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# Detection of serum antibodies to *Brucella* in Russian aquatic mammals

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**ABSTRACT.** A serologic survey of *Brucella* infection was performed in Caspian seals (*Pusa caspica*, n=71), Baikal seals (*P. sibirica*, n=7), ringed seals (*P. hispida hispida*, n=6), and beluga whales (*Delphinapterus leucas*, n=4) inhabiting Russian waters, by enzyme-linked immunosorbent assay (ELISA) using *Brucella abortus* and *B. canis* as antigens. The sera of 4 Caspian seals (4%) tested positive for *B. abortus*. The same sera samples demonstrated weaker yet detectable affinity for *B. canis* antigens. Several discrete bands against *B. abortus* and *B. canis* antigens were detected on Western blot analysis of the ELISA-positive seal sera; the bands against *B. canis* were weaker than those against *B. abortus*. The sera of 3 beluga whales (75%) were positive for *B. abortus* antigens but showed no binding to *B. canis* antigens in the ELISA. The positive whale sera showed a strong band appearance only against *B. abortus* antigens in the Western blot analysis. Many detected bands were discrete, while some of them had a smeared appearance. The present results indicate that *Brucella* infection occurred in Caspian seals and beluga whales inhabiting Russian waters, and that the *Brucella* strains infecting the seals and the whales were antigenetically distinct.

**KEY WORDS:** antibody, Baikal seal, beluga whale, *Brucella*, Caspian seal

*Brucella* are gram-negative intracellular pathogenic bacteria infecting many species of mammals and causing reproductive disorders including abortion. In terrestrial mammals, besides 6 classical *Brucella* species (*Brucella melitensis*, *B. abortus*, *B. ovis*, *B. canis*, *B. suis* and *B. neotomae* [6]), 3 new species of *Brucella*, i.e., *B. microti* [39], *B. inopinata* [40] and *B. papionis* [42] have been reported. In marine mammals, 2 novel species, *B. ceti,* which is preferentially associated with cetaceans, and *B. pinnipedialis*, which is preferentially associated with pinnipeds, have been identified so far [13]. Relatively little is known about the pathogenicity of *Brucella* infection in marine mammals. Some pathological changes in reproductive organs and nervous system were suggested as those induced by *B. ceti* infection [16, 19, 32, 36]. The abortion-inducing potential of *B. ceti* has been shown in captive and stranded dolphins and porpoises [11, 20, 25]. Apart from a report of placentitis in *Brucella*-infected northern fur seals (*Callorhinus ursinus*) [8], there is little evidence to suggest that *B. pinnipedialis* causes brucellosis or abortion.

*Brucella* infection in marine mammals has been reported to occur widely in various species inhabiting the European and North American sea [16, 19]. Although several species of marine mammals populate the coast of Russia and its inner lakes, little is known about their *Brucella* infection. Caspian seal (*Pusa caspica*) and Baikal seal (*P. sibirica*) are endemic species restricted exclusively to the Caspian Sea and the Lake Baikal, respectively [15, 26]. Caspian seal is registered as an endangered species in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (2017-3 version). Ringed seal (*P. hispida*) is a species of the circumpolar Arctic coasts with a broad geographic distribution and is classified into 5 distinct subspecies [17]. The subspecies *P. hispida hispida* has the largest population widely distributed in the Arctic Ocean. These 3 seal species belong to the same genus *Pusa*, as determined by the similarity in skull morphology and genetics [3, 38]. Beluga whale (*Delphinapterus leucas*), a member of the family Monodontidae, is supremely adapted to life in cold waters and inhabits the Arctic and sub-Arctic [30]. To obtain detailed knowledge of pervasiveness and character of *Brucella* infection in marine mammals inhabiting Russian waters, we conducted a serologic survey of Caspian seals, Baikal seals, ringed seals, and beluga whales found in this area.

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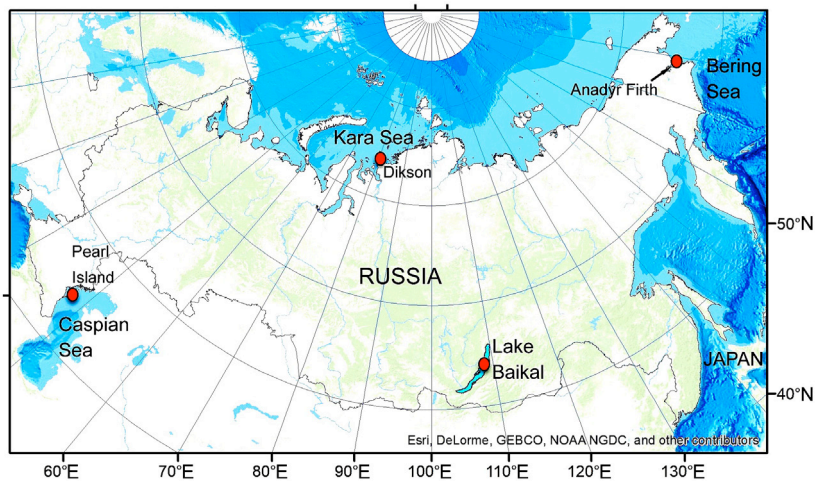


**Fig. 1.** Sampling sites included in the study.

## MATERIALS AND METHODS

### *Samples*

We have conducted the Russian-Japanese Joint Research Program for Biological and Environmental Studies with the permission from the Russian Federation and the local governments. This study was performed in accordance with the guidelines of the Animal Ethic Committee of the University of Tokyo. Sera samples from Caspian seals were obtained on the Pearl Island (Fig. 1; 45° 03' N, 48° 18' E) located in the north-west area of the Caspian Sea, between November 5–11, 1993; August 15–16, 1997; September 12–16, 1998; and September 24–October 8, 2000. Sera samples from 7 Baikal seals in the Lake Baikal (Fig. 1; 53° 46'–52' N, 108° 23'–31' E) were collected on May 21 and 22, 1998. The sera of six ringed seals were obtained at Dickson in the southern part of the Kara Sea from May 2 to 22, 2002 (Fig. 1; 73° 30' N, 80° 31' E). The ages of Caspian seals and ringed seals were estimated by counting the growth layers in both the dentine and cementum of the canine [4]. The 3 species of seals were all subjected to gross pathological observation. Sera from 4 beluga whales were collected at Anadyr Firth on July 20, 2001 (Fig. 1; 64° 83' N, 176° 72' E). The life stage of the beluga whales was estimated based on their body length [30].

### *Enzyme-linked immunosorbent assay*

Anti-*Brucella* serum antibody was detected in the enzyme-linked immunosorbent assay (ELISA) according to the protocol described previously [1]. Briefly, commercially available inactivated *B. abortus* strain 125 (Kaketsuken Co., Kumamoto, Japan) and *B. canis* strain QE-13B (Kitasato Institute Co., Tokyo, Japan) were solubilized and absorbed to the inner surface of each well (50 $\mu$g/50 $\mu$l/well) of a 96-well microtiter plate. The sera diluted at 1:100 were used as the primary antibody, and horseradish peroxidase-conjugated Protein A/G (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) diluted at 1:5,000 was used for detection. The absorbance at 405 nm was measured and the arithmetic mean of triplicate absorbance data points with the standard deviation (SD) was calculated. Serum samples showing the absorbance value higher than 0.2 against *B. abortus,* were regarded as positive, based on the values of serum samples from captive animals [1].

### *Western blot analysis*

Western blot analysis was performed according to the method described previously [36]. Proteins of the solubilized *B. abortus* strain 125 and *B. canis* strain QE-13B were respectively separated on a 10% polyacrylamide gel by SDS polyacrylamide gel electrophoresis (20 $\mu$g/lane) and blotted onto a polyvinylidene difluoride membrane (Millipore Co., Billerica, MA, U.S.A.). Serum samples diluted to 1:100 were used as the first antibodies, and then horseradish peroxidase-conjugated Protein-A/G (Thermo Fisher Scientific Inc.) diluted to 1:5,000 was used for detection.

## RESULTS

### *Anti-Brucella antibodies assessed by ELISA*

All serum samples were examined for antibodies against *B. abortus* and *B. canis* antigens by ELISA. Some samples from Caspian seals and beluga whales showed a value higher than 0.2 only for *B. abortus* antigens (Table 1). The sera of 4 among the examined 71 Caspian seals showed positive absorbance for *B. abortus* antigens (OD at 405 nm: 0.22–0.26) (Table 1). The absorbance for *B. canis* antigens in the same samples ranged from 0.12–0.19, which was relatively higher than that of other sera samples, though lower than 0.2 threshold. The age of the Caspian seals, estimated by counting the canine layers, ranged widely from less than 1 to 32 years. The 4 ELISA-positive Caspian seals were estimated to be adults at 11.5–31.5 years of age, based on

**Table 1.** Prevalence of serum antibodies against *B. abortus* in Caspian seals, Baikal seals, ringed seals and beluga whales

| Species | Year | Positive rate to *B. abortus* [a)] | | |
|---|---|---|---|---|
| | | Male | Female | Total (%) |
| Caspian seal (*Pusa caspica*) | 1993 | 0/9 | 0/3 | 0/12 |
| | 1997 | 1/4 | 0/3 | 1/7 (14) |
| | 1998 | 0/3 | 1/12 | 1/15 (7) |
| | 2000 | 2/14 | 0/23 | 2/37 (5) |
| | Total | 3/30 | 1/41 | 4/71 (6) |
| Baikal seal (*Pusa sibirica*) | 1998 | 0/6 | 0/1 | 0/7 |
| Ringed seal (*Pusa hispida hispida*) | 2002 | 0/3 | 0/3 | 0/6 |
| Beluga whale (*Delphinapterus leucas*) | 2001 | 1/1 | 2/3 | 3/4 (75) |

a) Absorbance greater than 0.2 at 405 nm in ELISA is regarded as positive.

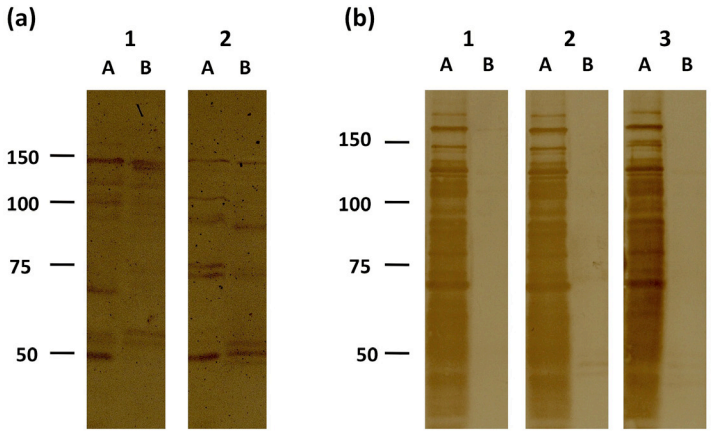


**Fig. 2.** Western blot analysis of the serum from ELISA-positive Caspian seals and beluga whales against *B. abortus* and *B. canis* antigens. (a), Caspian seals: Lane 1, sample ID, 98C46; and Lane 2, ID, 00C17. (b) Beluga whales: Lane 1, ID, No.1; Lane 2, ID, No. 10; and Lane 3, ID, No. 11. A, *B. abortus* antigen. B, *B. canis* antigen.

previous data [15].

The sera of 3 out of the 4 examined Beluga whales also demonstrated positive absorbance against *B. abortus* antigens (Table 1). Based on the body length, 2 of the examined whales appeared to be adults (395 and 350 cm) and the other 2 were juveniles (318 and 302 cm) [30]. One of the ELISA-positive whales was an adult and 2 were juveniles. The serum sample of the juvenile female whale showed high absorbance (OD at 405 nm=0.72), and that of the other 2 demonstrated lower absorbance (OD at 405 nm=0.26 and 0.27). No serum sample from the 4 examined whales reacted with *B. canis* antigens (OD at 405 nm=0.04–0.06).

### *Western blot analysis for antibody specificity against B. abortus and B. canis*

Since ELISA showed differences in antibody reactivity to *B. abortus* and *B. canis* in the antibody-positive serum samples between Caspian seals and beluga whales, the antigen specificity was further examined by Western blot using 2 available Caspian seal serum samples and all 4 beluga whale serum samples. The 2 Caspian seal sera (Serum ID: 98C46 and 00C17) previously showed the lowest absorbance in ELISA (OD at 405 nm: 0.22) out of the 4 positive serum samples. In Western blot analysis, they displayed similar band patterns: some weak yet sharp bands were formed as a response to both antigens of *B. abortus* and *B. canis* (Fig. 2a). The Western blot bands appeared to be slightly denser in case of *B. abortus* compared to *B. canis* antigens.

The Western blot band patterns of the 3 ELISA-positive serum samples from beluga whales (Serum ID: No. 1, No. 10 and No. 11) were similar to each other. Many bands, including the smeary bands, were detected in *B. abortus* Western blots while none were observed in case of *B. canis* (Fig. 2b). Western blot was also applied to the negative seal sera showing the absorbance close to the significance threshold of 0.2 (OD at 405 nm: 0.15–0.16) and 1 negative beluga whale serum (OD at 405 nm: 0.13), but no bands were observed (data not shown).

## DISCUSSION

The present serologic study demonstrated that *Brucella* infection occurred in Caspian seal and beluga whale populations inhabiting Russian waters. The presence of anti-*Brucella* antibodies in Caspian seals is consistent with the findings in the previous

report [9]. As Caspian seal pups are born from the middle of January to the middle of February [15], the examined yearling seals (n=16) in this study were estimated to be approximately 6–10 months old at the time of sampling. All of them were *Brucella*-antibody negative; however, the absence of anti-*Brucella* antibodies in these seals is not likely to be a result of immaturity of the acquired immunity, because the production of influenza virus antibodies in these yearlings was detected in our earlier research [31]. The age-dependent serological pattern of anti-*Brucella* antibodies has been reported in hooded seals (*Cystophora cristata*) in the North Atlantic population and the Eastern Pacific harbor seals (*P. vitulina richardsi*) [22, 28]. These studies show that yearling seals have a higher percentage of antibodies than pups or older seals. This may indicate that they were environmentally exposed to *Brucella* at the juvenile stage, which subsequently cleared in the later stages, as suggested by Nymo *et al.* [28]. It has been reported that *B. pinnipedialis* strains infecting hooded seal multiply neither in the seal macrophages nor epithelial cells, suggesting the absence of chronic infection in hooded seals [23, 24]. The present serologic data is, however, inconsistent with the age-dependency. The adult seals might have been infected at the early stage of life when *Brucella* spp. were epidemic in the past, and the infection might still persist in their later lives. Alternatively, the antibody-positive adult Caspian seals might be infected at an adult stage by a different transmission route in the hooded seals and harbor seals. To clarify how the *Brucella* spp. are transmitted in the Caspian seal populations and whether the infection persists, the *Brucella* must be isolated from Caspian seals and characterized in a future study. We found some females with a trace of abortion; however, they were *Brucella* antibody-negative. A previous study has shown that *B. pinnipedialis* was isolated from lungworms [14]. Although we observed a nematode infection in the lungs of several Caspian seals in the 2000 survey [31], there was no apparent relation between the histopathological observation of the parasites and anti-*Brucella* antibody positivity.

Anti-*Brucella* antibodies were not detected in the sera of the 7 examined Baikal seals, which is consistent with findings in earlier research [37]. All the Baikal seals were judged to be less than 1 year old based on their body length (85–103 cm) [26]. As Baikal seals deliver pups in February [26], the juveniles were estimated to be only 3 months old at the time of sampling. They seemed to be independent from their mothers. The lack of the specific antibody is likely not due to the immaturity of the immune system with impaired ability to produce antibodies, since previously we were able to detect specific antibodies to influenza virus in these animals [33]. Likewise, no anti-*Brucella* antibodies were detected in ringed seals in the present study. The age of the ringed seals ranged between 6–27 years, and all were thought to be adults [17]. Anti-*Brucella* antibodies were reported in the same subspecies *Pusa hispida hispida* inhabiting the North Atlantic, the west of Svalbard island and Baffin Island in the Arctic, and the Alaskan waters [12, 27, 29, 41]. As the sample size in the present study is very small, further survey is necessary to establish the distribution of *Brucella* in this species.

The beluga whales inhabiting the Anadyr Firth showed a high prevalence of the antibody, and 1 serum sample showed a strong antibody response. Anti-*Brucella* antibodies were previously found in beluga whales inhabiting Arctic Canada [27]. The Anadyr Firth is geographically connected to the Arctic Sea and the western North Pacific. Little data is available with regard to *Brucella* infection in the western North Pacific; however, we have previously shown that the infection occurred in 3 species of baleen whales and sperm whales based on a long-term and large-scale serologic study in the sea area [36]. The DNA analysis indicated that these *Brucella* were different from the typical *B. ceti* prevalent in European and American waters [34, 35]. Detailed DNA analysis of *Brucella* in the western North Pacific and the waters connected to the Arctic may provide valuable insight into the distribution of *Brucella* at a global level and the evolution of the bacteria.

The present ELISA and Western blot analyses indicated that the *Brucella* antigen in Caspian seals was different from that in beluga whales (Fig. 2). With regard to antigenicity, the types of *Brucella* detected in Caspian seals and beluga whales were more similar to *B. abortus* than *B. canis*. Further, compared to the seal *Brucella*, the beluga whale *Brucella* seemed to be more distant from *B. canis*. The presence of smeared bands might demonstrate that the beluga whale *Brucella* was a smooth colony (S) type possessing lipopolysaccharide (LPS) containing O-type polysaccharide (O-PS) in the outer membrane, as well as, *B. melitensis*, *B. suis*, *B. neotome*, and *B. ceti* [43]. On the other hand, the Caspian seal *Brucella* might be a rough colony (R) type that possesses LPS without O-PS, as well as, *B. canis*.

While *Brucella* species are known to preferentially infect their respective host mammalians, California sea lions (*Zalophus californianus*) were reported to be infected with terrestrial *Brucella* [5]. As Baikal seals and Caspian seals dwell in a land-locked aquatic area, they have a higher risk for the transmission of infectious agents from terrestrial animals, such as canine distemper virus—a causative agent of the mass-die-off of these seals [7]. Moreover, the reports of novel *Brucella* species are accumulating in animals other than mammals, such as fish and frogs [2, 10, 18, 21] and in the soil [39]. We may have to consider the possibility of interspecies transmission of *Brucella* in a greatly vast Russian Eurasia Continent, populated by various terrestrial and marine animals that form a huge ecosystem.

Recent global warming is bringing out an environmental change affecting animal distribution, especially the melting of ice in the Arctic sea. It may cause a critical ecological influence on the animals in the Arctic sea and the connecting North Asian waters including Anadyr Firth. We must not only continue the serologic survey of the inhabiting mammals but also accelerate the isolation and characterization of *Brucella* from them. In addition, pathological studies including reproductive disorders are warranted, although the evidence of abnormality of reproduction in seals and whales were not observed in the present study. These studies would contribute to the conservation of wild animals and their ecosystems.

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