Toxoplasma gondii infection in Amami spiny rat on Amami-Oshima Island, Japan

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ABSTRACT

The Amami spiny rat (Tokudaia osimensis) is an endangered rodent species that is endemic to the forests of Amami-Oshima Island, Kagoshima, Japan. In July 2018, a deceased adult male Amami spiny rat was found on the Yuwandake Mountain Trail on the south-central coast of Amami-Oshima Island. Histopathological observations revealed protozoan infections in the liver, lungs, and heart. Nested or semi-nested PCRs targeting the B1, SAG3, GRA6, and ROP18 genes successfully detected the genomic DNA of Toxoplasma gondii in the formalin-fixed and paraflin-embedded specimen. Sequence analyses of the SAG3, GRA6, and ROP18 genes suggested that the strain detected in the study specimen was related to the type II strain of T. gondii. This is the first confirmed case of T. gondii infection in an Amami spiny rat.

1. Introduction

Amami-Oshima Island is a subtropical island located ∼400 km south-west of Kyusyu, Japan. Reflecting the natural environment and geographical history of the island, this area is home to several endemic mammals, including the Amami spiny rat (Tokudaia osimensis), Ryukyu long-furred rat (Diplothrix legata), and Amami rabbit (Pentalagus furnessi). The Amami spiny rat is now categorized as endangered by the International Union for Conservation of Nature Red List of Threatened Species due to a rapid population decline as a result of diminishing habitats and predation by the non-native small Indian mongoose (Herpestes auropunctatus), and outdoor cats (Felis silvestris catus) and dogs (Canis lupus familiaris) (Ishii, 2016). For these above-mentioned reason, feral and stray cats are recognized as direct threats to the integrity of endemic mammals and natural ecosystems (Mameno et al., 2017).

Toxoplasma gondii (Apicomplexa: Sarcocystidae) is an intestinal coccidian parasite that targets members of the family Felidae as the final hosts, but also infects a wide range of warm-blooded animals as intermediate/paratenic hosts (Dubey and Odening, 2001). The prevalence of T. gondii in wildlife is correlated with the presence of final hosts since they contribute to environmental contamination via the excretion of oocysts in their feces (Lehrer et al., 2010; Fredebaugh et al., 2011). Recently, T. gondii antibodies were detected in 9.0% of feral and stray cats in Aammi-Oshima Islands (Matsuu et al., 2017), which indicated a potential risk of T. gondii transmission to endemic mammals.

In the present study, we report the first case of disseminated T. gondii infection in a deceased free-ranging Amami spiny rat.

2. Materials and methods

2.1. History

On July 8, 2018, a deceased adult male Amami spiny rat was found on a trail route on the Yuwandake Mountain Trail on the south-central coast of the Amami-Oshima Island (28°29′19.59″N, 129°31′80.6″N) (Fig. 1A). After collection, the specimen was preserved in a refrigerator for 3 days until a necropsy was performed at the Amami Wildlife Conservation Center. A death notification of the animal was submitted to the Agency...

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for Cultural Affairs.

2.2. Gross and histopathological examination

Tissue samples from the liver, spleen, lungs, kidneys, adrenal glands, heart, and digestive tracts were fixed in 10% neutral buffered formalin and processed into paraffin blocks for routine histopathologic processing. Paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for microscopic examination. Selected liver sections were subjected to immunohistochemical staining using the mouse *T. gondii* SAG1 (anti-SAG1) and BAG1 (anti-BAG1) antibodies diluted to 1:1,000. These antibodies were made (by author K. Ike) following previously described methods for *Neospora caninum* (Uchida et al., 2004; Kobayashi et al., 2013), except for use of recombinant *T. gondii* SAG1 and BAG1 proteins expressed with *Escherichia coli*. These antibodies were revealed using the EnVision Detection System (DaKo, USA). For the controls, we used sections without the primary antibodies, known *T. gondii*-negative tissues from a squirrel monkey (*Saimiri sciureus*) and mouse (*Mus musculus*), and positive liver tissues from a squirrel monkey that was spontaneously infected with *T. gondii*.

2.3. DNA extraction, PCR amplification, and sequence analyses

Total genomic DNA was extracted from the formalin-fixed and paraffin-embedded samples. Three 7 μm sections were collected from the liver and transferred to 1.5 ml tubes. Each tissue sample was deparaffinized with xylene three times, and genomic DNA was extracted with the DEXPAT Easy kit (TaKaRa, Japan) in accordance with the manufacturer’s instructions. For molecular confirmation, *B1* and *SAG3* genes were amplified using nested PCR primers, as previously described (Burg et al., 1989; Grigg et al., 2001). To further reveal the extent of genetic diversity of the *T. gondii* isolate, two polymorphic loci, *GRA6* (Fazaeli et al., 2000; Kyan et al., 2012) and *ROP18* genes (Khan et al., 2009), were selected using the following analyses.

Table 1

| Genes | Reaction | Primers (5′ to 3′) | PCR conditions |
|-------|----------|-------------------|----------------|
|       | First    | B1_Fext (GGAACTGCATCGGTTCATGAG) and B1_Rext (TCTTTAAGGGTTGTTGTGTC) | 40 cycles, 57 °C, 10 sec |
|       | Second   | B1_Fint (TGATAGGTGTGAGCTACGT) and B1_Rint (GGGAGGACATCTGGAATACCC) | 35 cycles, 62.5 °C, 10 sec |
| SAG3  | First    | SAG3_Fext (CACCTCACATCTCCAGACCC) and SAG3_Rext (GGCGGTTGATAGACAGACA) | 25 cycles, 60 °C, 30 sec |
|       | Second   | SAG3_Fint (TCCTGCGGATGTTCATCTCA) and SAG3_Rint (CAACAGAGACCGGAGGGAAGGA) | 25 cycles, 65 °C, 30 sec |
| GRA6  | First    | GRA6_Fext (ACACGCTGGCTCTGCTACGA) and GRA6_R (TCCGAAAGGGTCTGCTTAAAC) | 40 cycles, 58 °C, 30 sec |
|       | Second   | GRA6_Fint (CCATCTGAGCGAGAAGGTA) and GRA6_R | 25 cycles, 55 °C, 30 sec |
| ROP18 | First    | ROP18_Fext (TCGATGCGTTTCTGTCGCT) and ROP18_R (TGAGTCTGTTTCTGTCGCT) | 40 cycles, 60 °C, 30 sec |
|       | Second   | ROP18_Fint (TGCTCTGCGGGTCTTAAAMTG) and ROP18_R | 25 cycles, 50 °C, 30 sec |

Fig. 1. Gross morphology, histopathology, and immunochemistry of the Amami spiny rat. (A) Lateral view of the specimen at the time of discovery, showing loss of tissues from lips to cheeks. (B) Histopathology of the liver showing focal necrosis. H&E, scale bar = 200 μm. (C to E) Intracellular protozoa in the tissue of the liver (C), lung (D), and myocardium (E, arrowheads indicate the protozoa). H&E. (F and G) Immunostaining of anti-SAG1 (F) and anti-BAG1 (G). Scale bar = 20 μm.
performed at 72 °C for 7 min. Detailed information regarding the pri-
mers and amplification cycling conditions are shown in Table 1.

The amplicons from the second amplification of four loci were ana-
yzed by agarose gel electrophoresis, and SAG3, GRA6, and ROP18
amplicons were sent to Macrogen Company (Kyoto, Japan) where
the DNA sequencing was conducted using an ABI DNA sequencing system.
Phylogenetic analyses were performed with the following reference strains
that are available in INSD: archetypal type I (GT1, RH), type II
(BEVERLEY, DEG, PTG, Pruginaud, ME49), type III (CEP, CTG, NED,
VEG), atypical strains (BOF, CAST, CASTELLS, COUG, GUYDOS,
GUYKOE, GUYMAT, FOU, MAS, TgCatBr5, VAND), and strains isolated
from domestic animals in Okinawa Island, Japan (Ok24–Ok129)
(Kyan et al., 2012). Phylogenetic analyses were performed using MEGA soft-
ware, using a maximum-likelihood algorithm, with distances calculated
by the Tamura-Nei model for SAG3 and GRA6, and the kimura 2-
parameter model for ROP18. The stability of the topology was eval-
uated by bootstrapping with 1,500 replicates.

3. Results

3.1. Gross and histopathological examination

The animal weighed 88.7 g and measured 19.5 cm in total length
and 6.1 cm in tail length. There were no external injuries except for the
mouth part, which showed loss of tissues from the lips to the cheeks.
Gross examination of internal organs showed no significant changes
other than bile imbibition. Histologic postmortem changes were ob-
served in almost all tissues and were especially severe in the lungs.
Within the liver, multiple focal necrosis (Fig. 1B) and mild infiltration
of inflammatory cells around the Glisson's capsule and central veins,
and a number of intracellular protozoa, similar to T. gondii (Fig. 1C),
were observed. The lungs showed congestion of the alveolar capillary,
pulmonary edema, infiltration of inflammatory cells around large ves-
sels, and intracellular protozoa (Fig. 1D). The myocardium had a few
intracellular protozoa (Fig. 1E), and as a result, the section that was
immunostained with anti-T. gondii (Fig. 1F), was observed. Intracellular protozoa in the cytoplasm (Fig. 1F). In the section that was
immunostained with-anti-BAG1, strongly brown-staining cyst-like structures were observed, which enclosed hundreds of parasites (Fig. 1G).

3.2. Molecular analyses

Four genetic loci (B1, SAG3, GRA6, and ROP18) were successfully
amplified, of which 3 were successfully sequenced. The length of the
obtained sequences (excluding primer sequences) and the DNA Data
Bank of Japan accession numbers were: SAG3, 186 bp (LC474391);
GRA6, 107 bp (LC474392); and ROP18, 155 bp (LC474393),
respectively. In all phylogenetic trees, the sequences in the present
study were clustered with type II strains (Fig. 2). Although several
atypical strains (BOF, COUG, GUYKOE, FOU) also fell within the same
class, the phylogenetic position of these atypical strains were incon-
sistent in the trees that used GRA6 (Fig. 2B) and ROP18 (Fig. 2C) se-
quences.

4. Discussion

Based on the histopathological findings and molecular analyses, the
deceased Amami spiny rat was diagnosed with disseminated tox-
oplasmosis. Since the present case was a well-padded individual and
without any other lesions, the cause of death was presumably a result of
acute disseminated T. gondii infection.

In the Amami-Oshima Islands, there are no known endemic feline
species, and domestic cats serve as the only final hosts of T. gondii.
An estimated 600–1,200 feral cats roam the forested areas of the Amami-
Oshima Island (Shionosaki, 2016). In an epidemiological study in the
Amami-Oshima Island, seroprevalence of T. gondii among outdoor cats
in these mountain areas was shown to be higher than that in urban
areas (Matsuu et al., 2017). Furthermore, in such natural areas, the
Amami spiny rat, Ryukyu long-furred rat, and Amami rabbit are known
to be important prey species for feral cats (Shionosaki et al., 2015).
These data suggest that non-native cats and endemic mammals play
specific roles in the maintenance of T. gondii infection in these areas.
The prevalence of T. gondii in endemic mammals remains unknown, and
only a single suspected case of disseminated infection was previously
reported in an Amami rabbit (Kubo et al., 2013). A larger epidemi-
ological study on the prevalence of T. gondii in endemic mammals,
including endangered species, is urgently needed.

The source of T. gondii infection can vary among animal species,
feeding behaviors, and habitats. The main source of infection is from
the oocysts shed in the feces of a host felid or from tissue cysts of in-
termediate hosts (Dubey and Odening, 2001). Since the Amami spiny
rat is omnivorous and consumes seeds and insects (Iwasa, 2009), en-
vironmental contamination by oocysts from outdoor cats is considered
to be the most probable source of infection.

In general, the pathogenicity of T. gondii is determined by the strain
and host factors (Dubey and Odening, 2001). With regards to the strain,
type I strains are lethal in mice, whereas type II and III strains are
avirulent or have low virulence in the same animal (Sibley and
Boothroyd, 1992). Molecular analysis suggested that the sequences
determined in this study differed from the type I and III strains, but
were closely related to the type II strain. Unfortunately, there are no
other published reports on the molecular characterization of T. gondii
from the Amami-Oshima Island and, therefore, the strain could not be
compared with the present database of sequences. With regards to the
host factor, mice of any age are susceptible to T. gondii infection, and

Fig. 2. Mid-point phylogenetic trees using SAG3 (A), GRA6 (B), and ROP18
(C) sequences of Toxoplasma gondii de-
tected from the Amami spiny rat spe-
cimen and the references available in the public databases, *, type I strain;
**, type II strain; ***, type III strain;
****, atypical strain. Bars represents the number of nucleotide substitutions
per site.
more severe infections have been observed in pregnant or lactating mice than in nonlactating mice (Dubey and Odening, 2001). Adult rats are usually resistant, whereas some juveniles have shown clinical symptoms (Dubey and Odening, 2001). Furthermore, the consequences of infection vary between different host species. For example, hares (Lapins spp.) are considered to be more susceptible to T. gondii infection than domestic rabbits (Oryctolagus cuniculus), despite both belonging to the order Lagomorpha. An experimental infection study revealed that hares showed a high mortality rate at very low oocyst doses, whereas domestic rabbits were able to tolerate higher oocyst doses (Gustafsson et al., 1997; Sedlák et al., 2000). The different responses to varying levels of exposure to T. gondii infection among these lagomorphs is believed to be due to differences in the natural susceptibility to T. gondii infection or the negative impact of stress to the immune status of hares (Sedlák et al., 2000). The evolutionary history of hosts may be related to the susceptibility to T. gondii infection. Toxoplasma gondii can cause fatal infections in Australian marsupials and New World monkeys, and is thus these groups are considered to be the most susceptible groups of species to T. gondii infection. These animals have largely evolved separately from the felids, and hence also separately from exposure to T. gondii, and have thus not developed resistance to T. gondii infection (Innes, 1997; Carme et al., 2009). Such a hypothesis seems applicable to the Spiny rats which have a unique and ancient origin. Spiny rats that belong to the genus Tokudaia are distributed only in the three neighboring islands: Amami-Oshima Island for Amami spiny rat, Tokunoshima Island for Tokunoshima spiny rat (T. tokunoshimensis), and Okinawa Island for Okinawa spiny rat (T. muenkini). The divergence event for the genus Tokudaia and other Murinae (Apodemus, Micromys, Mus, and Rattus), distributed in the surrounding areas, is estimated to have occurred several million years ago (Suzuki et al., 2000; Sato and Suzuki, 2004). Since there are no endemic felids species in these islands, the Amami spiny rat is a certainly accidental intermediate host for T. gondii and plays no part in its natural history and evolution.

In conclusion, this is the first case report of T. gondii infection in the Amami spiny rat. Islands are of particular importance for the conservation of global rodent diversity (Amori et al., 2008). However, invasive predators, particularly cats, have directly contributed to population declines or the extinction of many native rodent species since these historically isolated, island animals lack evolved defenses against such predators (Medina et al., 2011). The present case demonstrates an example of the negative indirect impact of non-native predators. To reduce T. gondii infection in local wildlife, the populations of feral and stray cats should be controlled in these regions.

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Declarations of interest
None.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.06.001.

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