Brazilian Journal of Veterinary Parasitology
ISSN 1984-2961 (Electronic)
www.cbpv.org.br/rbpv
Braz. J. Vet. Parasitol., Jaboticabal, v. 27, n. 4, p. 481-487, oct.-dec. 2018
Doi: https://doi.org/10.1590/S1984-296120180059

Original Article

Isolation and genetic characterization of *Toxoplasma gondii* from free-ranging and captive birds and mammals in Pernambuco state, Brazil

Isolamento e caracterização genética de *Toxoplasma gondii* de aves e mamíferos de vida livre e cativeiro em Pernambuco

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Received May 21, 2018
Accepted July 11, 2018

Abstract

Recent genetic population studies on *Toxoplasma gondii* in Brazil have shown large genetic variability. The objective of the present study was to isolate and genotypically characterize *T. gondii* from free-ranging and captive wild mammals and birds in Pernambuco state, Brazil. Fragments of heart, brain, skeletal muscle and diaphragm tissue from 71 birds and 34 mammals, which were either free-ranging or captive, were collected. Samples from 32 of these animals were subjected to bioassays in mice. Samples from the remaining 73 animals underwent biomolecular diagnosis, using PCR technique, targeting a repetitive DNA fragment of 529 bp in *T. gondii*. A non-virulent isolate (TgButstBrPE1) was obtained from a free-ranging striated heron (*Butorides striata*) and, based on primary samples, seven animals were found to be positive. The primary samples and the isolate obtained were subjected to PCR-RFLP using the markers SAG1, 5'3'SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3. ToxoDB-RFLP genotype #13 from the striated heron isolate and Type BrIII genotype from a captive otter (*Lontra longicaudis*) (PS-TgLonloBrPE1) were obtained. The present study describes the first isolation and genotypic characterization of *T. gondii* in free-ranging striated heron, and the first genotypic characterization of *T. gondii* in a captive otter.

Keywords: Toxoplasmosis, genotyping, diversity, wildlife, northeastern Brazil.

Resumo

Recentes estudos genéticos nas populações deste parasita no Brasil têm mostrado grande variabilidade genética. O objetivo do presente estudo foi isolar e caracterizar genotipicamente *T. gondii* de aves e mamíferos de vida livre e de cativeiro no estado de Pernambuco, Brazil. Fragmentos de tecido do coração, cérebro, músculo esquelético e diafragma de 71 aves e 34 mamíferos de vida livre ou cativeiro foram colhidos. Amostras de 32 destes animais foram submetidas a bioensaios em camundongos. As amostras dos 73 animais restantes foram submetidas a diagnóstico biomolecular usando a técnica de PCR, tendo como alvo o fragmento repetitivo de 529 pb do DNA de *T. gondii*. Dentre os 32 bioensaios conduzidos, obteve-se um isolado não-virulento (TgButstBrPE1) de um socozinho (*Butorides striata*) de vida livre, e dentre as amostras

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primárias, sete animais foram positivos. As amostras primárias e o isolado foram submetidos a PCR-RFLP usando os marcadores SAG1, 5'3'SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico e CS3. Foram obtidos o genótipo ToxoDB-RFLP #13 do isolado do socozinho e o genótipo Type BrIII de uma lontra (*Lontra longicaudis*) de cativeiro (PS-TgLonloBrPE1). O presente estudo descreve o primeiro isolamento e caracterização genotípica de *T. gondii* em socozinho de vida livre, e a primeira caracterização genotípica de *T. gondii* em lontra em cativeiro.

Palavras-chave: Toxoplasmose, genotipagem, diversidade, fauna selvagem, nordeste brasileiro.

Introduction

Infections by *Toxoplasma gondii* are cosmopolitan, affecting endothermal animals, including humans (DUBEY, 2010). In Brazil, studies have revealed seroprevalence of approximately 90% in humans, especially in the southern region of the country, and also that clinical illness is almost always associated with cases of immunosuppression (DUBEY, 2010). Recent studies regarding the population genetics of *T. gondii* worldwide have shown that, although reproduction is almost always asexual, the genetic variability of this infectious agent is much greater than previously expected (PENA et al., 2008; SHWAB et al., 2014). This variability may be related to clinical conditions, which can range from unapparent infections to cases of encephalitis. Some of these conditions can end up causing the death of the host, even among immunocompetent individuals, whether these are humans or animals (DEMAR et al., 2008; CARME et al., 2009; DUBEY, 2010; WENDTE et al., 2011). Studies have suggested that the existence of a wild cycle for *T. gondii* with sexual reproduction in wild felids, together with genetic changes due to clonal reproduction (asexual) in multiple species of wild animals, could be intrinsically related to the existence of greater genetic variability of the parasite in South America (CARME et al., 2009; KHAN et al., 2011; WENDTE et al., 2011).

Demar et al. (2008) and Carme et al. (2009) suggested that this phenomenon may occur in Brazil, especially in areas bordering countries such as Guyana, French Guiana and Suriname, because of the continuous nature of the Amazon rainforest. Different genotypes of *T. gondii* found in isolates from Brazilian wild fauna reinforce this hypothesis (PENA et al., 2011; CAÑÓN-FRANCO et al., 2013; BARROS et al., 2014; VITALIANO et al., 2014). However, studies on the genetics of *T. gondii* among the wild fauna of northeastern Brazil are still scarce.

In the state of Pernambuco, Brazil, Pena et al. (2011) carried out isolation and molecular characterization of *T. gondii* from a young male red-handed howler monkey (*Alouatta belzebul*) and from an adult male jaguarundi (*Puma yagouaroundi*) at the zoo of Parque Estadual de Dois Irmãos, located in Recife, Pernambuco. In other municipalities of this state, seroepidemiological surveys of infection by *T. gondii* in wild animals kept in captivity were conducted among neotropical felids (RAMOS SILVA et al., 2001), wild birds (LEITE et al., 2007), capuchin monkeys (*Sapajus* spp.) (FERREIRA et al., 2015), and marine manatees (*Trichechus manatus*) (ATTADEMO et al., 2016). Investigations on anti-*T. gondii* antibodies in free-ranging wild animals were conducted among cattle egrets (*Bubulcus ibis*) in Fernando de Noronha (COSTA et al., 2012), and among rodents and marsupials in the Atlantic Rainforest (SIQUEIRA et al., 2013). These studies demonstrated that infection by this parasite among wild animals presents wide geographical distribution in Pernambuco, which demands research on isolation and genetic characterization of *T. gondii*.

Considering the wide array of wild species with positive results in serodiagnostic investigations on *T. gondii* infection and the relatively low number of published scientific papers on genetic characterization of this parasite from wild animals in northeastern Brazil, the objective of the present study was to isolate and characterize the genotypes of *T. gondii* strains in free-ranging and captive wild mammals and birds in the state of Pernambuco.

Material and Methods

Between March 2014 and September 2015, fragments of the brain, heart, skeletal muscle and diaphragm of 105 wild animals (71 birds and 34 mammals) were collected (Table 1). These specimens were either free-living (n=59) or kept in captivity (n=46) at the zoo and Conservation Unit of Parque Estadual de Dois Irmãos and from the Tangará wild animal screening center of Agência Estadual de Meio Ambiente de Pernambuco (CPRH). Sampling was performed according to convenience, after these animals had died due to different causes.

The samples thus collected were divided into two groups. Samples from thirty-two animals were used for bioassays in mice and those from 73 animals were used to make direct molecular diagnosis. For the bioassay, tissue samples (between 5 and 50 g depending on the size of the animal) were collected, pooled and processed in accordance with the technique described by Dubey (1998). Aliquots of 1.0 mL of the final homogenate were inoculated subcutaneously into each of three Swiss mice, which were kept under observation for 45 days regarding the appearance of clinical signs of toxoplasmosis. At the end of the 45-day period, blood was drawn from the surviving mice for serological evaluation through the Modified Agglutination Test (MAT, cut-off titre ≥ 25) (DUBEY & DESMONTS, 1987). The mice were then euthanized and tissue imprints of their lungs and brains were examined to search for tachyzoites or tissue cysts to confirm infection by *T. gondii* (DUBEY, 1998).

Tissue samples collected for direct molecular diagnosis were individually macerated into a 0.85% saline solution. An aliquot of 300 µL of each homogenate was subjected to DNA extraction using a commercial kit (Qiagen® DNeasy Blood & Tissue, Dusseldorf, Germany), following the recommendations of the manufacturer. *Toxoplasma gondii* DNA in the samples was screened through PCR using primers TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (CGCTGCAGACACAGTGCATCTGGATT),

Table 1. Free-ranging and captive wild birds and mammals from Pernambuco state, Brazil, used for isolation or direct molecular diagnosis of *Toxoplasma gondii*. 2014-2015.

Species	Popular name	N. of specimens	Origin
Birds			
Amazona aestiva	Blue-fronted amazon	3	captive
Amazona farinosa	Southern mealy amazon	1	captive
Anodorhynchus hyacinthinus	Hyacinth macaw	4	captive
Ara ararauna	Blue-and-yellow macaw	1	captive
Ara chloropterus	Red-and-green macaw	2	captive
Aramides cajaneus	Gray-nekced wood-rail	1	free-ranging
Eupsittula cactorum	Cactus parakeet	1	captive
Aratinga jandaya	Jandaya parakeet	2	captive
Ardea alba	Grat white egret	2	free-ranging
Asio clamator	Striped owl	2	free-ranging
Athene cunicularia	Burrowing owl	2	free-ranging
Butorides striata	Green-backed heron	2	free-ranging
Cacicus cela	Yellow-rumped cacique	2	captive
Caracara plancus	Southern caracara	2	free-ranging
Cochlearius cochlearius	Boat-billed heron	1	free-ranging
Colaptes melanochloros	Green-barred woodpeacker	1	free-ranging
Columbina talpacoti	Ruddy ground-dove	1	free-ranging
Crax alector	Black curassow	1	captive
Crax fasciolata	Bare-faced curassow	1	captive
Crax globulosa	Wattled curassow	1	captive
Falco peregrinus	Peregrine falcon	1	captive
Falco sparverius	American kestrel	1	free-ranging
Guaruba guarouba	Golden parakeet	2	captive
Icterus jamacaii	Campo troupial	1	captive
Nycticorax nycticorax	Black-crowned night-heron	3	free-ranging
Ortalis guttata	Speckled chacalaca	1	captive
Pionites leucogaster	Green-thighed parrot	1	captive
Pitangus sulphuratus	Great kiskadee	1	free-ranging
Porphyrio martinicus	Purple gallinule	1	captive
Pteroglossus inscriptus	Lettered araçari	1	captive
Puffinus puffinus	Manx shearwater	2	free-ranging
Pulsatrix perspicillata	Spectacled owl	4	3 free-ranging 1 captive
Rhea americana	Greater rhea	2	captive
Rupornis magnirostris	Roadside hawk	11	8 free-ranging 3 captive
Tyto furcata	Common barn-owl	5	4 free-ranging 1 captive
Vanellus chilensis	Southern lapwing	1	captive
Mammals			
Alouatta caraya	Black-and-gold howler monkey	1	captive
Ateles paniscus	Guiana spider monkey	1	captive
Bradypus variegatus	Brown-throated sloth	6	free-ranging
Callithrix jacchus	Common marmoset	4	free-ranging
Caluromys philander	Bare-tailed woolly opossum	1	free-ranging
Didelphis albiventris	White-eared opossum	1	free-ranging
Leopardus tigrinus	Northern tiger cat	1	captive
Lontra longicaudis	Neotropical otter	2	captive
Mazama gouazoubira	Gray brocket	2	captive
Nasua nasua	South american coati	1	free-ranging
Procyon concrivorus	Crab-eating raccoon	2	captive
Rattus rattus	House rat	9	free-ranging
Sapajus flavius	Blond capuchin monkey	2	captive
Tamandua tetradactyla	Southern tamandua	1	free-ranging
TOTAL		**105**	

N - number.

targeting a non-coding 529bp DNA fragment that is repeated 200-300 times in the *T. gondii* genome (HOMAN et al., 2000).

The DNA extracted from the positive samples was then subjected to genotyping through the PCR-RFLP technique using 11 markers: SAG1, 3'5'SAG2 + alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico (SU et al., 2010) and CS3 (PENA et al., 2008). Samples with complete RFLP genotyping were also analyzed using 15 microsatellite markers (MS): TUB2, W35, TgMA, B18, B17, M33, IV.1 and X1.1 (typing markers); and N60, N82, AA, N61 N83, M48 and M102 (fingerprinting markers), following the protocols of Ajzenberg et al. (2010), in order to increase genotyping precision. The results were analyzed using the software Genemapper® 4.1 (Applied Biosystems).

The present study was conducted while respecting biosafety and animal welfare norms under authorizations from SISBIO (No. 37855-1; Instituto Chico Mendes de Conservação da Biodiversidade, ICMBio); the Ethics Committee for the Use of Animals (CEUA) of Universidade Federal Rural de Pernambuco (UFRPE) (No. 109/2014); the Bioethics Committee of the School of Veterinary Medicine and Animal Science of Universidade de São Paulo (FMVZ-USP) (No. 1921061113); Parque Estadual de Dois Irmãos (PEDI) (No. 216/2014); and Agência Estadual de Meio Ambiente de Pernambuco (CPRH) (No. 010675/2014).

Results

Among the 32 samples animals that were used for the bioassay in mice, *T. gondii* was isolated from a free-ranging striated heron (*Butorides striata*) (3.1%) in the metropolitan region of Recife-PE. This isolate was named TgButstBrPE1, and ToxoDB-RFLP genotype #13 was identified in it. During the 45-day period of post-inoculation observation, none of the three mice inoculated in this group died or presented clinical signs of toxoplasmosis.

Among the 73 animals used for direct diagnosis by means of PCR, seven (9.6%) were positive for *T. gondii*. Heart was the positive tissue in six of these animals. Type BrIII genotype (ToxoDB-RFLP genotype #8) was diagnosed from a captive otter (*Lontra longicaudis*) at the zoo of Parque Estadual de Dois Irmãos (Table 2). This sample was identified as PS-TgLonloBrPE1. Table 3 lists the results from RFLP genotypic characterization of the *T. gondii* isolate from the free-ranging striated heron and from the primary sample of the captive otter.

The microsatellite analysis enabled genotyping of the isolate TgButstBrPE1, which presented the following sequence of alleles: TUB2 = 291, W35 = 242, TgMA = 205, B18 = 162, B17 = 342, M33 = 165, IV.1 = 278, XI.1 = 356, N60 = 142, N82 = 109, AA = 277, N61 = 87, N83 = 312, M48 = 213 and M102 = 164, which corresponds to Caribbean 1 type.

Discussion

Diagnoses of *T. gondii* infection obtained through isolation and molecular methods among wild birds in Brazil have been reported both from free-living wild specimens (GONDIM et al., 2010; LIMA et al., 2011; VILELA et al., 2011; BARROS et al., 2014; VITALIANO et al., 2014) and from captive specimens (VITALIANO et al., 2010). Nonetheless, research involving molecular diagnoses of *T. gondii* in northeastern Brazil is still

Table 2. Positive tissue samples for *Toxoplasma gondii* through direct PCR*, from free-ranging and captive wild birds and mammals in Pernambuco state, Brazil. 2014-2015.

ID	Species	Popular name	Origin	Geographical location		Positive tissue
				Latitude	Longitude	
32	*Sapajus flavius*	Blond capuchin	Captive	08º00'20.79"	34º56'51.85"	Diaphragm
54	*Pulsatrix perspicillata*	Spectacled owl	Captive	07º59'9.28"	34º55'54.89"	Brain Heart
64	*Lontra longicaudis*	Otter	Captive	08º00'20.79"	34º56'51.85"	Heart
96	*Sapajus flavius*	Blond capuchin	Captive	08º00'20.79"	34º56'51.85"	Heart
98	*Falco sparverius*	American kestrel	Free-ranging	07º55'17.23"	34º55'44.39"	Heart Muscle
101	*Butorides striata*	Striated heron	Free-ranging	08º2'5.37"	34º55'8.97"	Heart Brain Muscle
105	*Lontra longicaudis*	Otter	Captive	08º00'20.79"	34º56'51.85"	Heart Muscle

*529-bp fragment target (Homan et al., 2000). ID 105: *T. gondii* strain was named PS-TgLonloBrPE1.

Table 3. Multilocus genotyping of *Toxoplasma gondii* from the isolate TgButstBrPE1, from a free-ranging striated heron (*Butorides striata*), and from PS-TgLonloBrPE1, from a primary sample of a captive otter (*Lontra longicaudis*), obtained through PCR-RFLP. Pernambuco state, Brazil. 2014-2015.

Sample ID	PCR-RFLP markers												RFLP genotype
	SAG1	5'3'SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	APICO	CS3	
TgButstBrPE1	I	I	I	I	I	III	II	III	III	I	III	III	#13
PS-TgLonloBrPE1 (ID#105)	I	III	III	III	III	III	II	III	III	III	III	III	Type BrIII

scarce (GONDIM et al., 2010). In the case of wild mammals, isolation and molecular diagnosis seem to be more common, in relation to both captive and free-living specimens (SILVA et al., 2006; TRUPPEL et al., 2010; PENA et al., 2011; CABRAL et al., 2013; CAÑÓN-FRANCO et al., 2013; VITALIANO et al., 2014).

The ToxoDB-RFLP genotype #13 that was identified in the present study had been previously described in isolates from chickens, goats, a red-handed howler monkey and a fox (*Cerdocyon thous*) in northeastern Brazil (DUBEY et al., 2008; FEITOSA et al., 2017; RAGOZO et al., 2010; PENA et al., 2011; ALMEIDA et al., 2017). These findings corroborate the wide circulation of this genotype in this region. The present study provides the first description of a *T. gondii* isolate genotyped as #13 from a wild bird. This isolate caused no mortality in mice, thus corroborating other authors' findings that ToxoDB-RFLP genotype #13 is associated with low virulence in mice (DUBEY et al., 2008; RAGOZO et al., 2010; PENA et al., 2011). Moreover, a microsatellite analysis (which presented greater resolution) revealed the same sequence of alleles (unpublished data), that were found in the isolate from a red-handed howler monkey, also from Recife, Pernambuco (PENA et al., 2011). This indicates that there is wide circulation of this clone in the city of Recife. The fact the striated heron examined here was rescued a few days before its death indicates that the infection was acquired in the wild. This suggests that the isolates from domesticated animals and from captive wild animals are genotypically similar to those isolated from free-ranging wild animals.

Regarding direct molecular diagnosis based on primary samples, Dubey (2010) reported that this technique was very effective for the diagnosis, but not so much for genotyping, due to the small amount of genetic material usually found. Molecular diagnosing of *T. gondii* using primary samples from wild animals has been described in capybaras (TRUPPEL et al., 2010), sparrows (VILELA et al., 2011), several species of small neotropical felids (CAÑÓN-FRANCO et al., 2013) and several species of wild birds and mammals (VITALIANO et al., 2014). Cañón-Franco et al. (2013) also conducted genotyping using primary samples, individually, obtaining 14.6% (63/433) of success, similar to what was obtained in the present study (14.3%).

Two blond capuchins (*Sapajus flavius*), two otters (*L. longicaudis*), one spectacled owl (*Pulsatrix perspicillata*), one American kestrel (*Falco sparverius*) and one striated heron (*B. striata*) were positive for *T. gondii* using PCR. These are the first reports of molecular diagnosis using primary samples in those species. In the case of the blond capuchins, a critically endangered species, identification of this agent is important, since these specimens were part of a reproduction program for *ex situ* conservation (LYNCH ALFARO et al., 2014; FERREIRA et al., 2015; IUCN, 2018).

The otter in which the *T. gondii* Type BrIII genotype was identified did not present any clinical sign of toxoplasmosis. The death of this specimen was associated with senility and multiple organ failure due to neoplastic metastases. Type BrIII is a common clonal lineage circulating in Brazilian territory, and it is found in different animal hosts and also in humans.

The two striated herons that were positive for *T. gondii* in the present study were individuals from two different districts in Recife. They had been living near water courses in densely populated areas. The facts that this species inhabits urban areas and has a diet composed of fish and other aquatic organisms (GWYNNE et al., 2010) suggest that the transmission route was probably ingestion of *T. gondii* oocysts from water contaminated by domestic sewage. These results indicated that this species is an intermediate host and can serve as an indicator for *T. gondii* infection among the synanthropic wild fauna of Pernambuco.

Conclusion

The genotyping information obtained from the samples of wild animals of the present study corroborated the findings that ToxoDB-RFLP genotype #13 has wide circulation in northeastern Brazil and that Type BrIII is a widely distributed clonal lineage in Brazil.

Acknowledgements

The authors are grateful to Fundação de Amparo à Ciência do Estado de Pernambuco (FACEPE), Agência Estadual de Meio Ambiente de Pernambuco (CPRH) and the Superintendency of Instituto Brasileiro do Meio Ambiente e Recursos Naurais Renováveis in Pernambuco (IBAMA-PE) for their financial support and for conceding licenses for the use of biological material from wild fauna. S.M.G., R.A.M. and J.C.R.S. received fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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