Toxoplasmosis and genotyping of *Toxoplasma gondii* in *Macropus rufus* and *Macropus giganteus* in Argentina

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A B S T R A C T

Toxoplasma gondii infection is frequently asymptomatic; however, it can be severe or even fatal to some hosts. In this study, diagnosis of disseminated toxoplasmosis in one red kangaroo (*Macropus rufus*) and one great grey kangaroo (*Macropus giganteus*) from the La Plata Zoo, Argentina and the isolation and molecular characterization of *T. gondii* are reported. Both male kangaroos showed depression and sudden death. *Toxoplasma gondii* infection was diagnosed by fresh examination, histophatology, immunohistochemistry, PCR and bioassay in mice. During fresh examination many protozoan cysts were observed in diaphragm, heart and hind limb muscles of *M. rufus*. Cysts were also observed in samples from *M. giganteus*, although in lower number. Cysts from both kangaroos stained strongly with *T. gondii* anti-serum by immunohistochemistry. The *M. rufus* showed more considerable histopathological lesions like non-suppurative meningoencephalitis, myositis and myocarditis. All mice inoculated with tissues from both kangaroos developed IFAT titers to *T. gondii* (titer ≥800) and brain cysts at necropsy. Both *T. gondii* isolates were maintained by mice passages and the *M. rufus* isolate was also maintained in cell culture. *Toxoplasma gondii* DNA from tissue samples was analyzed by PCR-RFLP analysis using the markers 5′SAG2, 3′SAG2, BTUB, GRA6, SAG3, c22-8, L358, PK1, c29-2 and Apico. Genotyping revealed that the *T. gondii* isolate from *M. rufus* was clonal type III and the isolate from *M. giganteus* was clonal type II. This is the first report of disseminated toxoplasmosis in *M. rufus* and *M. giganteus* in Argentina caused by genotypes of *T. gondii* considered non-virulent in a mouse model.

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1. Introduction

Toxoplasma gondii infection occurs in a broad range of warm blooded animals including humans and is frequently asymptomatic; however, it can be severe or even fatal to some hosts. Some species like New World monkeys (Dietz et al., 1997), lemurs (Spencer et al., 2004), Pallas' cats (Basso et al., 2005), slender-tailed meerkats (Basso et al., 2009) and some Australian marsupials (Basso et al., 2007; Dubey et al., 1988) are considered highly susceptible to clinical toxoplasmosis, but little is known about *T. gondii* genotypes affecting these species. In the last years several fatal cases in macropods, principally wallabies, were reported (Adkesson et al., 2007; Basso et al., 2007; Bermudez et al., 2009; Dubey and Crutchley, 2008).

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Most *T. gondii* isolates from humans and animals from North America and Europe have been classified into one of the three genetic lineages (clonal types I, II, III) based on PCR-RFLP analysis (Howe et al., 1997; Howe and Sibley, 1995). These lineages have different virulence phenotypes in mice, with type I strains uniformly lethal in outbred mice and type II and III strains significantly less virulent (Sibley and Boothroyd, 1992). Recently *T. gondii* isolates from chickens in Brazil were characterized as "atypical" considering the classification above mentioned (Beck et al., 2009; Dubey et al., 2007). It is not known however, if the virulence phenotype in mice may be also observed in other animal species. Interestingly, clonal lineage type III was fatal in *Suricata suricatta* from Argentina (Basso et al., 2009) and caused severe disease in wallabies (Dubey and Crutchley, 2008).

This study reports the diagnosis, isolation and molecular characterization of *T. gondii* in captive *Macropus rufus* and *Macropus giganteus* from La Plata Zoo, Argentina.

2. Materials and methods

2.1. Animals

In March and April 2008, two dead adult male kangaroos from La Plata Zoo were received at the Faculty of Veterinary Medicine of the National University of La Plata, Argentina, with a clinical history of sudden death after a few days of appetite loss and depression. The animals were one red kangaroo (*M. rufus*) and one great grey kangaroo (*M. giganteus*). Both animals arrived at the zoo in October 2007 from a natural reserve in USA in good health. The animals were fed hay and vegetables distributed over the ground of the enclosure.

2.2. Necropsy, histopathological analysis and fresh examination

Complete necropsies were carried out and samples of hind limb muscles, heart, brain, diaphragm, tongue, liver, lungs and intestines were collected. Around 1 g of each tissue was fixed in 10% neutral buffered formalin and were routinely processed for histology; sections were cut to 5 μm thickness and stained with hematoxylin and eosin (H&E).

Ten grams of myocardium, diaphragm and hind limb muscle samples were processed by fresh examination. Muscles were minced in a meat grinder adding 50 ml of PBS, the solution was filtrated through sieve with sterile gauze and recovered in a 50 ml tube. The solution was centrifuged at 2500 rpm for 5 min, the supernatant was discarded and the pellet resuspended in 50 ml of fresh PBS. Finally, the solution was observed in a stereomicroscope at 20× to search for apicomplexan cysts.

2.3. Immunohistochemical analysis

Cysts obtained in fresh examination were extended and fixed into slides with Van der Griendt́s solution (2.5 ml formalin, 8.5 g NaCl and distilled water 1 l) during 8 h and with 100% ethylic alcohol for 10 min. Fixed samples were stained immunohistochemically for *T. gondii* with the LSAB + System HRP using a commercial kit according to the manufacturer's instructions (DakoCytomation, USA). A rabbit *T. gondii* hyperimmune anti-serum (gently provided by Dr. J.P. Dubey) was used at a 1:2.000 dilution (overnight at 4 °C) as the primary antibody. Positive controls were cysts in brain sections from a *T. gondii*-experimentally infected mouse and negative controls were cysts in heart sections of a *Neospora caninum*-naturally infected calf.

2.4. Isolation of T. gondii in mice and cell cultures

Brain and diaphragm samples from both kangaroos were homogenized in saline containing antibiotics and antimycotic (penicillin 1000 IU/ml, streptomycin 1 mg/ml and anphotericin B 2.5 μg/ml). Each sample was inoculated intraperitoneally in 2 female N:NIH Swiss mice. At 7 days post inoculation (dpi), peritoneal exudates were examined for *T. gondii* tachyzoites. Necropsy was performed in all mice that died after infection and brain homogenates from these mice were examined for *T. gondii* cysts. Brain samples were kept at −20 °C for *T. gondii* DNA detection.

Peritoneal exudates or brain homogenate from inoculated mice in which parasites had been detected were inoculated into bovine monocyte (BM) cultures and incubated with RPMI 1640 medium and 3% fetal calf serum at 37 °C and 5% CO_2 until tachyzoite growth was observed. Positive cultures were cryopreserved in RPMI 1640 medium with 20% fetal calf serum and 5% DMSO. Isolates were also maintained by mice passages.

2.5. Serologic studies

Inoculated mice were bled at 30 dpi. Sera were tested for antibodies to *T. gondii* beginning at a 1:25 dilution by the indirect fluorescent antibody test (IFAT) using culture-derived tachyzoites of the RH strain and a goat-anti mouse–IgG–FITC-conjugate (Sigma) at a dilution of 1:100.

2.6. DNA isolation

DNA was isolated from brain, heart and hind limb muscle samples from both kangaroos and brain from inoculated mice, with a commercial DNA-extraction kit (DNeasy® Tissue Kit QIAGEN), according to the manufacturer's recommendations.

2.7. Polymerase chain reaction (PCR) for T. gondii

The amplification was performed with the specific primer pair B22/B23 (Bretagne et al., 1993) for the B1 gene of *T. gondii*. One μl of genomic DNA from each sample was added to tubes of a PCR master mix containing 2.5 μl of 10× PCR buffer (Fermentas Life Sciences), 200 μM each of dATP, dTTP, dGTP and dCTP, 0.75 U of *Taq* DNA polymerase (Fermentas Life Sciences) and 0.4 μM of each primer in a final volume of 25 μl. The reaction was accomplished in a thermal cycler (PCR Sprint Thermo Electron Corporation) with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles with denaturation (94 °C; 30 s), annealing (60 °C; 30 s) and extension (72 °C; 60 s) and a final extension

step at 72 °C for 7 min. DNA from in vitro cultured RH-strain–T. gondii tachyzoites and sterile RNAsa-free water (Gibco) were used as positive and negative controls respectively. ÓRange ruler 50 bp DNA Ladder (Fermentas Life Sciences) was employed as size standard. The amplification products were visualized after electrophoresis with a 1.5% agarose gel and stained with ethidium bromide.

2.8. Restriction fragment length polymorphism (RFLP) analysis

DNA extracts were amplified by nested-PCR (n-PCR) followed by RFLP analysis. Markers based in 5′SAG2, 3′SAG2, BTUB, GRA6 and SAG3 loci and additionally, a set of 5 three-way markers: c22-8, L358, PK1, c29-2 and Apico of T. gondii were chosen for molecular characterization as described previously (Basso et al., 2009). DNA isolated from cultures of T. gondii strains RH, Me49 and NED were used as positive controls for the clonal types I, II and III respectively. Digestion products were analyzed after electrophoresis in a 3% agarose gel with ethidium bromide. A 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) was used as a size standard.

3. Results

3.1. Necropsy and fresh examination

Post mortem examination revealed poor body condition and depletion of fatty tissues in M. rufus. Gross lesions were not observed in M. giganteus. During fresh examination a large number of protozoan cysts was observed in diaphragm, heart and hind limb muscles of M. rufus (Image 1A). Cysts were elongated and measured 90–150 μm long (n = 16). Samples from M. giganteus also showed muscle cysts but in lower number and slightly shorter measures (80–120 μm, n = 5).

3.2. Histopathological studies and immunohistochemical staining

The main histopathological lesion observed in M. rufus was non-suppurative meningoencephalitis with gliosis foci, sateliosis and presence of tachyzoite groups and cysts (Fig. 2A and B). Other lesions were non-suppurative infiltration and focal necrosis in heart and striated muscles (Fig. 2C and D) with presence of tachyzoites and cysts resembling T. gondii. Groups of tachyzoites and cysts were also detected in sections of large intestine (Fig. 2E). Non-suppurative encephalitis was the only histopathological lesion detected in M. giganteus. Cysts from both kangaroos stained strongly with T. gondii anti-serum by immunohistochemistry (Fig. 1B).

3.3. Serological examination of mice and isolation of T. gondii in mice and cell cultures

All inoculated mice developed antibody titers for T. gondii by the IFAT (titer ≥800) and remained asymptomatic for at least 5 months post infection. Toxoplasma gondii tachyzoites were detected in peritoneal exudates of mice inoculated with brain (2/2) and diaphragm (2/2) of M. rufus; these mice died between 6 and 12 months post inoculation and showed a large number of brain cysts at necropsy. Tachyzoites were not detected in peritoneal exudates of 4/4 mice inoculated with samples from M. giganteus; however, these mice died between 5 and 12 months post inoculation and the necropsy revealed brain cysts in 4/4 mice. Tachyzoites and brain cysts were reinoculated in mice and isolates were maintained by mice passages until present.

Tachyzoites from mice inoculated with M. rufus tissue samples were inoculated in BM cultures and parasite growth was detected at 4 dpi. The isolate was cryopreserved. Parasite growth was not detected in BM cells
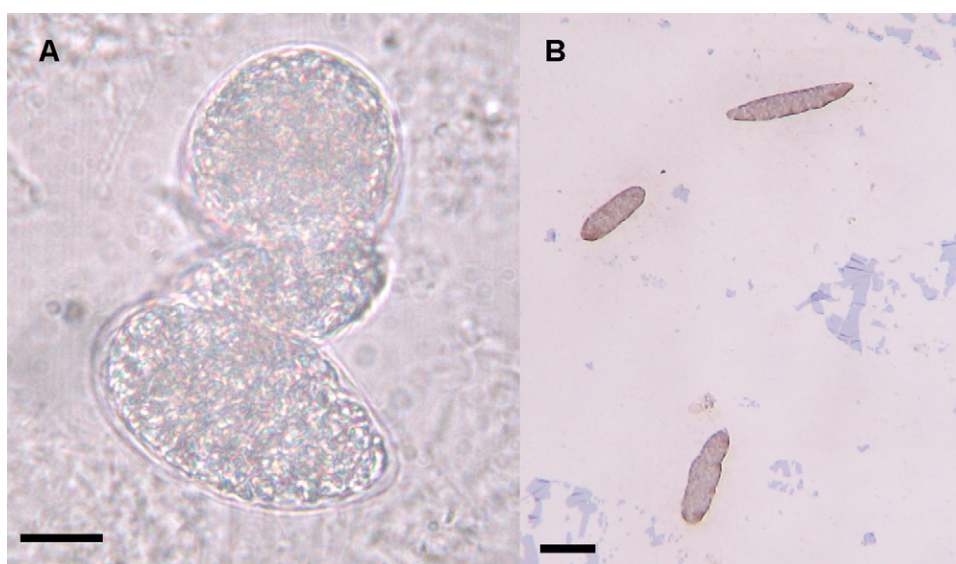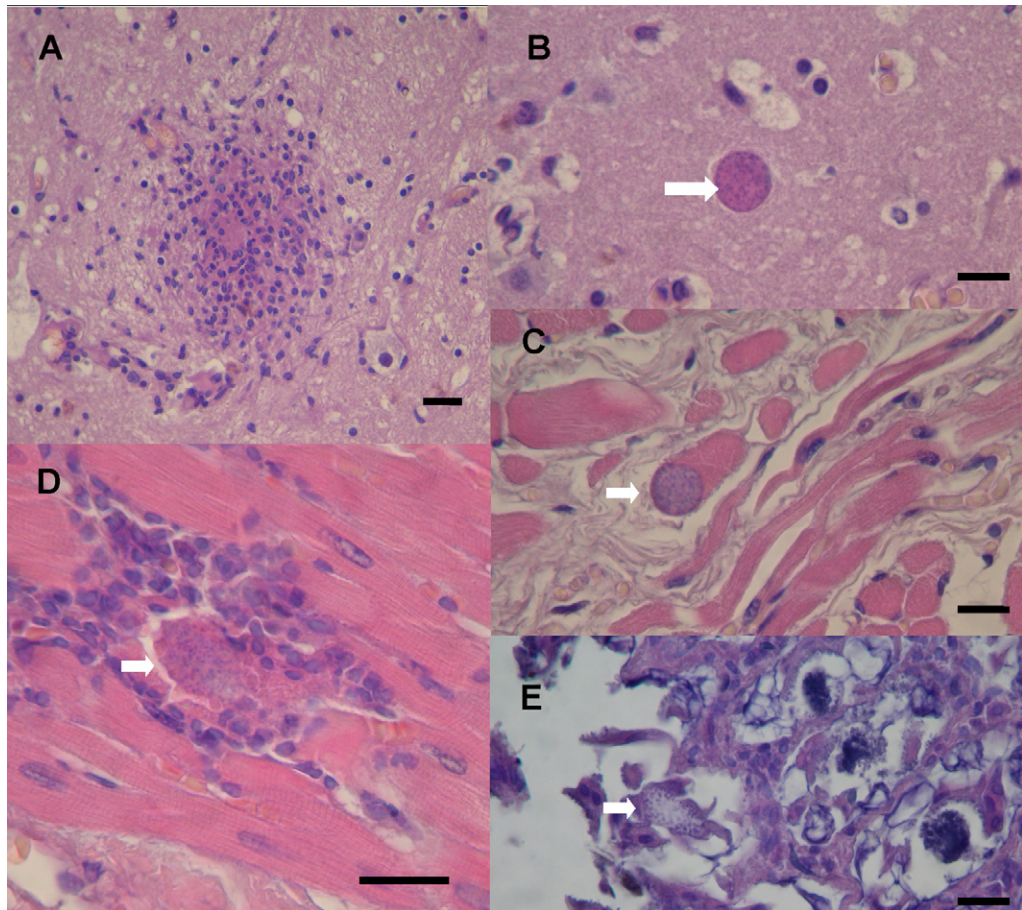

Fig. 1. (A) Muscle cysts from diaphragm of Mr observed in fresh examination. (B) Immunohistochemical staining using anti-T. gondii antibodies; fixed cysts from Mr stained positively. Scale bars: 20 μm.

Fig. 2. Sections of tissues from *Mr*, H&E staining. (A, B) severe non-suppurative inflammatory infiltration and *T. gondii* cysts (white arrow) in sections of central nervous system. (C) *T. gondii* cyst in a section of hind limb muscle (white arrow), (D) infiltration and presence of tachyzoites (white arrow) in myocardium and (E) presence of *T. gondii* tachyzoites (white arrow) in large intestine section. Scale bars: 30 μm.

inoculated with brain cysts from mice inoculated with tissues from *M. giganteus*.

3.4. PCR and RFLP-PCR

Amplification of the expected 115 bp target fragment with the *T. gondii*-specific primer pair B22–B23 was demonstrated in brain heart and hind limb muscles from both kangaroos and in all brain samples from inoculated mice (4 with *M. rufus* and 4 with *M. giganteus*). Nested-PCR of all *T. gondii* markers revealed amplicons corresponding to clonal type III in samples from *M. rufus*, and to clonal type II in samples from *M. giganteus*; genotyping was not performed in mice samples.

4. Discussion

Australian marsupials are one of the most susceptible animals to toxoplasmosis and frequently show severe lesions and signs including sudden death. In the present study both kangaroos showed lesions and widespread presence of *T. gondii* tachyzoites and cysts, especially in muscles. Wallabies, especially females, were considered more susceptible to acute toxoplasmosis than kangaroos

(Basso et al., 2007; Dubey et al., 1988); however both male kangaroos reported in the present study had similar lesions to those reported previously in *M. rufogriseus* (Adkesson et al., 2007; Basso et al., 2007; Bermudez et al., 2009; Dubey and Crutchley, 2008). In addition, the surprisingly high number of *T. gondii* cysts detected in muscles of *M. rufus* could be related to species or individual susceptibility, which could in turn be due to poor immune control of parasite multiplication. On the other hand, muscle cysts could be a potential source of human infection in countries where kangaroo meat is consumed. In consequence, muscle examination should be considered for diagnosis of toxoplasmosis in marsupials.

Molecular analysis using nine PCR-RFLP markers including SAG2, BTUB, GRA6, SAG3, c22-8, L358, PK1, c29-2 and Apico, revealed that the *T. gondii* isolate from *M. rufus* had genotype III and the isolate from *M. giganteus* had genotype II alleles at all loci and likely belong to the main clonal lineages of *T. gondii* (Howe et al., 1997; Howe and Sibley, 1995). Both genotypes corresponded with the most frequent genotypes reported in animal infections (Dubey and Jones, 2008). The widespread clonal type III of *T. gondii* was considered relatively non-virulent in a mouse model (Sibley and Boothroyd, 1992); however in the present

study severe disseminated toxoplasmosis was reported in *M. rufus* due to this genotype. Moreover, the same genotype was reported as cause of severe lesions in wallabies from USA (Dubey and Crutchley, 2008). Coexistence of tachyzoites and cysts of a unique genotype, especially in *M. rufus*, may suggest reactivation of a previous infection, possibly due to stress. On the other hand, *T. gondii* clonal type II, considered non-virulent in a mouse model (Sibley and Boothroyd, 1992) produced a low number of muscle cysts and tissue lesions limited to encephalitis in *M. giganteus*. However, whether distribution and severity of lesions were due to clonal type, infection dose or other factors could not be determined. Unfortunately, it was not possible to determine diagnosis in other live kangaroos since these two kangaroos were the only ones of their species present at the zoo and no other animals were housed in the same enclosure.

Possible sources of *T. gondii* infection in the present study include uptake of oocysts from the environment, contaminated food or water. *Toxoplasma gondii* infection was previously reported in Bennett's wallabies and slender-tailed meerkats in the same zoo and contamination of the zoo environment with oocysts from feral cat faeces were considered the most important source of infection (Basso et al., 2007, 2009). Moreover the *T. gondii* clonal type III isolated from *M. rufus* was also reported previously in slender-tailed meerkats, suggesting that this genotype may be frequent in the zoo environment (Basso et al., 2009). However, considering that both kangaroos arrived at the zoo 6 months before from a natural reserve in USA, a previous infection is possible. Despite of this our main recommendation to the zoo authorities was to build a black-out type enclosure to maintain the kangaroos or wallabies protected from oocyst contamination.

This is the first report of disseminated toxoplasmosis in *M. rufus* and *M. giganteus* in Argentina caused by genotypes of *T. gondii* considered non-virulent in a mouse model.

Acknowledgements

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