Parasitology International 64 (2015) 587–590

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Case report

# Isolation and molecular characterization of *Toxoplasma gondii* in a colony of captive black-capped squirrel monkeys (*Saimiri boliviensis*)

L. Pardini [a,c,*], A. Dellarupe [a,c], D. Bacigalupe [a], M.A. Quiroga [b], G. Moré [a,c], M. Rambeaud [a], W. Basso [e], J.M. Unzaga [a], G. Schares [d], M.C. Venturini [a]

[a] Laboratorio de Inmunoparasitología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118, La Plata 1900, Buenos Aires, Argentina
[b] Laboratorio de Patología Especial Veterinaria, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118, La Plata 1900, Buenos Aires, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas, CABA, Buenos Aires, Argentina
[d] Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Institute of Epidemiology, Südufer 10, 17493 Greifswald, Insel Riems, Germany
[e] Institute of Parasitology, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 266a, 8057 Zurich, Switzerland

ARTICLE INFO

*Article history:*
Received 11 July 2015
Received in revised form 7 August 2015
Accepted 18 August 2015
Available online 20 August 2015

*Keywords:*
*Toxoplasma gondii*
*Saimiri boliviensis*
Histopathology
Isolation
PCR
Genotyping

ABSTRACT

Toxoplasmosis is commonly asymptomatic; however, it can be a fatal multisystemic disease in some animal species, such as New World monkeys. An outbreak of acute fatal toxoplasmosis was reported in a colony of black-capped squirrel monkeys (*Saimiri boliviensis*) from the zoo of La Plata, Argentina. Post-mortem examination of two monkeys revealed macroscopical and microscopical lesions compatible with acute toxoplasmosis. The presence of *Toxoplasma gondii* was confirmed by immunohistochemistry on monkey tissues, bioassay in mice and PCR using the specific primers B22–B23. By PCR–RFLP analysis, *T. gondii* isolated in mice, deriving from both monkeys, showed the same restriction pattern, with most markers showing a type III restriction pattern, except for C22-8 (type II) and C29-2 (type I). To our knowledge this is the first report of fatal toxoplasmosis in *S. boliviensis* caused by a non-canonical or atypical genotype of *T. gondii*.



## 1. Introduction

*Toxoplasma gondii* is an apicomplexan parasite that affects a broad range of warm-blooded animals including human beings. Toxoplasmosis can be a fatal multisystemic disease in some animal species under captive conditions, such as New World monkeys, slender-tailed-meerkats, Australian marsupials, lemurs and Pallas' cats [1,2]. Transmission can occur horizontally by ingestion of water or vegetables contaminated with oocysts or raw or undercooked meat harboring tissue cysts, and vertically, during pregnancy [2]. Ante-mortem diagnosis is rare because clinical signs are not specific and therefore appropriate treatment is initiated late [2].

Three clonal lineages (type I, II and III) of *T. gondii* which differ in their virulence in laboratory mouse models have been described [2]. Non-canonical *T. gondii* with atypical genotypes has been associated with high virulence in mice and other animal species [3]. In the zoo of La Plata, Argentina, type III isolates were identified in a red kangaroo and tailed slender meerkats [2] and type II was isolated from a great gray kangaroo [1]. Interestingly, these isolates, considered avirulent in a mouse model, were fatal or caused severe disease in the affected animals.

Although reports of toxoplasmosis in captive squirrel monkeys are scarce, the relevance of this disease should be taken into consideration, since all reported cases worldwide were severe or fatal. Acute epidemic toxoplasmosis was reported in London Zoo, affecting an entire colony of squirrel monkeys (*Saimiri sciureus*), and killing one-third of the animals [2]. In French Guiana and Israel fatal outbreaks of toxoplasmosis were described in outdoor captive breeding colonies of squirrel monkeys [2]. A fatal case of toxoplasmosis was described in a young male red-handed howler monkey (*Alouatta belzebul*) in a Pernambuco State Zoo, Brazil [4]. The aim of this study was to identify, to isolate and to genotype *T. gondii* from an outbreak of acute toxoplasmosis in a colony of black-capped squirrel monkeys (*Saimiri boliviensis*) in the zoo of La Plata, Argentina.

## 2. Animals

Eight black-capped squirrel monkeys (*S. boliviensis*) were found dead in La Plata Zoo, Argentina, without previous clinical signs. Two of the animals (one adult [Sb1] and one 18-month-old [Sb2] male) were sent to the Special Veterinary Pathology Laboratory, Facultad de

* Corresponding author at: 60 y 118, La Plata 1900, Buenos Aires, Argentina.
*E-mail address:* laispardini@fcv.unlp.edu.ar (L. Pardini).

http://dx.doi.org/10.1016/j.parint.2015.08.009
1383-5769/

Ciencias Veterinarias, Universidad Nacional de La Plata, Argentina (FCV-UNLP) for diagnosis.

### 3. Necropsy, histopathology and immunohistochemistry

Complete necropsies were performed and samples of central nervous system (CNS), heart, liver, lung, spleen, kidney, and intestine were collected and preserved in 10% formalin in phosphate buffer solution (PBS). 5 μm tissue sample sections were stained with hematoxylin and eosin (H&E). Additionally, samples of heart, liver, lung and spleen were assayed by immunohistochemistry for *T. gondii* detection as previously described [5], using an LSAB + System HRP commercial kit (DakoCytomation, USA) according to manufacturer's recommendations and an anti-*T. gondii* rabbit sera as primary antibody diluted 1:2000. Positive controls were brain sections from a *T. gondii*-experimentally infected mouse and negative controls were heart sections of a *Neospora caninum*-naturally infected calf. Fatal toxoplasmosis was suspected and samples (Sb1 [pool of organs], Sb2 [heart]) of both animals were submitted to the Immunoparasitology Laboratory, FCV-UNLP for further examination.

### 4. Isolation of *T. gondii* in mice and cell culture

A pool of organs from Sb1 and heart from Sb2 were homogenized in saline containing antibiotics (penicillin 1000 IU/ml and streptomycin 100 μg/ml) and were inoculated intraperitoneally in two N:NIH Swiss mice (mice M1 and M2, respectively). Mice were observed daily and sacrificed upon appearance of clinical signs. Necropsies were performed and brain homogenates were examined microscopically. The brain from M1 was conserved at −20 °C for PCR detection. Peritoneal washing from successive mice passages of Sb2 (or M2) material was inoculated into VERO (kidney of African green monkey) cell cultures as previously described by Moré et al. [6]. Inoculated mice were maintained according to conditions established by the Institutional Committee for the Care and Use of Laboratory Animals-FCV-UNLP.

### 5. DNA isolation, PCR and *T. gondii* genotyping

DNA from the brain of M1, and from the cell culture inoculated with tachyzoites derived from peritoneal exudate from M2 was extracted with a commercial DNA extraction kit (Dneasy Tissue kit QIAGEN) according to the manufacturer's recommendations. Polymerase chain reaction (PCR) was performed using specific primers for *T. gondii* (B22–B23) as described previously [1]. Each amplification routine was conducted with a positive control (DNA of *T. gondii* RH strain), a negative control (control process sample DNA) and a no template control (NTC).

Genotyping was performed by nested-PCR (n-PCR) using nine markers: nSAG2, BTUB, GRA6, SAG3, L358, C22-8, C29-2, PK1 and Apico, followed by restriction fragment length polymorphism (RFLP) analysis. DNA from RH, Me49 and NED *T. gondii* strains were used as type I, II and III controls, respectively. The PCR and n-PCR/RFLP products were visualized in 1.5% and 2.5% (except for Apico [3%]) agarose gels (Biodynamics), respectively and stained with SYBR Safe (Invitrogen) using 100 bp standard (Cien Marker, Biodynamics) [6].

### 6. Necropsy

Post-mortem examination from both monkeys revealed lung congestion and fluid in chest cavity, hepatomegaly, splenomegaly, petechiae in liver and lymphadenopathy.

### 7. Histopathology and immunohistochemistry

Non-suppurative encephalitis with gliosis foci, satellitosis, perivascular hemorrhage, and neuronal necrosis, foci of coagulative necrosis randomly distributed with little evidence of inflammation in liver and spleen were observed in both monkeys (Fig. 1A). Pulmonary lesions

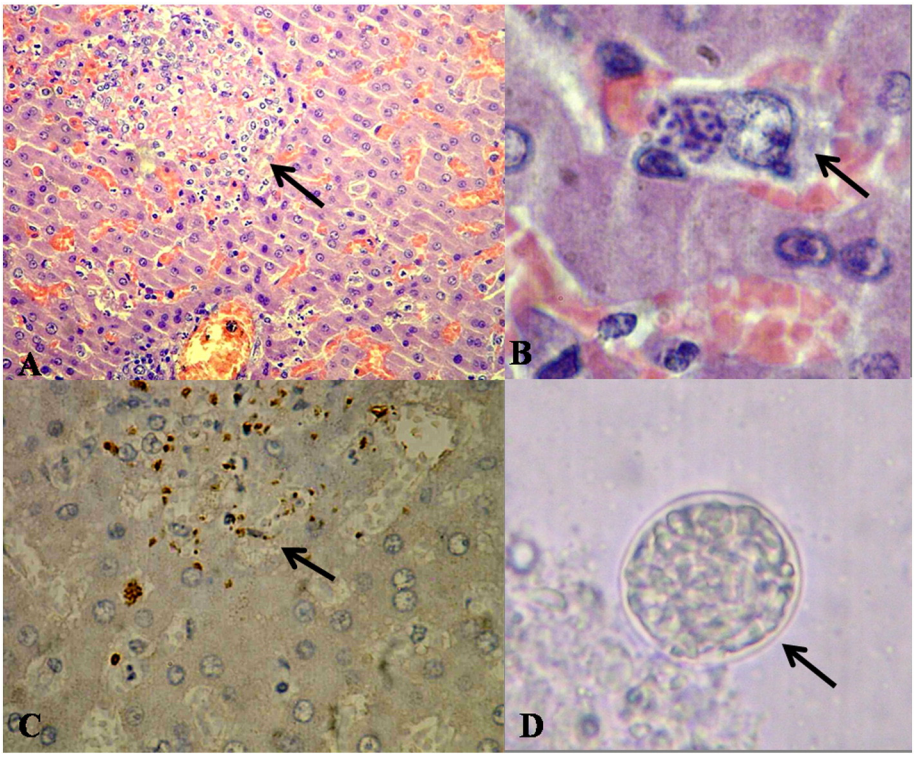


**Fig. 1.** A Liver: focus of coagulative necrosis in proximity of a centrilobular vein. H&E (20×), B liver: tachyzoites in cytoplasm of hepatocyte cell. H&E (100×), C liver: positive parasitic elements between hepatocytes and the area of necrosis with immunohistochemical staining (40×), D tissue cyst in mouse's homogenate brain (40×).

**Table 1**
*Toxoplasma gondii* genotyping results from isolate obtained from monkey Sb2 and *T. gondii* DNA derived from monkey Sb1.

| Sample/markers | SAG2 | BTUB | GRA6 | SAG3 | PK1 | L358 | C22-8 | C29-2 | Apico | TOXO DB |
|---|---|---|---|---|---|---|---|---|---|---|
| Tg DNA Sb1 | III | III | III | III | III | III | II | I | III | Similar to #163 |
| Tg isolate Sb2 | III | III | III | III | III | III | II | I | III | Similar to #163 |

included atelectasis and emphysema in Sb1, and edema, congestion, and macrophage proliferation in Sb2. Several foci of coagulative necrosis and mononuclear cell inflammation in the heart of Sb2 were also observed. Groups of tachyzoites were identified in CNS, liver, heart, myocardium and spleen of both monkeys by histopathology and confirmed as *T. gondii* by immunohistochemistry (Fig. 1A, B, C).

## 8. Mice and cells bioassays

Mice M1 and M2 were euthanized with neurological signs at days 33 and 20 post-infection respectively. Microscopic tissue cysts were observed in the brain homogenates (Fig. 1D). Tachyzoites were detected in VERO cells inoculated with peritoneal exudates from M2. The isolate was maintained by mice and cell culture passages.

## 9. DNA isolation, PCR and *T. gondii* genotyping

Amplification of the expected 115 bp fragment for *T. gondii* was demonstrated in DNA samples extracted from the brain of mouse M1 (inoculated with tissues of monkey Sb1) and from cell culture containing tachyzoites isolated from mouse M2 (inoculated with tissues of monkey Sb2). After genotyping, the *T. gondii* isolate obtained from monkey Sb2 was characterized as atypical or non-canonical and *T. gondii* DNA derived from monkey Sb1 showed an identical restriction pattern (Table 1).

## 10. Discussion

Fatal toxoplasmosis has been described in most genera of New World primates in several parts of the world [2]. These monkeys are among the most vulnerable species to this parasite. It has been suggested that such susceptibility is related to a “separate” evolution since the arboreal habitat of these monkeys would result in less frequent contact with *T. gondii* oocysts on the ground than other animal species. Susceptible species such as New World monkeys regularly showed acute disease, with sudden death or in some cases with nonspecific signs which include lethargy, malaise, depression, anorexia, diarrhea, hypothermia, serosanguineous nasal discharge and respiratory distress [2]. In the current study, a similar clinical presentation was observed. Tachyzoites in several organs and tissues were confirmed as *T. gondii* by immunohistochemistry. These findings were similar to other two cases of lethal acute toxoplasmosis in squirrel monkeys (*S. sciureus*) of Mexico City [7]. Although other outbreaks of acute toxoplasmosis have been reported in captive populations in London zoo, Israel, French Guiana and Brazil [2,4], only few studies have performed the isolation and a complete genotyping of the *T. gondii* isolates by nPCR–RFLP [4]. Genotyping of *T. gondii* among different animal populations is necessary to understand the epidemiology of this parasite. In this study, *T. gondii* with an identical result in genotyping was detected in two black-capped squirrel monkeys that died of fatal toxoplasmosis. The *T. gondii* isolate was characterized as atypical, similar to genotype #163, described in chickens from Brazil (http://toxodb.org/toxo/) for the nine markers used in our study.

In La Plata zoo, *T. gondii* type III isolates were found in a red kangaroo [1] and in slender-tailed-meerkats [2] and a type II isolate was described in a great gray kangaroo [1]. Additionally, non-canonical *T. gondii* with a type III restriction pattern for SAG2, SAG3, GRA6, BTUB, c22-8, L358, PK1 and Apico markers and a type I pattern for c29-2 marker was detected in Bennett's wallabies with fatal toxoplasmosis from the same zoo (Basso W. personal communication) [2]. The mentioned genotypes are different to the genotype found in the current outbreak; however all of them were fatal for the animals affected. An isolate obtained from an outbreak of toxoplasmosis in a colony of squirrel monkeys (*S. sciureus*) in Israel was described as type III but only using SAG2 marker [2]. In Mexico an isolate from *S. sciureus* was genetically characterized as type I but only using the SAG3 marker [7]. Additionally, in a Zoo of Recife, Brazil, an isolate obtained from a young male red-handed howler monkey (*A. belzebul*) was described as atypical by ten nPCR–RFLP markers similar to those used in the present study. However, the Brazilian isolate (TgRhHmBr1, #13 in ToxoDB), frequently described in other animals like chickens, cats, goats and even humans in Brazil [4], was not virulent in a mouse model and the genotype was different to the isolate of the present study in the markers SAG2, SAG3, BTUB and PK1. In French Guiana, *T. gondii* type II and an atypical genotype were reported in two outbreaks in a colony of squirrel monkeys [2]. However, microsatellite analysis was applied to characterize these isolates; therefore it is difficult to compare the results.

The source of infection in the present outbreak was not conclusively determined, but as all monkeys developed toxoplasmosis almost at the same time it is very likely that the infection was the result of a common point source exposure. Since *S. boliviensis* are herbivorous, the potential source of infection could have been oocysts, probably ingested through contaminated water or food. Stray cats which can shed oocysts contaminating the monkeys' environment were often observed in this zoological garden. Additionally, the transit of animal keepers between different habitats, could introduce oocysts potentially shed by the captive wild felids [2]. Infected wild mice and birds could also play a role in the occurrence of *T. gondii* outbreaks; although these animals did not represent a source of infection for monkeys, they could be ingested by stray cats perpetuating the existence of *T. gondii* in the zoo [2].

To our knowledge, this is the first report of fatal toxoplasmosis in *S. boliviensis* caused by an atypical genotype of *T. gondii* in Argentina. Further studies are necessary to clarify many aspects of *T. gondii* infection in New World primates especially referred to isolation and genetic characterization, as well as to biological characterization of atypical isolates. Genetic comparisons should be carried out using the same molecular analysis among different isolates to understand the epidemiology of toxoplasmosis in South America, and to obtain more accurate information in regards to phylogeny and virulence [8]. Finally, since toxoplasmosis in New World primates is predominantly acute and fatal, it is important to emphasize the need of rigorous prophylactic procedures and specific treatment to prevent the occurrence of fatal outbreaks of toxoplasmosis in captive monkey colonies.

## Acknowledgments

This research was funded by MINCyT (grant PICT 2012/0355). Also this study was partially financed with the program for the Scientific and Technological Cooperation between the Ministry of Science, Technology and Productive Innovation, Argentina (MINCyT; grant DA0808) and the German Academic Exchange Services (DAAD; grant D/08/11720).

## References

[1] G. More, L. Pardini, W. Basso, M. Machuca, D. Bacigalupe, M.C. Villanueva, et al., Toxoplasmosis and genotyping of *Toxoplasma gondii* in *Macropus rufus* and *Macropus giganteus* in Argentina, Vet. Parasitol. 169 (2010) 57–61.

[2] J.P. Dubey, Toxoplasmosis of Animals and Humans, Second ed. CRC Press, Boca Raton, 2010.
[3] H.F. Pena, S.M. Gennari, J.P. Dubey, C. Su, Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil, Int. J. Parasitol. 38 (2008) 561–569.
[4] H.F. Pena, M.F. Marvulo, M.C. Horta, M.A. Silva, J.C. Silva, D.B. Siqueira, et al., Isolation and genetic characterisation of *Toxoplasma gondii* from a red-handed howler monkey (*Alouatta belzebul*), a jaguarundi (*Puma yagouaroundi*), and a black-eared opossum (*Didelphis aurita*) from Brazil, Vet. Parasitol. 175 (2011) 377–381.
[5] W. Basso, G. More, M.A. Quiroga, L. Pardini, D. Bacigalupe, L. Venturini, et al., Isolation and molecular characterization of *Toxoplasma gondii* from captive slender-tailed meerkats (*Suricata suricatta*) with fatal toxoplasmosis in Argentina, Vet. Parasitol. 161 (2009) 201–206.
[6] G. More, P. Maksimov, L. Pardini, D.C. Herrmann, D. Bacigalupe, A. Maksimov, et al., *Toxoplasma gondii* infection in sentinel and free-range chickens from Argentina, Vet. Parasitol. 184 (2012) 116–121.
[7] C. Cedillo-Pelaez, C.P. Rico-Torres, C.G. Salas-Garrido, D. Correa, Acute toxoplasmosis in squirrel monkeys (*Saimiri sciureus*) in Mexico, Vet. Parasitol. 180 (2011) 368–371.
[8] C. Su, E.K. Shwab, P. Zhou, X.Q. Zhu, J.P. Dubey, Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*, Parasitology 137 (2010) 1–11.