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Genetic characterization of *Toxoplasma gondii* from Brazilian wildlife revealed abundant new genotypes

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ABSTRACT

This study aimed to isolate and genotype *T. gondii* from Brazilian wildlife. For this purpose, 226 samples were submitted to mice bioassay and screened by PCR based on 18S rRNA sequences. A total of 15 *T. gondii* isolates were obtained, including samples from four armadillos (three *Dasypus novemcinctus*, one *Euphractus sexcinctus*), three collared anteaters (*Tamandua tetradactyla*), three whited-lipped peccaries (*Tayassu pecari*), one spotted paca (*Cuniculus paca*), one oncilla (*Leopardus tigrinus*), one hoary fox (*Pseudalopex vetulus*), one lineated woodpecker (*Dryocopus lineatus*) and one maned wolf (*Chrysocyon brachyurus*). DNA from the isolates, originated from mice bioassay, and from the tissues of the wild animal, designated as "primary samples", were genotyped by PCR–restriction fragment length polymorphism (PCR/RFLP), using 12 genetic markers (SAG1, SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L258, PK1, CS3 and Apico). A total of 17 genotypes were identified, with 13 identified for the first time and four already reported in published literature. Results herein obtained corroborate previous studies in Brazil, confirming high diversity and revealing unique genotypes in this region. Given most of genotypes here identified are different from previous studies in domestic animals, future studies on *T. gondii* from wildlife is of interest to understand population genetics and structure of this parasite.

1. Introduction

Toxoplasma gondii is an intracellular protozoan parasite distributed worldwide capable of infecting virtually all warm-blooded animals, including birds, humans, livestock and marine mammals (Dubey, 2010). In Brazil, the prevalence of *T. gondii* infection in humans is especially high and can reach 100% in some areas (Bahia-Oliveira et al., 2003; Sobral et al., 2005; De Moura et al., 2006) and an average of 60% of the adult women have been exposed to this parasite (Neto et al., 1995). The interest in the evaluation of *T. gondii* infection has focused on domestic animals that cohabitate with or serves as food for humans, as these animals can act as reservoirs to human infections (Sogorb et al., 1972). Though wildlife may play an important role in transmission and maintenance of *T. gondii* in the environment, there is limited information on *T. gondii* circulating in wild animals (Yai et al., 2009; Dubey et al., 2011; Pena et al., 2011; Cabral et al., 2013; Cañón-Franco et al., 2013).

Genotypic studies on *T. gondii* from domestic animals in Brazil have shown high diversity of this parasite (Dubey et al., 2002, 2007a; Lehmann et al., 2006; Shwab et al., 2014). This genetic diversity is characterized by an epidemic population structure (Pena et al., 2008). Recent efforts to genetically characterize *T. gondii* isolates from the wildlife have shown that "exotic" or "atypical" strains are not insignificant anomalies in the population structure of this parasite, but rather important members of the gene pool that provide a much

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better representation of the vast host range utilized by this parasite. There is a need, therefore, to reconsider the established points of view on the population genetic structure and the relative roles of the various lifecycle stages of *T. gondii* in shaping the population biology of this important zoonotic pathogen (Wendte et al., 2011).

Constant human interference and the increasing urbanization of the Brazilian landscape have resulted in wildlife habitat lost and fragmentation, and in an increased interaction between humans, domestic and wild animals that can lead to a greater exchange of pathogens. Isolation of *T. gondii* from wildlife is difficult and time consuming because of several factors, including poor DNA material from naturally infected wildlife because of low density of *T. gondii* in tissues of asymptomatic animals, and difficulties in preserving and transporting tissue samples from remote areas (Dubey et al., 2011). In the present study, we successfully genotyped 22 *T. gondii* samples obtained from wildlife in different regions of Brazil, and provided new information on genetic diversity of the parasite.

2. Material and methods

2.1. Location and sampling

For three years (2009–2011), 226 samples (fragments of brain and heart) from free-living and captive wild animals were collected, by chance/convenience, from different locations in Brazil (Table 1). The locations were in four regions (North, Northeast, Midwest and Southeast), five states (Mato Grosso, Minas Gerais, Pará, Pernambuco and São Paulo) and covered the four major Brazilian ecosystems: Amazon Forest, Atlantic Forest, Cerrado and Pantanal. All sampling locations were on the Brazilian mainland, except for one on the island of Fernando de Noronha, 360 km off from the northeast cost. Wild animal samples were collected from both urban and rural areas, and each sample was from a single animal except for samples collected on Fernando de Noronha, which were each pooled tissues from five animals of the same species.

2.2. Bioassay

Fragments (brain and heart) of wild animal tissues, weighting from 5 to 50 grams (depending on the animal size), were mixed and homogenized, then digested in acidic pepsin and washed. Aliquots of homogenates were inoculated s.c. into five out-bred Swiss Webster (SW) mice (Dubey, 1998). Tissue imprints of lungs and brains of inoculated mice that died were examined for *T. gondii* tachyzoites (lungs) or tissue cysts (brain), by direct observation on microscope. Survivors were bled 45 days post infection (DPI) and a 1:25 dilution of serum was tested for *T. gondii* antibodies by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987) in order to ensure that these animals were not infected with *T. gondii*. Mice were killed 60 DPI and their brains were examined for tissue cysts as previously described (Dubey, 2010). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were detected in their tissues.

2.3. Molecular detection of T. gondii in wild animal tissues

DNA from 300 µL of the homogenate (prior to pepsin digestion) from tissues of wild animals (primary samples) was extracted with a commercial kit (Wizard® DNA Clean-Up System, Cat. A7280 – Promega, Madison, WI, USA), following manufacturer's instructions. *Toxoplasma gondii* was among the protozoans targeted with a nested PCR of 18S ribosomal DNA (PCR-18S) to detect parasites of the Sarcocystidae family in tissues of wild animals (data not published) performed using external primers Tg18s48F (5′CCATGCATGTCTAAGTATAAGC3′) and Tg18s359R (5′GTTACCCGTCACTGCCAC3′), and internal primers Tg18s58F (5′CTAAGTATAAGCTTTTATACGGC3′) and Tg18s348R (5′TGCCACGGTAGTCCAATAC3′) (Integrated DNA Technologies, USA). This amplification generates about 290 base pair (bp) product for *Sarcocystis neurona, N. caninum, H. hammondi* and *T. gondii*, and 310 bp for other *Sarcocystis* spp. The products of nested PCR were digested by two sets of restriction enzymes (set 1: *AluI* and *HhaI*, to differentiate *S. tenella* from *T. gondii, N. caninum* and *H. hammondi*; set 2: *DdeI, Hpy188III* and *MspI*, to differentiate all *Sarcocystis* species (da Silva et al., 2009). Twenty-eight positive samples for *T. gondii* were selected for genotyping analysis.

2.4. PCR/RFLP

DNA was extracted from lungs and brain of infected mice and from positive "primary samples" (tissue homogenate aliquots of wild animals). *T. gondii* strain genotyping was performed using the genetic markers SAG1, 5′ and 3′-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22–8, c29–2, L358, PK1, Apico and CS3 as described previously (Pena et al., 2008; Su et al., 2010). NeighborNet phylogenetic networks were inferred using the software SplitsTree4 (Huson, 1998; Huson and Bryant, 2006; Pena et al., 2008).

2.5. Animal ethics

This study was conducted after consultation with the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) and approval of the Ethical Committee of the Faculty of Veterinary Medicine of the University of São Paulo – USP (project no. 1588/2008). All experiments performed in mice were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation. All sampled wild animals died from diverse causes, such as road kills and other sources of trauma or illness. No wild animals were killed for this research.

Table 1
Sampling sites and animal data.

Local (municipality, state)	Geographic coordinates	I/DNA	FL/C	IDs
Araraquara, SP	21°47′41″ S, 48°10′36″ W	1/0	1/0	TgHoFBr1
Confresa, MT	10°38′40″ S, 51°34′4″ W	0/1	1/0	PS-TgSbaBr1
Fernando de Noronha, PE[a]	3°50′25″ S, 32°24′41″ W	0/2	2/0	PS-TgCaEgBr1; PS-TgCaEgBr2
Jaborandi, SP	20°54′0″ S, 47°16′0″ W	1/0	1/0	TgMWBr1
Jaboticabal, SP	21°15′19″ S, 48°19′21″ W	1/1	1/1	TgCantBr3; PS-TgTinBr1
Recife, PE	8°3′15″ S, 34°52′53″ W	1/0	0/1	TgOncBr1
Santarém, PA	2°26′22″ S, 54°41′55″ W	9/2	11/0	TgNbaBr1, 2, 3; TgCantBr1, 2; TgWlpBr1, 2, 3; TgSpPBr2; PS-TgSpPBr1; PS- TgNbaBr4
São Paulo, SP	23° 32′ 56″ S, 46° 38′ 20″ W	1/1	1/1	TgLWpBr1; PS-TgBHmBr1
Uberlândia, MG	18° 54′ 41″ S, 48° 15′ 44″ W	0/1	1/0	TgSbaBr2

I/DNA, *T. gondii* isolation in mice/DNA extracted directly from tissues of wild animals before mice bioassay; FL/C, free-living animals/captive animals.
[a] Island located 360 km from the Northeast coast.