Outbreak of toxoplasmosis in four squirrel monkeys *(Saimiri sciureus)* in Japan

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Abstract

Toxoplasma gondii is a protozoan parasite that causes fatal disease in New World monkeys. Several reports have described outbreaks of toxoplasmosis in squirrel monkeys. Here, we report the death of four squirrel monkeys in a captive colony from acute toxoplasmosis, one of which developed toxoplasmosis about 1 year after the initial outbreak. Serum anti–*T. gondii* antibody was detected by a latex agglutination test in the animals, and one presented seropositive before clinical signs were observed. Macroscopically, the lungs were severely affected and three animals showed pulmonary edema. Microscopically, interstitial pneumonia was observed in all animals. In the liver and heart, multifocal mononuclear cell infiltration with necrosis was detected. Parasite loading tended to be higher in the lungs, liver and heart than in the spleen, kidney and brain. The parasite was isolated from the brain of one animal and this isolate showed type II restriction patterns in the *SAG1*, *SAG2*, *SAG3*, *BTUB*, *GRA6*, *c22-8*, *c29-2* and *PK1* genes of *T. gondii* and type I restriction patterns in the *L358* and *Apico* genes by PCR-Restriction Fragment Length Polymorphism analysis. The clinical signs were reduced in mice infected with this isolate compared with those infected with reference type II strain PLK in a bioassay. To our knowledge, this is the first report of isolation of the parasite from squirrel monkeys in Japan and offers the opportunity for genomic and pathogenic analyses to aid our understanding of acute toxoplasmosis.

Keywords: *Toxoplasma gondii*, Squirrel monkey, Histopathology, Isolation

1. Introduction

Toxoplasma gondii is an apicomplexan parasite that infects warm-blooded animals including humans [1]. Members of the felid family, which are the definitive hosts of *T. gondii*, shed oocysts in their feces [2]. Because the oocysts are remarkably stable in the environment, transmission can occur horizontally by ingestion of water or vegetables contaminated with oocysts. Additionally, raw or undercooked meat harboring tissue cysts of *T. gondii* from intermediate hosts, such as sheep, goats, pigs and chickens, is a potential infectious source [3].

Although *T. gondii* infection is typically asymptomatic in adult humans and other animals [4], New World primates including squirrel monkeys (*Saimiri sciureus*) show high susceptibility to *T. gondii* and develop severe toxoplasmosis regardless of the strain involved, often dying without any clinical signs or with nonspecific signs such as anorexia and depression [5]. Although reports of toxoplasmosis in captive squirrel monkeys are scarce, the disease is severe or even fatal [6-8], indicating that toxoplasmosis in squirrel monkeys should be considered a risk.

In this study, we aimed to identify, isolate and genotype *T. gondii* from an outbreak of acute toxoplasmosis in a colony of squirrel monkeys in Hokkaido, Japan.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was

approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan (permit numbers 23-19, 24-1 and 29-43).

2.2. Necropsy, histopathology and immunohistochemistry

The principal tissues including the liver, spleen, kidney, heart, lung, brain, hilar lymph node and skeletal muscle were collected for histopathological analysis in 4 cases. After fixation with 15% phosphate buffered formalin solution, the tissues were routinely embedded in paraffin wax sectioned at 4 μm and stained with hematoxylin and eosin (HE). Immunohistochemistry for *T. gondii* was performed with anti–*T. gondii* polyclonal rabbit serum (Quartett, Berlin, Germany) as the primary antibody, and a secondary antibody conjugated with streptavidin–biotin–peroxidase (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). Briefly, after deparaffinization, tissue sections were placed in citrate buffer (pH 6), heated in a microwave for 10 min and blocked for endogenous peroxidase with 3% hydrogen peroxide in methanol. The sections were then incubated with the primary antibody diluted 1:200. After washing, sections were incubated with secondary antibody. The chromogen was developed with 3,3'-diaminobenzidine (Simple Stain DAB; Nichirei).

2.3. DNA extraction and real-time PCR

The lung and liver in case No. 1 and the liver, spleen, kidney, heart, lung and brain in case No. 2 and No. 3 were collected for quantification of parasites by real-time PCR. DNA was extracted from 1 g of tissue using a DNeasy Blood & Tissue Kit (Qiagen, Santa Clarita, CA, USA). In case No. 1, DNA was extracted from formalin-

fixed, paraffin-embedded tissues of the lung and liver with the QIAamp DNA FFPE Tissue Kit (Qiagen). The parasite load in tissues was quantified by real-time PCR for the *B1* gene (5'-AAC GGG CGA GTA GCA CCT GAG GAG A-3' and 5'-TGG GTC TAC GTC GAT GGC ATG ACA AC-3'), which is present in all known strains of this parasite species [9]. The PCR mixture (25 µL) contained 1 × SYBR Green PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 400 µM dUTP, 0.625 U of AmpliTaq Gold DNA polymerase and 0.25 U of AmpErase uracil-N-glycosylase (AB Applied Biosystems, Carlsbad, CA, USA), 0.5 µM of each primer and 50 ng of genomic DNA. Amplification was performed by a standard protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min). Amplification, data acquisition and data analysis were carried out in an ABI 7900HT Prism Sequence Detector (AB Applied Biosystems), and the cycle threshold values (Ct) were exported to Microsoft Excel for analysis. A standard curve was established from *T. gondii* DNA extracted from 1×10^5 parasites using 1 µl of a serial dilution ranging from 10,000 to 0.01 parasites. Parasite numbers were calculated by interpolation on a standard curve, with the Ct values plotted against a known concentration of parasites. After amplification, the PCR product melting curves were acquired via a stepwise temperature increase from 60°C to 95°C. Data analyses were conducted with Dissociation Curves version 1.0 f (AB Applied Biosystems).

2.4. Isolation of *T. gondii*

The lung and brain tissues (20 g) collected from case No. 2 were homogenized separately in 50 mL phosphate-buffered saline (PBS) containing acid pepsin solution

(Pepsin 1:20,000 from porcine stomach mucosa (Sigma, St. Louis, MO, USA) and 85.6 mM NaCl, pH 1.2) and incubated at 37°C for 45 min in a shaking water bath. After incubation, the samples were centrifuged at 500 × *g* for 10 min. The sediment was suspended in 10 mL of neutralizing solution (1.2% sodium bicarbonate, sodium bicarbonate pH 8.3 in PBS). The sediment was then washed with neutralizing solution twice at 500 × *g* for 10 min, and the remaining pellet was suspended in 1 mL of PBS. The samples prepared from the lung or brain tissue were inoculated intraperitoneally into interferon-gamma-deficient mice [10]. Mice were observed daily and euthanized upon the appearance of clinical signs. Peritoneal fluid from the mice was inoculated into human foreskin fibroblast (HFF) cells.

2.5. Bioassay in mice

The *T. gondii* isolate from case No. 2 (squirrel monkey isolate, OBYN-SM1) and strain PLK was propagated in HFF cells cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum. To purify the tachyzoites, parasites and host-cell debris were washed in ice-cold PBS, and the final pellet was re-suspended in cold PBS and passed through a 27-gauge needle and a 5.0-μm pore filter (Millipore, Bedford, MA, USA). To compare the pathogenicity of OBYN-SM1, BALB/c and C57BL/6 mice obtained from Clea Japan (Tokyo, Japan) were inoculated intraperitoneally with tachyzoites (1×10^3/mouse) of *T. gondii* OBYN-SM1 and PLK as a reference type II strain of *T. gondii*. All mice were monitored for survival and body weight until 30 days post-inoculation. Samples of serum and brain tissue were collected for serum antibody and quantitative analyses of *T. gondii* by real-time PCR, respectively, as detailed above. Serum antibody against

dense granule antigen protein 7 of *T. gondii* (TgGRA7) was detected by an enzyme-linked immunosorbent assay as described previously [11]

2.6. Restriction fragment length polymorphism (RFLP) analysis

Genotyping was performed using multilocus nested PCR-RFLP (Mn-PCR-RFLP) typing for 10 different genetic markers; SAG1, SAG2 (5′-SAG2, 3′-SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico [12]. The multiplex PCR reaction was carried out in 25 µl of volume consisting of 2.5 µl of 10× PCR buffer with 15 mM $MgCl_2$, 2.5 µl dNTPs (2 mM), 0.15 µl (50 µM) each of the forward and reverse external primers, 0.2 µl of AmpliTaq polymerase (5 U/µl) and 2 µl of DNA template. The reaction mixture was treated at 95℃ for 4 min, followed by 30 cycles of 94℃ for 30 sec, 55℃ for 1 min and 72℃ for 2 min. Positive controls consisted of tachyzoite lysate from *T. gondii* RH (type I), PLK (type II), and VEG (type III) strains. Negative control consisted of DNA-free water. Multiplex PCR amplified products were diluted (1 : 1) by adding 25 µl of nuclease-free water. The nested PCR amplification of each marker separately was carried out in 25 µl of volume consisting of 2.5 µl of 10× PCR buffer with 15 mM $MgCl_2$, 2.5 µl dNTPs (2mM), 0.3 µl (50 µM) each of the forward and reverse internal primers, 0.2 µl of AmpliTaq polymerase (5 U/ µl) and 2 µl of diluted multiplex PCR products. To reveal the RFLP pattern of each reference strain and samples, 5 µl of PCR products were mixed with 15 µl of digestion reaction containing 1×NEB buffer and volume of restriction enzymes was added following the manufacturer's instruction (New England BioLab, Ipswich, MA, USA). The digested PCR products were resolved in a 2.5% and 3% agarose gels by electrophoresis. Primers for Mn-PCR-RFLP,

appropriate restriction enzymes for different markers, incubation temperature and time were shown in Table 1.

2.7. Statistical analyses

The significance of the differences in mouse survival was analyzed by log-rank tests. Statistical analyses were performed using a two-way ANOVA followed by the Bonferroni test to estimate differences in body weight, with the data for each presented as a standard deviation of the mean. Because there was no normal distribution on the brain parasite number between PLK-infected C57BL/6 mouse and OBYN-SM1-infected animal (F test, $P = 0.0216$), the statistical difference was determined by Mann-Whitney's *U* test. All statistical analyses were performed with GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA) or Microsoft Excel. In the figure legends, the statistical significance levels are represented by asterisks, together with the name of the statistical test that was used. *P* values of < 0.05 were considered statistically significant.

3. Results

3.1. Clinical course and serum antibody

The four affected squirrel monkeys were kept with nine squirrel monkeys and one black-headed squirrel monkey (*Saimiri sciureus boliviensis*) in a zoo in Hokkaido, Japan. The first case (case No. 1) was detected in November 2011 (Table 2). The clinical signs were a cough and tremor, and the monkey died 2 days after developing symptoms. Case No. 2 was an offspring of case No. 1 and died without any clinical

signs 12 days after the death of its mother. After these deaths, the other 11 monkeys were tested using a latex agglutination test kit (Toxocheck-MT; Eiken-Kagaku, Tokyo, Japan) on December 19, 2011. Only case No. 3 gave a positive result (cut-off ≥ 64). Case No. 3 showed mild depression a month after the death of case No. 1 and died the next day (8 days after the antibody test). When the antibody test was performed a month after the death of case No. 3, the other 10 monkeys showed negative results. Ten months after the death of case No. 1, case No. 4 developed tachypnea and a tremor and died 2 days later. Case No. 4 was seropositive on the day of disease onset (Table 2).

3.2. Necropsy, histopathology, immunohistochemistry and detection of *T. gondii* DNA

Macroscopically, the lungs of cases 1, 2 and 3 showed a mixture of dark red (Fig. 1A) and pink regions and edema was evident in lung sections, along with foamy fluid in bronchi. In case No. 3, the spleen was enlarged.

In histopathological analysis, alveolar wall thickening with mononuclear cell infiltration and severe pulmonary edema was observed, and the alveolar epithelium appeared cuboidal. Additionally, in cases 1, 3 and 4, hyaline membrane formation was observed and the lungs showed interstitial pneumonia (Fig. 1B). In the liver and heart, multifocal inflammatory cell infiltration with necrosis of hepatocytes and myocardial cells was observed (Fig. 1C, D). The brain was examined in cases 1, 2 and 3 and scattered glial nodules were observed, with the predominant lesions present in case No. 2 (Fig. 1E). Severe necrotizing lymphadenitis was detected in the lymph nodes (Fig. 1F). Mild inflammatory cell infiltration in the interstitium of the kidney

198 was observed, but no histopathological changes were noted in the spleen. Tachyzoites
199 were detected in the tissues including liver, spleen, kidney, heart, lung and brain in all
200 cases by immunohistochemistry with anti–*T. gondii* polyclonal rabbit serum (Fig. 1G,
201 H).

202 The parasite load tended to be higher in the lungs, liver and heart compared with
203 the spleen, kidney and brain in cases No. 2 and No. 3 (Fig. 2). Although similar
204 parasite load was observed in all cases based on the immunohistochemistry, the
205 parasite load in the lungs and liver of case No. 1was lower than in cases 2 and 3 by
206 the quantitative real-time PCR. It may be due to the quality of DNA extracted from
207 formalin-fixed, paraffin-embedded tissues.

208

209 **3.3. Isolation and genotyping of *T. gondii***

210 We could culture the parasites derived from brain samples of case No. 2 in
211 HFF cells. In an immunofluorescent antibody test using an anti-NcGRA7 antibody,
212 an NcGRA7 signal was observed in the isolated parasites (Fig. 3). The three North
213 American clonal lineages of *T. gondii* (types I, II and III) differ in their activation of
214 immune responses and virulence in mice. Therefore, genotyping of the isolate was
215 performed by Mn-PCR-RFLP of the marker genes *SAG1, SAG2 (5′-SAG2, 3′-SAG2*
216 *and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1* and *Apico*, according
217 to a previous report [12]. DNA from RH, PLK and VEG *T. gondii* strains was used as
218 type I, II and III controls, respectively. OBYN-SM1 (*Toxoplasma* isolate from case
219 No. 2) showed restriction patterns corresponding to type Ⅱ *T. gondii* except for *L358*
220 and *Apico* indicating the type I patterns (Fig. 4, Table 3). Additionally, DNA of lung
221 tissues from squirrel monkeys (Cases No. 2 and No. 3) showed same patterns with

222 the OBYN-SM1 (Fig. 4, Table 3).

223

224 **3.4. Parasite virulence in mice**

225 No mice died in either group inoculated with OBYN-SM1, whereas the survival
226 rate of mice inoculated with PLK was 33.3% and 50% for BALB/c and C57BL/6
227 mice, respectively (Fig. 5A). Mice inoculated with OBYN-SM1 showed no obvious
228 change in body weight compared with the animals inoculated with PLK (Fig. 5B).
229 Although the parasite numbers in the brain were similar between OBYN-SM1 and
230 PLK in BALB/c mice, the number of parasites following inoculation with OBYN-
231 SM1 was significantly lower than with PLK in C57BL/6 mice (Fig. 5C). The
232 production of NcGRA7 antibody was confirmed at 2 and 4 weeks after inoculation
233 with OBYN-SM1 (Fig. 5D), indicating that the isolated OBYN-SM1 was active in
234 mice. These results suggested that OBYN-SM1 showed low pathogenicity in mice
235 compared with PLK.

236

237

238 **Discussion**

239 New World primates including squirrel monkeys appear to be particularly
240 vulnerable to *T. gondii* infection. The arboreal habitat of these monkeys might result
241 in less frequent contact with *T. gondii* oocysts compared with ground-dwelling
242 animals, therefore, exposure of the monkeys to *T. gondii* may induce acute disease. In
243 squirrel monkeys, death often occurs with no previous clinical signs or with
244 nonspecific signs such as lethargy and anorexia [5,13-17]. Toxoplasmosis in squirrel
245 monkeys is a systemic disease; however, predominate lesions are observed in the

246 lungs of many affected animals consisting of pulmonary edema, froth deposition in
247 the airways and pleural effusion [5,13,14,16,18,19]. Similar clinical presentation was
248 observed in the current study. Two of the four monkeys in this study showed
249 respiratory symptoms including a cough and tachypnea, and all animals died within 2
250 days of onset. In necropsy, cases No. 1, No. 2 and No. 3 showed mosaic-like patterns
251 and edema in the lungs. Histopathologically, severe pulmonary edema and interstitial
252 pneumonia were observed in the lungs. Additionally, multifocal inflammatory and
253 necrotic lesions were observed in many other organs including the liver, heart and
254 lymph nodes. Parasites were detected in all major organs by immunohistochemistry.
255 These findings were similar to three other cases of lethal acute toxoplasmosis in
256 squirrel monkeys in Japan [19], and cases in Mexico [5], Israel [13] and Argentina
257 [16].

258 Serum antibody against *T. gondii* is evaluated by a modified agglutination test, an
259 indirect hemagglutination test and a latex agglutination test in wild and captive
260 monkeys [13,20-23]. Although clinical manifestations have not been detected during
261 surveillance, relatively high seroprevalence has been observed in wild New World
262 primates, particularly *Cebus* primates (76.19%) [23]. In the present study, a serum
263 antibody test was performed using a commercial latex agglutination test kit in all
264 monkeys except for cases 1 and 2. Serum antibody was not detected in any of the
265 other clinically normal monkeys. Case No. 3 tested seropositive, without clinical
266 signs, and then developed toxoplasmosis 7 days after the antibody test. This finding
267 suggested that anti–*T. gondii* antibody was produced in the squirrel monkeys at least
268 one week before the onset of disease. In case No. 4, specific antibody was not
269 detected 8 months before the development of toxoplasmosis but was detected 1 day

before death. Thus, monitoring of anti–*T. gondii* antibody will be important for the survey of a colony of squirrel monkeys with outbreak of toxoplasmosis.

Although the infectious source was unclear in this case, the potential source of infection may be oocysts, probably ingested through contaminated water or food. These monkeys were kept indoors or outdoors and wild animals including stray cats were seen in the zoo. The seroprevalence of *T. gondii* among cats visiting animal hospitals in this area was 17.4% [24], stray cats may therefore be assumed to have a similar or higher prevalence of *Toxoplasma*. Cases No. 1, No. 2 and No. 3 were presumably infected by exposure to the same source because of the timing of disease occurrence. By contrast, the results of serum tests suggested that case No. 4 might have been infected with *T. gondii* separately from the main outbreak in 2011. In this zoo, one *Panthera leo* (30. 9. 1991 to 24. 2. 2014) and one *Panthera tigris altaica* (8. 1. 2011 to 7. 12. 2012) were reared. However, a causal relationship between two captive felids and toxoplasmosis in squirrel monkeys is unknown.

The outcome of *T. gondii* infection in mice is highly dependent on the parasite genotype with type I strains being uniformly virulent (LD_{100} = 1) and type II and III strains being nonvirulent (LD_{50} = 10^3 and 10^5, respectively) [25]. In the present study, *T. gondii* was isolated from the brain of case No. 2 and the isolate showed type II restriction patterns in the *SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2* and *PK1* genes of *T. gondii* and type I restriction patterns in the *L358* and *Apico* genes by PCR-RFLP. Some previous reports have described the genotypes of *T. gondii* that cause outbreaks of toxoplasmosis in squirrel monkeys. For example, in Mexico, a *T. gondii* isolate was characterized as type I based on the *SAG3* gene [5], and in Israel, a *T. gondii* isolate was described as type III based on the *SAG2* gene [13]. An isolate

obtained from black-capped squirrel monkeys in Argentina showed a type III restriction pattern in the *SAG2*, *SAG3*, *BTUB*, *GRA6*, *PK1*, *L358* and *Apico* genes but not *C22-8* and *C29-2* [16]. In French Guiana, *T. gondii* type II was reported in two outbreaks in a colony of squirrel monkeys [3]. Therefore, squirrel monkeys appear to be susceptible to severe toxoplasmosis irrespective of the strain or genotype involved, as shown in a mouse study. In our bioassay, isolate OBYN-SM1 was found to be infective in both BALB/c and C57BL6 mice, but showed low virulence compared with strain PLK (a type II reference strain). However, differences between host species should be taken into consideration. Whole genome analysis of OBYN-SM1 would be valuable in future studies to identify the virulence factors in this type II strain of *T. gondii*.

In the present study, we described the clinical course, pathological changes, parasite loads in each tissue, serum responses and pathogenesis as well as the genotype of isolate OBYN-SM1. To our knowledge, this is the first report of fatal toxoplasmosis in squirrel monkeys caused by an atypical genotype of *T. gondii* in Japan. Squirrel monkeys may provide a good primate model to understand the pathogenesis of acute toxoplasmosis because the pathology is similar to that in human acute toxoplasmosis [3,19]. Further studies are necessary to clarify the virulence factors of *T. gondii* in squirrel monkeys; this may aid our understanding of the mechanisms of onset of acute toxoplasmosis.

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330

331 **Conflict of interests**

332 None.

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Figure legends

Fig. 1. Macroscopic and microscopic changes and immunohistochemistry of *T. gondii*-infected tissue samples. (A) Macroscopic image of the lung isolated from case 1. Dark red and pink areas appeared mixed. (B) Lung tissue section from case 3. Interstitial pneumonia and edema with a hyaline membrane (arrow head). (C) Liver section from case 3. Multifocal inflammatory cell infiltration (arrow heads) with hepatocyte necrosis. (D) Heart section from case 3. Multifocal inflammatory cell infiltration (arrow heads) with myocardial necrosis. (E) Brain section from case 3. Scattered glial nodules (arrow head). (F) Hilar lymph node section from case 2. Severe necrotizing lymphadenitis. (G) and (H) Immunohistochemistry for *T. gondii* of the liver and lung samples from case 3, respectively. Positive signals indicating aggregation of tachyzoites with a parasitophorous vacuole (G) were detected in the tissues of all cases. Small signals indicating tachyzoites (H) were observed in the lungs (arrow heads).

Fig. 2. Parasite load in the tissues of three squirrel monkeys that developed toxoplasmosis. The parasite number in 50 ng of tissue was quantified by real-time PCR for the *B1* gene.

Fig. 3. Isolated parasite from the brain of a squirrel monkey (case 2). (A) Cultured parasite in HFF cells. (B) Immunofluorescent antibody test with an anti-NcGRA7 antibody.

Fig. 4. Genotyping of the isolated parasite, OBYN-SM1, using 10 different genetic

markers; SAG1, SAG2 (5′-SAG2, 3′-SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico by multiplex multilocus nested PCR-RFLP. DNA from RH, PLK and VEG *T. gondii* strains were used as type I, II and III controls, respectively. M: DNA marker (bp), 1: RH (type I), 2: PLK (type II), 3: VEG (type III), 4: DNA from OBYN-SM1, 5: DNA from lung of Case No. 2, 6: DNA from lung of Case No. 3.

Fig. 5. Bioassay in BALB/c and C57BL/6 mice infected with *T. gondii* strain PLK and the squirrel monkey isolate (OBYN-SM1) (n = 6). (A) Survival curves. *, Survival curves were generated using the Kaplan–Meier method. According to the log-rank test, the differences were significant ($P < 0.05$). (B) Body weight. *, Statistically significant differences were determined by two-way ANOVA plus Bonferroni *post hoc* analysis ($P < 0.05$). (C) Parasite burden in the brain at 30 days post-infection. *, Statistically significant differences were determined by the Mann-Whitney's U test in the same mouse strain ($P < 0.05$). (D) Serum antibody against TgGRA7 of mice infected with *T. gondii*. Sera were collected before inoculation with the parasite (Pre) and at 2 and 4 weeks after inoculation.

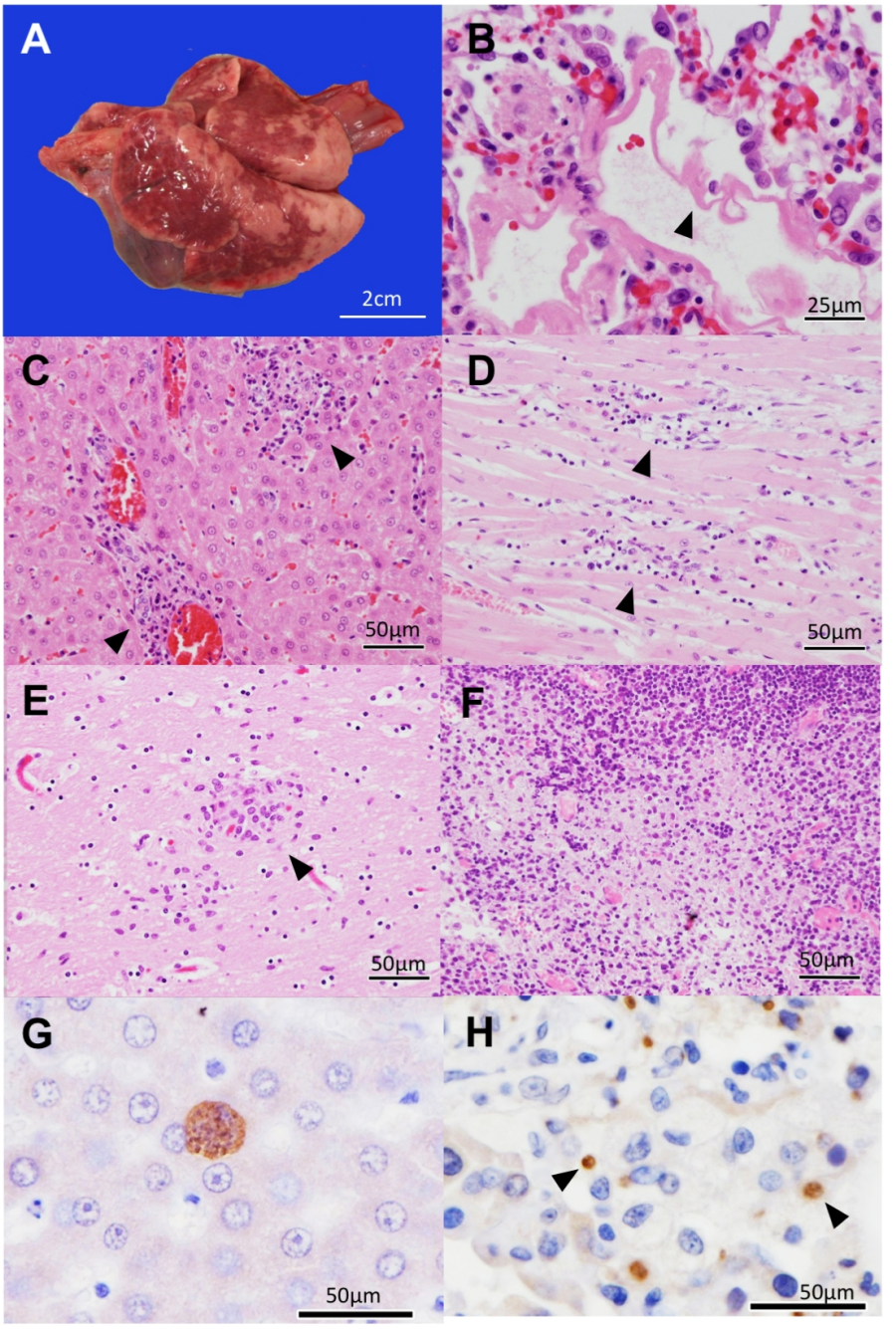


Fig. 1 Nishimura et al.

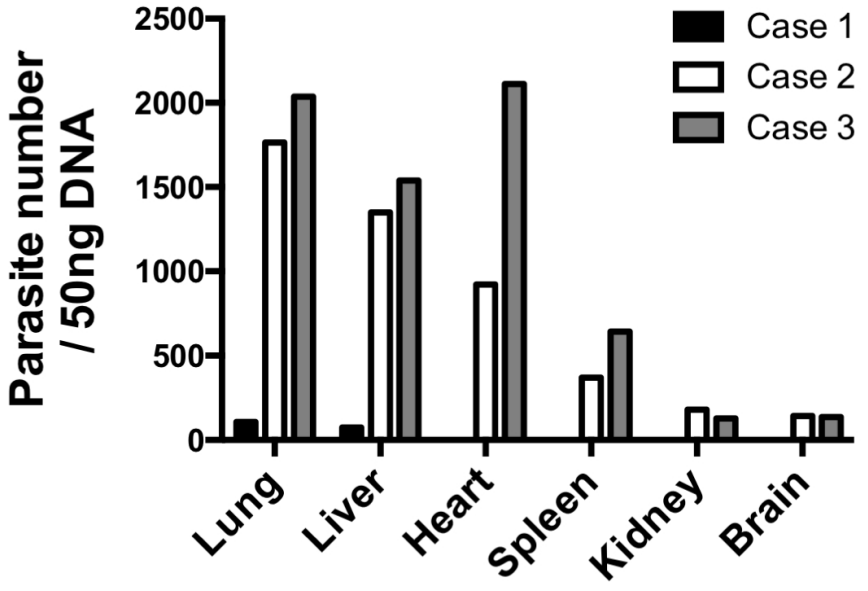


Fig. 2 Nishimura et al.

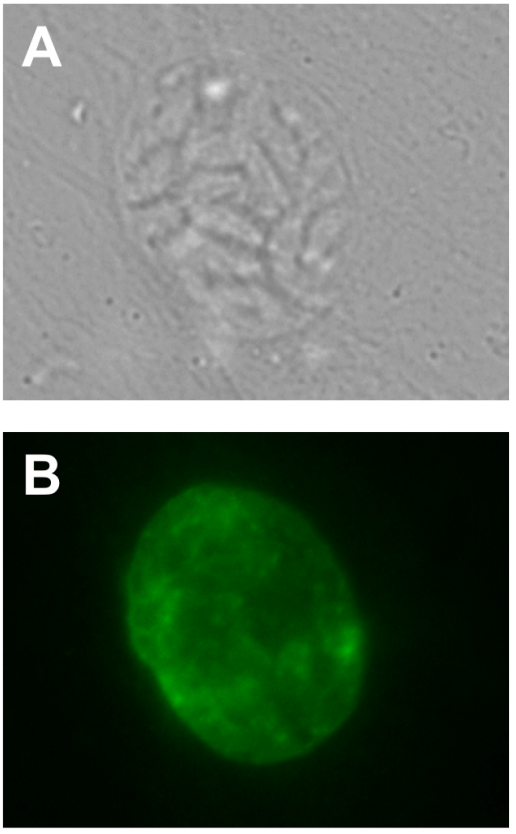
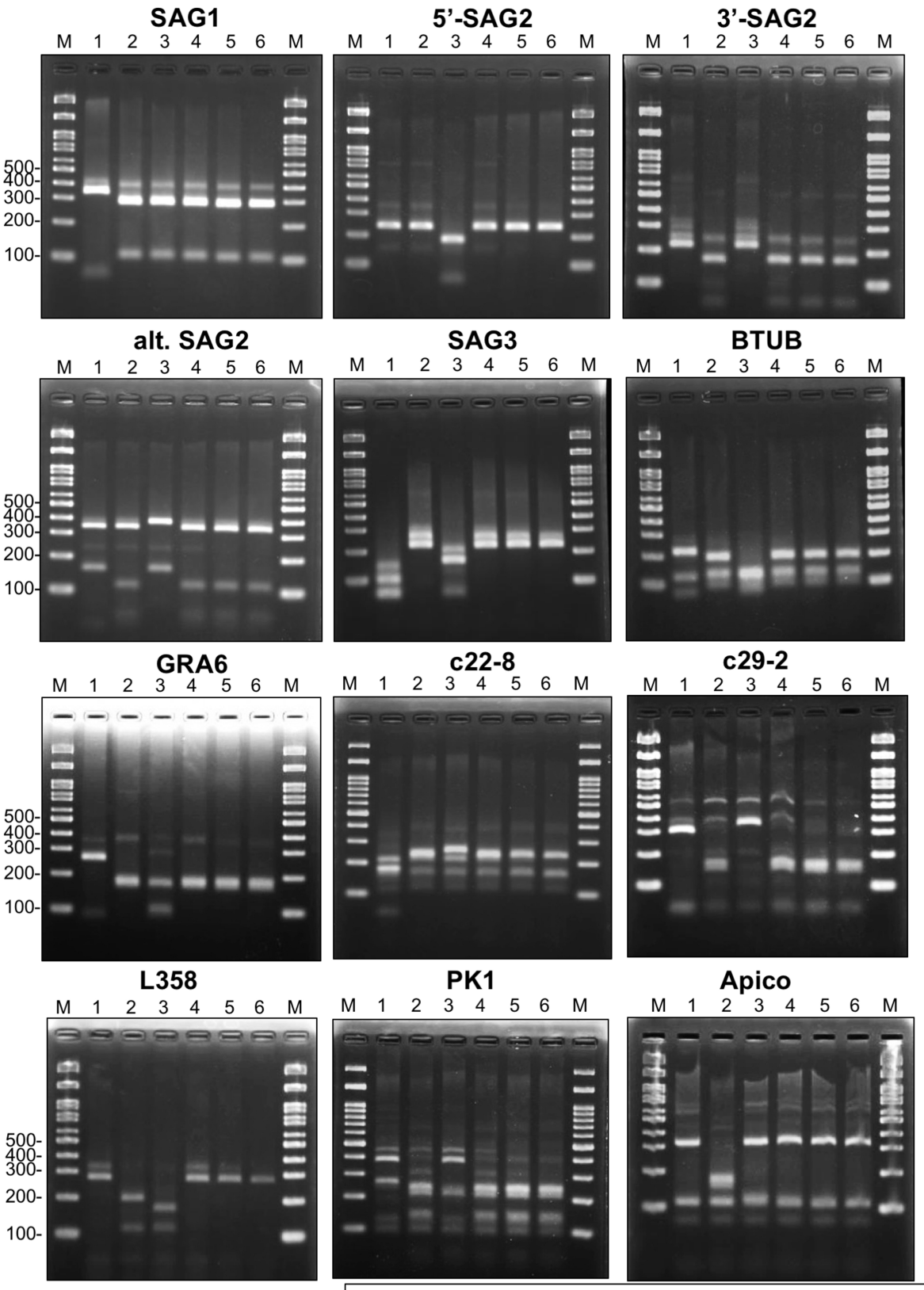


Fig. 3 Nishimura et al.



M: DNA marker (bp)
1: RH (type I), 2: PLK (type II), 3: VEG (type III)
4: OBYN-SM1, 5: Case No. 2, 6: Case No. 3

Fig. 4 Nishimura et al.

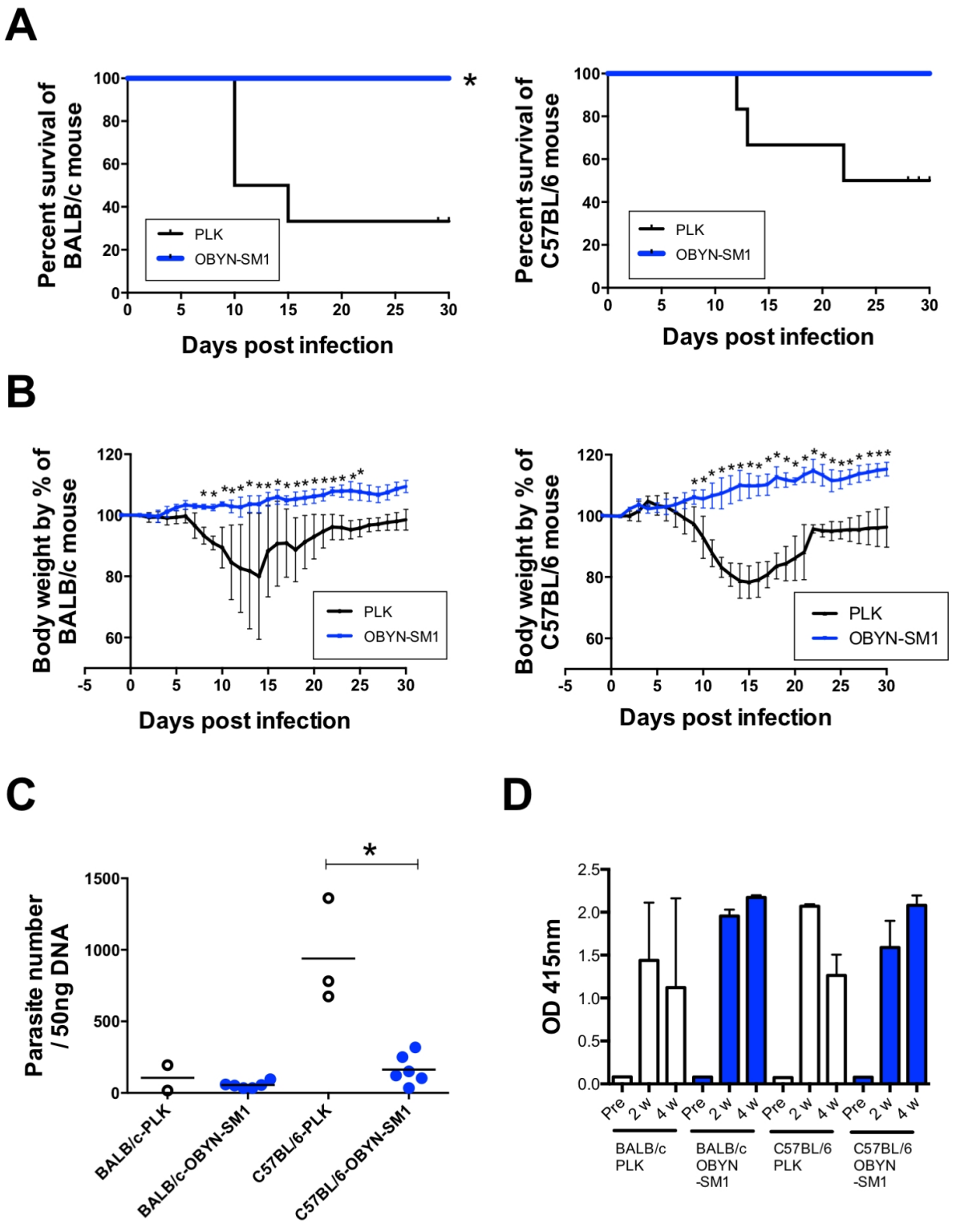


Fig. 5 Nishimura et al.

Table 1
Summary of primers for multiplex multilocus nested PCR-RFLP typing

Markers	Multiplex PCR primers (external primers)*	Nested PCR primers (internal primers)	Restriction enzymes	Incubation temperature	Incubation time
SAG1	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	Sau96I + HaeII (double digest)	37℃	1hr
5′-SAG2	Not needed. The DNA fragment for 5k-SAG2 is covered by the external primers of alt. SAG2.	F: GAAATGTTTCAGGTTGCTGC R: GCAAGAGCGAACTTGAACAC	MboI	37℃	1hr
3′-SAG2	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	HhaI	37℃	1hr
alt. SAG2	F: GGAACGCGAACAATGAGTTT R: GCACTGTTGTCCAGGGTTTT	F: ACCCATCTGCGAAGAAAACG R: ATTTCGACCAGCGGGAGCAC	HinfI+TaqI (double digest)	37℃, 65℃	30 min, 30 min.
SAG3	F: CAACTCTCACCATTCCACCC R: GCGCGTTGTTAGACAAGACA	F: TCTTGTCGGGTGTTCACTCA R: CACAAGGAGACCGAGAAGGA	NciI	37℃	1hr
BTUB	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	BsiEI+TaqI (double digest)	60℃	1hr
GRA6	F: ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F: TTTCCGAGCAGGTGACCT R: TCGCCGAAGAGTTGACATAG	MseI	37℃	1hr
C22-8	F: TGATGCATCCATGCGTTTAT R: CCTCCACTTCTTCGGTCTCA	F: TCTCTCTACGTGGACGCC R:AGGTGCTTGGATATTCGC	BsmAI+MboII (double digest)	37℃, 55℃	30 min, 30 min.
C29-2	F: ACCCACTGAGCGAAAAGAAA R: AGGGTCTCTTGCGCATACAT	F: AGTTCTGCAGAGTGTCGC R:TGTCTAGGAAAGAGGCGC	HpyCH4IV+RsaI (double digest)	37℃	1hr
L358	F: TCTCTCGACTTCGCCTCTTC R: GCAATTTCCTCGAAGACAGG	F: AGGAGGCGTAGCGCAAGT R: CCCTCTGGCTGCAGTGCT	HaeIII+NlaIII (double digest)	37℃	1hr
PK1	F: GAAAGCTGTCCACCCTGAAA R: AGAAAGCTCCGTGCAGTGAT	F: CGCAAAGGGAGACAATCAGT R: TCATCGCTGAATCTCATTGC	AvaI+RsaI (double digest)	37℃	1hr
Apico	F: TGGTTTTAACCCTAGATTGTGG R: AAACGGAATTAATGAGATTTGAA	F: GCAAATTCTTGAATTCTCAGTT R: GGGATTCGAACCCTTGATA	AflII+DdeI (double digest)	37℃	1hr

* F, forward primer; R, reverse primer.

Table 2

Clinical signs and serology results for the squirrel monkeys analyzed in this study

Case #	Age	Sex	date of occurrence	clinical signs	latex agglutination test for *T. gondii* antibody
1	4y*	♀	27.11.2011 (died 2 days after)	cough, tremor	ND
2	5m	♂	9.12.2011	none	ND
3	4y*	♂	26.12.2011 (died a day after)	mild depression	+
4	1.3y	♂	9.10.2012. (died 2 days after)	tachypnea, tremor, dog-sitting posture	+

* Age of monkeys for cases 1 and 3 is estimated.

ND: no data

Table 3

Summary of multilocus PCR-RFLP typing for *Toxoplasma* isolate from squirrel monkey

	Genetic markers										
T. gondii	SAG1*	(5'+3') SAG2[#]	alt. SAG2[$]	SAG3	BTUB	GRA6	C22-8	C29-2	L358	PK1	Apico
RH (type I)	I	I	I	I	I	I	I	I	I	I	I
PLK (type II)	II or III	II	II	II	II	II	II	II	II	II	II
VEG (type III)	II or III	III	III	III	III	III	III	III	III	III	III
OBYN-SM1	II or III	II	II	II	II	II	II	II	I	II	I
Case No. 2 (lung)	II or III	II	II	II	II	II	II	II	I	II	I
Case No. 3 (lung)	II or III	II	II	II	II	II	II	II	I	II	I

* Type II and type III are not distingusihable at SAG1 locus.

[#] SAG2 marker based on 5'- and 3'-ends of the gene sequence.

[$] A SAG2 marker based on the 5'-end of the gene sequence but different from 5'-SAG2.

As *Toxoplasma* reference strains, RH, PLK and VEG were used for type I, II and III, respectively.

OBYN-SM1: Toxoplasma isolate from squirrel monkey

Case No. 2 (lung) and Case No. 3 (lung): Lung tissues from squirrel monkeys (Cases No. 2 and No. 3)