The Journal of Veterinary Medical Science

FULL PAPER

Bacteriology

Molecular characterization of *Brucella ceti* from a bottlenose dolphin (*Tursiops truncatus*) with osteomyelitis in the western Pacific

Yuichi UENO[1)#*], Makio YANAGISAWA[2)#], Sayuri KINO[2)], Satoru SHIGENO[3)], Makoto OSAKI[1)], Daisuke TAKAMATSU[1,4)], Ken KATSUDA[1)], Tadashi MARUYAMA[5)] and Kazue OHISHI[6)*]

[1)]Division of Bacterial and Parasitic Disease, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[2)]Okinawa Churashima Foundation, 888 Ishikawa, Kunigamigun Motobucho, Okinawa 905-0214, Japan
[3)]Okinawa Prefectural Institute of Animal Health, 3-1 Kenekadan, Uruma, Okinawa 904-2241, Japan
[4)]The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagito, Gifu, Gifu 501-1193, Japan
[5)]School of Marine Biosciences, Kitasato University, 1-15-1 Kitasato, Minami Ward, Sagamihara, Kanagawa 252-0373, Japan
[6)]School of Engineering, Tokyo Polytechnic University, 1583 Iiyama, Atsugi, Kanagawa 243-0297, Japan

J. Vet. Med. Sci.
82(6): 754–758, 2020
doi: 10.1292/jvms.20-0015

Received: 8 January 2020
Accepted: 14 April 2020
Advanced Epub:
27 April 2020

ABSTRACT. Although the presence of *Brucella* spp. in the western Pacific has been suggested by epidemiological studies on cetaceans, it has not been confirmed by bacterial isolation. Here, for the first time, we report that a marine *Brucella* strain was isolated in the western Pacific from a bottlenose dolphin with osteomyelitis. The isolate from the lesion was confirmed to be *B. ceti* of sequence type 27 by multilocus sequence typing and Bruce-ladder PCR. Infrequent-restriction-site PCR and *omp2* gene sequencing revealed that molecular characteristics of this isolate were similar to those of *Brucella* DNA previously detected from minke whales in the western North Pacific. These results suggest that genetically related *Brucella* strains circulate in cetacean species in this region.

KEY WORDS: bottlenose dolphin, *Brucella ceti*, brucellosis, molecular characterization, western Pacific

Brucellosis is a worldwide zoonotic disease caused by *Brucella* spp. that affects terrestrial and marine mammals, although human cases caused by marine *Brucella* spp. are rare. [7, 8, 18, 19]. Among *Brucella* spp., *B. ceti* and *B. pinnipedialis* have been preferentially isolated from marine mammals, cetaceans, and pinnipeds, respectively [5, 7, 8, 19]. The microbiology and molecular biology of these two marine *Brucella* species differ from those of terrestrial species. Two molecular techniques, infrequent-restriction-site PCR (IRS-PCR) targeting marine *Brucella*-specific DNA fragments [2] and Bruce-ladder multiplex PCR [11] are useful for distinguishing between the marine and terrestrial *Brucella* spp. Furthermore, a recently developed multilocus sequence typing (MLST) scheme targeting 9 or 21 housekeeping genes has systematically classified *Brucella* strains and discriminated between *B. ceti* and *B. pinnipedialis* [18, 19] (PubMLST database: https://pubmlst.org/brucella/). Compared with brucellosis in terrestrial mammals, relatively little pathological evidence has been reported in marine mammals, despite a substantial number of pathological changes and abortions being witnessed in cetaceans [7, 8].

Most *B. ceti* strains have been isolated from cetaceans stranded on coastlines in European and North American waters. In previous epidemiological studies, common minke whales (*Balaenoptera acutorostrata*) in the western North Pacific showed high positive rate of antibodies to *Brucella* spp. [14, 16]. Those sero-positive whales often showed granular testes frequently seen in *Brucella*-infected ruminants [14, 16], and marine *Brucella*-specific DNA was detected from the testicles presenting granulomas by PCR [15]. Though these results strongly suggest that *Brucella* spp. are present in the western North Pacific, the pathogen has not been isolated. In this study, we report the first isolation of *B. ceti* in the western Pacific from a cetacean.

In October 2009, a male bottlenose dolphin (*Tursiops truncatus*) estimated to be 3 years old was captured off the Pacific coast

*Correspondence to: Ueno, Y.: yuueno@affrc.go.jp, Ohishi, K.: cie20910@syd.odn.ne.jp
#These authors contributed equally to this work.

(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

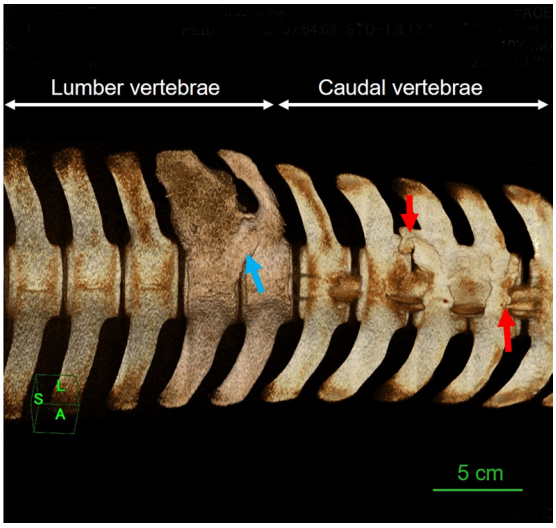

Fig. 1. Computed tomography image of the lesion site (abdominal). Major osteolysis with sequestrum formation (blue arrow) was observed on the transverse process and diapophysis of the second-from-last lumbar vertebra. Minor osteolysis areas were observed around the swelling of the peduncle lesion (red arrows).

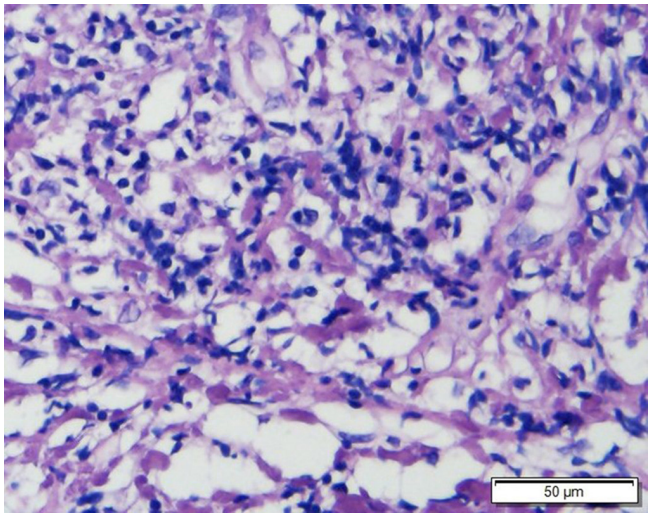

Fig. 2. Histopathological examination of biopsy samples from connective tissue around the lesion. Hematoxylin and eosin staining. Infiltration of inflammatory cell mainly comprising lymphocytes and macrophages was observed.

of Japan (33° 36′ N, 135° 57′ E) under the permission of the Fisheries Agency in Japan. It was transferred to Ocean Expo Park in Okinawa prefecture, where it has remained. During the last decade, leukocyte elevation was detected three times, and medical treatments were conducted each time, although the leukocyte number has remained normal for the past 3 years. Details of these medical treatments and clinical data would be reported in future. A slight swelling appeared on the left side of the waist in May 2018, though the animal was apparently healthy. X-ray and computed tomography examination revealed clear osteolysis with evidence of sequestrum formation. A marked lesion was observed on the second-from-last lumbar vertebra, and some minor lesions were seen on caudal vertebrae (Fig. 1). Biopsy samples from the connective tissue around the lesion were subjected to histopathological examination and bacterial isolation. Histopathological analysis showed inflammatory cell infiltration mainly comprising lymphocytes and macrophages (Fig. 2). According to the image exams and histopathological results, the animal was diagnosed with osteomyelitis. Anti-*Brucella* spp. antibodies in the dolphin serum samples were examined via indirect-ELISA according to a modified method reported previously [1]. The specific antibodies were detected in the dolphin sera collected both on the day of arrival to the park and on the day of the biopsy, indicating that marine *Brucella* spp. infected the dolphin in the western Pacific and remained latent in the dolphin's body. None of the other dolphins living with this dolphin were sero-positive. Captive medical and diagnostic treatments were conducted in accordance with the treatment plans determined by veterinarians with ample experience and approved by Aquarium Business Department of Okinawa Churashima Foundation.

The bacteria isolated from the biopsy specimens formed small, translucent, non-motile, and smooth colonies on tryptic soy agar supplemented with 1% glucose and 5% horse serum after 4 days incubation at 37°C under ambient and 10% CO_2-containing air conditions (Fig. 3A). Bacterial growth was promoted under the CO_2-containing conditions compared with that under ambient conditions. Colonies comprised gram-negative coccobacilli (Fig. 3B) and were judged as *Brucella* species from a biochemical examination using VITEK® 2 System (GN; bioMerieux, Marcy I'Etoile, France). The isolate was designated as BD1442, and its DNA was extracted by InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA). When the 16S rRNA gene sequence of BD1442 determined according to a previous study [9] (accession no. LC496781) was compared with those of other bacterial species using EzBioCloud server (https://www.ezbiocloud.net/) [21], it was found to be identical to those of marine and terrestrial *Brucella* spp., including the type strains of *B. melitensis*, *B. ceti*, and *B. pinnipedialis* (accession nos. AE008918, AM158982, and AM158981, respectively). The isolate BD1442 was analyzed by MLST, targeting 21 housekeeping genes as described in PubMLST database [19], and was identified as sequence type (ST) 27 (Fig. 4); therefore, we identified BD1442 as *B. ceti*. Information on the host animal and type of *B. ceti* BD1442 is summarized in Table 1, together with data on *Brucella* DNA from a minke whale in the western North Pacific, other ST27 strains and representative strains of other ST complexes.

B. ceti strains are composed of three distinct paraphyletic clusters, ST26 complex (ST26, ST55, ST56, and ST103), ST23 complex (ST23, ST50, ST98–ST100, and ST102), and ST27 (Fig. 4). Strains belonging to the ST26 and ST23 complexes appeared to prefer dolphins and porpoises, respectively. The remaining ST27 has some unique features (Table 1): (i) It comprises strains from multiple animal species; cetaceans, pinnipeds, and humans. (ii) The strains have often been associated with marked diseases. In marine mammals, the first ST27 strain was isolated from an aborted fetus of a bottlenose dolphin in California, USA [4, 18]. ST27 has also been found in the strains from human cases with neurobrucellosis [12, 17, 19]. (iii) The strains are geographically associated with Pacific waters, although there is an exceptional case from a dolphin in Croatia [3].

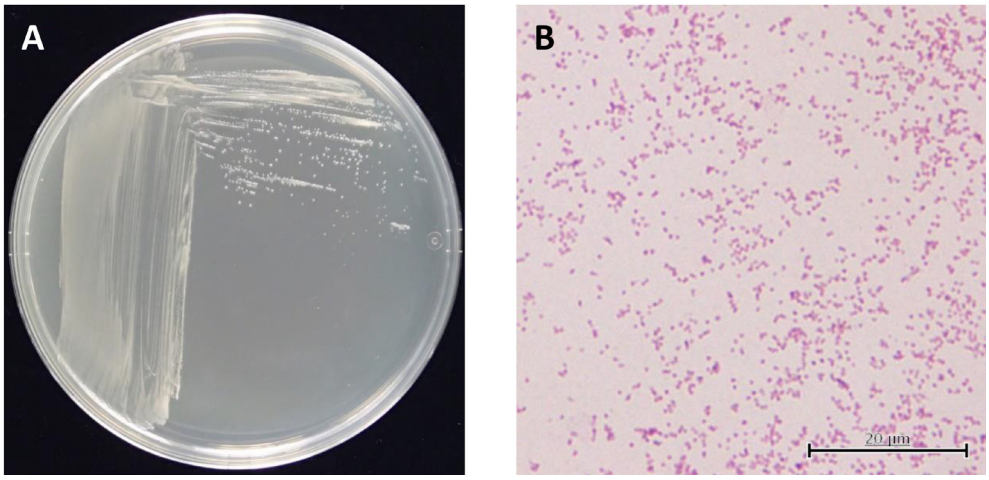

Fig. 3. Bacterial colony (A) and Gram staining (B) of strain BD1442. The strain BD1442 was cultured on tryptic soy agar supplemented with 1% glucose and 5% horse serum for 4 days at 37°C under 10% CO_2-containing air conditions.

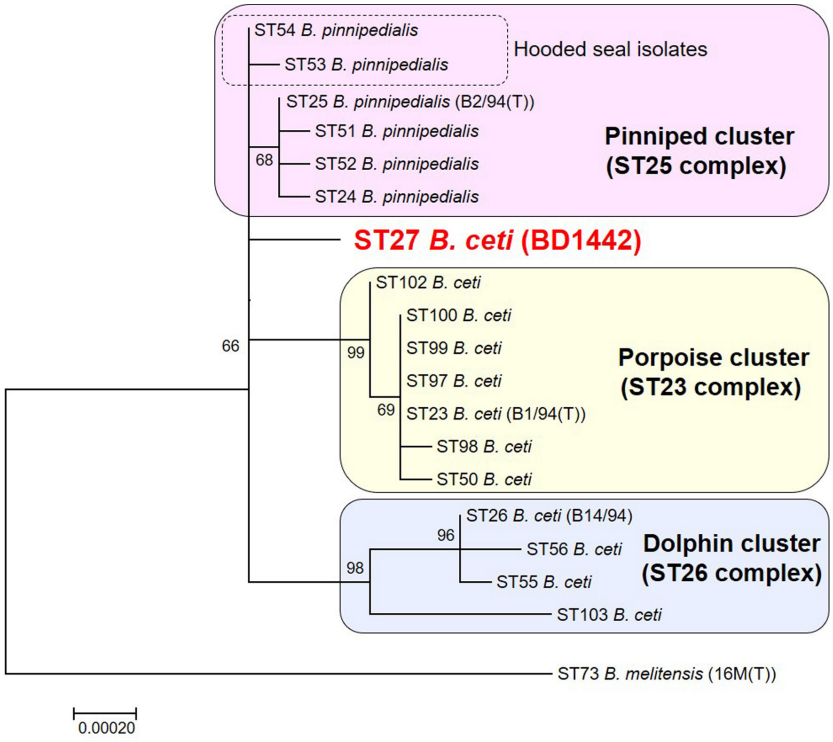

Fig. 4. Phylogenetic relationships of sequence types in marine *Brucella* based on concatenated sequence data of multilocus sequence typing 21. A Neighbor-Joining phylogenetic tree was constructed with concatenated 21 housekeeping gene sequences of marine *Brucella* isolates and the type strain of *B. melitensis*. The position of BD1442 in the tree is shown in red letters. Representative *Brucella* strains are shown in parentheses. The bootstrap values (1,000 replicates) are shown next to the branches. All nucleotide positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted using MEGA7: Molecular Evolutionary Genetics Analysis software, ver. 7.0 [10]. The bar represents substitutions per nucleotide position.

To investigate the relationship between BD1442 and previously detected *Brucella* DNA from minke whales in the western North Pacific [15], BD1442 was subjected to outer membrane protein gene sequencing [15], IRS-PCR [15] and Bruce-ladder multiplex PCR [11] according to the previous studies. IRS-PCR is a method to amplify four marine *Brucella*-specific DNA fragments (fragments I–IV) using four sets of PCR primers [2], whereas Bruce-ladder multiplex PCR can estimate *Brucella* species on the basis of the amplified fragment patterns [11]. PCR conditions and primers used in these molecular analyses are shown in Supplementary Table 1, and the results are summarized in Table 1.

Sequences of the outer membrane protein genes *omp2a* and *omp2b* of BD1442 (accession nos. LC484043 and LC484044, respectively) were identical to those of *Brucella* DNA obtained from Pacific minke whales [15] and some of the other ST27 strains (Table 1). IRS-PCR resulted in a specific product of approximately 300-bp from BD1442 only using primers for fragment I (Fig. 5A), and this was consistent with the results for *Brucella* DNA from Pacific minke whales [15] (Table 1). Using Bruce-ladder multiplex PCR analysis, five DNA fragments, including a 1,320-bp fragment, were amplified from BD1442 (Fig. 5B). The 1,320-bp fragment is known to originate from the marine *Brucella*-specific insertion of IS*711* post *bp26* [11], and the insertion has also been reported in minke whale *Brucella* DNA [15] (Table 1). Furthermore, BD1442 lacked the 794-bp fragment (Fig. 5B), a

Table 1. Comparison of isolate BD1442 with other sequence type (ST) 27 strains and representative *Brucella* strains

Strain[a)]	Species	Country	Sea area	Host	MLST 9	MLST 21	*Omp2* sequence[b)]		IRS-PCR[c)]	Bruce-ladder PCR[d)]	
							Omp2a	*Omp2b*	I/II/III/IV	IS*711* post *bp26*	794 bp fragment
BD1442	*B. ceti*	Japan	Pacific	Bottlenose dolphin	ST27	ST27	100 (LC484043)	100 (LC484044)	+/−/−/−	+	−
JM13/00[e)]		Japan	Pacific	Minke whale	NA	NA	100 (AB126348)	100 (AB126348)	+/−/−/−	+	NA
F5/99	*B. ceti*	USA	Pacific	Bottlenose dolphin	ST27	ST27	100 (DQ865282)	100 (DQ865283)	NA	+	−
F8/08–1	*B. ceti*	USA	Pacific	Bottlenose dolphin	ST27	ST27	NA	NA	+/−/−/−	+	−
F8/08–24	*B. ceti*	USA	Pacific	California sea lion	ST27	ST27	NA	NA	+/−/−/−	+	−
85A05748	*B. ceti*	Peru	Pacific	Human	ST27	ST27	NA[f)]	NA	NA	+	−
01A09163	*B. ceti*	Peru	Pacific	Human	ST27	ST27	NA[f)]	NA	NA	+	−
02/611	*Brucella* sp.	New Zealand	Pacific	Human	ST27	ST27	100 (DQ865280)	100 (DQ865281)	+/−/−/−	+	−
350/1	*B. ceti?*	Croatia	Adriatic sea	Bottlenose dolphin	ST27	NA	NA	NA	NA	+	NA
B1/94 (T)	*B. ceti*	UK, Scotland	Atlantic	Harbor porpoise	ST23	ST23	92.6 (AF300817)	94.9 (AF300816)	−/+/+ /−	+	+
B14/94	*B. ceti*	UK, Scotland	Atlantic	Common dolphin	ST26	ST26	90 (AF300815)	93.1 (AF300814)	−/−/−/+	+	+
B2/94 (T)	*B. pinnipedialis*	UK, Scotland	Atlantic	Common seal	ST25	ST25	100 (AF300819)	94.5 (AF300818)	+ /−/−/−	+	+
16M (T)	*B. melitensis*	USA		Caprine	ST7	ST73	98.6 (AE008917)	96.6 (AE008917)	−/−/−/−	−	+

MLST, multilocus sequence typing; IRS-PCR, infrequent-restriction-site PCR +, PCR positive; −, PCR negative; NA, not available. a) ST27 strains, representatives of each cluster and 16M (T) strain as an outgroup in Fig. 4 are listed. b) The sequence identity (%) to omp2 sequences of BD1442. Accession numbers are shown in parentheses. c) IRS-PCR targeting four fragments (fragment I–IV) reported by Clockaert *et al*. [2]. d) Bruce-ladder multiplex PCR reported by López-Goñi *et al*. [11]. e) Brucella DNA detected in a minke whale [15]. f) Partially determined sequence (519-bp) [17] is identical to that of BD1442.

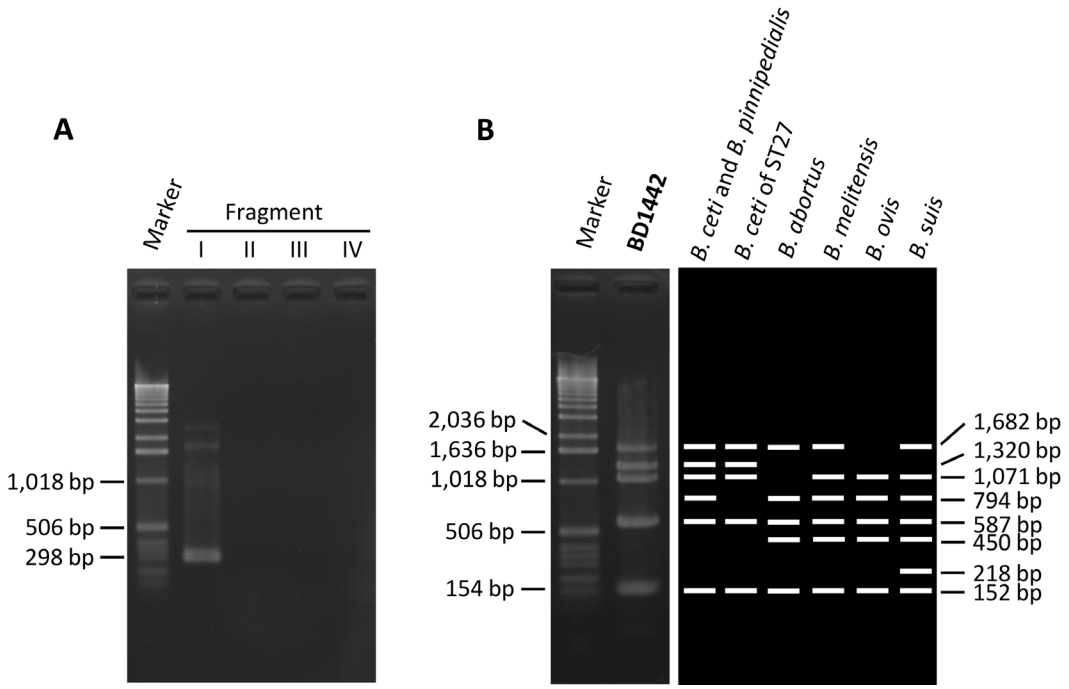

Fig. 5. Agarose gel electrophoresis images of infrequent-restriction-site PCR (A) and Bruce-ladder multiplex PCR (B) products of strain BD1442. (A) PCR products of fragments I–IV are shown. (B) PCR products of BD1442 are compared with the reported fragment patterns of other *Brucella* spp. shown in the diagram [11, 20].

characteristic feature of ST27 strains [20]. These results reinforced the MLST analysis results (Table 1) and strongly suggest that BD1442 is genetically close to *Brucella* DNA from the Pacific minke whale and other reported ST27 strains.

In this study, for the first time, we reported on the isolation of *B. ceti* in the western Pacific from a dolphin with osteomyelitis. We cannot conclude that the isolate directly caused osteomyelitis to the dolphin. However, similar osteomyelitis has been reported in another bottlenose dolphin infected with *Brucella* spp. [6]. It is important to further examine the relationship between the clinical signs and *Brucella* infection. DNA analysis showed that molecular characteristics of the isolate were closely related to *Brucella* DNA from minke whales in the western North Pacific. Although isolation from minke whales has not been successful [15], genetically related *Brucella* strains may circulate among species of cetacean in the western North Pacific. Virulence of these strains in cetaceans is not fully understood, however, reproductive disorders and neurologic diseases in cetaceans caused by *B. ceti* may significantly affect the ecology and population dynamics of cetaceans [7, 8]. Although there have been no reports of zoonosis in the western Pacific [13, 16], continuous monitoring is important for understanding the threat brucellosis poses to both marine mammals and humans.

REFERENCES

1. Abe, E., Ohishi, K., Ishinazaka, T., Fujii, K. and Maruyama, T. 2017. Serologic evidence of *Brucella* infection in pinnipeds along the coast of Hokkaido, the northernmost main island of Japan. *Microbiol. Immunol.* **61**: 114–122. [Medline] [CrossRef]
2. Cloeckaert, A., Grayon, M., Grépinet, O. and Boumedine, K. S. 2003. Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site-PCR and development of specific PCR identification tests. *Microbes Infect.* **5**: 593–602. [Medline] [CrossRef]
3. Cvetnić, Ž., Duvnjak, S., Đuras, M., Gomerčić, T., Reil, I., Zdelar-Tuk, M. and Špičić, S. 2016. Evidence of *Brucella* strain ST27 in bottlenose dolphin (*Tursiops truncatus*) in Europe. *Vet. Microbiol.* **196**: 93–97. [Medline] [CrossRef]
4. Ewalt, D. R., Payeur, J. B., Martin, B. M., Cummins, D. R. and Miller, W. G. 1994. Characteristics of a *Brucella* species from a bottlenose dolphin (*Tursiops truncatus*). *J. Vet. Diagn. Invest.* **6**: 448–452. [Medline] [CrossRef]
5. Foster, G., Osterman, B. S., Godfroid, J., Jacques, I. and Cloeckaert, A. 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int. J. Syst. Evol. Microbiol.* **57**: 2688–2693. [Medline] [CrossRef]
6. Goertz, C. E., Frasca, S. Jr., Bohach, G. A., Cowan, D. F., Buck, J. D., French, R. A., De Guise, S., Maratea, J., Hinckley, L., Ewalt, D., Schlievert, P. M., Karst, S. M., Deobald, C. F., St Aubin, D. J. and Dunn, J. L. 2011. *Brucella* sp. vertebral osteomyelitis with intercurrent fatal *Staphylococcus aureus* toxigenic enteritis in a bottlenose dolphin (*Tursiops truncatus*). *J. Vet. Diagn. Invest.* **23**: 845–851. [Medline] [CrossRef]
7. Guzmán-Verri, C., González-Barrientos, R., Hernández-Mora, G., Morales, J. A., Baquero-Calvo, E., Chaves-Olarte, E. and Moreno, E. 2012. *Brucella ceti* and brucellosis in cetaceans. *Front. Cell. Infect. Microbiol.* **2**: 3. [Medline] [CrossRef]
8. Hernández-Mora, G., Palacios-Alfaro, J. D. and González-Barrientos, R. 2013. Wildlife reservoirs of brucellosis: *Brucella* in aquatic environments. *Rev. - Off. Int. Epizoot.* **32**: 89–103. [Medline]
9. Hiraishi, A., Shin, Y. K., Ueda, Y. and Sugiyama, Y. 1994. Automated sequencing of PCR-amplified 16S rDNA on 'Hydrolink' gels. *J. Microbiol. Methods* **19**: 145–154. [CrossRef]
10. Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870–1874. [Medline] [CrossRef]
11. López-Goñi, I., García-Yoldi, D., Marín, C. M., de Miguel, M. J., Muñoz, P. M., Blasco, J. M., Jacques, I., Grayon, M., Cloeckaert, A., Ferreira, A. C., Cardoso, R., Corrêa de Sá, M. I., Walravens, K., Albert, D. and Garin-Bastuji, B. 2008. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. *J. Clin. Microbiol.* **46**: 3484–3487. [Medline] [CrossRef]
12. McDonald, W. L., Jamaludin, R., Mackereth, G., Hansen, M., Humphrey, S., Short, P., Taylor, T., Swingler, J., Dawson, C. E., Whatmore, A. M., Stubberfield, E., Perrett, L. L. and Simmons, G. 2006. 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.* **44**: 4363–4370. [Medline] [CrossRef]
13. Ohishi, K., Fujise, Y. and Maruyama, T. 2008. *Brucella* spp. in the western North Pacific and Antarctic cetaceans: a review. *J. Cetacean Res. Manag.* **10**: 67–72.
14. Ohishi, K., Bando, T., Abe, E., Kawai, Y., Fujise, Y. and Maruyama, T. 2016. Long-term and large-scale epidemiology of *Brucella* infection in baleen whales and sperm whales in the western North Pacific and Antarctic Oceans. *J. Vet. Med. Sci.* **78**: 1457–1464. [Medline] [CrossRef]
15. Ohishi, K., Takishita, K., Kawato, M., Zenitani, R., Bando, T., Fujise, Y., Goto, Y., Yamamoto, S. and Maruyama, T. 2004. Molecular evidence of new variant *Brucella* in North Pacific common minke whales. *Microbes Infect.* **6**: 1199–1204. [Medline] [CrossRef]
16. Ohishi, K., Zenitani, R., Bando, T., Goto, Y., Uchida, K., Maruyama, T., Yamamoto, S., Miyazaki, N. and Fujise, Y. 2003. Pathological and serological evidence of *Brucella*-infection in baleen whales (Mysticeti) in the western North Pacific. *Comp. Immunol. Microbiol. Infect. Dis.* **26**: 125–136. [Medline] [CrossRef]
17. Sohn, A. H., Probert, W. S., Glaser, C. A., Gupta, N., Bollen, A. W., Wong, J. D., Grace, E. M. and McDonald, W. C. 2003. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. *Emerg. Infect. Dis.* **9**: 485–488. [Medline] [CrossRef]
18. Whatmore, A. M. 2009. Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. *Infect. Genet. Evol.* **9**: 1168–1184. [Medline] [CrossRef]
19. Whatmore, A. M., Koylass, M. S., Muchowski, J., Edwards-Smallbone, J., Gopaul, K. K. and Perrett, L. L. 2016. Extended multilocus sequence analysis to describe the global population structure of the genus *Brucella*: phylogeography and relationship to biovars. *Front. Microbiol.* **7**: 2049. [Medline] [CrossRef]
20. Whatmore, A. M., Dawson, C., Muchowski, J., Perrett, L. L., Stubberfield, E., Koylass, M., Foster, G., Davison, N. J., Quance, C., Sidor, I. F., Field, C. L. and St Leger, J. 2017. Characterisation of North American *Brucella* isolates from marine mammals. *PLoS One* **12**: e0184758. [Medline] [CrossRef]
21. Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H. and Chun, J. 2017. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **67**: 1613–1617. [Medline] [CrossRef]