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Short Communication

Isolation and Characterization of *Toxoplasma gondii* Genotypes from Goats at an Abattoir in Okinawa

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SUMMARY: *Toxoplasma gondii* genotypes were isolated and characterized from cephalic muscle samples collected from 24 goats slaughtered at an abattoir in Okinawa between 2008 and 2009. Of the 24 samples assayed using latex agglutination, 18 were seropositive, 2 were pseudo-positive, and 4 were seronegative against *T. gondii* antibodies. The samples were then inoculated into laboratory mice to isolate the parasite. Among the isolated samples, 13 (72.2% of the 18 seropositive strains in the latex agglutination assay) were seropositive, 1 (50%) was pseudo-positive, and none were seronegative. However, after being frozen and stored at −20°C, all samples were found to be *T. gondii*-free. Of the 14 isolates of the *GRA6* genotype, 6 were of type I, 7 were of type II, and 1 was of type III; the genotype distribution ratio was similar to that of *T. gondii* strains isolated from locally raised pigs. Moreover, no sulfonamide-tolerant *dhps* gene mutant of *T. gondii* was detected.

The protozoan *Toxoplasma gondii* is an obligate intracellular parasite that infects almost all homoeothermic animals, including humans. Toxoplasmosis is a problematic zoonosis, particularly in vulnerable groups such as pregnant women and immunodeficient patients. One of the main modes of toxoplasmosis infection in humans is ingestion of undercooked and/or raw meat containing *T. gondii* cysts. *T. gondii* antibodies are prevalent in goats, and locally raised individuals are no exception. In fact, the prevalence rate in Okinawa was found to be as high as 57% (1,2). However, the symptoms of toxoplasmosis infection have rarely been detected during inspection of goat meat at abattoirs, and no cases of the meat not passing inspections made under the Meat Inspection Law have been reported either. Raw goat meat is a commonly served dish in Okinawa; therefore, this study focuses on the isolation of *T. gondii* in goat meat and on determining the storage conditions that may help control the spread of *T. gondii*. Furthermore, we intended to identify the *T. gondii* genotypes that may be used for epidemiological surveillance because they reflect specific geographical areas and animal species. This study also aims to identify the *dhps* gene mutant of *T. gondii*, which is tolerant to sulfonamide agents prescribed to patients with toxoplasmosis, and to characterize the different genotypes of *T. gondii* in the locally raised goat population.

T. gondii genotypes were isolated from 24 samples of cephalic muscles obtained from slaughtered goats in Okinawa between 2008 and 2009 and then characterized. Latex agglutination assay showed that of these 24 samples, 18 were seropositive, 2 were pseudo-positive, and 4 were seronegative for *T. gondii* antibodies. The muscles were minced using a food processor and 20-g samples were stored at 2 different temperatures for 18–22 h: (i) 24 samples at 4°C and (ii) 20 samples at −20°C. The samples were then digested with trypsin and inoculated intraperitoneally into 2 or 3 Charles River CD-1 mice (1 ml/mouse). Antibody tests were performed weekly for about 4 weeks. In this study, mice with antibody titers ≥64, and/or dead mice with cysts or tachyzoites, were considered positive for *T. gondii*. For the detection of tachyzoites, a drop of abdominal ascites or a drop of the saline used to rinse the peritoneal cavity was placed on a glass slide and was examined under a microscope. For the detection of cysts, we performed microscopic examination of brain impression smears or hematoxylin and eosin staining of sliced specimens.

Isolated cyst DNA was extracted from the brain samples using a DNA/RNA kit-Cell, Tissue (Dojindo, Tokyo, Japan), and these extracts were used to perform the nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays targeting the *GRA6* genes, which encode the dense granule antigens of *T. gondii*. The following PCR primer pairs were used for genotyping: first PCR cycle: GRA6FO (GGCAAACAAAACGAAGTG) and GTA6RO (CG ACTACAAGACATAGAGTG) and second PCR cycle: GRA6R (GTAGCGTGCTTGTTGGCGAC) and GRA6 (TACAAGACATAGAGTGCCCC). The obtained PCR products were digested using the limited enzyme *Mse*I, and the genotypes were determined according to the restriction pattern (3).

Sulfonamide agents, which are prescribed as remedial drugs for toxoplasmosis, cause bacteriostasis of *T. gondii* by acting on dihydropteroate synthetase (dhps) and

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Table 1. Isolation of *T. gondii* from goats in Okinawa

Stored		*T. gondii* infection antibodies of goats		
		Positive	Pseudo-positive	Negative
4°C	No. examined	18	2	4
	No. of mice positive (%)	13 (72.2%)	1 (50%)	0 (0%)
−20°C	No. examined	14	2	4
	No. of mice positive (%)	0 (0%)	0 (0%)	0 (0%)

Table 2. Isolation and characterization of *T. gondii* genotypes from goats in Okinawa

Sample no.	LAT titer of goat[1)]	No. of mice positive/ no. inoculated		LAT titer of mice[1)]	Detected *T. gondii* from mice		Genotype	Sulfonamide resistance
		4°C	−20°C		Tachyzoite	Cyst		
1	≧2.048	2/2	ND	256 (D), 256		+	I	W
2	1,024	3/3	ND	<16 (D), <16 (D), <16 (D)	+		II	W
3	≧2.048	3/3	ND	256, 256, 256		+	II	W
4	1,024	3/3	0/3	256, 512, 1.024		+	I	W
5	512	3/3	0/3	512 (D), 512, 1.024	+	+	I	W
6	1,024	3/3	0/3	64, 256, 512	+	+	III	W
7	256	0/3	0/3					
8	256	3/3	ND	128, 256, 512	+	+	II	W
9	256	3/3	0/3	128 (D), 128, 256	+	+	II	W
10	512	0/3	0/3					
11	512	0/3	0/3					
12	1,024	3/3	0/3	<16 (D), 256, 512	+	+	I	W
13	≧2.048	2/2	0/2	128, 512	+	+	I	W
14	1,024	2/2	0/2	1.024, 1.024	+	+	II	W
15	256	0/3	0/3					
16	512	0/3	0/3					
17	<16	0/3	0/3					
18	128	1/3	0/3	512		+	I	W
19	32	3/3	0/3	64, 256, ≧2.048		+	II	W
20	<16	0/3	0/3					
21	<16	0/3	0/3					
22	<16	0/3	0/3					
23	32	0/3	0/3					
24	128	3/3	0/3	128, 256, 1.024		+	II	W

[1)]: Latex agglutination test (LAT); 1:≧64 as positive; 1:32 as pseudo-positive; 1:<16 as negative.
ND, not done; D, death; W, wild type.

consequently inhibiting folic acid synthesis in *T. gondii*. Sulfonamide-tolerant strains are produced as a result of a 407-base substitution in the *dhps* genes. In this study, DNA extracts obtained from the brain samples of *T. gondii*-infected mice were examined using a kit for detecting tolerance to sulfonamide agents (version 1), developed by the Okinawa Prefectural Institute of Animal Health, where the nested-PCR and RFLP using the limited enzyme *Cfr*13I were successively performed (4).

Of the 18 seropositive muscle specimens stored at 4°C, 13 (72.2%) were positive and 1 was pseudo-positive for viable *T. gondii* (50%) (Table 1). In contrast, of the 20 muscle specimens stored at −20°C, none were positive for viable *T. gondii*. Meanwhile, in the seropositive or dead mice, tachyzoites were detected in 8, cysts in 13, and both tachyzoites and cysts in 7 (Table 2).

Most of the seropositive mice began to show an increase in antibody titer at around 2 weeks after inoculation. Clinical conditions varied across the seropositive mice and include cowlick, melancholy, abdominal dropsy, cyanosis, and forced breathing. Eventually, most mice recovered to a normal condition, but 5 died (Table 2). The clinical symptoms observed in the internal organs included tumefaction of the spleen, lung edema, and pneumonia. Other internal organs did not show significant lesions.

T. gondii genotypes differ according to geographical and host origin; therefore, these genotypes can be employed in epidemiological surveillance to determine the source and route of infectious diseases (5–7). In this study, of the 14 *T. gondii* strains with the *GRA6* genotype, which were isolated from the goat meat specimens, 6 were of type I, 7 were of type II, and 1 was of type III (Table 2). The observed genotypic distribution shows a striking similarity to the findings of a previous study on locally raised pigs (3,4).

The RFLP patterns of *dhps* genes from the 14 strains of *T. gondii* coincided with those of the wild type, which is susceptible to sulfonamide; this finding suggests that

no mutant strains tolerant to this agent exist (Table 2).

It is generally known that meat specimens from >50% of the locally raised goats from abattoirs in Okinawa carry *T. gondii* antibodies (1,2). Moreover, the risk of *T. gondii* infection in humans is a major concern because raw goat meat is a commonly served local dish in Okinawa. In the late 1970s, Ameku et al. (8) identified *T. gondii* in goat lymph node specimens collected in Okinawa. However, to the best of our knowledge, no research on the potential risks of *T. gondii* infection in goat meat intended for human consumption has previously been performed in Okinawa. In the present study, viable *T. gondii* was isolated at a high prevalence rate (70%) from the chilled and stored (4°C) goat meat specimens with seropositive and pseudo-positive antibodies. Therefore, we emphasize that the risk of *T. gondii* infection in humans after ingestion of raw goat meat cannot be overlooked.

Freezing meat is an effective measure against *T. gondii* contamination (9–11), which is a finding reconfirmed in this study: no *T. gondii* was observed in specimens frozen and stored at −20°C, the typical temperature of the freezing compartment of a domestic refrigerator.

The *T. gondii* genotypes type I and type II were equally predominant, while type III was prevalent to a lesser extent; the genotype distribution ratio shows a striking similarity with that observed for *T. gondii* in pigs (3,4). This finding suggests that the genotypes have specific geographic distributions. Furthermore, it also suggests that the hosts such as goats and pigs are not under a predation relation. The needs of pursuance into final hosts of which cats are strongly suspected to be the origin of *T. gondii* infections.

The 3 main genotypes isolated from *T. gondii* are known to express clear pathogenic differences in mice: type I expresses virulence factors causing death; type II expresses avirulence factors forming cysts; and type III expresses factors of both type I and type II (12). In the present study, goat muscle specimens collected from an abattoir did not show signs of *T. gondii* infection on inspection; therefore, we postulated that *T. gondii* exists only in the form of cysts, and consequently that type II would be isolated at a high rate. However, all the 3 types were detected, suggesting that unlike pigs, goats do not show pathogenic differences (4).

Unfortunately, this study focused on the host's clinical symptoms, but the latest findings have implicated a family of serine/threonine protein kinases found in rhoptries (ROPs) as important in mediating virulence differences between strain types. The difference in virulence between type I and II strains was explained by the existence of ROP5 (13). Types I and III showed presence of ROP18 (14), and types II and III had ROP16 (15). Further, it was explained that ROP16 and ROP18 affected the immune response of hosts (16–19).

The *T. gondii* strains isolated from goat muscle specimens showed presence of wild-type *dhps* genes, which do not express sulfonamide tolerance. Further, no respective base substitutions were observed, as was the case in a preceding study on *T. gondii* isolated from locally raised pigs. Therefore, it is considered unlikely that sulfonamide-tolerant *T. gondii* exists in Okinawa.

To date, there have been few studies on drug-tolerant strains of not only *T. gondii*, but also other protozoans; however, the kit developed by the Okinawa Prefectural Institute of Animal Health has enabled daily monitoring of *T. gondii*. Routine monitoring of livestock is vital because *T. gondii* infections are a serious problem not only in livestock but also in pregnant women and immunodeficient patients, especially when the parasite acquires drug tolerance.

It is extremely difficult to detect *T. gondii* infections in goat meat during onsite inspections. However, this study is the first to shed light on a novel methodology for identifying the potential risks of *T. gondii* infection by using laboratory mice inoculated with *T. gondii* and infected goat muscle specimens. A suitable preventive measurs, as has long been suggested, would be to convince people to refrain from eating raw goat meat and to instead serve the meat after it has been sufficiently cooked. Other alternatives for reducing the risk of infection include the following measures: (i) producing specific-pathogen-free goats such as *T. gondi*i-free goats; (ii) building and using abattoirs that fulfill the legal hygiene standards designed for the handling of fresh foods; or (iii) using frozen storage to reduce the risk of *T. gondii* contamination. The best option, however, would be to practice all of the above measures together.

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