北海道知床海岸にて大量座礁したシャチにおけるNeospora caninum,Toxoplasma gondii,ならびにBrucella属の感染調査

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Investigation for Presence of *Neospora caninum*, *Toxoplasma gondii* and *Brucella*-Species Infection in Killer Whales (*Orcinus orca*) Mass-Stranded on the Coast of Shiretoko, Hokkaido, Japan

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ABSTRACT. Twelve killer whale (*Orcinus orca*) were hemmed in by ice floes, and nine died on the Aidomari coast in the Nemuro Strait in Rausu, Shiretoko, Hokkaido, Japan on 8 February 2005. Tissue samples collected from 8 whales were tested for *Neospora caninum*, *Toxoplasma gondii*, and *Brucella* species DNA by polymerase chain reaction (PCR) assay. Gamma-globulin isolated from blood samples by ammonium sulfate precipitation was tested for antibodies to these pathogens by means of agglutination tests and immunoblotting. None of the 8 tissue samples had antibodies to the pathogens, when subjected to agglutination tests. In immunoblotting, one sample (sample No.5) showed antibody binding to *N. caninum* antigens. In the PCR assay, none of the samples was positive. Further study is necessary to examine the prevalence of the pathogens in marine mammals inhabiting this area.

KEY WORDS: infection, killer whale, mass-stranding.

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Toxoplasma gondii and *Neospora caninum* are apicomplexan protozoa with a worldwide distribution that cause neuromuscular disease and abortion through transplacental transmission in warm blooded hosts. *Brucella* species, a facultative, gram-negative intracellular pathogen also cause abortion in mammals, as well as sterility. Ingestion in contaminated food is one of the infection routes of these pathogens. Recently, *T. gondii* infection has been reported in wild marine mammals, including several species of cetaceans [1, 2, 4, 16, 18, 19]. As for *Brucella* species, infection has been observed in a wide range of species of marine mammals [6, 17, 22]. However, the source of these infections are still unclear and there has been no report of marine mammals being exposed to *N. caninum* in nature.

On 8 February 2005, 12 killer whale (*Orcinus orca*) were hemmed in by ice floes, and nine died on the Aidomari coast in the Nemuro Strait, Rausu, Shiretoko, Hokkaido, Japan (44° 1.1'N, 145° 11.1'E). The killer whales are predators, preying upon cold-blooded and warm-blooded animals. They are cautious and avoid shallow waters. They live in groups which show strong social cohesion.

We considered that the killer whales had a risk of exposure to the pathogens mentioned above, if the marine mammals which they had preyed upon were infected with these pathogens. In this regard, while there have been a number of studies on toxoplasmosis and *Brucella* infection in seals [9, 10, 14, 21] there have been few on the prevalence of the pathogens in killer whales.

Since mass-stranding of killer whales seldom occurs on the coast of this area, this was a good opportunity to study them as a pod. To investigate their exposure to the pathogens, antibody titers were obtained for blood samples from them by means of agglutination tests, and immunoblotting using *T. gondii* and *N. caninum* antigens. Tissue samples were tested for the presence of specific DNA fragments of the pathogens by the polymerase chain reaction (PCR).

Nine of the dead whales were available for study as they were beached. For 7 of them, necropsy samples and tissue samples were taken (No. 1, 2, 3, 4, 5, 6 and 9) on day 5 to 7 post mortem. The remaining 2 whales (No. 7 and 8) were frozen at –30°C and samples were taken on day 60 post mortem. The ages of the whales were roughly determined on the basis of their body length and sex (3). Whales of more than 5.2 m in body length were considered to be adult, milk in the mammary glands to indicate nursing, and no milk to indicate not nursing. Blood samples were taken from the venous cavity and centrifuged at 1,200 g for 20 min to obtain the fluid phase. The blood samples of all of the whales showed severe hemolysis and fibrinolysis, which made them unsuitable for direct serological testing. Therefore, the gamma-globulin fraction was isolated from the blood samples with a 33% saturated $(NH_4)_2SO_4$ solution in the final volume and stored at –80°C until use. The total protein concentration was measured using an assay kit (Coomassie Plus Protein Assay Reagent Kit: Pierce, Rockford, IL, U.S.A.), and adjusted to 2 mg/m*l* in PBS. The presence of IgG molecules in the samples was determined by immunoblotting. Antibodies to *T. gondii* were tested for using a commercial latex agglutination test (LAT) kit (Toxo Check: Eiken Chemical Co., Ltd., Tokyo, Japan) in triplicate for each sample. The manufacturer's instructions stated that porcine serum antibody titer of 1:64 or above should be regarded as positive.

Tachyzoites of the Beverley strain of *T. gondii* and tachyzoites of *N. caninum* isolated from sheep [12, 13] were maintained by continuous passage in Bovine angio-endothelial (BAE) cell cultures. The parasites were collected from the cell cultures and centrifuged 3 times at 1,200 g for 10 min while washing with phosphate buffered saline (PBS). To remove host cells and debris, the suspensions were passed through a 3-μm polycarbonate filter (Nuclepore; Corning Coster Corporation, Tokyo, Japan). An immunoblotting assay was conducted to determine the antigenicity of killer whale IgG, and specificity of the antibody binding to the parasites antigens as described elsewhere [18]. Briefly, Approximately 100 μl of the blood samples, or 10 mg of either *T. gondii* or *N. caninum* tachyzoites were mixed with 100 μl of SDS-PAGE sample buffer and incubated at 95°C for 5 min. Thereafter, 10 μl samples were applied with SDS-PAGE using a 10% gel and transblotted onto membranes (Immobilon-P; Millipore Inc., Tokyo, Japan). After blocking with chilled PBS containing 5% Skim milk(PBS-milk) overnight, the membranes onto which the parasite antigens had been transblotted were immersed in 500 μg/m*l* of the IgG separated from blood samples, diluted with PBS-milk and kept at 4°C for 18 hr. After washing in PBS cntaining 0.025% Tween 20, the membranes were allowed to react with diluted horseradish peroxidase-conjugated anti-bottlenose dolphin IgG (Bethyl Laboratories, Montgomery, Texas) at 4°C for 18 hr. Antibodies were detected by means of the peroxidase reaction using diaminobenzidine-4HCl in 0.1 M Tris-HCl containing 0.03% H_2O_2 (pH 7.4). The molecular weights (M.W.) of electrophoresed proteins were estimated based on the running distance compared with molecular weight markers (Kaleidoscope prestained standards: Bio-Rad Ltd, Hercules, California). Brain, liver, diaphragm, heart muscle and kidney were obtained from the 8 killer whales (No. 2~8), and testis from the 2 of them (No. 1 and 3). Samples weighing approximately 0.5 g were prepared and genomic DNA was extracted using the proteinase K-phenol method. For detection of *T. gondii* DNA, nested PCR was performed on the SAG2 locus according to the method of Howe *et al.* [11]. *N. caninum* DNA and *Brucella*-species DNA were detected by the method of Yamage *et al.* [24], and Watarai *et al.* [23], Erdenebaatar *et al.* [5], respectively.

There were 2 males and 7 females. According to the previously mentioned criteria, male (No. 1) was an adult, and 1 male (No. 3) and 2 females (No. 7 and 8) were calves. Three females (No. 2, 5 and 6) were determined to be nursing, because lactic fluid was detected in their mammary glands, and the other 2 females (No. 4 and 9) were not nursing. The parental relationships were unknown. Precise age determination through examination of the cementum glowth groups teeth is underway. The blood samples of 8 whales were tested for the presence of IgG molecules by immunoblotting using anti-Bottlenose dolphin IgG antibodies (Fig. 1). In this regard, since the mean IgG level in porcine serum is approximately 24.3 ± 0.94 mg/m*l* [20], an IgG molecule concentration of 1 mg/m*l* is a match for 1:24 fold diluted serum.

None of the 8 blood samples had antibodies to *T. gondii, N. caninum* and *Brucella* species, when subjected to agglutination tests at a concentration of 1 mg/m*l*, respectively. In immunoblotting, 1 of the 8 samples (sample No.5) showed antibody binding to *N. caninum* antigens, which showed molecular masses ranging from 42 to less than 18 kDa at concentration of 500 μg/m*l* (Fig. 2). No sample showed antibody binding to *T. gondii* antigens. In the PCR analysis, none of the 8 samples was positive for *T. gondii, N. caninum* or *Brucella*-species DNA.

Despite the positive result for 1 sample, these results suggest that killer whales are little exposed to these pathogens. Seal body parts were found in the stomachs of 6 whales (No. 1, 2, 4, 5, 6, and 9), leading us to speculate that the pathogens also have a low prevalence in seals in this area. In this regard, there has been no report of seals and dolphins in the Nemuro Strait being infected by these pathogens and little is known about marine mammal populations in this area. Not much is known about the migration behavior of killer whales either. Another possibility for the lack of *T. gondii* infection is that killer whales have high resistance against *T. gondii*. A recent study [7] found that though grey seals experimentally infected with *T. gondii* oocysts had high antibody titers, there was no evidence of *T. gondii* in their tissues. From these findings, they surmised that there were major differences in susceptibility to *T. gondii* between

Table 1. Sex, body measurements, age, antibody titers and PCR assays of killer whales stranded at Aidomari, Rausu, Hokkaido, Japan

Whale No.	Sex	Length (cm)	Body weight (t)	Age	*Brucella* PCR	*Brucella* BAT	*T. gondii* PCR	*T. gondii* LAT	*N. caninum* PCR	*N. caninum* ELISA
1	male	765	6.6	Adult	Neg.[a]	Nt.[b]	Nt.	Nt.	Nt.	Nt.
2	female	563	2.6	Nursing		<1[c]	Neg.	<1	Neg.	<1
3	male	271	0.7	Calf	Neg.	<1	Neg.	<1	Neg.	<1
4	female	658	4.7	Not nursing		<1	Neg.	<1	Neg.	<1
5	female	686	4.7	Nursing		<1	Neg.	<1	Neg.	<1
6	female	600	3.5	Nursing		<1	Neg.	<1	Neg.	<1
7	female	298	0.423	Calf		<1	Neg.	<1	Neg.	<1
8	female	274	0.364	Calf		<1	Neg.	<1	Neg.	<1
9	female	654	4.1	Not nursing		<1	Neg.	<1	Neg.	<1

a) Negative. b) Not tested. c) 1 mg/m*l*.

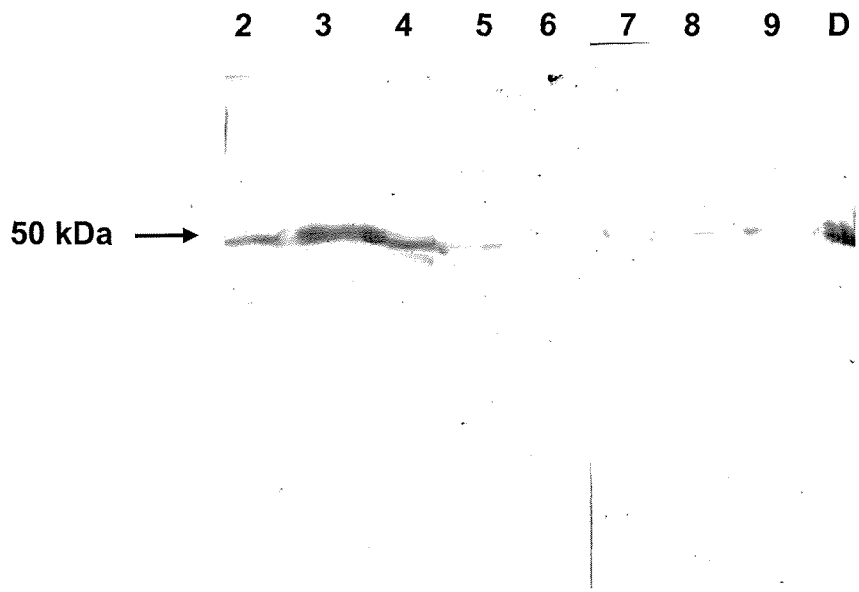

Fig. 1. Immunoblotting of the killer whale blood samples reacted with anti-Bottlenose dolphin IgG antibodies. The numbers are those assigned to the samples. D is bottlenose dolphin serum IgG. Arrow indicates m.w. of 50 kDa.

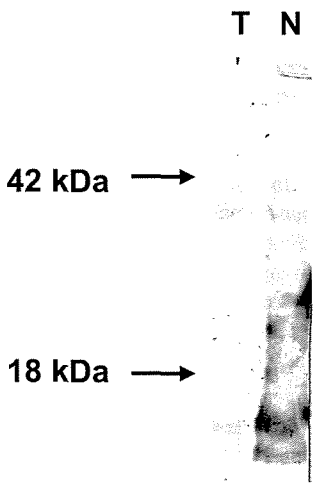

Fig. 2. Immunoblotting pattern of IgG antibodies to *T. gondii* and *N. caninum* antigens in killer whale blood. Membranes on to which antigens had been transblotted were immersed in diluted blood IgG samples of killer whale No. 5 at a concentration of 500 μg/ml. The arrows indicate m.w. 42, and 18 kDa, respectively. T: *T. gondii* antigens, N: *N. caninum* antigens.

marine mammals and terrestrial vertebrates. This should be checked through further study.

Regarding the single sample (No. 5) that seemed to show a positive reaction against *N. caninum*, the IgG antibody titer was rather low and the bands observed in immunoblotting were unclear. From the finding of the present study, we were unable to determine whether the reaction was due to the infection, or a cross reaction due to other stimuli. Therefore, further investigation of this type of infection will be necessary not only in killer whales but also in other marine mammals to clarify the prevalence of infection in this area.

Several theories have been advanced to explain mass strandings of whales and dolphins. They involve the effects of ocean currents, tides and coastal configurations, the migratory and social behavior of these mammals, food availability, echolocation or orientation failure, and long-standing wasting diseases [8]. Morimitsu *et al.* [15] suggested the parasitogenic neuropathy as a possible cause of mass stranding of dolphins. In the present study, intracellular pathogen did not appear to be involved in the stranding.

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