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Toxoplasma gondii in free-ranging wild small felids from Brazil: Molecular detection and genotypic characterization

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ABSTRACT

Brazil harbors the largest number of wild Neotropical felid species, with ten of the twelve species recorded in the American continent. Although these animals are considered to be definitive hosts for *Toxoplasma gondii*, there are few descriptions of the parasite in these species. Here, we performed a molecular detection of *T. gondii* by amplification of the marker ITS-1 from tissue samples obtained from 90 free-ranging wild small Neotropical felids from Rio Grande do Sul – Brazil. Of the sampled animals, 34.4% ($n = 31$) were positive including the species *Puma yagouaroundi* – jaguarondi (9/22), *Leopardus geoffroyi* – Geoffroy's cat (6/22), *Leopardus tigrinus* – oncilla (8/28), *Leopardus wiedii* – margay (6/10), *Leopardus pardalis* – ocelot (1/1) and *Leopardus colocolo* – Pampas cat (1/7). *Toxoplasma* DNA was detected with a frequency of 14.6% (63/433) in primary samples of tongue (16/56), brain (8/43), skeletal muscle (15/83), heart (7/63), diaphragm (3/56), vitreous humor (2/44), eye muscle (6/44) and eyeball (6/44). Multilocus PCR-RFLP genotyping of eleven small Neotropical felids using the molecular markers SAG1, 5′3′SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3 allowed the partial characterization of eight genotypes. We fully characterized two new genotypes that have not been described previously in Brazil (*Lw*#31Tn from *L. wiedii* and *Py*#21Sm from *P. yagouaroundi*) and one genotype *Py*#56Br from *P. yagouaroundi* that has been described previously in isolates from cats, dogs and capybaras from São Paulo state. This study constitutes the first detection and genotypic characterization of *T. gondii* in free-ranging felids in Brazil, demonstrating the occurrence of the parasite in wild populations and suggesting its potential transmissibility to humans and other domestic and wild animals.

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1. Introduction

Toxoplasma gondii is a protozoan of great medical relevance, especially for its impact on pregnant women, in whom severe clinical manifestations can occur, including miscarriage and fetal malformations. This parasite is also a major cause of death in immunocompromised individuals (Pappas et al., 2009; Innes, 2010). Water contaminated with *T. gondii* oocytes and the ingestion of raw or undercooked meat containing viable cysts of the parasite are the main routes of infection for humans and homeothermic animals, including birds and terrestrial, aquatic and flying mammals (Dubey, 1998).

Domestic cats and nearly all wild felid species worldwide are definitive hosts for *T. gondii* (Elmore et al., 2010; Jones and Dubey, 2010). The occurrence of Amazonian or Tropical toxoplasmosis in immunocompetent humans has been recorded in French Guiana (Ajzenberg et al., 2007), and the transmission of the disease has been linked to Neotropical felids; *T. gondii* was isolated and characterized from a *Panthera onca* (Carme et al., 2009). However, the zoonotic and ecological implications of toxoplasmosis in these wild felid species require further study.

In Brazil, serological surveys performed to date indicate that *T. gondii* circulates widely among Neotropical and exotic felid populations in zoos and conservation centers (Sogorb et al., 1997; Silva et al., 2007; Rivetti et al., 2008; André et al., 2010; Minervino et al., 2010; Ullmann et al., 2010). The isolation and genotypic characterization of the parasite in a *Puma yagouaroundi* from a Brazilian zoo confirms these observations (Pena et al., 2011).

Serological, molecular and biological studies on several wild animal species have documented the circulation of *T. gondii* within different trophic levels. Carnivorism is the primary means of parasite transmission within the food chain (de Thoisy et al., 2003; Wendte et al., 2011). Nevertheless, although wild felids are skilled predators of a wide variety of mammals, birds and insects, their participation in the wild life cycle of this parasite is poorly understood (Cañón-Franco et al., 2013).

Studies on wild and domestic animals in Brazil have shown wide genetic diversity in *T. gondii*, with genotypes diverging from the clonal types I, II and III found in other parts of the world (Dubey et al., 2002, 2007b; Lehmann et al., 2006). This genetic diversity is characterized by an epidemic population structure (Pena et al., 2008).

Genotype type II has been characterized in free-ranging wild felids in the United States (*Lynx rufus*) (Dubey et al., 2004) and Germany in *Felis silvestris silvestris* (isolate TgFsGER01), including isolates TgFsGER02 and TgFsGER03, which have been described as variants of type II (Herrmann et al., 2012). In North America, atypical genotypes have been described in *P. concolor* (type I–II) and *L. rufus* (type II–III), and the atypical X genotype has been detected in samples from both species (Miller et al., 2008). Subsequently the latter genotype was identified in *P. concolor vancouverensis* named as TgCgCa1, Cougar2, Cougar or COUG (Dubey et al., 2008). The atypical X type was renamed as haplotype 12 by Khan et al. (2011) and has recently been isolated from *L. rufus* in the United States (ToxoDB#5; Yu et al., 2013). In South America, another isolate (GUY-2004-JAG from *Panthera onca*) has been characterized as atypical using microsatellite markers. This isolate is responsible for Guianese toxoplasmosis (Demar et al., 2008).

Among captive felids, the clonal type II has been documented in France in *Phanthera tigris altaica* (Alerte, 2008; *unpublished results*) and *Felis margarita* (isolate TgSandcatQA1). In the latter species, three atypical genotypes have been described in Qatar and the United Arab Emirates, isolates TgSandcatUAE1-3 (Dubey et al., 2010). The first study of genotypic characterization of *T. gondii* in a small Neotropical felid in Brazil was in a *P. yagouaroundi* in the Recife zoo, named as TgJagBr1 (Pena et al., 2011).

The rDNA ITS-1 region is an important diagnostic target for closely related Toxoplasmatinae coccidians, as in the cases of the genera *Toxoplasma*, *Hammondia* and *Neospora* (Homan et al., 1997). The detection limit for PCR-ITS-1 on *H. heydorni* was 10 oocysts present in the feces of domestic dogs (=0.5 μl of DNA) (Šlapeta et al., 2002). This result was similar to what was established using seminested-PCR-AP10 on the same parasite (Soares et al., 2011). In this latter study, the sensitivity for the locus ITS-1 was ten times higher when it was amplified using nested-PCR. In this, at least one oocyst was detected, without involvement of analytical specificity, and all the Toxoplasmatinae species analyzed were detected (*H. hammondi* oocysts, tachyzoites of *N. caninum* and *N. hughesi* and tachyzoites of the strains RH, CTG and PTG of *T. gondii*).

The present study focuses on the molecular detection of *T. gondii* by amplifying the internal transcribed spacer (ITS-1) in various tissues from six species of free-ranging wild small Neotropical felids from Brazil: *P. yagouaroundi* (jaguarundi), *Leopardus geoffroyi* (Geoffroy's cat), *Leopardus tigrinus* (oncilla), *Leopardus wiedii* (margay), *Leopardus pardalis* (ocelot) and *Leopardus colocolo* (Pampas cat). This study also reports the genotypic characterization of *T. gondii* from primary tissue samples of these felids and thus provides information on the participation of these animals in maintaining the wild life cycle of *T. gondii* in southern Brazil.

2. Materials and methods

2.1. Sample collection

Ninety wild small Neotropical felids that had been fatally trampled between 1999 and 2010 were necropsied and kept frozen at −20 °C. A total of 433 tissue samples of skeletal muscle (quadriceps femoris), diaphragm, heart, tongue, brain, eyeball, eye muscle and vitreous humor (obtained by puncturing the posterior chamber of the eye) were obtained from these animals.

The sampled individuals belonged to five biological collections in the state of Rio Grande do Sul: Museum of Science and Technology of the Pontifical Catholic University of Rio Grande do Sul (Pontifícia Universidade Católica do Rio Grande do Sul – PUCRS), Laboratory of Cytogenetics and Molecular Evolution of the Federal University of Rio Grande do Sul (Universidade Federal do Rio Grande do Sul – UFRGS), Rio Grande do Sul Zoobotanical Foundation (Fundação Zoobotânica do Rio Grande do Sul – FZB),

Museum of Natural Sciences of the University of Caxias do Sul (Universidade de Caxias do Sul – UCS), and Museum of Natural Sciences of the Lutheran University of Brazil (Universidade Luterana do Brasil – ULBRA).

2.2. Epidemiological data

Information on the date of deposit in the collection, location within the state (midwestern, mideastern, metropolitan, northeastern, northwestern, southeastern, southwestern), species (*P. yagouaroundi*, *L. geoffroyi*, *L. tigrinus*, *L. wiedii*, *L. pardalis*, *L. colocolo*), gender (male, female) and age (adult, juvenile) of each felid was recorded when available. The associations between these variables and the presence of *T. gondii* were analyzed by the chi-square test with a significance level of $p = 0.05$ using the statistical software IBM SPSS Statistics19.

2.3. DNA extraction

As proposed by Pena et al. (2006), tissues were macerated in TE buffer, pH 8.0 (10 mM Tris–HCl, 1 mM EDTA), at a proportion of 1:4 and then subjected to enzymatic digestion with 1 mg/mL proteinase K (Invitrogen™, 20 units/mg). Genomic *T. gondii* DNA was obtained by means of phenol–chloroform extraction, resuspended in TE buffer, pH 8.0, and stored at −20 °C until further analysis.

2.4. PCR and genotyping

A ~500-bp fragment of ITS-1 was used for molecular detection of *T. gondii*. The fragment was amplified by PCR using the external primers JS4 and CT2c followed by nested PCR using the internal primers JS4b and CT2b, which target the 18S and 5.8S rRNA genes (Soares et al., 2011). Details of primers and PCR conditions are given in Supplementary Table S1.

Genotyping was performed by multilocus PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism), as described by Su et al. (2006), Dubey et al. (2007a) and Pena et al. (2008), using the molecular markers SAG1, 5′3′SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3. As positive controls, isolates RH88 (type I), PTG (type II) and CTG (type III) were added as references for archetypal strains, and MAS, TgCgCa1 and TgCatBr5 were added as references for atypical isolates. Human foreskin fibroblasts (HFF) and ultrapure water were used as negative controls.

3. Results

Based on ITS-1 amplification, *T. gondii* DNA was detected in 31 (34.4%) of 90 small Neotropical felids from Brazil. These individuals belonged to the species *P. yagouaroundi* (9/22), *L. geoffroyi* (6/22), *L. tigrinus* (8/28), *L. wiedii* (6/10), *L. pardalis* (1/1) and *L. colocolo* (1/7), as listed in Table 1. Most of the positive animals originated from the eastern (6/31), metropolitan (6/31) and northeastern (5/31) regions of Rio Grande do Sul. Male (20/41) and adult (24/52) felids showed the highest percentages of individuals positive for the parasite. According to a chi-square test, however, none of the analyzed variables (location, species, gender or age) was statistically significantly correlated with the presence of the parasite ($p > 0.05$).

Table 1
Detection of *Toxoplasma gondii* by amplification of ITS-1 in tissue samples from wild small Neotropical felids from Rio Grande do Sul ($n = 90$).

Variable	Number of animals		
	Examined	Positive	Percentage (%)
Species			
Puma yagouaroundi	22	9	40.9
Leopardus geoffroyi	22	6	27.3
Leopardus tigrinus	28	8	28.6
Leopardus wiedii	10	6	60.0
Leopardus pardalis	1	1	100.0
Leopardus colocolo	7	1	14.3
Gender			
Male	41	20	48.8
Female	15	5	33.3
Age			
Adult	52	24	46.2
Juvenile	4	1	25.0
Location			
Midwestern	2	0	0.0
Mideastern	15	6	40.0
Metropolitan	12	6	50.0
Northeastern	4	2	50.0
Northwestern	6	5	83.3
Southeastern	11	3	27.3
Southwestern	14	2	14.3
Date of deposit			
1999–2005	22	9	40.9
2006–2010	46	15	32.6

Toxoplasma DNA was obtained from 63 (14.6%) of 433 primary tissue samples, including tongue (28.6%; 16/56), brain (18.6%; 8/43), skeletal muscle (18.1%; 15/83) and heart (11.1%; 7/63). The parasite was detected less often in diaphragm (3/56), vitreous humor (2/44), eye muscle (6/44) and eyeball samples (6/44). The molecular data for ITS-1 are listed in Table 2.

As shown in Table 3, eight of the 63 primary samples that were positive for *T. gondii* were partially genotyped. These samples were taken from *L. geoffroyi* (*Lg*#24Sm), *L. wiedii* (*Lw*#30Sm), *L. tigrinus* (*Lt*#58Hr, 68Tn, 69Tn and 93Hr) and *P. yagouaroundi* (*Py*#36Sm and 47Sm). Furthermore, three samples were fully genotyped using the twelve available molecular markers. These samples were identified from *L. wiedii* (*Lw*#31Tn) and *P. yagouaroundi* (*Py*#21Sm and *Py*#56Sm), as shown in Table 4 and Fig. 1.

4. Discussion

Previous serological studies on *T. gondii* in native Brazilian felids held in captivity have shown prevalence rates between 52.8% and 66.7% (Silva et al., 2007; André et al., 2010; Ullmann et al., 2010). However, detection of the parasite by ITS-1 amplification reveals a lower frequency (34.4%) in the 90 free-ranging wild felids studied here. Free-ranging animals are an important source of toxoplasmic infection in conservation centers (Ullmann et al., 2010). Our results support this observation by demonstrating the presence of *T. gondii* in all six free-ranging wild small Neotropical felid species analyzed.

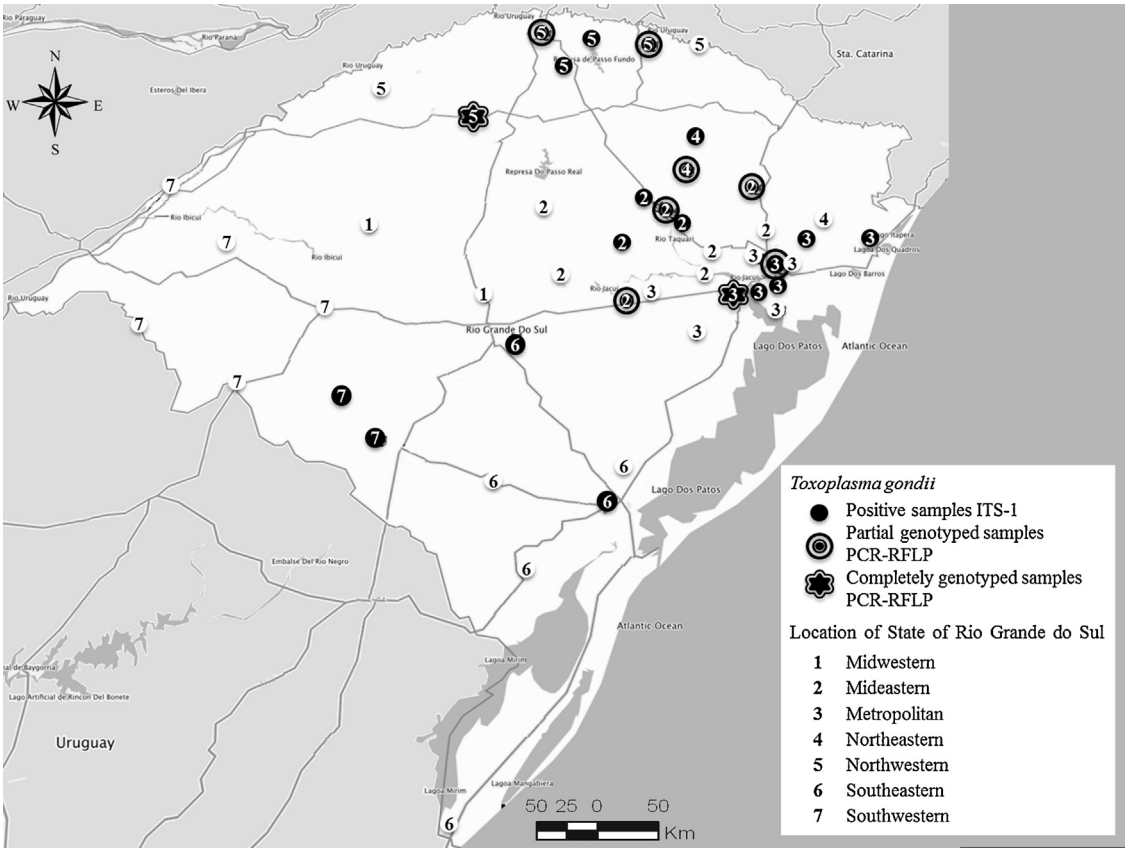

Fig. 1. Map of Rio Grande do Sul, Brazil showing the localities and samples of small Neotropical felids used for detection and genotypic characterization of *Toxoplasma gondii*.

Tests for anti-*T. gondii* antibodies in free-ranging *L. geoffroyi*, *L. pardalis* and *L. tigrinus* (Deem et al., 2004; Fiorello et al., 2006; Rendón-Franco et al., 2012; Uhart et al., 2012) have revealed frequencies similar to those found in captive animals, with infection susceptibility being unrelated to gender, age or species. With respect to predator-prey relationships, the diet and behavioral habits of wild felids may have a greater effect on *T. gondii* transmissibility (Cañón-Franco et al., 2013).

Dubey et al. (2004) have emphasized the need to replicate primary samples in mice because the amount of *T. gondii* DNA in the tissues may be lower than 250 μg/100 g of tissue. This observation may explain the low positivity rates obtained in the analyzed tissue samples.

The natural infection among the capybaras was confirmed by means of PCR detection of *T. gondii* in several tissue and blood samples. The diagnostic sensitivity of ITS-1 was slightly higher than that obtained through B1 gene amplification, thus resulting in molecular frequencies of 11.5% and 7.7%, respectively. This was similar to what was observed for ITS-1 in *N. caninum* (21.9%) when compared with Nc5 locus amplification (9.5%) in samples from the same rodent (Truppel et al., 2010a, 2010b).

Indeed, several protocols and techniques used for detection of *T. gondii* DNA have shown divergent diagnostic sensitivity (Ivović et al., 2012). The natural and experimental studies (Truppel et al., 2010a, 2010b; Soares et al., 2011) show variations in diagnostic sensitivity between different loci. This can be attributed to greater or lesser numbers of copies of each target sequence present in the DNA of the parasite. Through this, other molecular frequencies for *T. gondii* will be obtained.

Additionally, the nature of the animals' trampling deaths, their time of permanence on the road prior to collection and the storage temperature are critical to the stability of the genetic material. Nonetheless, *T. gondii* DNA was detected from 40.9% (9/22) of the felids deposited between 1999 and 2005 and from 32.6% (15/46) of those deposited between 2006 and 2010.

In the analyzed wild felids, *T. gondii* was detected in tongue (28.6%), brain (18.6%), skeletal-muscle (18.1%) and heart samples (11.1%). As in domestic felines, brain, skeletal muscle and heart samples are considered appropriate for obtaining viable parasite isolates because these organs exhibit higher numbers of cysts (Dubey, 2010). Pena et al. (2011) have conducted bioassays in mice and have noted the usefulness of skeletal muscle from *P. yagouaroundi* in obtaining *T. gondii* isolates.

Ocular toxoplasmosis is widely described in humans (Commodaro et al., 2009) and dogs (Swinger et al., 2009). In domestic felines, the infection causes partial or total blindness and anisocoria (Dubey and Carpenter, 1993) as a consequence of chorioretinitis and uveitis (Powell and Lappin, 2001). Further, Dubey et al. (2010) have identified

Table 2
Numbers of examined (*E*) and positive (*P*) samples and percentages of positive samples (%) for *Toxoplasma gondii* by amplification of ITS-1 according to tissue and species in wild small Neotropical felids from Rio Grande do Sul.

Species	Skeletal muscle			Tongue			Diaphragm			Heart			Brain			Vitreous humor			Eye muscle			Eyeball		
	E	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%	*E*	*P*	%
Leopardus colocolo	6	1	16.7	3	1	33.3	2	0	0.0	4	1	25.0	2	1	50.0	2	1	50.0	2	1	50.0	2	1	50.0
Leopardus geoffroyi	21	2	9.5	13	3	23.1	14	0	0.0	14	1	7.1	10	1	10.0	11	1	9.1	11	0	0.0	11	1	9.1
Leopardus pardalis	1	0	0.0	1	0	0.0	0	0	0.0	0	0	0.0	1	1	100.0	0	0	0.0	0	0	0.0	0	0	0.0
Leopardus tigrinus	26	4	15.4	16	6	37.5	17	1	5.9	21	2	9.5	12	1	8.3	12	0	0.0	13	1	7.7	12	1	8.3
Leopardus wiedii	8	1	12.5	8	4	50.0	9	1	11.1	10	2	20.0	7	0	0.0	7	0	0.0	7	1	14.3	7	0	0.0
Puma yagouaroundi	21	7	33.3	15	2	13.3	14	1	7.1	14	1	7.1	11	4	36.4	12	0	0.0	11	3	27.3	12	3	25.0
Subtotal	83	15	18.1	56	16	28.6	56	3	5.4	63	7	11.1	43	8	18.6	44	2	4.6	44	6	13.6	44	6	13.6

Table 3
Toxoplasma gondii multilocus genotypes and partial genetic characterizations in primary samples from wild small felids in Brazil based on PCR-RFLP.

Species/sample ID	Location	PCR-RFLP genotype												Identity with Brazilian isolates
		SAG1[a]	SAG2[b]	Alt. SAG2[c]	SAG3	BTUB	GRA6	C22-8	C29-2	L358	PK1	APICO	CS3	
Leopardus geoffroyi														
Lg#24Sm	Cristal do Sul	u-1	I/III	NA	III	I?	NA	u-1	NA	X?	NA	I	II	
Leopardus tigrinus														
Lt#58Hr	Caxias do Sul	I	III	NA	III	I/II	III	NA	NA	I	u-1	NA	NA	
Lt#68Tn	Marques de Souza	u-1	I	X	III	I	NA	u-1	NA	III	NA	I	NA	
Lt#69Tn	Dois Lageados	u-1	I	NA	III	I?	III	u-1	NA	I	III	III?	NA	
Lt#93Hr	Morungava	I	I/II	NA	III	I	II	NA	NA	NA	NA	NA	NA	
Leopardus wiedii														
Lw#30Sm	Pântano Grande	NA	I/III	NA	III	NA	NA	u-1	NA	NA	NA	NA	NA	
Puma yagouaroundi														
Py#36Sm	Gaurama	I	I	I	I	II	I	I	I	I	NA	I	I	
Py#47Sm	Unknown	u-1	I	I	III	II	III	I	I	I	NA	I	I	

u-1: novel allele different from the clonal types I, II and III; Lg: *Leopardus geoffroyi*; Lt: *Leopardus tigrinus*; Lw: *Leopardus wiedii*; Py: *Puma yagouaroundi*; Hr: heart; Tn: tongue; Sm: skeletal muscle; NA: product not amplified.

[a] It is not possible to distinguish between types II and III at the SAG1 locus.

[b] SAG2 marker based on the 5′and 3′ ends of the gene (Howe et al., 1997).

[c] Modified SAG2 marker based on the 5′ end of the gene sequence (Su et al., 2006).

Table 4
Toxoplasma gondii multilocus genotypes and complete genetic characterizations in primary samples from wild small felids in Brazil based on PCR-RFLP.

Species/isolate ID	Location	PCR-RFLP genotype											Identity with Brazilian isolates
		SAG1[a]	SAG2[b]	Alt. SAG2[c]	SAG3	BTUB	GRA6	C22-8	C29-2	L358	PK1	APICO	CS3	
Leopardus wiedii														
Lw#31Tn	Arroio dos Ratos	u-1	I	II	III	III	II	II	I	I	I	I	I	New
Puma yagouaroundi														New
Py#21Sm	Ijuí	u-1	I	II	III	II	III	u-1	I	I	III	I	I	
Py#56Br	Unknown	I	III	III	III	I	III	I	III	III	u-1	III	u-1	TgCatBr76 (SP)[d]
														TgDogBr16 (SP)[e]
														TgCpBr33 (SP)[f]

u-1: novel allele different from the clonal types I, II and III; Lw: *Leopardus wiedii*; Py: *Puma yagouaroundi*; Tn: tongue; Sm: skeletal muscle; Br: brain; NA: product not amplified.

[a] It is not possible to distinguish between types II and III at the SAG1 locus.

[b] SAG2 marker based on the 5′and 3′ ends of the gene (Howe et al., 1997).

[c] Modified SAG2 marker based on the 5′ end of the gene sequence (Su et al., 2006).

[d] Pena et al. (2008).

[e] Dubey et al. (2007b).

[f] Yai et al. (2009).

T. gondii cysts in the eye muscles of *Felis margarita* with no manifestation of ocular lesions. This study constitutes the first recorded detection of *T. gondii* DNA in the vitreous humor (Vh) of two wild Neotropical felids, *P. yagouaroundi* (#20Vh) and *L. geoffroyi* (#67Vh). Eyeball and eye-muscle samples were equally positive in these animals.

Erechim, in the state of Rio Grande do Sul, is known worldwide for its high frequency (greater than 17.7%) of patients with ocular toxoplasmosis (Glasner et al., 1992). Molecular studies in this region have revealed the circulation of genotype type I and type-I recombinants in humans and pigs (Vallochi et al., 2005; Khan et al., 2006; Belfort-Neto et al., 2007), linking this pathology to the consumption of raw or undercooked pork meat (Jones et al., 2006). In the current study, one felid *P. yagouaroundi* (*Py*#36Sm) obtained from the city of Gaurama, 17 km from Erechim in northwestern Rio Grande do Sul, exhibited type-I alleles at 10 of the 12 analyzed markers and a type-II allele at BTUB, indicating the circulation of this genotype in the wild, in domestic animals and in humans.

The genetic structure of the *T. gondii* population in Brazil is beginning to show a wide diversity of genotypes from domestic and wild animals and is characterized by the clonal expansion of atypical genotypes that are distinct from the clonal types present elsewhere in the world (Dubey and Su, 2009).

Multilocus genotyping revealed the presence of an atypical recombinant (type I, u-1, III) in a *P. yagouaroundi* (*Py*#56Br), which has been previously described by Dubey et al. (2007b) in dogs from the city of São Paulo (TgDogBr16), by Pena et al. (2008) in cats from Ribeirão Preto (TgCatBr76) and by Yai et al. (2009) in capybaras from Valparaíso (TgCpBr33), all cities in São Paulo state. Notably, these locations are distant from Rio Grande do Sul (approximately 1100 km away). According to the cited studies, this isolate is characterized by high mortality in mice.

Additionally, two novel atypical genotypes were identified as *Lw*#31Tn in *L. wiedii* (margay) and *Py*#21Sm in *P. yagouaroundi* (jaguarundi). The results obtained here suggest that atypical genotypes of *Toxoplasma* may circulate widely in Brazil and that the studied wild small felids play a role in *T. gondii* transmission across the interface between wild and domestic animals, a phenomenon known as "spill-over" or "spill-back".

Urban-adapted wild hosts facilitate the transmission of several pathogens, increasing the risk to vulnerable human, domestic-animal and wild-animal populations (Bradley and Altizer, 2007). In French Guiana, Mercier et al. (2011) have demonstrated the hybridization of wild and anthropized *T. gondii* genotypes, which are potential human pathogens. Considering the frequent incursions of wild Brazilian small felids into urban and peri-urban areas, these animals may play an important role in the toxoplasmosis cycle as definitive hosts, thus allowing the parasite to circulate between susceptible hosts.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2013.07.019.

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