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Journal of Wildlife Diseases, 41(2), 2005, pp. 454–458

Prevalence of *Toxoplasma gondii* and *Neospora caninum* in Sika Deer from Eastern Hokkaido, Japan

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ABSTRACT: Brain and serum were collected from 120 and 12 free-ranging sika deer (*Cervus nippon yesoensis*), respectively, from six regions in eastern Hokkaido during controlled hunts in the autumn of 2003. Brains were tested for *Neospora caninum* and *Toxoplasma gondii* DNA by polymerase chain reaction (PCR) assays. Antibodies to *Toxoplasma gondii* were measured by means of a latex agglutination test. No brain tested positive for either type of DNA, and no antibody to *Toxoplasma gondii* was detected in serum, suggesting a low prevalence of infection with these organisms in free-ranging sika deer from eastern Hokkaido. Further examination of multiple tissues by PCR and serologic surveys will be necessary to confirm this.

Key words: Free-ranging sika deer, LAT, neosporosis, northeastern Japan, PCR, toxoplasmosis.

There are approximately 120,000 free-ranging sika deer (*Cervus nippon yesoensis*) in Hokkaido, and in recent years, the deer population has been increasing (Hokkaido Government, 1998). Such large numbers of deer cause substantial damage to agriculture and forestry lands, have adverse effects on urbanization, and alter ecological systems. Moreover, they can harbor zoonotic diseases posing a potential public health risk in Japan (Asakura et al., 1998; Sato et al., 2000). *Toxoplasma gondii* and *Neospora caninum* are apicomplexan protozoa with worldwide distributions that can cause neuromuscular disease and abortion in different animal species (Dubey and Beattie, 1988; Dubey, 1999). *Toxoplasma gondii* causes disease in human beings (Dubey and Beattie, 1988), and although there is no evidence of natural infection of human beings with *N. caninum,* there is concern about the zoonotic potential because it can infect nonhuman primates (Dubey, 1999). Following oral infection, cats and dogs, the definitive hosts of *T. gondii* and *N. caninum,* respectively, shed oocysts in the feces, which can serve as a major source of infection for other species (Dubey and Beattie, 1988; Dubey, 1999). Transplacental transmission can also occur (Dubey and Beattie, 1988; Dubey, 1999). Oral infection can occur by ingesting raw meat contaminated with the parasites or their sporulated oocysts, and meat from domestic animals and game, such as deer, is considered a potential source of human infection with *T. gondii* (Sacks et al., 1983; Zarnke et al., 2000; Kutz et al., 2001).

There are reports of the prevalence of antibodies to *T. gondii* in white-tailed deer (*Odocoileus virginianus*) (Smith and Frenkel, 1995; Vanek et al., 1996) and neosporosis has been reported in California black-tailed deer (*Odocoileus hemionus columbianus*) (Woods et al., 1994; Dubey et al., 1996). Serosurveys of the prevalence of antibodies to *N. caninum* in white-tailed deer (*Odocoileus virginianus*) in the southeastern United States determined that more than 40% of deer had high levels of antibodies (Dubey et al., 1999; Lindsay et al., 2002). However, little is known about the prevalence of these parasites in wildlife from Hokkaido, northern Japan.

In addition to serologic testing, the polymerase chain reaction (PCR) has also been used to detect infection with these organisms in a variety of species (Almer'a et al., 2002; Aspinall et al., 2002; O'Handley et al., 2002; Masala et al., 2003). The objective of the present study was to determine the prevalence of *T. gondii* and *N. caninum* in free-ranging sika deer from eastern Hokkaido, through direct detection of the parasites in brain tissue using PCR. A limited number of sera was also tested for antibodies to *T. gondii.*

Tachyzoites of *N. caninum* isolated from sheep (Kobayashi et al., 2001; Koyama et al., 2001) and tachyzoites of the Beverley strain of *T. gondii* were maintained by continuous passage in BAE cell cultures. After tachyzoites were collected from cell cultures, they were suspended in phosphate buffered saline and the host cells and debris removed by passing suspensions through a 3 μm polycarbonate filter (Nuclepore, Corning Coster Corporation, Tokyo, Japan). DNA was extracted from the purified tachyzoites using proteinase K digestion and phenol–chloroform–isoamyl alcohol.

Brains of 120 free-ranging sika deer were obtained during controlled hunts held in the autumn of 2003 from six different regions in eastern Hokkaido (Fig. 1). The number of samples collected was determined by assuming a population of 50,000 animals in the test area and prevalences for either *T. gondii* or *N. caninum* of approximately 50% to maximize the sample size and obtain a minimal confidence of 95% with a relative precision of 20%. The sex and age of the animals were inferred from the antlers and teeth, and recorded. They were kept refrigerated during shipment and storage. The cerebrum and cerebellum were removed from each brain within 5 days after hunting. Brain samples weighing approximately 0.5 g were prepared, and genomic DNA was extracted using the proteinase K-phenol method. Parasite DNA was amplified by PCR using *N. caninum* specific oligonucleotide primers, Np21-plus (5′–gtgcgtccaatcctgtaac–3′) and Np6-plus (5′–cagtcaacctacgtcttct–3′) (Liddell et al., 1999) and the technique described by Yamage et al. (1996) and using a *T. gondii* B1 gene primer set (5′–ggaactgcatccgttcatgag–3′ and 5′–cagacgaatcaacggaactg–3′) as described by Burg et al. (1989). The PCR product sizes were 328 and 500, respectively. For the PCR reaction, 1 μg of template DNA was used in a 50 μl reaction conducted using the High Pure PCR Template Preparation Kit (Roche Diagnostic, GmbH, Manhein, Germany). Thermocycling consisted of 40 cycles of 1 min at 94 C, 1 min at 50 C, and 2 min at 72 C, with a final extension at 72 C for 8 min. The χCR products were visualized on a 2% agarose gel run at 80 V for 30 min. The gels were stained with ethidium bromide, and photographed in a short-wave ultraviolet light box. Appropriate positive (*N. caninum* and *T. gondii* tachyzoite DNA) and negative (H_2O) controls were included in each set of reactions. The sensitivity of the reaction in measuring the parasite levels in the background host DNA was assessed by addition of progressively smaller numbers of *N. caninum* or *T. gondii* tachyzoites to 0.5g of uninfected deer brain, followed by DNA extraction as described above (Fig. 2).

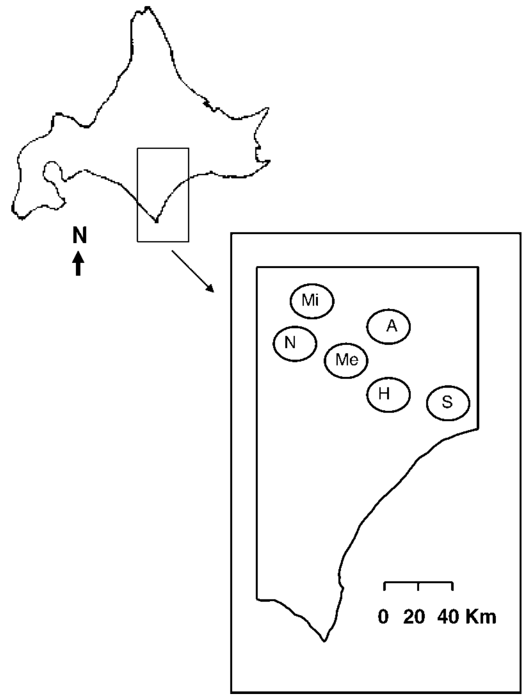

FIGURE 1. Map of eastern Hokkaido and regions sampled in this study. A indicates Ashoro, H is Honbetsu, Me is Meto, Mi is Mikuni, N is Nukabira, S is Shiranuka.

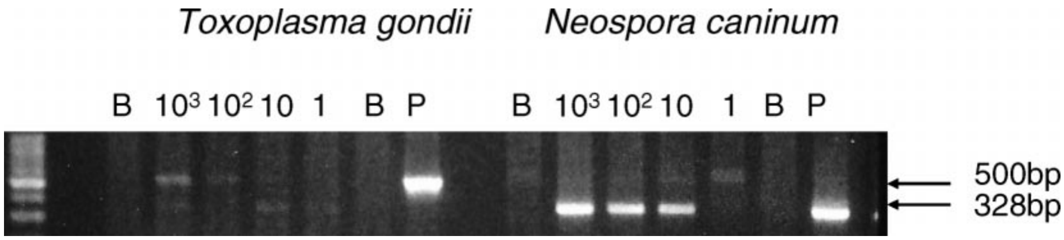

FIGURE 2. Sensitivity of the PCR reactions and ability to detect parasite DNA in the background of host DNA. Lane P, genome DNA; lane B, deer brain DNA; lane number means number of tachyzoites added in the brain.

Serum samples were collected from 12 sika deer in the Ashoro region during the same period. Sera were tested in triplicate for antibodies to *T. gondii* using a commercial LAT kit (Toxo Check, Eiken Chemical Co., Ltd., Tokyo, Japan). An antibody titer of 1:64 or above was regarded as positive.

Eighty-four of the brain samples were classified according to region (Ashoro, Honbetsu, Meto, Mikuni, Nukabira, and Shiranuka), age (yearling, fawn and adult), and sex (except for yearlings); these parameters were not available for the other 36 samples (Table 1). Of the 120 brain samples, none was positive for *N. caninum* or *T. gondii* DNA. None of the 12 serum samples had antibody to *T. gondii* as detected by LAT at a dilution of 1:16 (data not shown). These results suggest that the prevalence of *N. caninum* and *T. gondii* in free-ranging sika deer from eastern Hokkaido is currently low. There are no feral dogs or cats in Hokkaido, which suggests that a sylvatic cycle of *N. caninum* and *T. gondii* mediated by the definitive hosts might be rare in wildlife in Hokkaido at present.

Masala et al. (2003) showed that although *T. gondii* DNA could be amplified from a variety of tissues from sheep and goat abortions, detection varied among tissues, and placenta was the tissue with the highest detection rate. Thus, PCR detection in this study could have been low because brain alone was used as the target organ. In addition, failure to detect antibodies to *T. gondii* could stem from the small number of samples examined that all came from the same area. In view of this, it will be necessary to examine multiple tissues from animals by PCR and expand the serologic analyses to confirm the low prevalence of *T. gondii* and *N. caninum* in wildlife from Hokkaido. The presence of *T. gondii* has been reported in domestic

TABLE 1. Number of brains from hunted deer in eastern Hokkaido analyzed using PCR.

Region	Yearling Unknown Sex	Fawn		Adult		Total (%)[a]	PCR Positive
		Male	Female	Male	Female		
Ashoro	0	0	0	7	5	12 (14)	0
Honbetsu	7	5	2	7	5	26 (31)	0
Meto	3	1	1	6	5	16 (19)	0
Mikuni	0	2	0	0	0	2 (2)	0
Nukabira	1	5	0	1	2	9 (11)	0
Shiranuka	4	2	4	6	3	19 (23)	0
Subtotal (%)[a]	15 (18)	15 (18)	7 (8)	27 (32)	20 (24)	84	0
Others[b]						36	0
Total (%)						120	0

[a] The proportions of the 84 for each classification are represented as percentages.
[b] Brains from animals of unknown region, age, or sex.

cats in Japan (Hagiwara, 1977; Fujinami et al., 1983) and recent studies have shown positive rates ranging between 5.4% (Maruyama et al., 2003) and 6.0% (Nogami et al., 1998). Sawada et al. (1998) reported that 15 of 48 dogs (31.3%) raised on dairy farms having cases of cattle abortion due *N. caninum* infection were positive for antibody to *N. caninum*, whereas the occurrence of antibodies in dogs from an urban area was 7.1% (14 of 198 dogs). If wildlife populations continue to grow, and urbanization continues to expand, this will increase the chance of contact between domestic animals and wildlife. Because very little is known about the prevalence of *T. gondii* in domestic and feral cats and *N. caninum* in domestic and feral dogs from eastern Hokkaido, further investigations on these infections in both domestic and wildlife species is warranted.

This work was supported by Grant-in-Aid for Scientific Research (The 21st Century Center-of-Excellence Program) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received for publication 24 April 2004.