FULL PAPER *Parasitology*

Seroepidemiology of *Toxoplasma gondii* and *Neospora caninum* in Seals around Hokkaido, Japan

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ABSTRACT. Serological analysis was performed to detect *Toxoplasma gondii* and *Neospora caninum* infection in seals in Hokkaido. Serum samples were collected from 322 Kuril harbor seals (*Phoca vitulina stejnegeri*) at Nosappu, Akkeshi and Erimo, from 46 spotted seals (*P. largha*) at Nosappu, Erimo, Yagishiri Island, Hamamasu and Syakotan, and from 4 ribbon seals (*P. fasciata*) and a bearded seal (*Erignathus barbatus*) at Nosappu between 1998 and 2006. Recombinant surface antigen of *T. gondii* (SAG2t) and *N. caninum* (NcSAG1t) were used as antigens for ELISA to detect antibodies. Antibodies against SAG2t were detected from 4% of 77 Kuril harbor seals at Nosappu in 2005. Antibodies against NcSAG1t were detected from 2% (1/66) in 2003, 5% (4/79) in 2004 and 10% (8/77) in 2005 of Kuril harbor seals and 11% of 9 spotted seals in 2004 sampled at Nosappu. Eight percent of 12 Kuril harbor seals from Akkeshi and 25% of 4 spotted seals from Erimo in 2005 also contained antibodies against NcSAG1t. These suggest sporadic infection of *T. gondii* and *N. caninum* in Kuril harbor seals and spotted seals in Hokkaido. Of the ELISA-positive seals, 2 seals having anti-SAG2t antibodies and 3 seals having anti-NcSAG1t antibodies in 2005 were judged to be juveniles that have no maternal antibodies. These suggest that the protozoan infections have occurred in recent years. Infection of terrestrial protozoa such as *T. gondii* and *N. caninum* in seals indicates that the sea environment has been contaminated with protozoa.

KEY WORDS: Japan, *Neospora caninum*, seal, seroepidemiology, *Toxoplasma gondii*.

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Toxoplasma gondii and *Neospora caninum*, which are apicomplexan protozoa with worldwide distribution, cause neuromuscular disease and abortion through transplacental transmission in warm-blooded hosts [6, 37]. *T. gondii* and *N. caninum* infections have been reported mainly in terrestrial mammals and birds, although recently they have been found in wild marine mammals, including several species of seals [8]. *T. gondii* infection is known to be a major cause of encephalitis in sea otters (*Enhydra lutris*) [4, 8]. The ingestion of oocysts in contaminated food or water and the ingestion of infected tissues of intermediate hosts are the two main sources of postnatal *T. gondii* and *N. caninum* infection. Felids and dogs are the only known host that can excrete environmentally resistant oocysts of *T. gondii* and *N. caninnum*, respectively [6, 37]. Miller *et al.* [20] presented evidence that land-based surface runoff was a significant risk for *T. gondii* infection in sea otters. It is possible that oocysts of these protozoa wash into the sea in runoff contaminated by excrement of cats and dogs. In Hokkaido, influx of runoff contaminated with substances such as pesticides and organic matters into environmental water has been known, and it is particularly serious in the snowmelt season [29, 30]. *Cryptosporidium parvum* oocysts that are genetically similar to *C. parvum* infected in cattle have been detected from river in Hokkaido [38]. If the coastal Hokkaido has been contaminated by terrestrial protozoa, marine mammals may have been infected.

Around Hokkaido, there are 5 species of seals: The Kuril harbor seal (*Phoca vitulina stejnegeri*), the spotted seal (*P. largha*), the ringed seal (*P. hispida*), the ribbon seal (*P. fasciata*) and the bearded seal (*Erignathus barbatus*) [16]. Kuril harbor seals are distributed along the northwestern Pacific coast [17]. These seals haul out year round on rocky reefs near land and have a strong fidelity to their particular hauling-out site [28]. The four other species migrate in winter and spring, drifting south to Hokkaido with the packed ice [16]. The four species are distributed in the Sea of Japan and the Sea of Okhotsk mainly, although some spotted seals can be found along the Pacific coast all the year round [15, 23, 24]. Miller *et al.* [21] reported antibodies against *N. caninum* from an encephalitic Pacific harbor seal (*P. v. richardsi*) that had demonstrable *T. gondii* and *Sarcocystis neurona* in encephalitic lesions. For proper management and conservation of seals, information on the prevalence of the protozoa, which can be the death of seals, is indispensable. The infection of pathogenic protozoa in the seals indicates protozoan contamination of the sea environment, which is a problem not only for marine mammals but also human health. In this study, we surveyed *T. gondii* and *N. caninum* infection in seals around Hokkaido.

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MATERIALS AND METHODS

Samples: Serum samples were collected from Kuril harbor seals (231 seals), spotted seals (25), ribbon seals (4) and bearded seal (1) at Nosappu; from Kuril harbor seals (16) at Akkeshi; from Kuril harbor seals (75) and spotted seals (6) at Erimo; from spotted seals (2) at Yagishiri Island; from spotted seals (12) at Hamamasu; and from spotted seal (1) at Syakotan (Fig. 1). All samples from Nosappu and Akkeshi were obtained from seals by-caught in salmon fixed nets in the coastal waters from late August to November. Of the samples from Erimo, 46 samples from Kuril harbor seals and 5 samples from spotted seals were collected from seals taken in investigative capture from late June to early July; the remaining 30 samples from Erimo were collected from seals by-caught in salmon fixed nets from late August to November. Samples from Yagishiri Island and Syakotan were collected from seals stranded in May and in April, respectively. One sample from Hamamasu was collected from a spotted seal stranded in January, and others samples from Hamamasu were collected from seals by-caught in gill nets from March to April. Whole blood was collected from the heart of dead seal in fishnet and of stranded dead seal or from the vein of living seal taken in investigative capture, centrifuged, serum separated, and stored at –20°C or –80°C. Capture of living seals was conducted under license from the Japanese Ministry of Environment and the Hokkaido Government. To check the condition of animals, the respiration rate and body temperature of the seals were monitored while the living seals were restrained. Body length (nose to tip of tail) was used as index of age [1, 2, 9, 26, 36]. Seals measuring 125 cm or below were judged to be juveniles (age <2 years).

Antigens: Surface antigen 2 (SAG2) of *T. gondii* [13, 14] and surface antigen1 of *N. caninum* (NcSAG1) [3] were recombined as antigens for enzyme-linked immunosorbent assay (ELISA) to detect antibodies against *T. gondii* and *N. caninum*, respectively. The template DNA for polymerase chain reaction (PCR) was extracted from tachyzoites of the *T. gondii* RH strain [7] and *N. caninum* Nc-1 strain [6] as described previously [3, 13]. Two oligonucleotide primers, 5'-ACGAATTCGTCCACCACCGAGACG-3' and 5'-ACGAATTCTTCTTGCCCGTGAGA-3', were used to amplify the truncated SAG2 (SAG2t) gene without sequences encoding a highly hydrophobic signal peptide and C-terminus by PCR [33]. The truncated NcSAG1 (NcSAG1t) gene without sequences encoding a hydrophobic signal peptide and a C-terminus was amplified by PCR with two primers, 5'-ACGAATTCATCAGAAAAATCACCT-3' and 5'-ACGAATTCGACCAACATTTTCAGC-3' [3]. The SAG2t gene or NcSAG1t gene was inserted into *Eco*RI site of the bacterial expression vector, pGEX-4T-3 (Promega, U.S.A.). Each resulting plasmid was designated as pGEX/SAG2t or pGEX/NcSAG1t. pGEX/SAG2t or pGEX/NcSAG1t was expressed as glutathione S-transferase (GST) fusion proteins (GST-SAG2t or GST-NcSAG1t) in *Escherichia coli* (DH5 α strain) and the proteins were purified by glutathione sepharose 4B according to method of Chahan *et al.* [3].

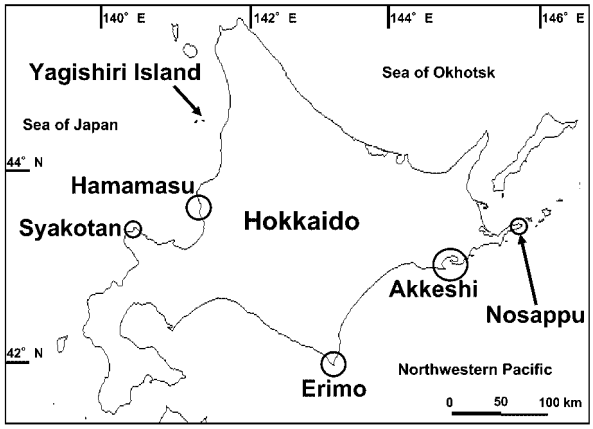

Fig. 1. Map of sampling area. Serum samples were collected from 322 Kuril harbor seals at Nosappu, Akkeshi and Erimo, from 46 spotted seals at Nosappu, Erimo, Yagishiri Island, Hamamasu and Syakotan, and from 4 ribbon seals and a bearded seal at Nosappu between 1998 and 2006.

Enzyme-linked immunosorbent assay (ELISA): ELISA was performed according to modified procedure described previously [3, 10, 13, 14]. GST-SAG2t, GST-NcSAG1t or GST was diluted to 5 μg/m*l* in phosphate-buffered saline (PBS). The antigens were absorbed into each well (50 μ*l*/well) of a 96-well microtiter plate at 4°C for at least 2 hr. After the wells were blocked with 1% bovine serum albumin (BSA) in PBS for 1 hr at room temperature (RT), they were washed with PBST (PBS that contains 0.05% concentration of Tween 20). Fifty μ*l* of sera diluted to 1:100 in PBS containing 0.5% BSA and 0.05% Tween 20 (BSA-PBST) was added to each well. After incubation at RT for 1 hr, the plates were washed with PBST. Fifty μ*l* of Peroxidase-conjugated Protein G (Sigma, U.S.A.) diluted to 1:200 in BSA-PBST was added to each well, and the plate incubated for 1 hr at RT. The plates were washed with PBST, and 100 μ*l* of substrate solution (0.05 M citrate buffer pH 4.0, 0.008% hydrogen peroxide; 40 mM 2.2'-azino-di-3-ethyl-benzothiazobine-6-sulfuric acid) was added to each well. After incubation for 30 min at RT, optical density (OD) of each well was read with a spectrophotometer using a 405 nm filter. The ELISA result was determined for each sample by taking the mean OD value of two readings with GST-SAG2t or GST-NcSAG1t minus the mean value of two readings with GST. The samples were considered positive if the calculated OD value was >0.1. The cutoff value of 0.1 was explained experimentally to be available for ELISA using recombinant SAG2t in cats and using NcSAG1t in cattle [3, 13, 14].

RESULTS

Antibodies against SAG2t were detected only in Kuril harbor seals from Nosappu in 2005 (Table 1). The inci-

Table 1. Number of anti-SAG2t antibodies positive sera collected from seals around Hokkaido, Japan

Year	Nosappu							Akkeshi		Erimo				Yagishiri Island		Hamamasu		Syakotan
	Kuril Harbor seal		Spotted seal		Ribbon seal		Bearded seal	Kuril harbor seal		Kuril harbor seal		Spotted seal		Spotted seal		Spotted seal		Spotted seal
	Male	Female	Male	Female	Male	Fmale	Male	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Female
1998	0/2[a)]	0/7	0/1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1999	–	–	–	–	–	–	–	–	–	0/6	0/2	–	–	–	–	–	–	–
2003	0/31	0/35	0/3	0/3	–	0/2	0/1	–	–	0/9	0/6	–	–	–	–	–	–	–
2004	0/47	0/32	0/7	0/2	–	–	–	0/2	0/2	0/16	0/12	0/2	–	–	–	–	–	–
2005	1/45	2/32	0/6	0/3	0/1	0/1	–	0/7	0/5	0/15	0/9	0/3	0/1	0/1	0/1	–	–	–
2006	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/4	0/8	0/1

SAG2t is recombinant truncated surface antigen 2 of *Toxoplasma gondii* without hydrophobic signal peptide.
a) Number of positive/number of sample.

Table 2. Number of anti-NcSAG1t antibodies positive sera collected from seals around Hokkaido, Japan

Year	Nosappu							Akkeshi		Erimo				Yagishiri Island		Hamamasu		Syakotan
	Kuril Harbor seal		Spotted seal		Ribbon seal		Bearded seal	Kuril harbor seal		Kuril harbor seal		Spotted seal		Spotted seal		Spotted seal		Spotted seal
	Male	Female	Male	Female	Male	Fmale	Male	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Female
1998	0/2[a)]	0/7	0/1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1999	–	–	–	–	–	–	–	–	–	0/6	0/2	–	–	–	–	–	–	–
2003	0/31	0/35	0/3	0/3	–	0/2	0/1	–	–	0/9	0/6	–	–	–	–	–	–	–
2004	2/47	2/32	1/7	0/2	–	–	–	0/2	0/2	0/16	0/12	0/2	–	–	–	–	–	–
2005	4/45	4/32	0/6	0/3	0/1	0/1	–	0/7	1/5	0/15	0/9	1/3	0/1	0/1	0/1	–	–	–
2006	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/4	0/8	0/1

NcSAG1t is recombinant truncated surface antigen 1 of *Neospora caninum* without hydrophobic signal peptide.
a) Number of positive/number of sample.

dence was 4% (3/77). The range of OD for ELISA-positive sera was 0.11–0.79 (Table 3). Mean OD of negative sera plus three standard deviations was 0.07, which was <0.1.

Antibodies against NcSAG1t were detected in Kuril harbor seals from Nosappu in 2003, 2004 and 2005, in a spotted seal from Nosappu in 2004, in a Kuril harbor seal from Akkeshi in 2005 and in a spotted seal from Erimo in 2005 (Table 2). The incidence of the antibodies in Kuril harbor seals from Nosappu was 2% (1/66) in 2003, 5% (4/79) in 2004 and 10% (8/77) in 2005. That in spotted seals from Nosappu in 2004 was 11% (1/9). That in Kuril harbor seals from Akkeshi in 2005 was 8% (1/12). That in spotted seals from Erimo in 2005 was 25% (1/4). The range of OD for ELISA-positive sera was 0.11–1.38 (Table 3). Mean OD value of negative sera plus three standard deviations was 0.08, which was <0.1.

No seals had antibodies against both SAG2t and NcSAG1t (Table 3). Of 3 seals having antibodies against SAG2t, 2 seals were judged to be juveniles from their body length (≤125 cm) (Table 3). Of 16 seals having antibodies against NcSAG1t in 2005, 1 Kuril harbor seal from Nosappu, 1 Kuril harbor seal from Akkeshi and 1 spotted seal from Erimo were juveniles. The juvenile Kuril harbor seals having antibodies against the protozoa were taken between September and November (Table 3). Because birthing season of Kuril harbor seals is from mid to late May [28], all juvenile Kuril harbor seals having antibodies against the protozoa were older than 3 months of age. A juvenile spotted seal having antibodies against protozoa was taken in late August. As birthing season of spotted seals is mid to late March [26], the spotted seal having antibodies against the protozoa was older than 5 months.

DISCUSSION

Antibodies against SAG2t or NcSAG1t were detected in seals from Hokkaido. In previous studies using cats and cattle, recombinant SAG2t of *T. gondii* and NcSAG1t of *N. caninum* were validated as useful antigens which promised a highly sensitive and specific ELISA [3, 13, 14]. The mean OD value of negative sera plus three standard deviation was <0.1, which attests to the appropriateness of the cutoff value of 0.1. The results of ELISA with SAG2t suggest sporadic infection of *T. gondii* in Kuril harbor seals from Nosappu. The results of ELISA with NcSAG1t suggest sporadic infection of *N. caninum* in Kuril harbor seals and in spotted seals from Nosappu, in Kuril harbor seals from Akkeshi and in spotted seals from Erimo. Of the seals having antibodies against the protozoa, 2 seals having antibodies against SAG2t and 3 seals having antibodies against NcSAG1t in 2005 were judged to be juveniles that were older than 3 months of age. Although little is known about immunity in newborn seals, maternal antibodies (IgG) were transferred to seal pups via the colostrum primarily [34], like cats. Omata *et al.* [31] showed that maternal antibodies against *T. gondii* in newborn cats were no longer detectable at 2–3 months. These suggest that antibodies against the protozoa detected in juvenile seals that were older than 3 months were

Table 3. Anti-SAG2t or -NcSAG1t antibodies positive seals sampled at Hokkaido, Japan

	ID of seals	Species[c]	Sampling year	Sampling date	Sampling area	Sex[d]	OD[e]	Maturity[f]	Body length (cm)
Anti-SAG2t antibodies positive seals[a]	NZ0504	Kuril	2005	3 September	Nosappu	F	0.11	J	125
	NZ0567	Kuril	2005	13 October	Nosappu	F	0.30	A	146
	NZ0575	Kuril	2005	18 October	Nosappu	M	0.79	J	120
Anti-NcSAG1t antibodies positive seals[b]	NZ0323	Kuril	2003	9 September	Nosappu	M	1.38	A	133
	NZ0404	Kuril	2004	1 September	Nosappu	M	1.25	A	152
	NZ0455	Kuril	2004	7 October	Nosappu	F	0.67	A	136
	NZ0461	Kuril	2004	19 October	Nosappu	M	0.12	A	142
	NZ0481	Kuril	2004	11 November	Nosappu	F	0.15	A	144
	NG0407	Spotted	2004	4 November	Nosappu	M	0.53	A	126
	NZ0511	Kuril	2005	5 September	Nosappu	M	0.31	A	167
	NZ0519	Kuril	2005	6 September	Nosappu	F	0.66	A	147
	NZ0526	Kuril	2005	7 September	Nosappu	M	0.11	A	152
	NZ0533	Kuril	2005	12 September	Nosappu	F	0.86	A	133
	NZ0554	Kuril	2005	26 September	Nosappu	F	0.14	A	129
	NZ0560	Kuril	2005	1 October	Nosappu	M	0.93	A	129
	NZ0574	Kuril	2005	18 October	Nosappu	F	1.24	A	137
	NZ0586	Kuril	2005	17 November	Nosappu	M	0.35	J	118
	AZ0515	Kuril	2005	14 September	Akkeshi	F	0.15	J	108
	EG05101	Spotted	2005	28 August	Erimo	M	0.12	J	121

a) SAG2t is recombinant truncated surface antigen 2 of *Toxoplasma gondii* without hydrophobic signal peptide.
b) NcSAG1t is recombinant truncated surface antigen 1 of *Neospora caninum* without hydrophobic signal peptide.
c) Kuril or Spotted indicates Kuril harbor seal or spotted seal, respectively.
d) M or F indicates male or female, respectively.
e) Optical density read in ELISA using SAG2t or NcSAg1t with 405 nm filter.
f) Seals measuring 125 cm or less, were judged to be juvenile (age <2 years). J or A indicates juvenile or adult (age ≥2 years), respectively.

not maternal antibodies. Then the protozoan infections are thought to have occurred in recent years. The incidence of antibodies against NcSAG1t was higher than that of antibodies against SAG2t. The antibodies against *N. caninum* had been detected in a killer whale (*Orcinus orca*) stranded at Aidomari, Shiretoko, Hokkaido [32]. The marine mammals around Hokkaido may be exposed more commonly to *N. caninum* than to *T. gondii*. For definitive diagnosis, detection of the protozoa or protozoan DNA from the tissue of seals is required.

The infections of terrestrial protozoa were suggested in Kuril harbor seals from Nosappu and Akkeshi. Kuril harbor seals are distributed from Erimo to Nosappu along the Pacific coast of Hokkaido. The distance from Erimo to Akkeshi is about 170 km, and there are no hauling-out sites for Kuril harbor seals between the two areas [17]. Analysis of mtDNA indicated that movement of the Kuril harbor seals between Erimo and eastern Hokkaido (from Akkeshi to Nosappu) is restricted [39]. The difference in incidence of Kuril harbor seals with antibodies against the protozoa between eastern Hokkaido and Erimo may relate to the exiguity of contact between the Kuril harbor seals inhabiting eastern Hokkaido and the Kuril harbor seals inhabiting Erimo. The movement range of Kuril harbor seals in eastern Hokkaido is presumed to be between Akkeshi and southern Kuril Islands, because most of the Kuril harbor seals bycaught in fixed salmon nets at Nosappu are considered to be from the Habomai Islands [12]. It is thought that there is place somewhere in the movement range (from Akkeshi to southern Kuril Islands) contaminated by the protozoan that may infect Kuril harbor seals. Spotted seals are kwon for the great range of movement [22], and the spotted seals tagged at Erimo were recovered at Akkeshi (Saito, unpublished). Therefore, although a spotted seal sampled at Erimo had antibodies against *N. caninum*, it is possible that the spotted seal was not infected at Erimo. The spotted seal might have been infected with *N. caninum* somewhere excluding Erimo before moving to Erimo. The antibodies against *T. gondii* and *N. caninum* were not found in seals from the Sea of Japan in this study. However, we must note that few samples were collected from the area, and all were from spotted seals. Due to the fidelity to costal area, Kuril harbor seals may be more prone to contamination from land than other species.

Runoff is considered to carry oocysts of terrestrial protozoa such as *T. gondii* and *N. caninum* from land into the sea [5, 20]. The infection of the terrestrial protozoa in the Kuril harbor seals of eastern Hokkaido suggests that range of Kuril harbor seals in eastern Hokkaido contains water contaminated with terrestrial protozoa. *T. gondii* has been reported in domestic cats in Japan and recent studies have shown 4% positive rates in Hokkaido [19]. Sawada *et al.* [35] reported 31% positive rates on dogs in dairy farms suffering from cattle abortion due *N. caninum* and 7% in urban area in Japan. Epidemiological investigation in animals at waterfront area is required to estimate risk of contamination with the protozoa from land to sea. If infections of these protozoa increase and spread on land or the flow of proto-

zoa-contaminated water from land to the sea increase, contamination of sea environment also will increase.

There are three possible routes by which seals could become infected with *T. gondii* or *N. caninum*: ingestion of sporulated oocysts, ingestion of bradyzoite cysts in the tissues of intermediate hosts, or vertical transmission. The infection via intermediate host is unlikely because seals do not prey on warm-blooded animals, which are recognized being intermediate hosts. It is unknown whether vertical transmission of the protozoa in seals is a significant path of infection. In sea otters, vertical transmission does not seem to be the primary cause of *T. gondii* infection [5]. Gajadhar *et al.* [11] demonstrated experimentally that oocysts that are shed by cats and sporulate are capable of establishing infection in seals. The infective oocysts of *T. gondii* can survive for months in seawater [18]. Several studies have demonstrated that oocysts and cysts of pathogenic protozoa including *T. gondii* are concentrated by clams, mussels and oysters during filter-feeding activity [5]. For sea otters, shellfishes that concentrate the oocysts are considered to be a route of infection [5]. Because Kuril harbor seals and spotted seals feed mainly on fish and cephalopod [27], shellfishes are not thought to be a principal route of *T. gondii* and *N. caninum* infection in these seals. North Pacific giant octopus (*Paroctopus dofleini*) is the most important prey for Kuril harbor seals and spotted seals at Nosappu [27]. As these octopuses feed on shellfishes [25], the seals are potentially to ingest secondary the oocysts concentrated by shellfishes via the octopuses. For confirming the route of protozoan infection in the sea, status of contamination in various marine organisms by protozoa must be researched.

For both marine mammal and human health, contamination of marine environment and marine organisms by pathogenic protozoa should be monitored, and appropriate runoff management is necessary. Feces of domestic animals including cats and dogs must be disposed properly to prevent environmental contamination with protozoan oocysts contained in the feces.

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