the Tb phage (Foster et al., 2002; Tryland et al., 2005; Foster et al., 2007).

Other characteristics can be observed in Table 2.3 for both species, but identification by phenotypic methods is not always possible. Based on this, molecular markers have an important role in distinguishing terrestrial species of *Brucella* from marines (Cloeckaert et al., 2000). The comparison of *B. ceti* and *B. pinnipedialis* with the terrestrial *Brucella* species have been shown by DNA–DNA hybridization, to be related to the six classical *Brucella* species at a level of >77% DNA–DNA relatedness (Foster et al., 2007).

Table 2.3. Differential characteristics of *Brucella ceti* and *Brucella pinnipedialis*

Characteristics	***B. ceti***	***B. pinnipedialis***
Preferred host	Cetaceans (porpoises, dolphins and whales)	Pinnipeds (seals, sea lions and walruses)
Colonies on Columbia sheeps' blood agar and Farrell's medium	Visible after 3–4 days	Visible after 7–10 days or absent
Supplementary CO_2 for growth	-	+
Oxidation of:		
L-Alanine	-	-
L-Arabinose	+	-
L-Arginine	-	-
L-Asparagine	-	-
meso-Erythritol	V	+
D-Galactose	+	-
L-Glutamic acid	+	+
L-Lysine	-	-
DL-Ornithine	-	-
D-Ribose	+	+
D- Xylose	+	-

*+ positive; - negative; V variable. Adapted from Foster et al. (2007) and CFSPH (2009b)

The *bp*26 gene has been identified as an immunodominant antigen in *Brucella* infections of cattle, sheep, and humans and it is a molecular marker for an identification of brucellosis (Vizcaíno et al., 1996; Cloeckaert et al., 2001; Xin et al., 2013). A PCR performed on the bp26 gene of the reference strains of the terrestrial *Brucella* species produced a

product of the expected size (1,029 bp), while a PCR performed on DNA of three representative *B. ceti* strains from marine mammals (a seal, a dolphin, and a porpoise) produced a larger product, of about 1,900 bp. The nucleotide sequences of the 1,900-bp PCR products, of the three marine *Brucella* strains (B2/94, B1/94, and B14/94), were determined and they revealed the presence of an insertion sequence, IS711, downstream of the bp26 gene. Therefore, this difference in amplification of the bp26 gene can be used to distinguish the *Brucella* species from terrestrial to marine mammal strains (Cloeckaert et al., 2000).

The symptoms of *Brucella* infection in marine mammals are not recognized since most of the research developed is with dead animals stranded at the coast. However, a range of associated pathology has been found, which includes sub-blubber abscessation, hepatic and splenic necrosis, macrophage infiltration in liver and spleen, possible abortion, epididymitis, spinal discospondylitis and meningitis (Foster et al., 2002). In a study with striped dolphins (*Stenella coeruleoalba*) infected by *B. ceti*, González et al. (2010) observed lesions in the heart, liver, lungs, joints and placenta of animals, suggest that *B. ceti* has the ability to cause chronic infection of multiple organs before it crosses the blood–brain barrier. González et al. (2010) described meningoencephalitis associated with *Brucella* spp. in three young striped dolphins (*Stenella coeruleoalba*) and Hernández-Mora et al. (2008) isolated *B. ceti* from cerebrospinal fluid of 6 dolphins and 1 fetus along the Costa Rica, Pacific coast. Consequently, *S. coeruleoalba* constitutes a highly susceptible host and a potential reservoir for *B. ceti* transmission. Another case of meningoencephalitis caused by *B. ceti* in an adult male striped dolphin that was found stranded on the Mediterranean Sea coast (Alba et al., 2013).

Since the initial reports of *Brucella* spp. in marine mammals, the method of transmission between individuals and populations are still unclear, but in a study developed by Maio et al. (2014), *B. ceti* was isolated and identified by phenotypic and molecular methods in tissues of the respiratory system and lungworms of harbour porpoises (*Phocoena phocoena*) that stranded on the Dutch coast between 2008 and 2011. These results suggest that respiratory exposure may represent a transmission

route of classical *Brucella* spp. as well as, lungworms may act as vector for indirect transmission. In addition, Dagleish et al. (2008) described the pathology associated with an isolate of *B. ceti* that was cultured and identified from testis and epididymis of an adult harbour porpoise (*P. phocoena*). This animal presented an abscess in the fibrous tissue capsule and adjacent tissues, and no spermatozoa were present, which suggests the potential for sexual transmission and/or sterility to sequelae to infection similar to those reported in terrestrial animals (Dagleish et al.,2008; Von Bargen et al., 2012; Büyükcangaz et al., 2013; Van der Henst et al., 2013).

In the North Atlantic Ocean (Greenland Sea), *B. pinnipediae* was isolated from various tissues (tonsils, lung, lung lymph nodes, spleen, liver, kidney, ovary and epididymis) from 38% of the investigated hooded seals (*Cystophora cristata*) (Tryland et al., 2005). All animals were apparently healthy and were caught in their natural habitat. The hooded seal is commercially hunted and consumed in Norway, as well as other marine mammals in other countries, consequently the pathological impact of *B. ceti* e *B. pinnipediae* and zoonotic potential must be considered (Tryland et al., 2005; Maquart et al., 2009).

Brucella microti

The first isolation of *B. microti* was in the Czech Republic from two clinical specimens of diseased wild common voles (*Microtus arvalis*) during an epizootic event between 1999-2003. These animals presenting acute infections were characterized by edema of extremities, occasionally with colliquating abscesses, arthritis, lymphadenitis, perforations of the skin resulting from colliquated abscesses, orchitis, and peritoneal granulomas (Hubalek et al., 2007). These two isolates from *M. arvalis* were considered a novel species of the genus *Brucella* (Scholz et al. 2008b; Rónai et al., 2015).

A wild boar (*Sus scrofa*) was recently reported as infected by *B. microti*. The strain was isolated from the submandibular lymph node of a hunted female wild boar in Hungary. This lymph node did not show any gross pathological, or histological changes, and was negative for *B. abortus, B. suis* and *B. canis* specific sera; the colonies appeared in pure

culture, after two days incubation on *Brucella* selective agar, and after enrichment in *Brucella* selective broth (Rónai et al., 2015). Rapid growth is a typical characteristic of *B. microti*, it is a non-fastidious species, different from other *Brucella* species.

The biochemical profile of *B. microti* is closer to that of *Ochrobactrum* spp. than from another genus of α-Proteobacteria. The close phylogenetic relationship of *Brucella* spp. and *Ochrobactrum* spp., and the high metabolic activity of *B. microti* suggests that the soil may be a reservoir of *Brucella* spp. In the Czech Republic, this strain was found in all samples recovered from soil samples from areas where common voles with *B. microti* contamination occurs. Isolation of *B. microti* directly from soil reflects its ability to persist for a long time in the environment outside a mammalian host and supports the idea that soil can be a reservoir of infection (Scholz et al., 2008a).

Non-fastidious, *B. microti* grows well at 28 and 37ºC on meat peptone agar (MPA), forming transparent to whitish colonies, 1–2 mm in diameter, after 1–2 days of incubation. H_2S was not produced. Good growth was also observed on blood agar and on standard nutrient agar at 28 and 37ºC. Colonies were slightly concave, smooth, with very light brown exopigment and continuous edges. After 72 h of growth at 37ºC, cultures appeared as large colonies (6–9 mm) with noticeable brownish pigmentation. Growth occurs without supplementary CO_2, serum or blood. No haemolysis was observed (Scholz et al. 2008b; Rónai et al., 2015).

The metabolism is aerobic, non-fermentative and non-spore-forming. Oxidase, catalase and urease positive. Nitrate and nitrite are reduced (with gas formation from nitrate). No production of H_2S and the Voges–Proskauer reaction is positive. Reaction on Simmons' citrate is negative and no growth in broth containing 6.5% NaCl occurs. Growth is not inhibited in the presence of 20 μg/mL thionin and basic fuchsin. Oxidation of D-glucose, maltose, L-arabinose, D-mannose, adipic acid and malic acid are positive. Acid is produced from glucose, maltose, fructose and xylose. Cultures are lysed by Tb, F1 and F25 phages but not by RTD and by Wb phages. Bacteria agglutinate with monospecific M and A antiserum. Cells are sensitive to gentamicin, tobramycin, cotrimoxazole and ofloxacin, but

resistant to colistin, piperacillin, ceftazidime and tazobactam (Scholz et al. 2008b; AI Dahouk et al., 2012; Rónai et al., 2015).

In the IS711-based AMOS multiplex-PCR, a 1900 bp fragment, not amplified from other *Brucella* species, are generated with the *B. ovis*-specific primers (Hubalek et al., 2007). Isolates from differential natural habitats and host preferences (soil, common voles and wild red foxes) were found to possess identical 16S rRNA, recA, outer membrane protein (omp) 2a, and omp2b gene sequences and identical multilocus sequence analysis profiles at 21 different genomic loci. The correct identification was thought to be genus-specific bcsp31 PCR for genus *Brucella* and a IS711-based AMOS-PCR for *B. microti* (AI Dahouk et al., 2012).

Common voles (*Microtus arvalis*), wild red foxes (*Vulpes vulpes*), and wild boar (*Sus scrofa*) are host for *B. microti*. The pathogenicity of *B. microti* for humans and livestock has not been proven yet, but experimental inoculations of *B. microti* in chicken embryos showed a rapid multiplication and provoked marked gross lesions (hemorrhages and necroses) and 100% mortality, between the 2nd and 4th day post-inoculation. All inoculated embryos presented necroses in liver, kidneys, lungs, spleen, gastrointestinal tract, spinal meninges, yolk sac and chorioallantoic membrane (Wareth et al., 2015).

In experimental cellular and murine infections, *B. microti* exhibited a high pathogenic potential and an enhanced capacity for intramacrophagic replication, compared with that of *B. suis*. Infection of spleen and liver with *B. microti* peaked at day 3, compared with *B. suis* infection, which peaked at day 7. The results confirmed that *B. microti* multiplied in human monocytes and in human and murine macrophage-like cells (Bagüés et al., 2010).

Brucella inopinata

B. inopinata (BO1) is a unique and unusual species of the *Brucella* genus isolated firstly from a breast implant wound of a 71-year-old patient with brucellosis symptoms of unknown origin. This species exhibits the general characteristics, microbiological, biochemical and molecular, of the genus *Brucella*, but is distinct from any previously described members of

this species (De et al., 2008). *B. inopinata* is the most unique species within the genus *Brucella* because it has similar DNA sequences in various housekeeping genes and genes encoding outer-membrane proteins, when compared with all other recognized *Brucella* species (Scholz et al., 2010).

The growth occurs on *Brucella* agar, MacConkey Agar, Trypticase Soy agar, Sheep Blood agar and Standard Nutrient agar at 25–42ºC. Colonies are formed within 18 h, with a diameter of approximately 1–2 mm, smooth and opaque. Good growth does not require CO_2, supplementary serum or blood and no haemolysis is observed.

This strain is positive for oxidase and catalase, urease reaction, nitrate and nitrite reduction, H_2S formation and Voges–Proskauer reaction. It is negative for hydrolysis of aesculin, gelatine liquefaction, and production of indole and citrate utilization. Furthermore, growth occurs on cetrimide and Salmonella–Shigella (SS) agar (Scholz et al., 2010). The BO1 strain is susceptible to antimicrobial agents, doxycycline (0.12 µg/mL), tetracycline, (0.25 µg/mL), streptomycin (2 µg/mL), gentamicin, (1 µg/mL), and trimethoprim-sulfamethoxazole (<0.5 and 9.5 µg/mL). The antimicrobial susceptibility pattern of BO1 was similar to profiles of other *Brucella* spp. isolates (De et al., 2008).

The DNA sequencing of the full-length 16S rRNA gene of BO1 demonstrated that it was 99.6% identical to the consensus sequence of *Brucella* spp. with five base differences at positions 167 to 170 and 234. A dendrogram indicates that BO1 and the *Brucella* consensus sequence (represented by the 16S rRNA gene sequence for *B. ovis*) cluster together (De et al., 2008).

Brucella papionis

B. papionis was first isolated from baboons (*Papio* spp.) and phylogenetic analysis confirmed that they represent a well-separated lineage related most closely to *B. ovis* (Figure 2.1). Growth is comparable to that of classical species of *Brucella*. There were two strains isolated, in 2006 and 2007, from two cases of stillbirth and retained placenta in baboons at a primate research centre in Texas, USA. The baboons did not have contact with each other and were captured in Tanzania. This is the

first report of *Brucella* isolated in association with two cases of stillbirth in nonhuman primates (Whatmore et al., 2014; Scholz et al., 2016) and presented abortions, a typical symptom of *Brucella* infection.

These bacteria are non-motile and non-spore-forming and presented resistant to decolourization with 0.5% acetic acid. The metabolism is aerobic and does not require supplementary CO_2 for growth. The growth occurs at 30–37ºC. Colonies on SBA (Sheep Blood agar) and Farrell's agar are visible at 3–4 days and are small (0.5-1 mm in diameter), raised, circular, entire and convex. It is non-haemolytic and greyish in colour or honey-coloured (on Farrell's agar). Isolates do not grow in the presence of thionin, or basic fuchsin at 1/50,000 in MacConkey agar and in broth with 6.5% NaCl. The strains agglutinate with monospecific anti-A serum but not anti-M or anti-R serum. Cells are lysed by Wb, Bk2 and Fi phage. Cells are sensitive to doxycycline, rifampicin, ciprofloxacin and streptomycin antibiotics. *B. papionis* are positive for Vogues–Proskauer reaction, catalase and urease. However, they are negative for oxidase, indole hydrolysis and nitrates are not reduced. No production of H_2S, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, b-galactosidase, b-glucosidase or gelatinase. Positive for fermentation of L-arabinose and D-glucose at 37ºC while D-sorbitol is variable. At 37ºC, the strains are not able to undergo fermentation or oxidation of D-mannitol, inositol, L-rhamnose, sucrose, melibiose or amygdalin at 30ºC. They are not able to assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, maltose, N-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenyla*cetic* acid (Whatmore et al., 2014).

Brucella vulpis

Currently, *B. vulpis* has two strains identified (F60 and F965) and they were isolated in 2008 in Eastern Austria from mandibular lymph nodes of two wild red foxes (*Vulpes vulpes*), a known indicator species for natural foci of brucellosis. Both strains are characterized by the presence of additional genetic material derived from soil-associated bacteria such as *Ochrobactrum* and *Rhizobium*. This suggests that the soil may be a

reservoir for *B. vulpis* and for gene acquisition by horizontal gene transfer. Atypical brucellae strains, such as *B. vulpis*, *B. inopinata* and as yet the unclassified *Brucella* strain BO2, based on the phylogenetic reconstructions (Figure 2.1) represent ancestral species relative to the host-adapted classical *Brucella* species (Hofer et al., 2012; Scholz et al., 2016).

B. vulpis are Gram-negative coccobacilli or short rods, with approximately 0.5–0.7 µm in diameter and 0.4–1.3 µm long, arranged singly and, occasionally, in pairs or small groups. These bacteria are non-motile and non-spore-forming. They are aerobic and slow-growing (typical of classical the *Brucella* species). They do not require CO_2 for growth and occur at 30–37ºC on standard solid media and on *Brucella* selective medium. The colonies have a diameter of approximately 0.5 mm after 72 h of incubation on Columbia agar and are greyish, round, convex, glossy, smooth, and non-haemolytic. Red rods are observed on Modified Ziehl–Neelsen stained smears. Strains grow in the presence of 20 mg/mL of thionin or basic fuchsin after 3–4 days of incubation in aerobic conditions. Agglutination occurs with monospecific anti-A serum, but not with monospecific anti-M serum. The strains are positive for catalase, urease, and Voges–Proskauer reactions and negative for H_2S production, oxidase and nitrate reductase. Positive physiological reactions are visible with different peptidases and hydrolyses D-fructose. Strong lytic activity against *B. vulpis* is presented by Tb, Fi, Bk2, Wb, R/C, Iz, F1 and F25 phages (Hofer et al., 2012; Scholz et al., 2016).

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