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Short communication

## Fatal toxoplasmosis associated with an atypical *Toxoplasma gondii* strain in a Bennett's wallaby (*Macropus rufogriseus*) in Spain



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### ABSTRACT

Toxoplasmosis is often fatal in captive wallabies, but the causes of this high susceptibility are not well understood. Here, we report fatal toxoplasmosis in a Bennett's wallaby (*Macropus rufogriseus*) due to an atypical *Toxoplasma gondii* strain for the first time in Europe. The wallaby was from a colony of 7 Bennett's wallabies that died over a 17-month period at a safari-zoological park in northeastern Spain. Only one of these wallabies was examined at necropsy. *T. gondii*-like organisms were detected by histological examination in several tissues and the diagnosis was confirmed through detection of *T. gondii* DNA by PCR. A nested PCR-based assay detected the 200- to 300-fold repetitive 529 bp DNA fragment of *T. gondii* in a sample of brain tissue. Genotyping analysis with 15 single-copy microsatellite markers was performed on this positive DNA sample and revealed an atypical genotype. Atypical genotypes are frequently associated with severe forms of toxoplasmosis in humans. The present report highlights the possible implications of the introduction of new atypical, more pathogenic *T. gondii* strains, to non-endemic areas.

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## 1. Introduction

*Toxoplasma gondii* is a zoonotic intra-cellular apicomplexan protozoan parasite of worldwide distribution that

infects most species of warm-blooded animals, including humans (Dubey, 2010). Felids are the only definitive hosts of *T. gondii* and the only hosts that excrete resistant oocysts into the environment. The clinical presentation of *T. gondii* infection varies widely depending on the strain of the parasite, the host-species susceptibility to the parasite, and other factors such as the immune status of the host, the infective dose or the parasite life-cycle stage ingested (Dubey and Crutchley, 2008; Dubey, 2010). New

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World primates, pro-simians of Madagascar and marsupials from Australasia are all highly susceptible species to *T. gondii* infection and severe cases of toxoplasmosis have been reported in these species worldwide. Among Australian marsupials, wallabies appear to be one of the most susceptible species and the presentation of the disease is often acute and fatal in these animals (Bermúdez et al., 2009; Sós et al., 2012), especially in zoological collections as shown by many recent cases reported worldwide (Basso et al., 2007; Dubey and Crutchley, 2008; Bermúdez et al., 2009; Sós et al., 2012). Although fatal toxoplasmosis has been widely described in marsupials, there have been few attempts to genotype the strains involved in this severe form of toxoplasmosis.

Globally, diverse *T. gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages (Su et al., 2012). In the Northern Hemisphere and especially North America and Europe, *T. gondii* exhibit a striking clonal population with a few predominant genetic lineages, type I, II, III and the recently haplogroup 12 described in North America (Dubey et al., 2011; Su et al., 2012). In contrast, in parts of South America and Asia there is a divergent genetic diversity of the parasite, characterized by an assemblage of genotypes different from the predominant archetypes in Europe and North America that show greater evidence of recombination (Su et al., 2012). These strains have been historically considered as atypical in order to differentiate them from the more commonly described archetypes. It is now clearly established that early genetic studies have led to incorrect strain classification of certain isolates because they were based on a single genetic locus or on a limited set of genetic markers (Herrmann et al., 2010). A recent re-evaluation of the population structure of *T. gondii* in North America using sequenced-based phylogenetics and population genetics analyses found that previously reported atypical isolates from sea otters and other marine mammals in fact correspond to a fourth major clonal lineage, type 12, which accounts for 46% (79/169) of the wildlife isolates and is the dominant strain in wildlife from North America (Dubey et al., 2011).

In Europe, type II strains are predominant in animals and human infections (Ajzenberg et al., 2002; Herrmann et al., 2010; Montoya et al., 2008; Prestrud et al., 2008; Aubert et al., 2010). Atypical strains of *T. gondii* have been only described in oocysts from domestic cats (Herrmann et al., 2010), in arctic foxes (Prestrud et al., 2008) and in humans that presented with an uncommon but severe clinical toxoplasmosis (De Salvador-Guillouët et al., 2006; Elbez-Rubinstein et al., 2009; Delhaes et al., 2010).

In the present report we describe an atypical *T. gondii* strain found in a fatal case of toxoplasmosis in a Bennet's wallaby (*Macropus rufogriseus*).

## 2. Materials and methods

The wallaby was a male of nine years and was part of a colony of 7 Bennet's wallabies in a safari-zoological park in North-East (NE)-Spain. Between late May 2010 through October 2011, a colony of 6 original Bennet's wallabies (W1–W6) and a young wallaby (W7) born during

that period in the park in NE-Spain died. The age of the animals ranged from 11 months to nine years and both sexes were present. Affected animals had similar non-specific clinical signs such as weakness, depression, sialorrhoea and acute diarrhoea and all died within a few days or up to two months from the beginning of the first clinical signs. Due to past recurrent problems with toxoplasmosis in the safari-zoological park, the wallabies were suspected to have died of toxoplasmosis but diagnosis was confirmed in only one wallaby (W4) examined at necropsy. The histopathological lesions were consistent with a disseminated protozoan infection with multifocal nonsuppurative inflammatory lesions in many organs such as adrenal glands, brain, heart, liver, stomach, intestine and mesenteric lymph nodes. *T. gondii*-like organisms were observed in hematoxylin-eosin stained sections associated with those lesions in multiple tissues.

Sera samples were collected from 3 remaining wallabies (W5, W6 and W7) before clinical symptoms were evident. Antibodies against *T. gondii* were measured using the modified agglutination test (MAT) described by Dubey and Desmonts (1987). A commercial positive control (Toxotrol-A, Biomerieux, France) and negative controls were included in each test. Agglutination titers of 1:25 were considered positive and doubtful results were re-assayed.

Brain samples from W4 were collected for PCR analysis. DNA was obtained from 0.5 g of brain tissue as previously described by Almería et al. (2002). Briefly, the tissues were washed once with a lysis buffer containing saponin to eliminate red blood cells. Subsequently, samples were incubated in proteinase K buffer (200 µg of proteinase K/ml) at 37 °C overnight. Proteinase K was inactivated by incubating the samples at 95 °C for 10 min. Samples were then extracted with phenol–chloroform–isoamyl alcohol (25:24:1), followed by a second extraction with chloroform–isoamyl alcohol (24:1). DNA was precipitated with 100% ethanol, washed once with 70% ethanol and the pellet resuspended in 200 µL of molecular biology grade water (Sigma–Aldrich, UK).

For PCR-based diagnosis of *T. gondii*, a nested PCR for detection of the 529 bp repetitive fragment (AF487550) of *T. gondii* was performed as previously described by Su and Dubey (2009) using primers Tox-8 and Tox-5 as external (amplified fragment of 450 bp) and Tox-9 and Tox-11 as internal primers (amplified fragment of 162 bp). A minimum of two PCR reactions for each extracted sample were performed. Positive and negative parasite controls were included in each test. Non template control samples for PCR contamination were obtained by performing PCR reactions without template DNA. PCR products were run by electrophoresis through a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

PCR positive samples from W4 were sequenced using the internal *T. gondii* primers Tox-9 and Tox-11 mentioned above. Sequencing reactions with fluorescent dideoxynucleotides were performed following the manufacturer's recommendations using the Big Dye 3.1 Terminator Kit (Applied Biosystems) and analyzed on an ABI 3100 Genetic Analyzer (Applied Biosystems) in the sequencing facility of the Universitat Autònoma de Barcelona, Bellaterra, Spain. Sequences of the putative strains were

**Table 1**

Genotyping results of *T. gondii* DNA with 15 microsatellite markers in a single multiplex PCR assay from a wallaby (W4) collected at a safari-zoo in North-East Spain and from 10 reference strains collected in animals from America, Europe, and Africa.

Type	Isolate <sup>1</sup>	Origin	Host	Microsatellite markers <sup>2</sup>														
				TUB2	W35	TgM-A	B18	B17	M33	IV.1	XI.1	M48	M102	N60	N82	AA	N61	N83
Atypical	W4	Spain	Wallaby	291	242	203	156	336	NA	274	356	215	174	130	109	291	95	310
I	CT1	USA	Cow	291	248	209	160	342	169	274	358	209	168	145	119	265	87	306
II	PTG	USA	Sheep	289	242	207	158	336	169	274	356	215	174	142	111	265	91	310
II	TgA32132	France	Sheep	289	242	207	158	336	169	274	356	221	174	138	111	277	91	312
III	CTG	USA	Cat	289	242	205	160	336	165	278	356	215	190	147	111	269	89	312
Atypical	CASTELLS	Uruguay	Sheep	287	242	207	158	358	169	274	356	239	164	138	109	283	87	324
Atypical	TgCgCa01	Canada	Cougar	289	242	205	158	336	169	274	354	219	174	151	119	259	79	332
Atypical	TgCatBr5	Brazil	Cat	291	242	205	160	362	165	278	356	237	174	140	111	265	89	314
Atypical	TgA18001	French Guiana	Jaguar	289	242	203	160	336	169	274	358	209	176	142	111	257	107	340
Africa 1	TgA105001	Gabon	Chicken	291	248	205	160	342	165	274	354	223	166	147	111	269	89	306
Africa 3	TgA105004	Gabon	Chicken	291	242	207	160	342	165	278	354	223	166	142	111	277	97	310

<sup>1</sup> PTG is a clone of the ME49 strain; TgA32132 is also known as FR-OVI-ARI061 strain; CTG is also known as CEP or C strain; TgCgCa01 is also known as COUGAR or COUG strain; TgA18001 is also known as GUY-2004-JAG strain; TgA105001 is also known as GAB3-2007-GAL-DOM002 strain; TgA105004 is also known as GAB2-2007-GAL-DOM002 strain. All reference strains except CTG, CASTELLS, and TgCgCa01 are available at the *Toxoplasma* Biological Resource Center (<http://www.toxocrb.com>).

<sup>2</sup> NA, not amplified; Black boxes indicate atypical alleles.

examined and purged of errors using Chromas Pro 1 (Larkin et al., 2007) and compared with other GenBank partial sequences using the nucleotide Blast utility (<http://www.ncbi.nlm.nih.gov/BLAST>).

The positive *T. gondii* DNA sample from W4 was genotyped using 15 microsatellite markers distributed over 10 of 14 chromosomes, as described previously (Ajzenberg et al., 2010). Briefly, for each primer pair, the forward primer was 5'-end labeled with fluorescein to allow sizing of PCR products electrophoresed in an automatic sequencer. PCR was carried out in a 25 µL reaction mixture consisting of 12.5 µL of 2X QIAGEN Multiplex PCR Master Mix (Qiagen, France), 5 pmol of each primer and 5 µL of DNA. Cycling conditions were 15 min at 95 °C; 30 s at 94 °C, 3 min at 61 °C, and 30 s at 72 °C (35 cycles); and 30 min at 60 °C. One microliter of the PCR product was mixed with 0.5 µL of a dye-labeled size standard (ROX 500, Applied Biosystems) and 23.5 µL of desionised formamide (Applied Biosystems). This mixture was denatured at 95 °C for 5 min and then electrophoresed using an automatic sequencer (ABI PRISM 3130xl, Applied Biosystems). The sizes of the alleles in bp were estimated using GeneMapper analysis software (version 4.0, Applied Biosystems).

### 3. Results

*Toxoplasma gondii* DNA was detected by nested PCR for detection of the 529 bp repetitive fragment PCR in brain tissue from the only wallaby with tissue specimen available (W4). The 154 band sequence showed 100% similarity to *T. gondii* repetitive DNA sequences from GenBank (e.g. EF648168.1 and DQ779195.1 among others).

The genotyping results of *T. gondii* DNA from W4 with 14 of 15 microsatellite markers are shown in Table 1 and

revealed an atypical genotype clearly different from the three main clonal lineages I, II, and III. Although some allelic combinations were common to those observed in certain South-American strains, the full combination with 15 markers was unknown in the database of the French National Reference Center for toxoplasmosis. Therefore it was not possible to match this genotype with known strains that are endemic in America, Africa, and especially in Europe where it was collected.

Antibodies (MAT ≥ 1:25) against *T. gondii* were found in the 3 wallabies analyzed; 1 had a titer of >3200 and the other two wallabies had a titer of 50. These animals died afterwards showing similar signs to the previous wallabies which have died, but no tissues were collected at necropsy.

### 4. Discussion

In the present report, an atypical genotype of *T. gondii* was confirmed to be involved in the death of a Bennet's wallaby. The clinical signs of the diseased wallabies were similar to fatal cases of toxoplasmosis in wallabies previously reported in other studies, with sudden death after a few days of non-specific clinical signs (Bermúdez et al., 2009; Sós et al., 2012). The histopathological evidence of a disseminated protozoan infection in the fourth sequentially dead wallaby in the park was confirmed by specific *T. gondii* PCR, sequencing and genotyping. Unfortunately, tissue samples were only collected from that wallaby. However, when only three other wallabies remained in the zoo (W5, W6 and W7) and they were tested for *T. gondii* antibodies before the appearance of clinical symptoms, all were seropositive, an indication of prior contact with the parasite. The possibility of different genotypes and/or

re-infection in the other affected wallabies cannot be discarded.

There is scant information concerning the isolation and genotyping of *T. gondii* associated with fatal toxoplasmosis in macropods (reviewed by Dubey and Crutchley, 2008). Recently, in cases of fatal toxoplasmosis in one red kangaroo (*Macropus rufus*) and one great gray kangaroo (*Macropus giganteus*) from a zoo in Argentina, analyses revealed that the *T. gondii* isolate from *M. rufus* was clonal type III and the isolate from *M. giganteus* was clonal type II (Moré et al., 2010). In 4 cases of toxoplasmosis in Bennett's wallabies in USA, genotype III was found in the two available isolates, as well as in another isolate in a Tammar wallaby (*Macropus eugenii*) (Dubey and Crutchley, 2008). Genotypes II and III are relatively common strains considered of low pathogenesis in the mouse model. In another Tammar wallaby from the USA the isolate was an atypical strain with Type I at 4 of the 10 RFLP markers (Dubey et al., 2011). The numerous fatal toxoplasmosis reports in Australian marsupials in comparison to other species highlights a particular susceptibility to toxoplasmosis. Genotyping of strains from wild and captive marsupials in Australia has showed a broad predominance of atypical strains or non archetypal type II-like strains circulating in this country (Parameswaran et al., 2010). Strains bearing atypical alleles U-2, U-6 at the polymorphic *B1* locus and Type I alleles were shown to cause infections that resulted in neurological disease, including one clinical case in a wallaby. However, the majority of clinical cases were from captive animals (Parameswaran et al., 2010). On the other hand, a moderate seroprevalence of *T. gondii* has been observed in some wild free-ranging Australian marsupials, indicating that at least some species can survive with *T. gondii* infection in the wild (Parameswaran et al., 2009). Therefore, strain type may predict disease severity but other factors such as the stress associated with captivity could be relevantly linked to clinical toxoplasmosis in Australian marsupials.

In humans, atypical genotypes are more frequently associated with severe forms of toxoplasmosis (Ajzenberg et al., 2002; Delhaes et al., 2010), such as the newly described 'Amazonian toxoplasmosis' reported in French Guiana, which is characterized by severe cases and atypical strains linked to a neotropical forest-based cycle (Demar et al., 2012). In Europe, there is a large predominance of type II strains which accounts for more than 95 percent of isolates in human infection and in the domestic cycle of the parasite (Ajzenberg et al., 2002; Halos et al., 2010). The isolation of atypical strains is uncommon in Europe and likely suggests contamination by non-European strains either during residence abroad or after consumption of imported horse meat (Pomares et al., 2011). In the present study, it was not possible to match the *T. gondii* genotype observed in the wallaby with any known strains that are endemic in America, Africa and Europe. These results suggest that this strain has been imported into Europe even though its geographic origin remains unknown based on the genetic data. There is a growing body of evidence that these atypical strains imported to Europe are more likely to cause severe toxoplasmosis in immunocompetent human patients and in congenital toxoplasmosis than are type II

strains (De Salvador-Guillouët et al., 2006; Delhaes et al., 2010; Pomares et al., 2011). It has also been demonstrated that atypical strains can lead to reinfection and congenital toxoplasmosis in pregnant women with a past immunity to type II strains (Elbez-Rubinstein et al., 2009). These findings highlight the importance of detecting atypical strains and understanding how these newly identified atypical genotypes are possibly being introduced into non-endemic areas.

To our knowledge this is the first report of an atypical *T. gondii* strain related to death in wallabies in Europe. The presence of an atypical strain as the cause of death of wallabies in Spain is difficult to explain because the analyzed wallaby was born in the park. One scenario may be through the ingestion of sporulated oocysts from food or water contaminated by feces of wild felids present in the same safari park-zoo area. Imported meat from countries where atypical strains of *T. gondii* are common has been suggested as a possible way of contracting clinical toxoplasmosis in humans (Elbez-Rubinstein et al., 2009; Pomares et al., 2011). However, the carnivores present in the zoo were only fed with locally produced meat. On the other hand, among wild felids in the park, there were jaguars (*Panthera onca*) originally from South America. If these animals were infected and were eliminating *T. gondii* oocysts at the time of the outbreak, this could explain the presence of an atypical strain of *T. gondii* in Spain, where to date, although there have been very few genotyping studies, only type II and III have been observed in humans (Fuentes et al., 2001), and in cats (Montoya et al., 2008, Almeria et al., unpublished data). Another explanation would be that this novel atypical strain had recently emerged in Spain after a recombination event in local cats. It has been demonstrated that sexual recombination, by reshuffling existing alleles between two parents, may yield progeny with very different biological properties such as hypervirulence (reviewed by Grigg and Sundar, 2009). The new genotypes with the greatest fitness can then be clonally expanded by self-mating in felids and asexual reproduction in intermediate hosts. In this case, although there is a control program of rodents and feral cats in the safari-zoological park, the nearby forest areas are only separated by an accessible fence and feral cats were seen in the safari park-zoo areas. However, the genotyping data provided with the 15 microsatellite markers are insufficient for detecting such recombination events since the evolutionary analysis of this *T. gondii* strain can only be achieved by sequencing a large number of loci using whole-genome SNP analysis (Minot et al., 2012).

Alternative environmental or host factors may also have exacerbated the infection. The animal could have had a latent infection reactivated by stress or immunodepression. However, as indicated above, latent infections are not common in macropods in captivity due to the high mortality rate of the disease in this species, and in this case the animals had sudden deaths without preceding clinical signs. To our knowledge the animal had not been recently moved or introduced into a new environment but the possibility of environmental or host factors exacerbating the infection cannot be ruled out.

Future studies should include the genotype analysis of *T. gondii* strains and their relationship with clinical

toxoplasmosis, particularly in wildlife species. The present report also highlights the possible implications of introducing new atypical, more pathogenic *T. gondii* strains, in non-endemic areas.

### Conflict of interest

The authors declare that they have no conflict of interest.

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