

Long-term and large-scale epidemiology of *Brucella* infection in baleen whales and sperm whales in the western North Pacific and Antarctic Oceans

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ABSTRACT. In a long-term, large-scale serologic study in the western North Pacific Ocean, anti-*Brucella* antibodies were detected in common minke whales (*Balaenoptera acutorostrata*) in the 1994–2010 offshore surveys (21%, 285/1353) and in the 2006–2010 Japanese coastal surveys (20%, 86/436), in Bryde's whales (*B. edeni brydei*) in the 2000–2010 offshore surveys (9%, 49/542), in sei whales (*B. borealis*) in the 2002–2010 offshore surveys (5%, 40/788) and in sperm whales (*Physeter macrocephalus*) in the 2000–2010 offshore surveys (8%, 4/50). Anti-*Brucella* antibodies were not detected in 739 Antarctic minke whales (*B. bonaerensis*) in the 2000–2010 Antarctic surveys. This suggests that *Brucella* was present in the four large whale populations inhabiting the western North Pacific, but not in the Antarctic minke whale population. By PCR targeting for genes of outer membrane protein 2, the *Brucella* infection was confirmed in tissue DNA samples from Bryde's whales (14%, 2/14), sei whales (11%, 1/9) and sperm whales (50%, 2/4). A placental tissue and an apparently healthy fetus from a sperm whale were found to be PCR-positive, indicating that placental transmission might have occurred and the newborn could act as a bacterial reservoir. Marked granulomatous testes were observed only in mature animals of the three species of baleen whales in the western North Pacific offshore surveys, especially in common minke whales, and 29% (307/1064) of total mature males had abnormal testes. This study provides an insight into the status of marine *Brucella* infection at a global level.

KEY WORDS: antibody, *Brucella*, testis, whale

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Members of the genus *Brucella* are gram-negative intracellular bacteria causing brucellosis with reproductive disorders including abortion in mammals [1, 6]. In terrestrial mammals, *Brucella melitensis*, *B. abortus*, *B. ovis*, *B. canis*, *B. suis*, *B. neotomae*, *B. microti*, *B. inopinata* and *B. papionis*, are currently identified [1, 6, 36]. Among them, *B. melitensis*, *B. abortus* and *B. suis* have been frequently reported to be zoonotic [5].

Since the first isolation of *Brucella* sp. from marine mammals [8, 29], many isolates have been reported from various species, mainly along European coasts [11, 13]. These isolates from marine mammals have microbiological and molecular characteristics distinct from those of land mammals [11, 13]. Based on their preferred hosts, the marine isolates are classified as *B. ceti* and *B. pinnipedialis* at present [10]. However, it should be noted that the isolated strains are geographically biased [11, 13] and that bacterial isolation from wider sea areas, including the Pacific Ocean and the Southern hemisphere, is mandatory for understanding brucellosis in marine mammals worldwide. As *B. ceti* has been isolated from placentas and aborted fetuses in captive [20] and stranded dolphins [12, 14], they are also thought to have the potential to induce abortion in dolphins, similar to

what has been observed in terrestrial animals infected with *Brucella* [6].

Extensive *Brucella* sero-surveys have been performed on cetaceans in the sea around Europe [15, 32], North America [23], South America [33] and South Central Pacific [31]. The serologic studies and numerous bacterial isolations from cetaceans showed that bacterial infection was prevalent in various cetacean species in many oceans [11, 13]. We previously reported an epidemiological study of *Brucella* infection in the western North Pacific in 2000 and in the Antarctic Sea in 2000/2001 where anti-*Brucella* antibodies were found in 38% (15/40) of the common minke whales (*Balaenoptera acutorostrata*) and 9% (4/43) of the Bryde's whales (*B. edeni brydei*) inhabiting the western North Pacific [25]. In contrast, no anti-*Brucella* antibodies were detected in the 104 Antarctic minke whales (*B. bonaerensis*) examined [25]. We found marked granulomatous testes in 48% (13/27) of the mature male minke whales in the western North Pacific. Anti-*Brucella* antibodies were detected in 62% (8/13) of the mature whales with the abnormal testes [25]. Such lesions in testes are common in *Brucella*-infected ruminants [28]. However, they have rarely been observed in *Brucella*-infected marine mammals inhabiting waters around Europe and North and South America [9, 11, 13] except in one study [7].

In the present study, we conducted a large-scale serologic investigation of whales in the western North Pacific and Antarctic Oceans from 1994 to 2010. For the confirmation of *Brucella* infection, PCR targeting outer membrane protein 2 genes was performed. We report the distribution of antibodies against *Brucella* in large Pacific whales and propose a hypothesis on the transmission and maintenance of this

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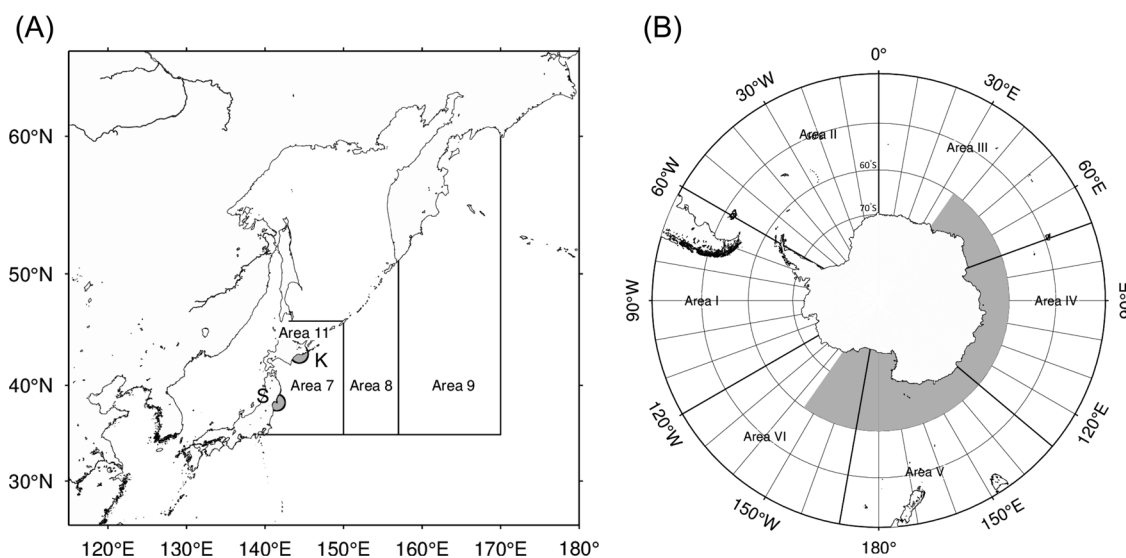


Fig. 1. Sampling sites in the western North Pacific (A) and Antarctic (B) Oceans. Numbered areas on the map denote the International Whaling Commission Research subarea numbers. The two gray zones labeled S and K in (A) are the research areas of the JARPN II coastal component off Sanriku and Kushiro, respectively. The gray zone in (B) is research area of JARPA/JARPA II.

bacterium in the investigated whale species.

MATERIALS AND METHODS

Samples: Common minke whales, Bryde's whales, sei whales (*B. borealis*) and sperm whales (*Physeter macrocephalus*) were captured legally under the Japanese Whale Research Program in the western North Pacific (JARPN) during 1994–1999, and in the offshore (2000–2010) and the coastal (2006–2010) components of JARPN II. Antarctic minke whales and fin whales (*B. physalus*) were taken under the Japanese Whale Research Program under Special Permit in the Antarctic (JARPA, 2000/01–2004/05 and JARPA II, 2005/06–2009/10). These programs were authorized by the Government of Japan and are in full compliance with the relevant international treaty, the International Convention for the Regulation of Whaling. The sampling sites are shown in Fig. 1.

Blood samples from the captured dead whales were obtained from their veins on the flippers or upper jaws, or by partially cutting their flukes or tip of upper jaws. The blood was centrifuged ($1,458 \times g$, 10 min), and the separated sera were immediately frozen at -80°C for the offshore researches or put in liquid nitrogen for the coastal researches. Thereafter, they were stored at -50°C until further analysis. Serum samples from 1,353 common minke whales, 542 Bryde's whales, 788 sei whales and 50 sperm whales, collected offshore, and sera from 229 and 207 common minke whales, respectively, collected at Sanriku and Kushiro in the coastal researches, were included in the study. Serum samples from 739 Antarctic minke whales and 12 fin whales were also used for the test. The maturity of males was determined by body length based on previous reports in common minke whales [16], sei whales [19], Bryde's whales [17],

sperm whales [24] and Antarctic minke whales [2]. The maturity of females was determined by the presence of at least one corpus luteum or corpus albicans in the ovaries [18].

Four tissue samples (three placentas and one fetus spleen) from three sperm whales collected in 2001, fourteen tissues (twelve placentas and two fetus spleens) from twelve Bryde's whales collected in 2001 and 2002, and nine tissues (five testes, two placentas and two fetal spleens) from seven sei whales collected in 2002, were used for detection of *Brucella* DNA. Autopsy was performed on a research vessel immediately after the capture, and the collected tissue samples were stocked at -80°C . Because abnormal testes were not very frequently observed in these whales, and because placental and spleen tissues are generally known to be *Brucella*-susceptible tissues [13], these tissues were also used in addition to testes. No pathological changes were observed in the used tissues. Among the whales, of which tissue DNAs were examined, two sperm whales and two sei whales were antibody-positive by agglutination test. A sperm whale, four Bryde's whales and five sei whales were in an intermediate zone between positive and negative.

Detection of anti-*Brucella* antibodies in serum samples: Anti-*Brucella* serum antibody was detected in the agglutination test using commercially available inactivated *B. abortus* strain 125 (Kaketsuken Co., Kumamoto, Japan) as described previously [25]. The sera showing more than 30 international units (IU) and 15–30 IU of agglutinins in the test were regarded as positive and intermediate, respectively. The specificity of the serum antibodies to *Brucella* antigen was examined in a standard Western blot analysis. Anti-*Brucella* antibody positive sera from five common minke whales, four Bryde's whales, four sei whales and two sperm whales, were examined. Sera from four seronegative Antarctic minke whales and from a *B. abortus* infected cow, were used as

Table 1. Profiles of antibody-positive whales inhabiting the western North Pacific, based on sex, maturity and sea area

		Offshore (JARPN, JARPN II)				Coastal (JARPN II)	
		Common minke whale 1994–2010 ^(c)	Bryde's whale 2000–2010 ^(c)	Sei whale 2002–2010 ^(c)	Sperm whale 2000–2010 ^(c)	Common minke whale (S) ^(a) 2006–2010 ^(c)	Common minke whale (K) ^(b) 2006–2010 ^(c)
Sex	Male	21% (251/1,180)	10% (24/238)	6% (22/370)	7% (1/14)	15% (14/93)	24% (33/136)
	Female	20% (34/173)	8% (25/304)	4% (18/418)	8% (3/36)	18% (25/136)	20% (14/71)
Maturity	Mature	20% (236/1,162)	6% (22/376)*	4% (25/590)	10% (4/39)	19% (9/47)	22% (14/65)
	Immature	26% (49/191)	16% (27/166)*	8% (15/198)	0% (0/11)	16% (30/182)	23% (33/142)
Area	7	21% (103/481)	8% (17/209)	8% (2/26)	9% (2/23)	–	–
	8	26% (64/246)	8% (11/146)	5% (10/210)	0% (0/10)	–	–
	9	20% (108/546)	11% (21/187)	5% (28/552)	12% (2/17)	–	–
	11	13% (10/80)	–	–	–	–	–
Total		21% (285/1,353)	9% (49/542)	5% (40/788)	8% (4/50)	17% (39/229)	23% (47/207)

Percentages were calculated as antibody-positive individuals in the total examined. The coastal component of JARPN II was performed at Sanriku (a) and Kushiro (b). (c) Study period. –, No samples collected. * the statistically significant difference at 95% CI.

negative and positive controls, respectively. Commercially available *B. abortus* (strain 125) and *B. canis* (strain QE-13B) (Kitasato Institute Co., Tokyo, Japan) were dissolved in sample buffer (2% sodium dodecyl sulfate [SDS], dithiothreitol 770 mg and 4.5 ml of 0.5 M Tris-HCl, pH 6.8, in 10 ml of phosphate-buffered saline [PBS]). The bacterial proteins (20 µg/lane) were separated on 10% polyacrylamide gel by SDS polyacrylamide gel electrophoresis. The separated proteins on the gel were then blotted onto a polyvinylidene difluoride membrane (Millipore Co., Billerica, MA, U.S.A.). After blocking with Block Ace solution (4 µg/100 ml distilled water: Yukijirushi Co., Tokyo, Japan), the membrane was reacted with whale sera diluted 1:100. After washing three times with PBS containing 0.1% Tween-20, the membrane was reacted with horseradish peroxidase-conjugated Protein A/G diluted 1:5,000 (ThermoFisher Scientific Co., Waltham, MA, U.S.A.). The bands were visualized by color development using diaminobenzidine (DAB) solution (a tablet containing 80mg DAB in 50 ml of 0.05 M Tris-Cl pH8.0) (Wako Pure Chemical Industries, Osaka, Japan) and 30 µl 30% H₂O₂.

Molecular identification of *Brucella* in whale tissues: Polymerase chain reaction (PCR) targeting *omp2a* and *omp2b* was conducted using tissue DNA samples. DNA was extracted from the tissue samples using a commercial DNA extraction kit (DNeasy Tissue Kit; Qiagen, Boston, MA, U.S.A.) and amplified by PCR using a commercial PCR kit (Ex Taq Kit; Takara, Kyoto, Japan). As positive controls, we used DNA samples from granulomatous testes in common minke whales, in which *Brucella* DNA had previously been detected [26]. The following specific primer sets were used for PCR: for *omp2a*, A1 (forward primer; 5'-GGCT ATTCAAAATTCTGGCG-3') and A2 (reverse primer), 5'-TATGCTTAACGTCGATCTGA-3'); for *omp2b*, B1 (forward primer, 5'-CCTTC AGCCAAATCAGAATG-3') and B2 (reverse primer, 5'-TGTATCCAAT CCGTAATGGA-3'). A 1 µM concentration of each primer was included in amplification reaction mixtures. Amplification was carried out in a thermal cycler (TC-312W: Techne Ltd., Staffordshire, U.K.) under the conditions: 96°C for 1 min; 30 cycles of 96°C for

20 sec, 57°C for 30 sec and 72°C for 1 min; followed by a final extension at 72°C for 10 min. When we obtained only a faint band, we repeated the PCR again with 35 cycles. The amplified DNA fragments were purified using a spin column (Wizard SV Gel and PCR Clean-up Kit; Promega Inc., Madison, WI, U.S.A.). Nucleotide sequences were analyzed by dye terminator method with the same primer sets as those for PCR, using a commercial kit (BigDye Terminator v3.1 Cycle sequencing kit: Thermo Scientific Inc.). After purification with filtration cartridge (DTR Gel filtration cartridge: EdgeBio Inc., Gaithersburg, MD, U.S.A.), the sequences were determined using a sequencer (3,130 × 1 Genetic analyzer: Applied Biosystems Inc., Life Technologies, Carlsbad, CA, U.S.A.).

Statistic analysis: Statistical significance was determined by overlapping of confidence interval (CI). That was calculated based on mean, standard deviation and sample size for desired confidence level of 95%, using Microsoft Excel, Confidence function.

RESULTS

Long-term changes in serum anti-*Brucella* antibodies in three species of baleen whales and sperm whales in the western North Pacific: In common minke whales, a high prevalence (9–38%) of anti-*Brucella* serum antibodies was detected in each year examined from 1994 to 2010 (Table 1, Fig. 2A). In Bryde's whales (during 2000–2010) and sei whales (during 2002–2010), anti-*Brucella* antibodies were also detected in every examined year, but with a lower prevalence of 2–20% and 1–10%, respectively (Table 1, Fig. 2B and 2C). Although the number of serum samples from sperm whales was limited, 4 of the total 50 serum samples collected between 2000 and 2010 were antibody positive (Table 1). No significant difference in antibody prevalence was found between male and female, between mature and immature, and among the inhabited sea areas. However, immature Bryde's whales showed significantly higher percentages of antibody-positive individuals (average 16%, 95% CI:11–23%) than mature whales (average 6%, 95% CI:4–9%) (Table 1). Anti-

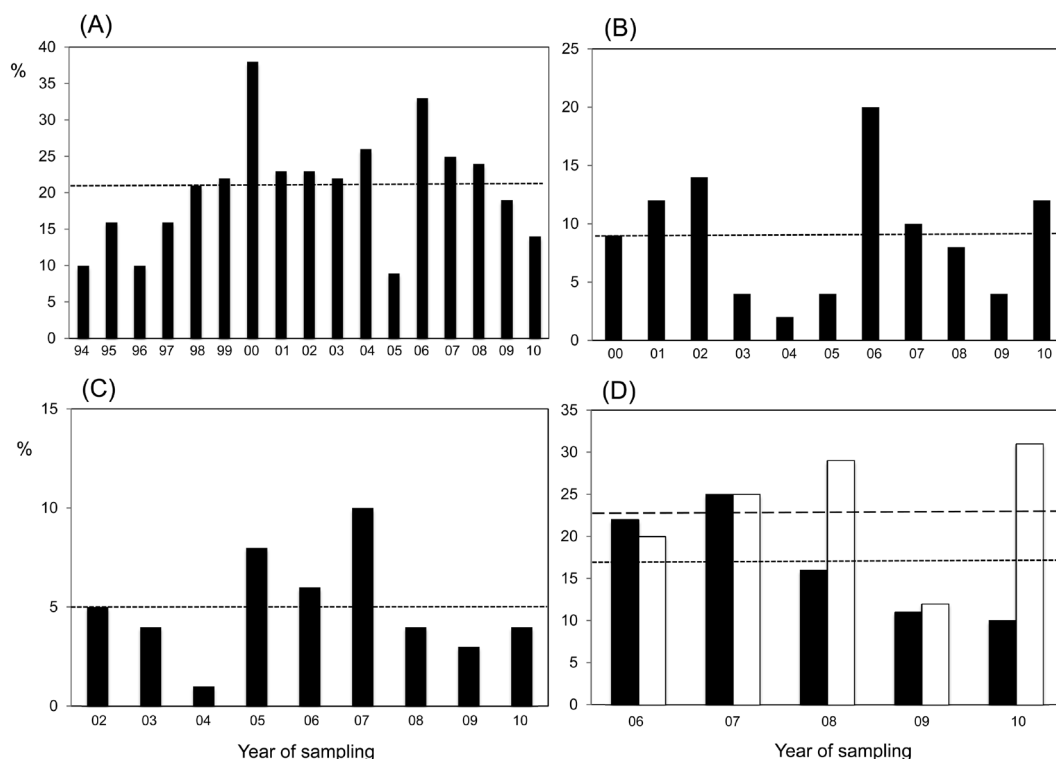


Fig. 2. Long-term changes in anti-*Brucella* antibodies in baleen whales inhabiting the western North Pacific. (A), common minke whale; (B), Bryde's whale; (C), sei whale from the offshore component; and (D), common minke whale from the coastal component of the JARPN II. Horizontal axis, year of sampling. Vertical axis, percentage of antibody-positive individuals among total whales examined. In (D), black bars, percentage at Sanriku; white bars, percentage at Kushiro. Dotted lines in (A)–(C), average percentage during the study periods; dotted line and dashed line in (D), average percentage at Sanriku and Kushiro, respectively. Examined serum sample numbers are, (A): 21 (94), 100 (95), 77 (96), 100 (97), 100 (98), 100 (99), 40 (00), 100 (01), 100 (02), 100 (03), 100 (04), 100 (05), 99 (06), 100 (07), 59 (08), 43 (09), 14 (10), 1353 (total), (B): 43 (00), 50 (01), 49 (02), 50 (03), 50 (04), 50 (05), 50 (06), 50 (07), 50 (08), 50 (09), 50 (10), 542 (total), (C): 39 (02), 50 (03), 100 (04), 100 (05), 99 (06), 100 (07), 100 (08), 100 (09), 100 (10), 788 (total), (D): Sanriku 46 (06), 52 (07), 45 (08), 45 (09), 41 (10), 229 (total), Kushiro 25 (06), 48 (07), 35 (08), 57 (09), 42 (10), 207 (total).

Brucella antibodies were also detected in common minke whales collected in the coastal component of JARPN II at Sanriku and Kushiro during 2006–2010 (Table 1, Fig. 2D). Their antibody prevalence (10–25% at Sanriku and 12–31% at Kushiro) was similar to that found in the offshore of common minke whales (Table 1, Fig. 2A and 2D).

After assessment in the agglutination test, the results were confirmed by Western blot analysis using agglutination-positive sera from each whale species. While all of the examined agglutination test-positive sera showed the similar band patterns, the results of one serum sample from each of 3 whale species are shown in Fig. 3. Many bands of the *B. abortus* antigens were found (lanes 1, 5, 7, 9 and 12), while few, much weaker bands were detected against *B. canis* antigens (lanes 2, 6, 8, 10 and 11). No band was observed in the sera from the agglutination test-negative Antarctic minke whales (lanes 3 and 4). These results indicate that the antigenicities of the Pacific cetacean *Brucella* strains are closer to those of *B. abortus*, but different from those of *B. canis* (Fig. 3).

Appearance of abnormal testes in common minke whales in the western North Pacific: As markedly granulomatous

testes with caseation and mineralization were found in many mature common minke whales in the 2000 survey [25], we carefully examined the presence or absence of such lesions. Similar lesions were found in a substantial number of common minke whales in each year of the study period (Figs. 4 and 5). Abnormal testes were found only in mature males throughout the survey period. In common minke whales, 29% (307/1,064) of total mature males had abnormal testes. In Bryde's whales, similar testis lesions were found in 12 out of 239 (5%) males examined in the study period. Although 2 of the 12 whales with lesions were judged to be immature from their body length, they were on the borderline of maturity. In 371 male sei whales, only one abnormal testis was observed in a mature male. Thus, abnormal testes were almost always found in mature whales, although anti-*Brucella* antibodies were detected both in mature and immature animals.

In common minke whales, anti-*Brucella* antibodies appeared in 116 among 307 mature males with abnormal testes (average 38%, 95% CI: 32–44%), whereas they appeared in 105 among 757 mature males with normal testes (average 14%, 95% CI: 12–17%). This indicated that the appearances

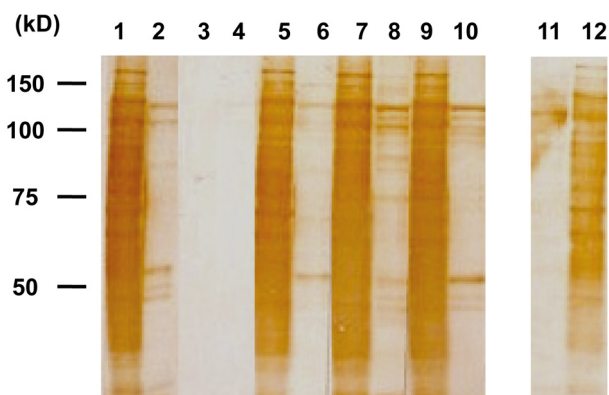


Fig. 3. Western blot analysis of antibodies to *Brucella* antigens in whale serum samples. The electrophoresed *B. abortus* (lanes 1, 3, 5, 7 and 9) and *B. canis* (lanes 2, 4, 6, 8 and 10) (approximately 20 μ g protein /lane) antigens under reduced conditions were reacted with agglutination test-positive whale serum samples diluted 1:100 from common minke whale (lanes 1 and 2), Antarctic minke whale (lanes 3 and 4), Bryde's whale (lanes 5 and 6), sei whale (lanes 7 and 8), and sperm whale (lanes 9 and 10). As a control, *B. abortus* infected cow serum was reacted to *B. canis* (lane 11) and *B. abortus* (lane 12). Peroxidase-conjugated Protein A/G diluted 1:5,000, was used for detection.

of granulomatous testes and of anti-*Brucella* antibodies were related. In the twelve Bryde's whales with the granulomatous testes, only one was shown to be antibody-positive, and eleven were negative. The sei whale with the abnormal testis was antibody-negative.

We also observed similar lesions in female gonads in a very limited number of common minke whales, Bryde's whales and sei whales, as we previously reported in the 2000 survey [25]. The pathology of the female gonads will be reported elsewhere.

Absence of serum anti-Brucella antibody and granuloma lesions in Antarctic minke whales and fin whales inhabiting the Antarctic Sea: No anti-*Brucella* antibody was detected in a total of 739 serum samples from Antarctic minke whales and 12 serum samples from fin whales collected under JARPA and JARPA II during 2000/01–2009/10. In addition, no abnormal gonads with granulomatous lesions were found in these whales.

Brucella DNA in tissues from whales in the western North Pacific: Presence of *Brucella* DNA was confirmed by PCR targeting *Brucella omp2a* and *omp2b* genes using tissue DNA samples from Bryde's whales, sei whales and sperm whales. We successfully amplified and determined the sequences of the *omp2a* and *omp2b* genes in two placental tissues (JARPN II/2001:B13 and B39) of Bryde's whales, and in a placental and the fetal spleen tissues from a sperm whale (JARPN II/2001: S03). Only the *omp2a* gene was successfully detected in a testis sample (JARPN II/2002: SE13) from a sei whale. All of the PCR-positive tissues were originated from whales, of which anti-*Brucella* antibody titers were in an intermediate zone between positive and negative by the agglutination test, although they were regarded as se-



Fig. 4. Appearance of granulomatous testes in mature common minke whales. Typical abnormal testes were shown (M090, JARPNII 2006). Many and large granulomas (arrows) were observed in the right testis, whilst only small ones were seen in the left testis. L: left, R: right.

ronegative in the present study. The nucleotide sequences of amplified DNA fragments were identical between those from two Bryde's whales, and they were also identical between those from maternal placenta and from fetal spleen samples of the sperm whale. The nucleotide sequences with accession numbers were deposited with the DNA Data Bank of Japan. The *omp2a* sequences from a sperm whale (LC032015), a Bryde's whale (LC032017) and a sei whale (LC032018), were identical to that of common minke whales (AB126348) [26]. They were also identical to the recently reported 500-bp partial sequence of *Brucella omp2a* from the sperm whale neonate stranded on Hawaii [35].

The sequences of *omp2b* from a sperm whale (LC032014) and a Bryde's whale (LC032016) were identical. Blast search in databases, DDBJ/GenBank/EMBL, showed that they have higher nucleotide identity (99.5%) to that of *B. melitensis* strain 16M (AE009569), than those of common minke whale *Brucella* (AB126348) (96.1%) and of *B. pinnipedialis* B2/94 strain (AF300818) (94.9%).

DISCUSSION

The present study showed that anti-*Brucella* antibodies were stably maintained for more than 10 years in three species of baleen whales and sperm whales inhabiting the western North Pacific Ocean (Table 1, Fig. 2). These findings suggest that a stable *Brucella* transmission occurred in these whales. Anti-*Brucella* antibodies were continually detected in sera from immature animals in the three species of baleen whales. Western blot analysis showed that antigenicities of *Brucella* in the three baleen whales and sperm whale were close to *B. abortus*, but dissimilar to *B. canis* (Fig. 3). Previous studies have reported that the smooth colony types of *B. abortus* and *B. ceti* have lipopolysaccharides (LPS) containing O-type polysaccharide in outer membrane,

whilst the rough colony type of *B. canis* has LPS without it [23, 38]. Although the LPS compositions of *Brucella* in the large whales inhabiting the western North Pacific are not known, results from the current study suggest that they are smooth types, like as *B. ceti* reference strain [9] and a North Atlantic minke whale isolate [4]. This indicates that the conventional agglutination test, the ruminant *Brucella* diagnosis kit used in the present study, is valid for the detection of anti-*Brucella* antibodies in the Pacific whales. To identify the *Brucella* species in the Pacific large whales, the bacterial isolation and the characterization are mandatory. Previous PCR analysis using DNA extracted from granulomatous testes of common minke whales showed that the Pacific whale *Brucella* possessed marine *Brucella* specific sequences and the insertion of transposon, and showed higher similarity to *B. pinnipedialis* rather than to *B. ceti* [26]. The detailed studies of the Pacific whale *Brucella* will give an important insight into the bacterial evolution and the status of marine *Brucella* infection at a global level.

We detected *Brucella* DNA in the maternal placental and fetal tissues in one sperm whale sample. This fetus was apparently healthy, with a body length of approximately 3 m, and estimated to be 11 months old according to the method of Best [3]. This is an evidence of a transplacental transmission of *Brucella* in a large whale, and its size strongly suggested that the fetus could have been delivered alive and could possibly have acted as a reservoir of *Brucella*. Recently, a similar case has been reported in a neonate sperm whale stranded on a coast of Hawaii estimated to be only 1–2 days old. From several tissues of the neonate whale, *Brucella* was detected by cultivation and PCR [35]. These data together indicate that mother-to-calf transmission via the placenta or milk may take place in large whales. The tropism of *Brucella* to the placenta and fetus in marine mammals has been shown in captive [20] and stranded dolphins [12, 14]. *Brucella* has previously been detected in the milk and mammary glands of stranded dolphins [12, 14], however, these were not examined in the present study. Though the abortion rate and overall health of *Brucella*-positive whale progenies are not known, it is noteworthy that, in a bovine model, experimental infection of *B. abortus* shortly before parturition showed that 38% of the animals delivered healthy calves [37]. The bacteria were isolated from the lung and spleen in more than 30% of the healthy progenies [37].

Brucella infection is closely associated with the fundamental mammalian events of placental development in the mother and subsequent nursing. This association may contribute to transmission of the bacteria over many generations. The immune response in a pregnant female would be suppressed to protect the fetus [22, 30], and *Brucella* infection during the fetal to newborn stage may induce immune tolerance against *Brucella* [34]. This early life stage-related immunosuppression might increase the probability of the bacterial transmission via the maternal route. To address this possibility, we must accumulate information on the interaction between *Brucella* and cetacean immunity in early life, as well as the chronic infection stage in adults. We do not exclude the possibility of other transmission routes. Sexual

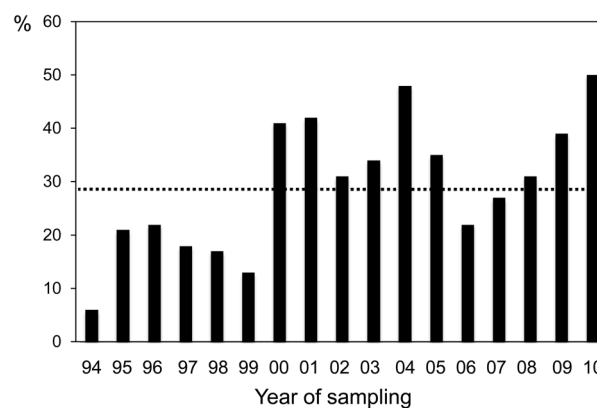


Fig. 5. Time course of the appearance rate of abnormal testes in common minke whales offshore the western North Pacific. Horizontal and vertical axes indicate the year of the sampling and percentage of whales with abnormal testes among the total mature male population each year, respectively. Dotted line, average percentage in total samples in the periods examined. Observed mature male numbers are, 17 (94), 89 (95), 58 (96), 77 (97), 75 (98), 62 (99), 27 (00), 84 (01), 78 (02), 82 (03), 90 (04), 75 (05), 78 (06), 78 (07), 51 (08), 33 (09), 10 (10), 1064 (total).

transmission from the *Brucella*-positive male might occur. Exposure to *Brucella* may also occur through the diet, secretion, discharges or parasites, such as lungworm [27]. It is noteworthy that these environmental transmissions can induce interspecies transmission.

In the present study, we observed granulomatous testes in common minke whales in every year of the survey (Figs. 4 and 5). We do not know why such lesions frequently develop only in adult whales in the western North Pacific. As common minke whales reach maturity at around 6–10 years of age [16], the accumulation of some unknown factors or sexual maturation may be involved in the development of the granulomatous lesions in the testes. At present, we cannot conclude that the granulomatous testes were directly caused by *Brucella* infection, although the relation between appearance of granuloma testes and anti-*Brucella* antibodies was found. If we postulate that the abnormal testes were directly caused by *Brucella* infection, the inconsistencies that some whales with the abnormal testes were anti-*Brucella* antibody negative and that some antibody positive whale did not have the granuloma, may be explained as followings. If the development of abnormal testes takes a long time after *Brucella* infection even after the brucellosis is cured, the anti-*Brucella* antibody would decrease to below the detection threshold. Even after recovery of the brucellosis, the lesions may persist longer time. The granuloma lesion, on the other hand, might not be always induced in testes of *Brucella*-infected whales, even anti-*Brucella* antibodies raised. The average appearance of abnormal testes during 2000–2010 (JARPN II) (average 35%, 95% CI: 31–39%) was significantly higher than that during 1994–1999 (JARPN) (average 18%, 95% CI: 14–22%) (Fig. 5). It is difficult to explain the reason of the difference, because no apparent change was observed in

the ecology or environmental conditions of western North Pacific minke whales during JARPN and JARPN II. To understand the pathology caused by *Brucella* in the baleen whales, more studies including the bacterial isolation and its characterization, and a study of tissue-specific bacterial growth, are necessary.

In contrast to whales in the North Pacific, no *Brucella*-specific antibody was detected in 739 serum samples of Antarctic minke whales. This strongly suggested that the Antarctic minke whales are virgin populations in terms of *Brucella* infection. The common minke whale and Antarctic minke whale inhabiting the Northern and Southern hemispheres, respectively, were classified as a single species until 2000, but currently are regarded as two independent species based on genetic and morphological differences. The clear difference in the prevalence of anti-*Brucella* antibodies between the two species, also supports the notion that they are geographically isolated and have no contact with each other during the dynamic seasonal migration. In the Southern hemisphere, however, serologically positive dolphin populations along the coasts of Peru and of the Solomon Islands [31, 33], and pinniped populations in the Antarctic [13], have been reported. These data indicate that interspecies transmission is very low, and/or that opportunities for interspecies contact do not exist. Although the features of *Brucella* strains from these animals in the Southern hemisphere are unknown as they have not yet been isolated, the low bacterial cross-infection rate among animals may have resulted in greater host specificity of *Brucella* in the evolution of each lineage. However, a careful watch must be kept to detect *Brucella* in large whales in the Antarctic, since once *Brucella* invades a virgin animal population, it may spread rapidly and severely affect the populations of the new host mammals.

Recent global warming is changing the marine environment and may affect migration patterns of whales, which may increase the chance of contact and risk of bacterial transmission between species. In the near future, the Pacific and Atlantic oceans will likely be connected due to the melting of Arctic Ocean ice [21]. As baleen whales perform a seasonal migration from the equatorial to the polar region, they must be regarded as a long-distance vector for the transmission of infectious diseases. The accumulation of information on diseases, such as *Brucella* infection in marine mammals, will provide deeper insight into the effect of diseases on populations of cetaceans and marine ecosystems.

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