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NOTE

Parasitology

Seroprevalence of *Toxoplasma gondii* in free-ranging and feral cats on Amami Oshima Island, Japan

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ABSTRACT. On Amami Oshima Island, free-ranging and feral cats are harmful to wildlife populations. In this study, the seroprevalence of *Toxoplasma gondii* in these cats was examined using a newly developed *Gaussia* luciferase immunoprecipitation system assay. Of 1,363 cats, 123 cats (9.0%) was positive for *T. gondii*. The prevalence was significantly different in different areas; among cats in the rural area, where many wild animals live, including endangered species, *T. gondii* infection was more prevalent than in the urban area of the island. This finding indicates a possible risk to wildlife of infection from free-ranging and feral cats. Therefore, management of cats is important for wildlife conservation.

KEY WORDS: free-ranging and feral cat, *Toxoplasma gondii*, zoonotic diseases

Amami Oshima Island is the largest island among the Amami Islands, which are subtropical islands of Japan (Fig. 1). Its climate is subtropical, with an average annual temperature of 21.5°C and annual rainfall of 2,914 mm [11]. It has an area of ~712.35 km^2, and a population of ~60,000 people. Amami Oshima Island is known to have many endemic animal species, including the Amami rabbit (*Pentalagus furnessi*), Amami Ishikawa's frog (*Odorrana splendida*), and Ryukyu long-haired rat (*Diplothrix legata*). Most of these species are considered endangered according to the IUCN Red List of Threatened Species (http://www.iucnredlist.org/details/16559/0). Lately, the free-ranging and feral cats (*Felis catus*) on the island have been found to be harmful to wildlife populations [4, 8]. Not only do the free-ranging and feral cats prey on wild animals, there is concern that the cats may act as a reservoir for several pathogens that can be transmitted to native wildlife.

Toxoplasma gondii is an obligate intracellular parasite, a member of the phylum Apicomplexa, and the most prevalent parasite in humans and most warm-blooded animal species. Felids are the only definitive hosts of *T. gondii*; they begin shedding oocysts 3–10 days after infection and continue shedding for ~10–14 days, allowing the parasite to spread beyond its host into the environment. Contaminated soil, water bodies, and agricultural crops may serve as the sources of infection for wild or domestic animals. Free-ranging and feral cats may serve as a reservoir for this parasite and play an important role in the risk of its transmission between animals and humans. Therefore, we conducted an epidemiological study to better understand the distribution of *T. gondii* infection among free-ranging and feral cats on Amami Oshima Island. A latex agglutination test (LAT) has been used as the major method to diagnose *T. gondii* serologically; however, the commercial LAT kit was discontinued recently in Japan. Therefore, for this epidemiological study, we developed a new *Gaussia* luciferase immunoprecipitation system (GLIPS) assay using samples of a recombinant dense granule antigen 7 (TgGra7) protein derived from *T. gondii*.

In total, 1,363 serum samples were obtained from unowned free-ranging and feral cats on Amami Oshima Island were trapped by Trap-Neuter-Return from 2013 to 2017. All of the serum samples were stored at −80°C until use. Among these samples, 371 and 210 samples were obtained from cats living in the Naze and Setouchi areas, respectively, which are the largest towns on the island. To be precise, 110, 252, 133, 215 and 72 samples were obtained from the Kasari, Tatsugo, Sumiyo, Yamato, and Uken areas, respectively; these areas along the coast or near the forest have a low population density and are considered rural (Fig. 1). Additionally, 123 samples from domestic cats in 13 prefectures (Miyagi, Tokyo, Kanagawa, Saitama, Chiba, Osaka, Hyogo, Tottori, Okayama, Yamaguchi, Oita, Kumamoto and Kagoshima) in mainland Japan were examined as controls. These samples were provided by a commercial laboratory (Adtec Co., Ltd., Usa, Japan). This study was approved by the Institutional Animal Care and Use committee, Kagoshima University (permission number: VM17031), and carried out according to the guidelines of the

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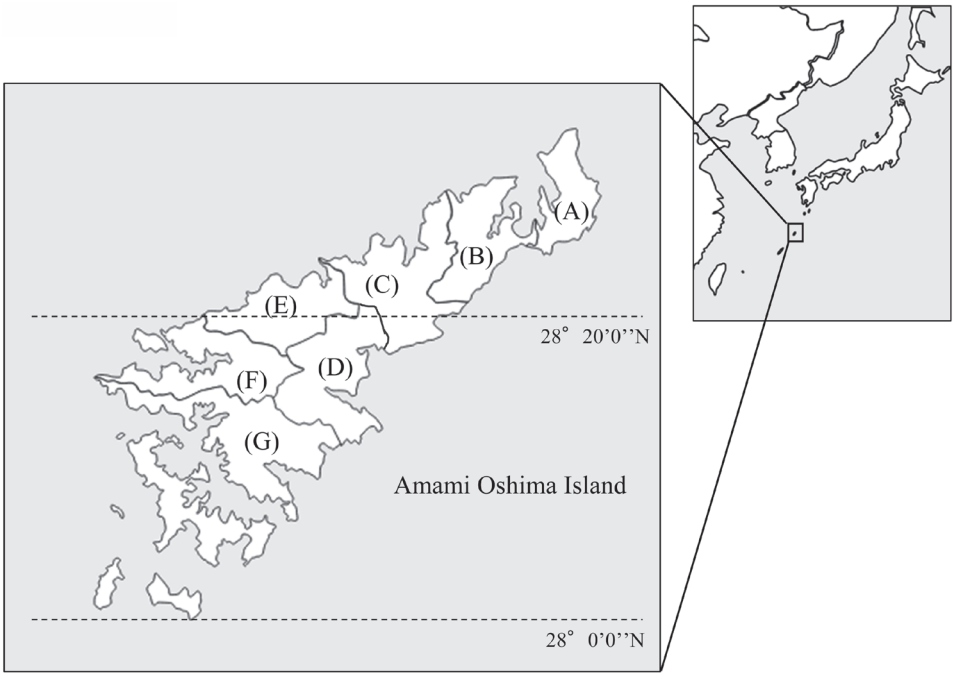

Fig. 1. Geographical locations where serum samples were collected in this study. (A) Kasari, (B) Tatsugo, (C) Naze, (D) Sumiyo, (E) Yamato, (F) Uken and (G) Setouchi areas.

committee.

To evaluate the levels of antibody against *T. gondii* in cat serum samples, a GLIPS assay was performed. The pGLIP plasmid vector for the GLIPS assay was constructed as described previously [5]. Total DNA was extracted from the RH strain of *T. gondii* using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, U.S.A.). TgGra7 DNA was amplified by PCR using forward and reverse primers described elsewhere [9]. After the TgGra7 PCR product was subcloned into the pGLIP vector using *Bam*HI and *Eco*RI, DNA sequencing was used to confirm the correctness of the DNA constructs. Plasmid DNA was then prepared using a commercial kit (PureLink HiPure Plasmid Midiprep Kit, Invitrogen, Carlsbad, CA, U.S.A.) and transfected into 293T cells with Lipofectamine 2000 (Thermo Fisher, Waltham, MA, U.S.A.). Crude protein extracts were obtained for use as an antigen. Crude protein extracts were also prepared from cells transfected with an empty plasmid to serve as a negative control. Serum samples were first diluted 1:50 in GLIP buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, and 1% Triton X-100) in a 96-well polypropylene plate. In this plate, 45 μl of GLIP buffer, 5 μl of diluted serum, and 10^7 light units (LU) of a crude 293T cell extract containing either *Gaussia* luciferase (Gluc)-TgGra7 or Gluc alone as control antigens was added to each well. Next, the plate was incubated for 30 min at room temperature on a shaker. Then, in 5 μl of a 20% suspension of UltraLink protein A/G beads (Thermo Fisher) in GLIP buffer and then in phosphate-buffered saline, LUs were measured on a microplate illuminometer (Promega, Fitchburg, WI, U.S.A.) using a *Renilla* luciferase assay system (Promega).

In this assay, a cutoff of 5.0×10^4 LU was chosen based on background LU levels and a receiver-operator characteristic (ROC) curve analysis, which indicated the most appropriate positive and negative cutoffs for a western blot assay (data not shown). The sensitivity and specificity of the GLIPS assay were 81.8 and 77.8%, respectively. To confirm the diagnostic accuracy of the GLIPS assay, a LAT was performed on 200 randomly chosen serum samples using a commercial kit (Toxocheck-MT; Eiken Chemical, Tokyo, Japan). The results of the LAT and GLIPS assay were evaluated by means of the percentage agreement and kappa values with a 95% confidence interval. The results of the GLIPS assay of 200 cat serum samples showed a kappa value of 0.75 (95% confidence interval, 0.57 to 0.75), and compared favorably with those of the commercial LAT kit. Agreement between the GLIPS and LAT assays was high.

In the screening of 1,363 serum samples from free-ranging and feral cats on Amami Oshima Island, 123 cats (9.0%) tested seropositive for anti-TgGra7 antibody (range 5.1×10^4 to 4.6×10^6, mean 2.5×10^5, median 1.1×10^5 LU). Four (3.3%) among one hundred and twenty-three domestic cats in mainland Japan tested positive for the same antibody (range 6.4×10^4 to 1.1×10^6, mean 3.5×10^5, median 9.8×10^4 LU; Fig. 2). The prevalences of cats positive for the *T. gondii* antibody on Amami Oshima Island and in mainland Japan were compared by χ^2 testing, and the prevalence of *T. gondii* on Amami Oshima Island was significantly higher than that in mainland Japan ($P<0.05$). The prevalence of anti-TgGra7 antibody in cats on Amami Oshima Island was also examined by cats' gender, age, and location (area) by χ^2 testing. There were no significant differences in rates by gender or age; however, we observed area-dependent variation in *T. gondii* prevalence ($P<0.01$). Cats in the Sumiyo area showed the highest antibody positivity, and the difference between this prevalence and those in the Kasari, Naze, Yamato and Setouchi areas was significant ($P<0.01$). The prevalence in the Tatsugo area was also significantly higher than that in the Kasari and Setouchi ($P<0.05$)

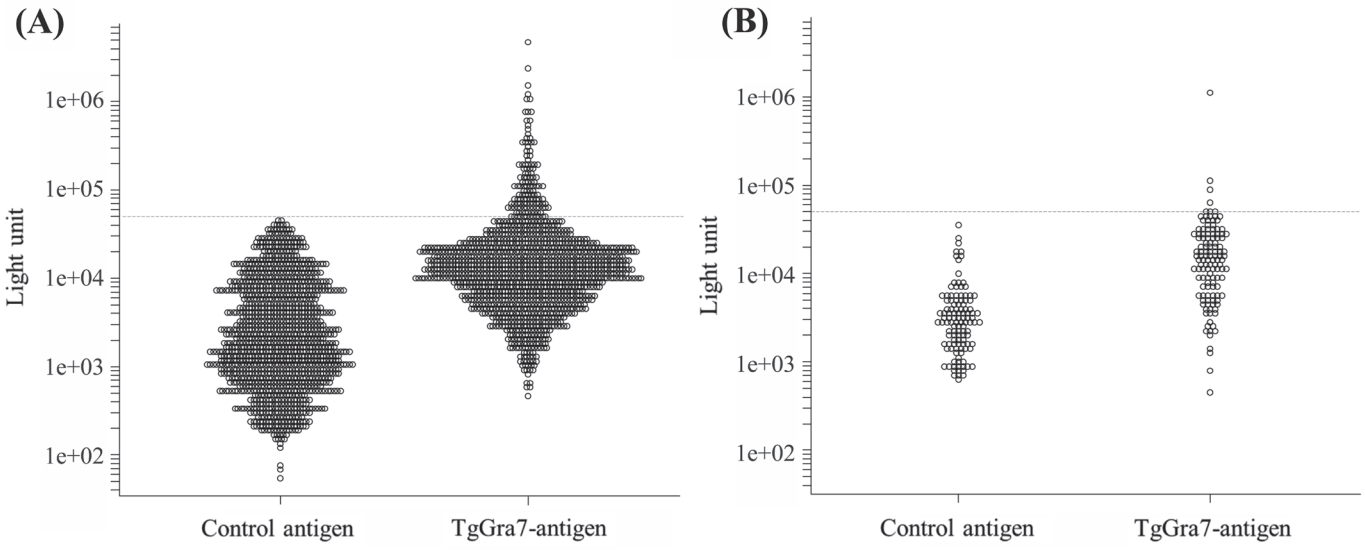

Fig. 2. *Gaussia* luciferase immunoprecipitation system (GLIPS) detection of anti-TgGra7 antibody in cats. Serum samples from 1,363 free-ranging cats on Amami Oshima Island (A) and 123 domestic cats in mainland Japan (B) were screened for the presence of anti-TgGra7 antibodies using TgGra7-GLIP antigen in a GLIPS assay. All of the serum samples were also analyzed for antibodies against a control antigen to determine the cutoff limit (dashed line).

Table 1. Seroprevalence of *Toxoplasma gondii* in cats on Amami Oshima Island

Parameters		No. tested	No. positive	Positivity rate (%)
Area	Kasari (A)[a)]	110	6	5.5
	Tatsugo (B)	252	31	12.3 [b,c)]
	Naze (C)	371	19	5.1
	Sumiyo (D)	133	26	19.6 [d)]
	Yamato (E)	215	19	8.8
	Uken (F)	72	8	11.1
	Setouchi (G)	210	14	6.7
Cat gender	Male	545	45	8.3
	Female	543	52	9.6
	Unkwon	275	26	9.5
Cat age	Juvenile	29	3	10.3
	Young	142	8	5.6
	Adult	852	84	9.8
	Unknown	340	28	8.2
Total		1,363	123	9

a) Letters in parentheses correspond to those in Fig. 1. b) $P<0.05$ vs. Kasari and Setouchi, c) $P<0.01$ vs. Naze, d) $P<0.01$ vs. Kasari, Naze, Yamato and Setouchi.

and Naze areas ($P<0.01$) (Table 1).

Dense granule antigens of *T. gondii* are secretory proteins abundant in the parasitophorous vacuoles surrounding the parasite. TgGra7 belongs to this protein family and has been shown to be a good diagnostic marker for the detection an anti-*T. gondii* antibody in acute and chronic infections of humans and pigs [2, 9]. In our present study, anti-TgGra7 antibody in cats was detected by the GLIPS assay, which is based on *Gaussia* luciferase-tagged antigens produced in mammalian cells. The sensitivity of the assay agreed substantially with that of the LAT kit; therefore, we employed the GLIPS assay for this surveillance. The overall prevalence of the antibody among free-ranging cats on Amami Oshima Island was higher than that in domestic cats in mainland Japan. The prevalence varied by area of the island (5.1–19.6%). In epidemiological studies in other countries, the *T. gondii* antibody seroprevalence among cats in rural areas has tended to be higher than that in urban cats [6, 10]. In our present study, there was a similar tendency. Naze is a main town on Amami Oshima Island, and more than a half of the human population on this island is concentrated in this area. Setouchi is the second largest town. Cats from these two areas showed comparatively low seroprevalences (5.1 and 6.7%). In the rural areas, the prevalence of *T. gondii* tended to be high (8.8–19.6%) in the mountainous forest areas of Tatsugo, Sumiyo, Yamato and Uken, which are habitats of both invasive and native rodents. Feral cats living

these areas had been found to prey on these animals [8]. For cats, hunting is recognized as risk factor of *T. gondii* infection [1, 7]. Cats living in these mountainous forested areas meet wild animals at a high frequency, and this might be related to their higher prevalence of *T. gondii* antibody. In particular, the Sumiyo area retains some primeval forest and is known as a habitat for the Amami rabbit and Ryukyu long-haired rat (Japanese Ministry of the Environment http://www.env.go.jp/nature/kisho/hogozoushoku/amaminokurousagi.html). Our present data raise the concern that wild animals living in this area are also at risk of *T. gondii* infection. Unfortunately, research on the prevalence of *T. gondii* in wild animals on this island is scarce. In the only study to examine this, an adult female Amami rabbit was found to have a systemic protozoal infection, and toxoplasmosis was suspected [3]. Therefore, we also examined stored serum samples from twelve wild animals (one Ryukyu long-haired rat and eleven Amami rabbits). These samples were taken to an animal hospital on Amami Oshima Island between 2013 and 2017 and tested using a commercial LAT kit (Toxochek-MT). One seropositive Amami rabbit was identified (data not shown). Although a larger epidemiological study on wildlife is needed, *T. gondii* is suspected to be present in wild animals, including endangered species, on the island.

In this study, we examined the epidemiology of *T. gondii* infection in free-ranging and feral cats on Amami Oshima Island for the first time. Outdoor cat management is important for conserving ecosystems and biodiversity, and, recently, various countermeasures have been undertaken by federal or local governments. Understanding epidemics and the spread of infectious diseases in cats is important because of the risk of the zoonosis to people, in addition to domestic animals and wildlife.

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