

Brucella neotomae Infection in Humans, Costa Rica

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Several species of *Brucella* are known to be zoonotic, but *B. neotomae* infection has been thought to be limited to wood rats. In 2008 and 2011, however, *B. neotomae* was isolated from cerebrospinal fluid of 2 men with neurobrucellosis. The nonzoonotic status of *B. neotomae* should be reassessed.

Members of the genus *Brucella* are the infectious agents of brucellosis, a neglected disease responsible for economic losses resulting from abortion and low performance in production animals (1). The 4 species mainly responsible for this widespread bacterial zoonosis are *B. melitensis*, *B. abortus*, *B. suis*, and to a lesser extent *B. canis*. Underdiagnosis and limited awareness of the disease among healthcare practitioners is common in many countries (1).

B. neotomae, isolated in 1957 from wood rats (*Neotoma lepida*) in North America (2), has been considered nonzoonotic (3). It has been isolated from target organs of experimentally infected mice and guinea pigs (4,5). We report the isolation of *B. neotomae* from cerebrospinal fluid samples from 2 human patients with neurobrucellosis.

The Study

In 2008, a *Brucella* sp. isolate was submitted to the Tropical Diseases Research Center at the Universidad de Costa

Rica. This isolate (denoted strain bneohCR2) was cultured from a cerebrospinal fluid sample obtained from a 64-year-old male patient at one of the main hospitals in San José, Costa Rica. In 2011, another isolate (denoted strain bneohCR1) was recovered from a cerebrospinal fluid sample from a 51-year-old male patient at a regional hospital in Costa Rica. As is common for other patients with brucellosis, the blood leukocyte count for each patient was almost within the reference range, and C-reactive protein level was within reference range. Both patients showed clinical signs compatible with neurobrucellosis (6), had positive Rose Bengal test results, and recovered after receiving 1 month of streptomycin (750 mg/d) and 3 months of doxycycline (100 mg/12 h).

Further bacteriologic analysis (7,8) confirmed that the isolates were a *Brucella* sp. (Table). Real-time PCR high-resolution melting analysis (9) confirmed genus designation but was inconclusive regarding species designation. Bruce-ladder multiplex PCR (10) and multiple loci variable number of tandem repeats–16-loci panel analysis (<http://mlva.u-psud.fr/brucella/>; Figure 1) indicated that the profiles for both DNA isolates corresponded to profiles for *B. neotomae*. Analysis of bneohCR2 by multiplex single-nucleotide polymorphism (SNP) primer extension assay (11) and by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of protein extracts (12) (online Technical Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/23/6/16-2018-Techapp1.pdf>) confirmed that the isolate was *B. neotomae*.

We performed whole-genome sequencing of bneohCR1 and bneohCR2 and resequencing of reference strain *B. neotomae* 5K33. Sequencing data were deposited at the European Nucleotide Archive (<http://www.ebi.ac.uk/ena/>) under accession codes ERS1563929 (bneohCR1), ERS1563928 (bneohCR2), and ERS1624467 (SK33). To place the bneohCR1 and bneohCR2 in a phylogenetic context, publicly available reads from 51 *Brucella* whole-genome sequences (online Technical Appendix Table 2) were aligned and then mapped to *B. suis* 1330 by using SMALT version 0.5.8 (<ftp://ftp.sanger.ac.uk/pub/resources/software/smalt/>). Reads from bneohCR1 and bneohCR2 genomes mapped to 98.6% of the *B. suis* 1330 genome. SNPs were called from the alignment by use of Samtools (<http://samtools.sourceforge.net/>), and 34,307 variable sites across all isolates were extracted by using SNP sites (13). The resulting alignment of SNPs

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Table. Differential biochemical profile of *Brucella* isolates from 2 men with neurobrucellosis, Costa Rica, 2008 and 2011

Analysis	bneohCR1	bneohCR2
Biochemical tests		
Oxidase	-	-
Citrate utilization	-	-
Nitrate reduction	+	+
CO ₂ required	-	-
H ₂ S production	+	+
Urease activity, h	0–0.5+	0–0.5+
Growth in presence of dyes		
Thionin 20 µg/mL	-	-
Basic fuchsin 20 µg/mL	-	-
Agglutination using monospecific serum		
A	+	+
M	-	-

was used for maximum-likelihood phylogenetic reconstruction by use of RAxML version 7.0.4 (<https://github.com/stamatak/standard-RAxML>). The generated phylogenetic tree confirmed that the bneohCR isolates clustered together with *B. neotomae* reference strain 5K33 (ENA accession no. JM5C01, assembly accession no. GCA_00742255.1) (Figure 2). Isolates bneohCR1 and bneohCR2 differed from the reference genome by 174 and 160 SNPs, respectively. This number of SNPs is smaller than that between *B. abortus* 9–941 and *B. abortus* 2308 (214 SNPs), which are 2 well-recognized strains of the same *Brucella* species.

Analysis of 23 previously reported genomic islands or anomalous genomic loci (14) was performed for both bneohCR genomes. For this analysis, a “genomic-island pseudo-molecule” was constructed by concatenation of 23 genomic regions obtained from different *Brucella* genomes.

BLAST (https://github.com/sanger-pathogens/Farm_blast) comparison of this pseudo-molecule and the bneohCR draft genomes, generated by assembly with Velvet (15), showed that the genomic loci known as 26.5 kb, 12 kb, and GI-6 that are absent in *B. neotomae* (14) are also absent in the queried genomes.

Conclusions

This report of *B. neotomae* as a cause of zoonotic disease raises questions about possible underrepresentation of reported cases. This study also has implications for brucellosis diagnosis. Specifically, the differences among *B. neotomae* and the other *Brucella* species at the biochemical level are subtle. The major difference between *B. neotomae* and *B. abortus*, the main cause of human brucellosis in Costa Rica, is the presence of oxidase activity in *B. abortus*, which is assessed subjectively (7,8). Because *B. neotomae* has not, until now, been considered zoonotic, some cases of brucellosis reported as being caused by atypical zoonotic classical *Brucella* might have been misdiagnosed cases of *B. neotomae* infection. The introduction of whole-genome sequencing into the clinical field will thus improve diagnosis.

A lack of epidemiologic information with regard to the 2 patients reported here precluded the investigation of exposure or contact with hosts known to harbor *B. neotomae*. Further studies are needed to establish which animals may act as reservoirs for *B. neotomae* in Costa Rica.

In summary, *B. neotomae* should be considered a zoonosis risk for infection in humans. Incorporation of molecular techniques for diagnosis will help resolve the

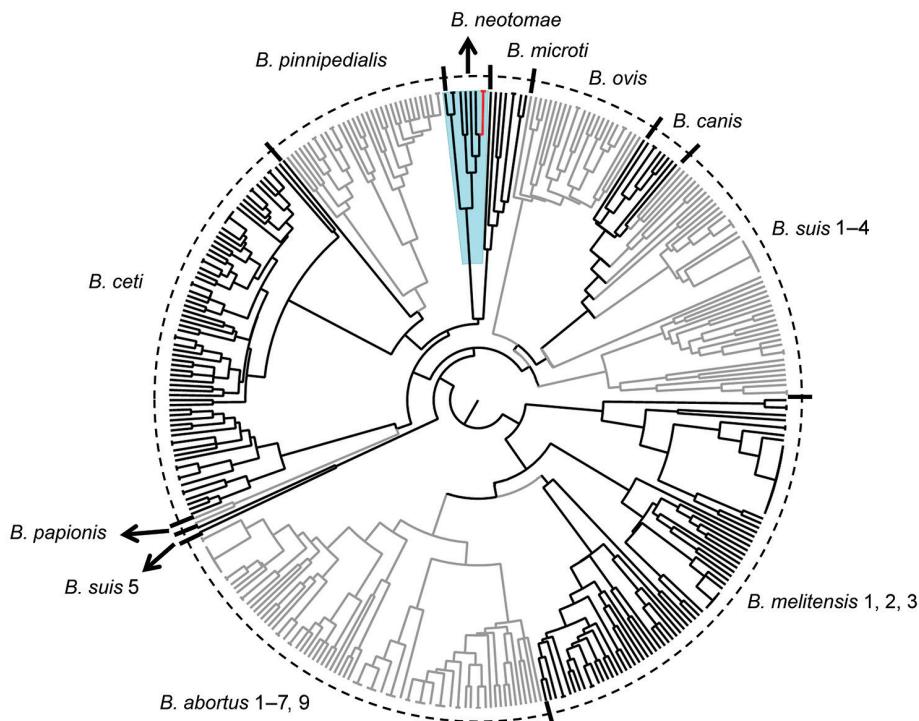


Figure 1. Dendrogram based on multiple locus variable number of tandem repeats–16-loci panel analysis of *Brucella* spp. (<http://mlva.u-psud.fr/brucella/>) and clinical isolates from human cerebrospinal fluid samples from 2 patients with brucellosis. The isolates bneohCR1 and bneohCR2 (red branches) showed a pattern consistent with previously reported profiles for *Brucella neotomae* (blue shading). Black, gray, and tic marks are used to differentiate between adjacent species. Arrows separate small neighboring clusters and indicate the *B. neotomae* cluster.

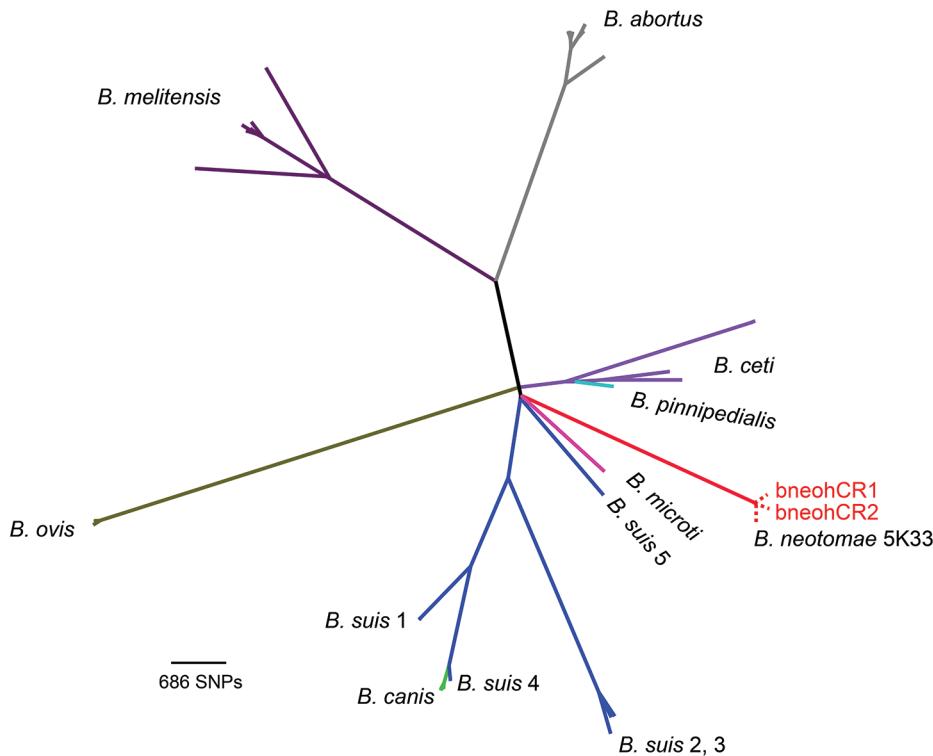


Figure 2. Phylogenetic tree based on 34,307 single-nucleotide polymorphisms (SNPs) found among 51 *Brucella* genome sequences. The clinical isolates bneohCR1 and bneohCR2 cluster with *B. neotomae* 5K33 and differ by 164 SNPs. A different color is used to represent each *Brucella* species. Dotted red lines denote the 3 *B. neotomae* isolates, which overlap at the tip of the branch because of the high identity among them.

Brucella genus homogeneity obtained when only biochemical assays are used.

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All procedures involving live *Brucella* were carried out according to the “Reglamento de Bioseguridad de la CCSS39975-0,” year 2012, after “Decreto Ejecutivo no. 0965-S,” year 2002 and protocol approved by SIA0434-14 Universidad Nacional, Costa Rica. These genetic resources were accessed in Costa Rica according to the Biodiversity Law no. 7788 and the Convention on Biological Diversity, under the terms of respect to equal and fair distribution of benefits among those who provided such resources.

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References

1. World Health Organization. The control of neglected zoonotic diseases. In: NZD4 Organising Committee, editor. WHO conference report. Geneva: WHO Press; 2014. p. 23–35.
2. Stoenner HG, Lackman DB. A new species of *Brucella* isolated from the desert wood rat, *Neotoma lepida* Thomas. Am J Vet Res. 1957;18:947–51.
3. Moreno E. Retrospective and prospective perspectives on zoonotic brucellosis. Front Microbiol. 2014;5:213. <http://dx.doi.org/10.3389/fmicb.2014.00213>
4. Stoenner HG. The behavior of *Brucella neotomae* and *Brucella suis* in reciprocal superinfection experiments in mice and guinea pigs. Am J Vet Res. 1963;24:376–80.
5. Gibby IW, Gibby AM. Host–parasite relationships with *Brucella neotomae*. J Bacteriol. 1965;89:9–16.
6. Ceran N, Turkoglu R, Erdem I, Inan A, Engin D, Tireli H, et al. Neurobrucellosis: clinical, diagnostic, therapeutic features and outcome. Unusual clinical presentations in an endemic region. Braz J Infect Dis. 2011;15:52–9. [http://dx.doi.org/10.1016/S1413-8670\(11\)70140-4](http://dx.doi.org/10.1016/S1413-8670(11)70140-4)
7. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the brucellosis laboratory. Paris: Institut National de la Recherche Agronomique; 1988. p. 39–41.
8. Weyant R, Popovic T, Bragg S. Basic laboratory protocols for the presumptive identification of *Brucella* species. Atlanta: Centers for Disease Control and Prevention; 2001. p. 1–16.
9. Winchell JM, Wolff BJ, Tiller R, Bowen MD, Hoffmaster AR. Rapid identification and discrimination of *Brucella* isolates by use of real-time PCR and high-resolution melt analysis. J Clin Microbiol. 2010;48:697–702. <http://dx.doi.org/10.1128/JCM.02021-09>
10. García-Yoldi D, Marín CM, de Miguel M-J, Muñoz PM, Vizmanos JL, López-Goñi I. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella*

- melitensis* Rev1. Clin Chem. 2006;52:779–81. <http://dx.doi.org/10.1373/clinchem.2005.062596>
11. Scott JC, Koylass MS, Stubberfield MR, Whatmore AM. Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. Appl Environ Microbiol. 2007;73:7331–7. <http://dx.doi.org/10.1128/AEM.00976-07>
 12. Isidoro-Ayza M, Ruiz-Villalobos N, Pérez L, Guzmán-Verri C, Muñoz PM, Alegre F, et al. *Brucella ceti* infection in dolphins from the western Mediterranean Sea. BMC Vet Res. 2014;10:206. <http://dx.doi.org/10.1186/s12917-014-0206-7>
 13. Page AJ, Taylor B, Delaney AJ, Soares J, Seemann T, Keane JA, et al. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. Microb Genomics. 2016 Apr 29. <http://dx.doi.org/10.1099/mgen.0.000056>
 14. Mancilla M. The *Brucella* genomic islands. In: López-Goñi I, O’Callaghan D, editors. *Brucella: Molecular Microbiology and Genomics*. Poole (UK): Caister Academic Press; 2012. p. 36–57.
 15. Page AJ, De Silva N, Hunt M, Quail MA, Parkhill J, Harris SR, et al. Robust high-throughput prokaryote de novo assembly and improvement pipeline for Illumina data. Microb Genomics. Microbiology Society; 2016 Aug 25. <http://dx.doi.org/10.1099/mgen.0.000083>

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Technical Appendix

Technical Appendix Table 1. Protein mass values of *Brucella* reference strains and bneohCR2*

Peak N°	Mass	Bce	Bpi	Bme	Bab	Bsu	Bca	Bov	Bmi	BneK	BneCR
1	2771	0	1	0	0	0	0	0	0	0	0
2	2919	0	0	0	0	1	0	0	0	0	0
3	3026	0	1	1	1	1	1	1	0	0	0
4	3181	1	1	1	0	1	1	0	0	0	0
5	3407	0	0	0	1	0	0	1	0	0	0
6	3700	1	1	1	1	1	1	1	1	1	1
7	3831	0	0	0	0	0	1	1	0	0	0
8	4126	0	0	0	1	0	0	1	0	0	0
9	4541	0	1	1	1	0	1	1	0	1	1
10	4558	1	0	0	1	1	0	0	1	0	0
11	4854	1	1	1	1	1	1	1	1	1	1
12	4898	0	0	1	0	1	1	0	0	0	0
13	5112	0	0	0	1	0	0	1	0	0	0
14	5135	1	1	1	1	1	1	0	0	1	1
15	5169	1	0	1	0	1	1	1	1	1	0
16	5546	1	1	1	0	0	0	0	0	0	0
17	5686	1	1	1	0	1	1	1	1	1	1
18	5772	1	1	0	0	0	1	0	0	0	0
19	5834	0	0	0	1	1	1	0	0	0	0
20	5876	1	1	0	1	0	0	1	0	1	1
21	5940	0	0	0	1	0	0	0	1	1	0
22	6039	0	0	1	0	1	0	0	1	0	0
23	6049	0	0	0	1	0	0	0	0	1	1
24	6150	0	0	0	1	1	0	0	0	0	0
25	6289	1	1	1	1	1	1	1	1	1	1
26	6322	0	0	1	0	1	0	0	0	0	0
27	6369	1	1	0	1	0	1	0	0	0	0
28	6420	0	0	1	1	1	0	0	0	0	0
29	6542	1	1	1	1	1	1	0	1	1	0
30	6676	1	1	1	1	1	1	1	0	1	1
31	7056	1	1	1	0	1	1	1	1	1	1
32	7183	1	1	1	1	1	0	0	0	1	0
33	7270	0	0	0	1	0	0	0	0	0	0
34	7309	0	0	0	0	0	1	0	0	0	0
35	7362	0	0	0	0	0	0	0	1	0	0
36	7400	1	1	1	0	1	1	1	1	1	1
37	7518	1	1	1	0	1	1	1	1	1	1
38	7660	1	0	1	0	1	1	1	0	1	1
39	7785	1	0	1	0	1	1	1	0	0	0
40	8045	1	1	1	0	1	1	1	1	0	0
41	8121	0	1	0	0	0	0	0	0	0	0
42	8192	1	0	1	1	1	1	1	0	1	0
43	8247	1	1	1	0	1	0	1	0	1	1
44	8589	0	0	0	0	1	0	0	0	0	0
45	8685	1	1	1	1	1	1	1	0	1	1
46	8730	1	1	1	1	1	1	0	1	1	1
47	8823	1	1	1	0	1	1	1	1	0	0
48	9082	0	1	1	0	0	1	1	0	1	1
49	9113	1	0	0	1	1	0	0	1	0	0
50	9319	1	1	1	1	1	1	1	1	1	0
51	9800	0	0	1	0	1	1	1	0	1	1
52	9827	0	0	0	1	0	0	0	1	0	0
53	9970	0	0	1	0	0	0	1	0	0	0
54	10085	1	1	0	0	1	1	0	1	1	1
55	10227	0	0	0	1	0	0	1	0	1	1
56	10271	1	1	1	1	1	1	0	1	0	0
57	10417	1	1	1	1	1	1	1	1	1	0

Peak N°	Mass	Bce	Bpi	Bme	Bab	Bsu	Bca	Bov	Bmi	BneK	BneCR
*Bce, <i>B. ceti</i> bmarCR17; Bpi, <i>B. pinnipedialis</i> B2/94; Bme, <i>B. melitensis</i> Rev1; Bab, <i>B. abortus</i> 2308; Bsu, <i>B. suis</i> s2; Bca, <i>B. canis</i> CR12; Bov, <i>B. ovis</i> PA; Bmi, <i>B. microti</i> CCM4915; BneK, <i>B. neotomae</i> 5K33, BneCR, <i>B. neotomae</i> bneohCR2; 0, absence; 1, presence.											

Technical Appendix Table 2. General information and accession numbers of the genomes included in the phylogenetic analysis*

Strain ID	Sample	Host	Country	Accession number
<i>B. abortus</i> 9–941	ND	Bovine	USA	NC_006932.1 & NC_006933.1
<i>B. abortus</i> 2308	Aborted fetus	Bovine	USA	NC_007618.1 & NC_007624.1
<i>B. abortus</i> 2308W	Aborted fetus	Bovine	USA	ERS668782
<i>B. abortus</i> 01–0065	ND	Bison	USA	GCA_000413795.1
<i>B. abortus</i> 104M	Placenta	Cattle	China	GCA_001296965.1
<i>B. abortus</i> 134	Blood	Human	China	GCA_000298635.1
<i>B. abortus</i> CNBG 1432	Blood	Human	Argentina	GCA_000366525.1
<i>B. abortus</i> CNBG 308	Blood	Human	Argentina	GCA_000366545.1
<i>B. abortus</i> 3196	ND	ND	ND	GCA_000740945.1
<i>B. abortus</i> 63/144	ND	Human	France	GCA_000370025.1
<i>B. abortus</i> 63/59	ND	ND	Poland	GCA_000366605.1
<i>B. abortus</i> 63/75	ND	ND	ND	GCA_000740295.1
<i>B. abortus</i> 64/108	ND	Goat	India	GCA_000370085.1
<i>B. canis</i> ATCC 23365	Allantoic fluid of aborted puppy	Dog	ND	NC_010103.1 & NC_010104.1
<i>B. canis</i> HSK A52141	Blood	Dog	South Korea	GCA_000238195.1
<i>B. canis</i> Oliveri	ND	ND	Colombia	GCA_000530495.1
<i>B. canis</i> RM6/66	ND	ND	ND	GCA_000740335.1
<i>B. canis</i> SVA13	Aborted material	Dog	Sweden	GCA_000691585.1
<i>Brucella ceti</i> TE10759–12	Brain and spleen	Striped dolphin	Italy	CP006896 & CP006897
<i>B. ceti</i> TE28753	Brain	Striped dolphin	Italy	CP006898.1 & CP006899.1
<i>B. ceti</i> F23–97	ND	Bottle nose dolphin	France	NZ_AQKR00000000.1
<i>B. melitensis</i> 16M ATCC 23456	ND	Goat	USA	NC_003317.1 & NC_003318.1
<i>B. melitensis</i> ATCC 23457	ND	Goat	Turkey	NC_012441.1 & NC_012442.1
<i>B. melitensis</i> M28	ND	Sheep	Vaccine in China	NC_017244.1 & NC_017245.1
<i>B. melitensis</i> M5–90	Vaccine	Vaccine from M28	China	NC_017246.1 & NC_017247.1
<i>B. melitensis</i> NI	Aborted fetus	Bovine	China	NC_017248.1 & NC_017283.1
<i>B. melitensis</i> bv. 3 str. Ether	ND	ND	ND	NZ_CP007760.1 & NZ_CP007761.1
<i>B. melitensis</i> 20236	ND	ND	ND	NZ_CP008750.1 & NZ_CP008751.1
<i>B. melitensis</i> 2008724259	ND	ND	ND	NZ_CP016983.1 & NZ_CP016984.1
<i>B. melitensis</i> bv. 2 str. 63/9	ND	ND	ND	NZ_CP007789.1 & NZ_CP007788.1
<i>B. microti</i> CCM 4915	Systemic infection	Common voles	Czech Republic	NC_013119.1 & NC_013118.1
<i>B. neotomae</i> 5K33	Pooled tissue: lung, spleen, liver and kidney	Desert wood rat	Utah, USA	JMSC01 GCA_000742255.1
bneohCR1	CSF	Human	Costa Rica	ERS1624467†
bneohCR2	CSF	Human	Costa Rica	ERS1563929†
<i>B. ovis</i> ATCC 25840	Tissue	ND	Australia	ERS1563928†
<i>B. ovis</i> IntaBari-2008–114–542	ND	ND	Argentina	NC_009505.1 & NC_009504.1
<i>B. ovis</i> IntaBari-1993–758	ND	ND	Argentina	GCA_000365985.1
<i>B. ovis</i> IntaBari-2009–88–4	ND	ND	Argentina	GCA_000366005.1
<i>B. ovis</i> F8/05B	ND	ND	Argentina	GCA_000366045.1
<i>Brucella pinnipedialis</i> B2/94	Spleen	Common seal	Scotland	GCA_000367085.1
<i>B. suis</i> 1330	ND	Swine	ND	NC_015857.1 & NC_015858.1
<i>B. suis</i> ATCC 23445	ND	Swine	Denmark	NC_004310.3 & NC_004311.2
				NC_010169.1 & NC_010167.1

Strain ID	Sample	Host	Country	Accession number
<i>B. suis</i> VBI22	Milk	Bovine	USA	NC_016797.1 & NC_016775.1
<i>B. suis</i> bv.1 str. S2	Vaccine	Vaccine in China	China	NZ_CP006961.1 & NZ_CP006962.1
<i>B. suis</i> bv. 2 PT09143	ND	Wild boar	Spain/Portugal	NZ_CP007691.1 &NZ_CP007692.1
<i>B. suis</i> bv. 3 str. 686	ND	ND	ND	NZ_CP007719.1 & NZ_CP007718.1
<i>B. suis</i> bv. 5 str. 513	ND	ND	ND	NZ_DS999724.1
<i>B. suis</i> bv. 4 str. 40	ND	ND	ND	NZ_GG703793.1
<i>B. suis</i> F5/05-4	ND	ND	ND	NZ_KB850877.1
<i>Brucella</i> sp. F5-99	Aborted fetus	Bottle nose dolphin	USA	NZ_ACF00000000

*CSF, cerebrospinal fluid; ND, no data available.

†Isolates and/or WGS described in this study.