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NOTE

Parasitology

Prevalence of serum antibodies to *Toxoplasma gondii* in free-ranging cats on Tokunoshima Island, Japan

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ABSTRACT. The prevalence of *Toxoplasma gondii* infection in free-ranging cats on Tokunoshima Island was assessed by testing 125 serum samples using anti-*T. gondii* IgG indirect enzyme-linked immunosorbent assay. The overall seropositivity rate was 47.2% (59/125). Seropositivity rates in cats with body weight >2.0 kg (57.4%) were significantly higher than in those with body weight ≤2.0 kg (12.5%, $P<0.01$). Analysis of the number of seropositive cats by settlement revealed the presence of possibly-infected cats in 17 of 23 settlements, indicating the widespread prevalence of *T. gondii* on the island. This is the first study to show the seroprevalence of *T. gondii* in free-ranging cats on Tokunoshima Island. The information revealed in this paper will help to prevent the transmission of *T. gondii* among cats and also in both wild and domestic animals and humans on the island.

KEY WORDS: free-ranging cat, seroprevalence, Tokunoshima Island, *Toxoplasma gondii*

Toxoplasma gondii is a zoonotic protozoan parasite, which is able to infect diverse species of warm-blooded animals, including humans. Oocysts of *T. gondii* are shed in feces of infected domestic cats and wild felids, the definitive host, and ingestion of oocysts-contaminated feces, soil, and water is one of the main routes of infection in both cats and other animals that act as intermediate hosts. Since oocysts are distributed widely by infected cats, and *T. gondii* can infect various hosts in various ways, the impact extends to wildlife and livestock production. Tokunoshima Island in the Nansei Islands is located in a subtropical area, has a surface area of approximately 247.85 km^2 and about 23,600 residents [7]. The forest area of Tokunoshima Island harbors many endemic mammals, such as the Amami rabbit (*Pentalagus furnessi*), Ryukyu long-haired rat (*Diplothrix legata*), and Tokunoshima spiny rat (*Tokudaia tokunoshimensis*). Currently, conserving endemic animals is one of the main issues on this island. In addition, a case of *T. gondii* infection in the endemic Amami spiny rat (*T. osimensis*) [18] and suspected toxoplasmosis of Amami rabbit [9] has been reported in the adjacent Amami-Oshima Island. Free-ranging cats (stray, feral, and owned-outside cats) are frequently found not only in town, but also in forest areas and farmlands on the Island [10]. This study surveyed *T. gondii* infection in free-ranging cats on Tokunoshima Island for the first time, as a first step towards inferring the distribution of the infection on the island.

One hundred and twenty-five serum samples of free-ranging cats were provided from the population control program on Tokunoshima Island, carried out by the local Tokunoshima government and the Ministry of the Environment. In the program, free-ranging cats were captured by traps from all towns on the Island; Amagi-cho, Isen-cho, Tokunoshima-cho. Captured cats are either released or adopted by new owners after being neutered. Information on the samples such as the date of capture, area of capture, sex, and body weight were also provided. All serum samples were stored at −20°C until further analysis. Ten serum samples from specific pathogen free (SPF) cats were used as negative controls. Eight serum samples of naturally-infected cats from the Okinawa Island, which were previously confirmed to be infected by commercial latex agglutination test (LAT) kits (Toxocheck-MT; Eiken Chemical, Tokyo, Japan), were used as positive controls. Serum anti-*T. gondii* antibody was measured by indirect enzyme-linked immunosorbent assay (ELISA). Antigen was prepared from *T. gondii* RH Ankara strain tachyzoites as described previously [3].

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Briefly, tachyzoites were obtained from peritoneal fluids of BALB/c mice infected 72 hr prior intraperitoneally. After washing three times with sterile phosphate buffered salts (PBS, pH: 7.4), the pellet was suspended with 20% sodium dodecyl sulfate (SDS) and waited for 30 min, then centrifuged at 14,000 *g* for 5 min. The supernatant was collected and kept at −20°C. The tests were conducted following the method reported previously [2], with some modifications. Ninety six-well microtiter plates were coated with 100 µl per well of an antigen suspension containing 3.0×10^5/ml samples of *T. gondii* RH Ankara strain tachyzoites in PBS and incubated overnight at 4°C. In other procedures, incubations were performed at room temperature. Plates were washed with PBS-T (PBS-0.05% Tween 20) and blocked for 1 hr with 200 µl of 1% bovine serum albumin (BSA) in PBS-T per well. The serum samples were 1:100 diluted in dilution buffer (0.1% BSA in PBS-T) and applied at 100 µl per well for 1 hr, after being washed. Next, plates were washed and 100 µl 1:20,000-diluted Goat anti-cat IgG horseradish peroxidase (HRP) conjugated antibodies (Life Technologies, Frederick, MD, USA) in dilution buffer were applied. Subsequent to 1-hr incubation and washing, 10 min reaction with 100 µl of substrate (TMB microwell Peroxidase Substrate System; SeraCare Life Sciences, Milford, MA, USA) was carried out and stopped after by 50 µl of 2[N] H_2SO_4 per well. The absorbance was measured at 450 nm by a microplate reader (SpectraMax Paradigm, Molecular Device, Sunnyvale, CA, USA). All samples were analyzed in duplicates. The optical density (OD) values of each sample, negative controls, and positive controls were calculated. The cut-off value was considered as the mean OD values of negative controls plus three standard deviations. The Fisher's exact test was performed to detect any differences in the rates of positivity in both genders (male and female), weight, and towns. The level of maturity of free-ranging cats was considered by bodyweight conventionally; cats weighing 2.0 kg and less were categorized as young, while cats over 2.0 kg were categorized as adults. *P*- values less than 0.01 were considered significant. All data analysis and map drawing of Tokunoshima Island with GPS points were performed using R version 3.6.3 [16]. To show the representative points of settlements, the map and the information of land use were obtained from National Spatial Planning and Regional Policy Bureau, Ministry of Land, Infrastructure, Transport and Tourism of Japan [14]. To confirm the diagnostic accuracy of ELISA assay, western blotting was performed on 66 randomly chosen serum samples. Crude tachyzoite lysate was prepared as previously reported [1]. *T. gondii* PLK strain maintained in Vero cell cultures was collected and passed through a 27 G needle, three times. The cells were pelleted at 2,000 rpm for 10 min, washed in PBS, and then passed through a 5 µm filter. Parasites were then repelleted at 2,000 rpm for 10 min and lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Crude tachyzoite lysate was 3:1 diluted with SDS sample buffer and boiled 95°C for 5 min. Ten µg of samples per well were loaded on 12.5% polyacrylamide gel and separated by electrophoresis. Then antigens were transferred onto polyvinylidene difluoride western blotting membrane (Roche Diagnostics GmbH, Mannheim, Germany) using the Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad, Hercules, CA, USA), and blocked in Block Ace (DS Pharma Biomedical, Osaka, Japan) overnight at 4°C. After washing in PBS-T, membranes were incubated in cat serum diluted 1:500 in dilution buffer (1% BSA PBS-T) at room temperature for 1 hr. Then, membranes were again incubated in 1:10,000-diluted Goat anti-cat IgG HRP conjugated antibodies (Life Technologies) in dilution buffer for 1 hr subsequent to wash. Next, membranes were washed, and chemiluminescent images were developed using Amersham ECL Western Blotting Detection Reagent (GE Healthcare UK Ltd., Buckinghamshire, UK) and LAS-3000 mini (Fujifilm, Tokyo, Japan).

The overall seropositivity rate in free-ranging cats on Tokunoshima Island was 47.2% (59/125) with the cut-off value set at 0.438 (Fig. 1A). The seropositivity rate was then compared by sex, maturity, and towns (Table 1). No significant difference was observed between males and females (*P*=0.35) (Fig. 1B). Adult cats had a significantly higher seropositivity rate (57.4%) than young cats (12.5%, *P*<0.01) (Fig. 1C). No significant difference was found in the seropositivity rates between the 3 towns (*P*=0.85). The three towns on Tokunoshima Island can be divided into 44 settlements. A total of 125 samples were obtained from 23 settlements. In 17 of those 23 settlements, 1 or more samples tested positive (Fig. 2). Thirty-seven out of 66 samples were positive (56.1%) by western blotting analysis (Fig. 3 and Table 2). Compared to the seropositivity rate in ELISA and western blotting, the results are reasonably in accord (Table 3), and the seroprevalence based on ELISA using PLK strain antigen with positive/negative controls and randomly selected samples was substantially concordant with the result of ELISA using RH Ankara strain antigen (data not shown).

This study showed a considerably high prevalence of anti-*T. gondii* IgG in free-ranging cats, indicating some risk of *T. gondii* infection to other animals and humans on Tokunoshima Island. The result may be the highest among all studies of seroprevalence in cats conducted previously in Japan. The seropositivity rates have been reported in cats that visited animal hospitals; 5.4% (78/1,477)

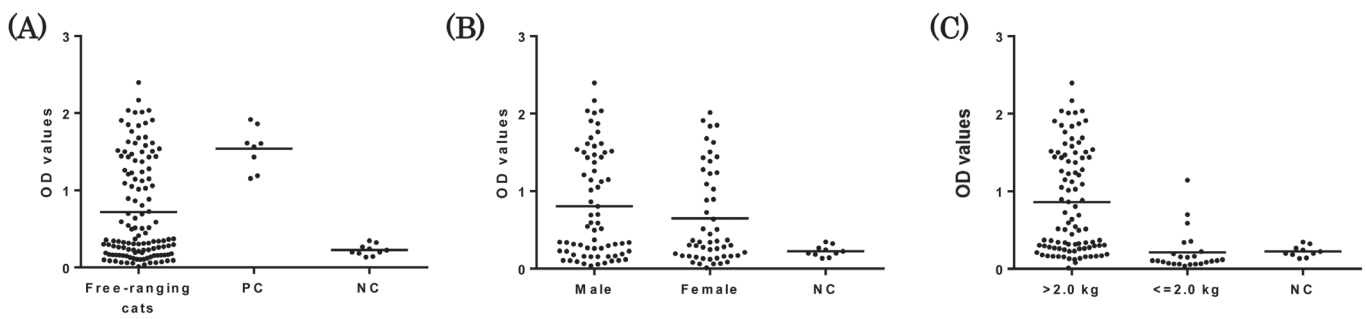

Fig. 1. Anti-*Toxoplasma gondii* IgG optical density values with mean (line) in (A) all 125 samples from free-ranging cats, (B) male and female cats, (C) cats >2.0 kg and ≤2.0 kg of body weight. PC: positive controls, NC: negative controls.

Table 1. The number of samples categorized by sex and body weight from 3 towns on Tokunoshima Island

Town	Total	Sex			Body weight		
		Male	Female	Unknown	>2.0 kg	≤2.0 kg	Unknown
Amagi-cho	34 (15)	17 (8)	17 (7)	0 (0)	25 (14)	9 (1)	0 (0)
Isen-cho	48 (24)	30 (16)	18 (8)	0 (0)	37 (22)	11 (2)	0 (0)
Tokunoshima-cho	42 (20)	21 (12)	15 (7)	6 (1)	31 (18)	4 (0)	7 (2)
Unknown	1 (0)	0 (0)	1 (0)	0 (0)	1 (0)	0 (0)	0 (0)
Total	125 (59)	68 (36)	51 (22)	6 (1)	94 (54)	24 (3)	7 (2)

Numbers in parentheses indicate positive samples.

Table 2. The number and the ratio of positive in samples tested both ELISA and western blotting

No. of tested	ELISA	Western blotting
	Positive (%)	Positive (%)
66	34 (51.5)	37 (56.1)

Table 3. The comparison of the results of ELISA and western blotting

Western blotting	ELISA		Total
	Positive	Negative	
Positive	31	6	37
Negative	3	26	29
Total	34	32	66

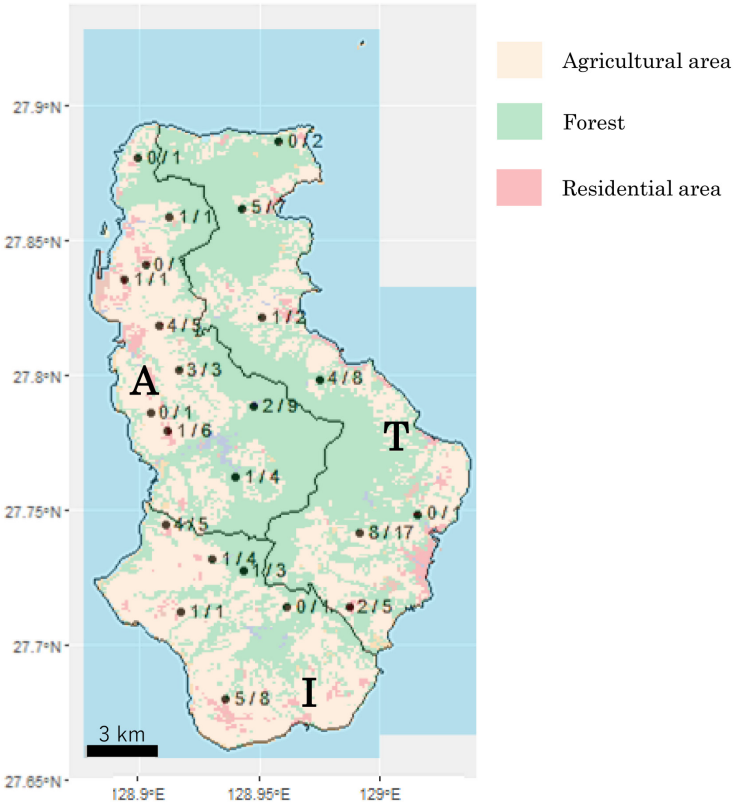

Fig. 2. A map of land use on Tokunoshima Island with points that represent the 23 settlements where the cats were captured. The numbers beside the points stand for the following ratio: (the number of positive samples in the settlement) / (the number of captured cats in the settlement). Big characters are the initials of towns; A: Amagi-cho, I: Isen-cho, T: Tokunoshima-cho. The black lines on the island are borders of the three towns.

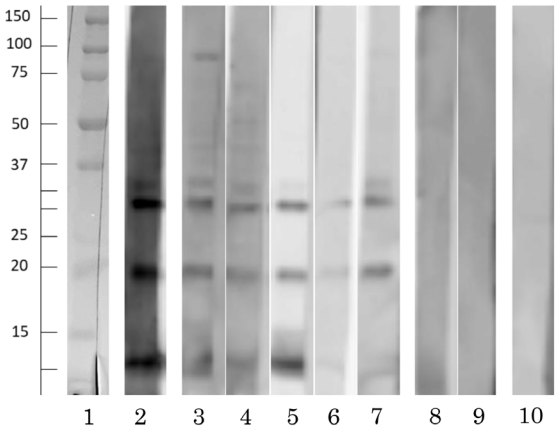

Fig. 3. Representative data of western blotting. Lane1: Marker, Lane2: Positive control, Lane3–Lane 9 are samples from Tokunoshima Island: sample No. Fc17025, Fc17030, Fc17037, Fc17047, Fc17054, Fc17004, Fc17020, respectively, Lane10: Negative control. Lane from 3 to 7 were determined as positive.

in cats from 17 prefectures (Hokkaido, Miyagi, Niigata, Ishikawa, Tochigi, Saitama, Kanagawa, Shizuoka, Aichi, Kyoto, Osaka, Hyogo, Shimane, Tokushima, Saga, Kagoshima, Okinawa) in 1994 and 1999 [11], and 6.0% (48/800) in those from 16 prefectures (Hokkaido, Gunma, Saitama, Tokyo, Kanagawa, Gifu, Aichi, Mie, Kyoto, Osaka, Tottori, Okayama, Hiroshima, Ehime, Fukuoka,

Kumamoto) in 1997 [15]. The seroprevalence in free-ranging cats in Japan were reported as 9% in 2013–2017 in Amami-Oshima Island [12], and 13.4% in 1998–1999 in Chiba prefecture [6]. In Tokachi subprefecture, Hokkaido in 2013–2014 showed significantly higher seroprevalence in cats allowed to roam outdoors or reared at the farm (30.0%) than in cats reared indoors (7.5%) [17]. Various seroprevalence values, including much higher results in free-ranging cats have been reported from other countries, such as La Rioja and Madrid in Spain (36.4%) [13], Tasmania, Australia (84.2%) [5], and Izmir, Turkey (34.4%) [2]. As those studies mentioned, the tendency that free-ranging cats have higher seropositivity than house-kept cats might also be true in Tokunoshima Island. A dietary analysis using fecal samples obtained from free-ranging cats on Tokunoshima Island has proved that 17.7% and 30.8% of those samples contained forest-living species such as the Amami rabbit, Ryukyu long-haired rat, and Tokunoshima spiny rat, and farmland animals such as the black rat (*Rattus rattus*) and shrews (*Crocidura* spp.), respectively [10]. Thus, cats that roam around both forest areas and farmlands may have some opportunities to bring oocysts to and contaminate such environments.

In this study, the seropositivity rate in adult cats was significantly higher than young cats. This result is consistent with some studies that reported that seropositivity rates in adult cats are higher than those in kittens or juvenile cats, indicating more opportunity of exposure to *T. gondii* [5, 11, 13, 17]. Anti-*T. gondii* IgG remains for long period after infection, at least 6 years in cats [4].

The result also showed that possibly infected free-ranging cats are found in 17 of the 23 settlements (Fig. 2), suggesting *T. gondii* infection widely occur on the island, and the introduction of *T. gondii* to this island is not a recent incident. Although it is not clear how long cats have been established on the island, cattle industry, which creates population sources of free-ranging cats on this island, has flourished for the last 50 years [8], suggesting that free-ranging cats have become abundant since the last several decades. A case of *T. gondii* infection was reported in a deceased Amami spiny rat on Amami-Oshima Island, revealing the risk of *T. gondii* infection to wildlife [18]. No significant difference was found in the seropositivity rate between three towns on the island, and also between samples from cats captured in forest area and residential area including farmland (data not shown), indicating that the forest area, as well as the residential area, might be contaminated with oocysts shed by those free-ranging cats.

Interestingly, the reported seroprevalence of *T. gondii* in free-ranging cats was much lower on Amami-Oshima Island [12], which is located about 42 km northeast of Tokunoshima Island (nearest coastline distance). Amami-Oshima Island (712 km^2) is the largest of the Amami Islands, more than 2.5 times larger than Tokunoshima Island. The disagreement in seroprevalence despite their proximity might have resulted from geographical features or cat density, due to the smaller size of Tokunoshima Island. Furthermore, Tokunoshima Island thrives more with livestock production compared to Amami-Oshima Island. Cattle barns are possibly one of the major factors of the population of free-ranging cats, because a study on Tokunoshima Island suggested that feeding cats in cattle barns contributes to maintaining those population [8]. To identify the reasons behind the high seroprevalence in free-ranging cats on Tokunoshima Island, more comprehensive studies that include analysis of natural and artificial geographical features are required. Because of wide host range and complicated route of infection, one health viewpoints are essential for epidemiologic research of *T. gondii* transmission, which analyzes human, animal and wildlife integrally.

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