



Short communication

Seroprevalence of *Toxoplasma gondii* in wild sika deer in JapanTokio Hoshina^{a,b,*}, Shinya Fukumoto^c, Hiroka Aonuma^a, Erisha Saiki^a, Seiji Hori^b, Hirotaka Kanuka^{a,*}^a Department of Tropical Medicine, The Jikei University School of Medicine, Tokyo, Japan^b Department of Infectious Diseases and Infection Control, The Jikei University School of Medicine, Tokyo, Japan^c National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan

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ABSTRACT

Toxoplasmosis is a food-borne infection that is widespread around the world, causing congenital disorders and opportunistic infections. Ingestion of undercooked meat is one of the risk factors for infection with the causative agent, *Toxoplasma gondii*. Japanese people occasionally eat rare meat as a traditional cuisine style called “Sashimi”. A rapid increase in venison consumption in Japan has occurred mainly due to enhanced population control of wild Japanese deer (*Cervus nippon*) in recent decades. In particular, Yezo-sika deer (*C. n. yezoensis*) in Hokkaido (the northernmost and largest prefecture in Japan) is frequently supplied to markets as branded game/bushmeat. To study the possible burden of *Toxoplasma gondii* among wild Yezo-sika deer, plasma samples of Yezo-sika deer hunted during two seasons, 2010–2012, in Eastern Hokkaido were investigated. A total 80 samples were examined using the Sabin–Feldman dye test, which is highly specific and sensitive for identifying the development and persistence of antibodies after primary *Toxoplasma* infection, demonstrating that 38 cases (47.5%) were seropositive (cut-off titer < 1:16). Antibody prevalence of *T. gondii* in female deer was higher than in males. Adult deer aged 3 years or over showed higher seroprevalence compared with younger animals. The overall seroprevalence fluctuated significantly according to the season when the deer were hunted. These results indicated widespread infection of *T. gondii* among Japanese wild Yezo-sika deer, suggesting that both appropriate handling and treatment of bushmeat are required to prevent food-borne toxoplasmosis in Japan.

Toxoplasma gondii is a protozoan parasite in the phylum Apicomplexa, which has the most successful strategies to infect various homeotherms, including approximately one-third of the human population [1]. A leading cause of toxoplasmosis in humans is food-borne infection, and every edible homeotherm vertebrate has the potential to be a source of infection. A previous epidemiological study in the 1980s pointed out the correlation between raw meat consumption and *Toxoplasma* infection in Japan [2]. Currently, *Toxoplasma* seroprevalence in livestock in Japan is fairly low compared with other countries [3]. On the other hand, infection by *T. gondii* in wild animals is uncontrollable. One of the largest proportions in the game market in Japan is wild sika deer. *Cervus nippon yezoensis* (Yezo-sika deer) is a dominant subspecies (approximately 75%) of hunted deer in Japan.

The Sabin–Feldman dye test (SFDT), which was first developed by Sabin and Feldman in 1948 [4], has been considered as the gold standard for the detection of anti-*T. gondii* antibodies in humans [5]. Although the SFDT requires live parasites (tachyzoite stage) and serum plus accessory factor (human complement), severely limiting its availability, SFDT continues to be the primary reference test to confirm the

accurate diagnosis of *T. gondii* infection.

In this report, the seroprevalence of *Toxoplasma* infection in Japanese wild sika deer, particular in Yezo-sika deer in Hokkaido, was examined using SFDT. Our results suggest that Japanese deer meat could be considerable source of *Toxoplasma* transmission to humans.

Plasma from hunted wild Yezo-sika deer (*C. n. yezoensis*) was collected during two hunting seasons, 2010–2011 and 2011–2012, in Eastern Hokkaido. Hunters who belong to ELEZO Co., Ltd. performed hunting for commercial purposes in accordance with the guideline published by the Ministry of the Environment and the Ministry of Agriculture, Forestry and Fishers of Japan. The hunters recorded the sex, age, hunted date, and location of the deer. Age of the deer was determined using horn morphology for males, and body constitution in maternal line cluster for females, respectively. Within 2 h after shooting, deer blood samples were placed into tubes containing EDTA-2K and centrifuged at 2400 rpm for 15 min. The plasma fraction was stored at –20 °C and used for further analysis. Eighty serum samples were randomly picked up from approximately 400 samples, which were collected during the two hunting seasons.

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A type 1 *T. gondii* (RH strain) used in this study was a gift from Dr. K. Nagamune. The parasites were maintained by serial passage in human foreskin fibroblast cells (HFF) cultured in D10 medium (Dulbecco's modified Eagle medium (Wako) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei), 2 mM glutamine (Sigma), 10 mM HEPES buffer (Sigma) and 10 µg/ml gentamicin (Sigma)) at 37 °C. The preparation of purified tachyzoites for the Sabin–Feldman dye test was based on a previous report [6].

SFDT was performed in accordance with the protocol described previously [7], as follows. Briefly, plasma samples were inactivated at 56 °C for 30 min and then diluted 1:4, 1:16, 1:64, 1:256, and 1:1024 with DPBS (Wako). The concentration of purified tachyzoites was adjusted to 4×10^6 parasites/ml in DPBS-Ca. Accessory factor (AF) was *Toxoplasma*-specific antibody-negative serum collected by volunteers, and previously checked for suitability with the test. Solution A was made by mixing 400 µl of AF, 60 µl of Alsever's solution (Sigma), and 40 µl of the tachyzoite suspension. Then, 15 µl of solution A was added into 15 µl of each diluted plasma sample and incubated for 1 h at 37 °C. After incubation, 15 µl of alkaline methylene blue solution (10% methylene blue alcohol saturated solution (Hayashi) in alkaline sodium borax buffer (0.53% Na₂CO₃, 9.73 ml + 1.91% Na₂B₄O₇/10H₂O 0.27 ml)) was added to each sample. After incubation for 10 min at room temperature, the reacted samples were dispensed into a cell counter (C-Chip, NanoEntek) and the parasites were examined at 400× magnification using a light microscope (Leica DM 2500). The numbers of blue-stained and unstained tachyzoites were counted in 16 compartments of C-Chip (1 × 1 µm), which usually included > 100 tachyzoites. Dilutions for which ≥ 50% of the observed tachyzoites remained unstained were considered a positive titer. A cut-off titer of 1:16 or higher was considered positive for *T. gondii* infection.

Statistical analysis was done by using R version 3.3.3. Twoby2 in package “Epi” was used to calculate Fisher's exact test, relative risk, odds ratio, and 95% confidence interval between two groups. For analysis of the correlation between reciprocal titers of SFDT and case numbers was calculated using Spearman's rank correlation coefficient.

All experiments using human serum (accessory factor) were performed in accordance with institutional guidelines and approved by the Ethics Committee of The Jikei University School of Medicine (Permit Number: 27-024 (7908)). All donors provided written informed consent for collection of samples and subsequent analyses.

To assess the prevalence of *T. gondii* infection in meat from wild Japanese deer destined for human consumption, the focus of this study was meat from Yezo-sika deer hunted in Eastern Hokkaido, because this is one of the major sources of venison in Japan. In 2016, approximately 120,000 Yezo-sika deer were hunted throughout Hokkaido, and half of these (60,000) were from the Eastern Hokkaido, including Tokachi, Hidaka, and Kushiro subprefecture. About 10% of hunted deer were slaughtered for meat production. The characteristics of hunted deer are shown in Table 1. Hokkaido prefecture is located in the northern part of Japan; the latitude and longitude of Obihiro city, the approximate center of the hunting site is 42°-55'N and 143°-11'E, which is situated in

a boreal wet forest biome according to the Holdridge life zones system of bioclimatic classification. Plasma from 80 wild Yezo-sika deer was obtained during two hunting seasons. Forty-five cases (56.25%) were female, and their average age was 2.8 years old. Thirty-five cases (43.75%) were male, with an average age of 2.6 years old. In total, 75% of the deer were hunted in the Toyokoro and Urahoro areas (Table 1).

To examine infection by *T. gondii* in wild Yezo-sika deer, SFDT, which is an accurate method for identifying antibodies to the *Toxoplasma* parasite was employed in this study. SFDT is a robust serological measure to evaluate anti-*Toxoplasma*-specific immunoglobulins (IgA, IgM, and IgG), which cause a prompt increase in titer within about 2 weeks after infection [19]. All 80 serum samples were examined using the SFDT, resulting in the observation that 38 cases were seropositive: the number of cases at each titer level of 1:16, 1:64, 1:256, and ≥ 1:1024 were 20, 13, 4, and 1, respectively (Fig. 1A). The highest seropositivity (60%) was observed in the Toyokoro area (Table 1). *Toxoplasma* seroprevalence in this study was higher than in previous reports from Japan [8–10]. Positive serum with a titer 1:16 or higher generally indicates sub-acute infection (3–4 weeks after infection). Positive but lower titers (1:16 or less) mostly represent chronic infection that persists for several months to years, and occasionally acute infection (a week or two after infection). These results suggested active infections of *T. gondii* in the Yezo-sika deer population in Eastern Hokkaido.

To examine the relationship between seroprevalence and potential risk factors associated with *T. gondii* infection in wild Yezo-sika deer, the sex and age of the hunted deer were investigated. Seroprevalence of *T. gondii* was significantly higher in female deer ($p = .037$) (Fig. 1B) and in those aged 3-years-old or more ($p = .035$) (Fig. 1C). The correlation between seroprevalence and age suggested multiple rounds of *T. gondii* infection in older deer, which was also reported in a previous study in goats [11]. According to the previous report [12], sexual differences in behavioral patterns and eating behaviors might affect the seropositivity ratios. There was also a significant difference of seroprevalence between the two hunting seasons. In the season from October 2010 to January 2011, plasma samples were obtained from 30 deer (15 males, 13 females, and two unknown). In the second season from October 2011 to January 2012, plasma samples were collected from 50 deer (16 males, 32 females, and two unknown). Seroprevalence was higher in the latter season ($p = .021$) (Fig. 1D). High rainfall precipitation and humidity are known to increase the seroprevalence of *T. gondii* in wild deer [13]. The summer of Eastern Hokkaido in 2010 was extremely hot and dry, presumably causing a reduction in the infection rate of the *Toxoplasma* parasite in Yezo-sika deer.

In this study, the high prevalence of *T. gondii* in wild Yezo-sika deer in Eastern Hokkaido was demonstrated using the SFDT, which was established by Drs. Sabin and Feldman in 1948 as the first reliable serologic examination method for *Toxoplasma* infection [4]. The limitation of the SFDT is that it involves complicated serologic steps, which make it unsuitable for use as a routine test. Despite these disadvantages, the field of serologic diagnosis of toxoplasmosis still demands the SFDT as

Table 1
Seroprevalence of *Toxoplasma gondii* in wild Yezo-sika deer hunted in Eastern Hokkaido, Japan.

	n	Male (%)	Female (%)	Unknown sex (%)	Age ^a (range)	Female age ^a (range)	Male age ^a (range)	SFDT+ (%)
Erimo	2	2 (100)	0 (0)	0 (0)	1.5 (1–2)	–	1.5 (1–2)	0 (0)
Samani	2	2 (100)	0 (0)	0 (0)	2.5 (2–3)	–	2.5 (2–3)	0 (0)
Hiroo	6	2 (33.3)	4 (66.7)	0 (0)	2.8 (1–5)	3.5 (2–5)	1.5 (1–2)	2 (33.3)
Urakawa	1	1 (100)	0 (0)	0 (0)	4.0 (4)	–	4.0 (4)	1 (100)
Toyokoro	40	13 (32.5)	27 (67.5)	2 (5)	2.9 (1–7)	2.8 (1–4)	3.5 (1–7)	24 (60)
Obihiro	2	0 (0)	2 (100)	0 (0)	2.5 (2–3)	2.5 (2–3)	–	0 (0)
Urahoro	20	10 (50)	10 (50)	1 (5)	2.3 (1–5)	2.7 (1–4)	2.0 (1–5)	9 (45)
Onbetsu	2	2 (100)	0 (0)	0 (0)	3.0 (1–5)	–	3.0 (1–5)	0 (0)
Unknown area	5	3 (60)	2 (40)	1 (20)	2.0 (1–3)	3.0 (3)	1.5 (1–2)	2 (40)
Total	80	35 (43.75)	45 (56.25)	4 (5)	2.7 (1–7)	2.8 (1–5)	2.6 (1–7)	38 (47.5)

^a Years old.

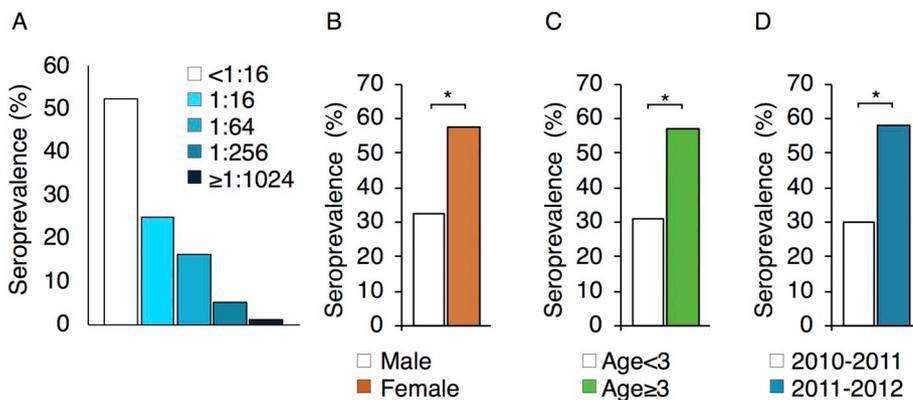


Fig. 1. Seroprevalence of *Toxoplasma* parasite infection in Yezo-sika deer. A cut-off titer of 1:16 or higher was considered positive for *T. gondii* infection (A). *Toxoplasma* seroprevalence related to sex, age, and hunting season. (B) Correlation between *T. gondii* seroprevalence and deer sex. The percentages of SFDT-positive males and females were 32.3% (10/31) and 57.8% (26/45), respectively ($*p = .037$, RR 1.791, 95% confidence interval 1.015, 3.161). Female Yezo-sika deer showed significantly higher seroprevalence than male deer. (C) Correlation between *T. gondii* seroprevalence and deer age. The seroprevalence was 31.0% (9/29) in deer aged under 3 years old and 57.1% (28/49) in deer aged 3 years old or more ($*p = .035$, RR 1.841, 95% confidence interval 1.016, 3.336). Older Yezo-sika deer showed significantly higher seroprevalence than young deer. (D) Correlation between *T. gondii* seroprevalence and hunting season. The seroprevalence was 30.0% (9/30) in the 2010–11 season and 56.0% (29/50) in the 2011–12 season ($*p = .021$, RR 1.933 95% confidence interval 1.066, 3.506).

reference test due to its sufficient sensitivity and specificity; performance of the SFDT is known to be equivocal for *Toxoplasma* IgG western blotting in human diagnosis [14]. In a study of experimental infection of elk (*Cervus canadensis*) with *T. gondii*, an indirect hemagglutination antibody test failed to detect the infection [15].

The proportion of seropositive wild deer we identified in this study was 47.5%. The seroprevalence observed in this study was almost equivalent to the results of surveillance in wild roe deer in France [12] and farmed red deer in Scotland [16]. Compared with other investigations of sika deer in Japan, the seroprevalence in this study was considerably higher. A possible explanation for the discrepancy may be that these previous studies employed a latex agglutination test (LAT) instead of the SFDT to survey the seroprevalence of *T. gondii* in sika deer. The LAT is a simple and rapid diagnostic test, but its sensitivity and specificity are lower than or similar to those of the SFDT. In addition, the only LAT kit available in Japan (Toxocheck-MT, Eiken-Kagaku, Tokyo) was originally developed to assess *Toxoplasma* infection in swine. There has been only one study comparing sensitivity and specificity of the LAT, an enzyme-linked immunosorbent assay (ELISA), and western blotting using deer samples [17], which did not include the SFDT as a reference test. The difference in sensitivity and specificity among these two methods might cause have underestimation of *Toxoplasma* parasite numbers in deer when the LAT was employed for diagnosis.

Another possibility for the higher *Toxoplasma* seroprevalence in the present study may be cross-reactivity of the SFDT with other species of parasites infecting the animals. Cross-reactivity among antigens of different protozoan parasites such as *Sarcocystis* spp. and *Neospora* spp. detected by the SFDT was first reported in sheep in the 1960s [18]. Experimental infection of calves with *Sarcocystis cruzi* showed transient low positivity titers in the SFDT [19]. On the other hand, another study using the SFDT observed no cross-reaction among *T. gondii*, *Sarcocystis* spp., and *Neospora* spp. in rats, rabbits, and ewes [20]. Further studies are needed in order to examine whether the numbers of parasite-infected sika deer in Japan have been underestimated or not.

In summary, this is the first report to evaluate the seroprevalence of *T. gondii* in wild Japanese deer using the SFDT, which can diagnose both active and persistent infections of *T. gondii* accurately compared with other serological examinations. Most *Toxoplasma* parasite-infected deer identified during this study were considered to be in a chronic infection phase, suggesting that SFDT-positive deer could be a reservoir in nature capable of transmitting *T. gondii* to other animals and humans. Sustainable, quantitative risk assessment of *T. gondii* infection in wild sika deer in Japan may facilitate the prevention of Toxoplasmosis in humans.

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