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NOTE

Parasitology

Toxoplasma gondii antibody prevalence and isolation in free-ranging cats in Okinawa, Japan

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ABSTRACT. Cats are an important host of *Toxoplasma gondii* from an epidemiological perspective because they are the only definitive hosts that excrete oocysts in their feces. In this study, 201 free-ranging cats in Okinawa were examined for *T. gondii* infection. Using the latex agglutination test, we detected antibodies against *T. gondii* in 26.9% (54/201) of the cats. Oocysts of *T. gondii* were not detected upon microscopic examination of the feces of 128 cats. *T. gondii* was isolated from the tissues of 9 out of 24 seropositive or pseudo-seropositive cats with a bioassay using laboratory mice. Genotyping for the *GRA6* gene revealed that five and four of the isolates were type I and II, respectively.

KEY WORDS: cat, isolation, Okinawa, seroprevalence, *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects almost all homeothermic animals, including humans. Upon infection, *T. gondii* forms cysts in the host tissue, and the parasites colonize the host for life. Consequently, toxoplasmosis, classified as a zoonosis, is a public health concern, especially in vulnerable individuals, such as immunodeficient patients and pregnant women [2].

The major route of *T. gondii* infection is the consumption of undercooked meat containing *T. gondii* cysts or food and water contaminated by *T. gondii* oocysts. The Felidae family (cats) are important to the epidemiology of *T. gondii* because they are definitive hosts that excrete environmentally resistant oocysts in their feces [2].

The Ministry of Agriculture, Forestry and Fisheries revealed that the Okinawa Prefecture, a chain of islands far off the southwest coast of Japan, had the highest frequency of swine toxoplasmosis in the country [15]. Furthermore, more than 50% of slaughtered goats were found to have *T. gondii* antibodies in Okinawa [8]. It is important to clarify the infiltration status of oocysts to prevent *T. gondii* infection in domestic animals and humans. Thus, in this study a survey was conducted assessing the presence of antibodies against *T. gondii* in free-ranging cats in Okinawa, and the parasites were isolated from infected cats.

In this survey, 201 free-ranging/stray cats, injured or sick, originating from 29 of the 41 municipalities in Okinawa were sampled. The cats were accommodated in the Animal Conservation and Management Center in Okinawa Prefecture by the Act on Welfare and Management of Animals between 2011 and 2013. Since cats are euthanized under the Act when their owners do not appear or a new owner cannot be found for the animals during the public notice period, 7 days after being accommodated, the use of cats in this study did not require approval by the ethics committee responsible for animal experiments. Specimens, namely serum, feces, brain, and heart, were collected after euthanasia by carbon dioxide gas inhalation, according to the Standards relating to the Methods of Destruction of Animals. The collected brain and heart tissues were stored at 4°C. Body weight of the sampled cats ranged from 0.2 to 4.5 kg.

Detection of T. gondii antibodies in cat sera

Sera obtained from the 201 cats were subjected to the latex agglutination test (LAT) using a commercially available kit (Toxocheck; Eiken Chemical Co., Ltd., Tokyo, Japan). Titers measured by LAT ranged from 1:8 to 1:2,048. A titer of 1: ≥64 was considered positive, while a titer of 1:32 was considered pseudo-seropositive, meaning near the positive and negative boundary.

Examination of cat feces for T. gondii oocysts

Feces from 128 cats were morphologically examined using the saturated sucrose liquid flotation assay.

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Bioassay in mice

T. gondii were isolated from the brains and hearts of seropositive or pseudo-seropositive cats (n=24, wherein 23 were seropositive and 1 was pseudo-seropositive). Brain and heart tissues (1−5 g) were cut into small pieces with scissors, digested with trypsin (FUJIFILM; Wako Pure Chemical Co., Ltd., Osaka, Japan), and inoculated intraperitoneally into 3–4 mice (1 ml/mouse). For the bioassay, 4–6-week-old female ICR mice were obtained from Kyudo Co., Ltd. (Tosu, Japan). The bioassay was considered positive when mice showed any clinical signs or died and *T. gondii* cysts/tachyzoites were found in any of the tissues [11]. For the detection of tachyzoites, a drop of abdominal ascites was prepared on a glass slide and subjected to microscopic analysis without staining. The lung or spleen was cut with a scalpel and the cross-section was stamped on a glass slide and stained with acridine orange. For the detection of cysts, a section of the mouse cerebrum was sandwiched between a glass slide and coverslip and microscopically examined without staining. The infection bioassay experiment was conducted with the approval of the Animal Care and Use Committee of the Okinawa Prefectural Institute of Health and Environment (Approval no. 24-11 and 25-9).

Genotyping of T. gondii

DNA was extracted from the cysts in mouse brain tissue using a QIAmp DNA Mini kit (QIAGEN, Venlo, Netherlands). The extracts were subjected to nested polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) analysis [3, 17], targeting the *GRA6* gene of *T. gondii*. The following PCR primer pairs were used: first PCR, GRA6FO (GGCAAACAAAACGAAGTG) and GTA6RO (CGACTACAAGACATAGAGTG); second PCR, GRA6R (GTAGCGTGCTTGTTGGCGAC) and GRA6F (TACAAGACATAGAGTGCCCC) [3, 17]. The obtained PCR products were digested with *Mse*I (New England Biolabs Inc., Ipswich, MA, USA) for 10 min at 37°C, and the genotypes were determined according to their restriction pattern [3, 17].

Antibodies against *T. gondii* were found in 54 of the 201 (26.9%) cats in samples from 23 of the 29 municipalities, including the remote islands. The seroprevalence in kittens, weighing less than 1 kg, was 0% (0/39); in cats weighing more than 1 kg, it was 33.3% (54/162) (Table 1). Oocysts of *T. gondii* were not detected upon microscopic examination of cat fecal matter, regardless of the presence or absence of the antibody in the serum.

T. gondii isolated from 9 of the 24 seropositive or pseudo-seropositive cats were confirmed to be viable using a bioassay conducted in laboratory mice. Clinical signs of *T. gondii* infection in mice included cowlicks, melancholy, abdominal dropsy, cyanosis, and forced breathing. Clinical signs in the internal organs consisted of spleen tumefaction, lung edema, and pneumonia. Other internal organs did not show any significant lesions. Eventually, three of the infected mice recovered, while the remaining six died. Of the nine *GRA6*-positive *T. gondii* strains isolated from the seropositive cats, five isolates were type I and four were type II. Type III or other atypical genotypes were not detected (Table 2). Of the six *T. gondii* strains that killed the mice, five were of type I

Table 1. Seroprevalence of *Toxoplasma gondii* in cats of Okinawa

Weight (kg)	No. of cats examined	No. of cats positive (%)
<1.0	39	0 (0%)
1.0–1.9	35	9 (25.7%)
2.0–2.9	79	26 (32.9%)
≥3.0	48	19 (39.6%)
Total	201	54 (26.9%)

Table 2. Isolation and characterization of the *GRA6* genotype of *Toxoplasma gondii* from cats in Okinawa

Sample no.	Titer value from latex agglutination test	Isolated from cat tissue		*GRA6* genotype	Mice	References
		Brain	Heart			
1	512	+	NT	I	D	
2	256	-	NT			
3	32	-	NT			
4	128	-	NT			
5	128	-	NT			
6	1,024	-	NT			
7	256	-	NT			
8	256	-	NT			
9	256	-	NT			
10	≥2,048	+	NT	I	D	
11	256	+	NT	I	D	[4]
12	1,024	-	NT			
13	256	-	NT			
14	128	+	NT	II	L	
15	128	-	NT			
16	128	-	NT			
17	512	+	NT	II	L	[4]
18	256	-	NT			
19	128	-	NT			
20	≥2,048	+	NT	I	D	
21	64	+	+	I	D	[4]
22	512	-	+	II	L	
23	256	-	-			
24	256	-	+	II	D	[4, 14]

NT, not tested; D, dead; L, alive.

and one was type II.

In this study, 26.9% of cats were positive for antibodies against *T. gondii* in Okinawa, indicating that the prevalence of *T. gondii* infection is substantially higher than that reported in other prefectures in Japan (9–13%) [9, 10]. We also found that the parasite had a wide distribution, since antibodies were detected in samples from 23 of 29 municipalities, including the remote islands. This high seroprevalence of *T. gondii* in cats in Okinawa suggests that people and domestic animals living in this area are at an increased risk of infection.

We confirmed the viability of *T. gondii* isolated from the tissues of seropositive cats by conducting a bioassay in mice. Historically, *T. gondii* has been considered to be clonal and to exhibit less genetic diversity, presenting only three genotypes (type I, II, and III) in North America and Europe [5, 12]. The genotype of *T. gondii* differs according to geographical location and pathogenicity in mice. Thus, the parasite is genotyped in epidemiological surveillance studies investigating the source and route of infection [1]. The strains isolated from mice in our bioassay were predominantly of the type I and II genotype. This is the first report on *GRA6*-based genotyping of *T. gondii* from cats in Okinawa. Previously, these genotypes have been detected in similar proportions in pigs and goats in Okinawa; however, type III parasites were less prevalent [8, 16, 17], suggesting the possibility that the source of *T. gondii* infection in domestic animals is cats in Okinawa.

In recent study, with advances in gene analysis methods, there has been an increase in the number of known genotypes and details of the genetic diversity of this parasite [6, 7, 13]. Some of the strains in this study have been analyzed in detail [4, 14]. However, in order to characterize Okinawa's *T. gondii*, it is necessary not only to sequence the *GRA6* gene for more strains but also to analyze other genes.

CONFLICT OF INTEREST. The authors have no conflicts of interest to declare.

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