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Isolation and genetic characterization of *Toxoplasma gondii* from a captive black-and-gold howler monkey (*Alouatta caraya* Humboldt, 1812) in Brazil

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ABSTRACT

Toxoplasma gondii was isolated in mice from different tissues of a captive black-and-gold howler monkey (*Alouatta caraya*) kept in a colony at the Primatology Center of Rio de Janeiro State, Brazil, and it was genotypically characterized based on using PCR-RFLP and Microsatellite Analysis (MS), later on. *T. gondii* was successfully isolated from inocula deriving from heart, liver and tissue pool (heart, liver, lungs, axillary lymph nodes and cerebellum) samples. The isolate was named TgBgHmBrRJ1. The high virulence of the aforementioned strain was observed in infected mice. Non-archetypal genotype (ToxoDB PCR-RFLP #206) was obtained through PCR-RFLP. This genotype had been previously described in 12 isolates from different hosts, also in Southeastern Brazil, a fact that indicates likely high circulation of this genotype in this region. The isolate was also classified as non-archetypal, based on MS genotyping, as well as presented genotypic identity close to that of strains isolated from free-range non-symptomatic chickens (TgCkBr244,245,278,279) in Espírito Santo State. It is worth emphasizing that despite the large number of reports about clinical toxoplasmosis in neotropical primates in Brazil, this is just the second isolate of this parasite ever reported in this group of animals.

1. Introduction

Brazil holds the greatest neotropical non-human primate (NHP) biodiversity in the world (Rylands and Mittermeier, 2009). Among these primates, howler monkeys (*Alouatta* spp.) are classified as threatened species in major Brazilian ecosystems (IUCN, 2020). Several parasites can infect neotropical non-human primates (NHP) and lead to symptomatic infections with different prognoses; *Toxoplasma gondii* stands out among these biological agents (Catão-Dias et al., 2013).

Toxoplasmosis is a zoonotic infection caused by *T. gondii* that affects mammals and birds worldwide (Dubey, 2010). Cats and wild felids are definitive hosts of this protozoan, because they can shed resistant oocysts in the environment through their feces. Domestic and wild mammals, as well as birds, are intermediate hosts that can develop cysts in their tissues in chronic infections (Dubey et al., 2020). Clinical toxoplasmosis is associated with host factors, such as immune status and

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genetic profile, as well as with parasite factors, such as dose, stage and strain (Dubey, 2010). Neotropical NHPs are one of the mammalian groups mostly susceptible to *T. gondii* infection and they often develop fatal disease (Epiphanio et al., 2003; Catão-Dias et al., 2013; Dubey et al., 2021). Reports of acute toxoplasmosis in captive neotropical NHPs were found in Brazil (Santos et al., 2013; Paula et al., 2020; Santana et al., 2021).

Toxoplasma gondii presents high genetic diversity in Central and South America, where almost 200 genotypes were already identified, whereas only few types of it, mainly classical types II and III, circulate in North America, Europe and Asia, (Shwab et al., 2014). In total, 177 PCR-RFLP genotypes were identified in Brazil among almost 720 samples genotyped from domestic and wild animals, as well as from humans, in dozens of published studies - BrI, BrII and BrIII are the most prevalent clonal Brazilian lineage types (Pena et al., 2008).

Given the vulnerability of howler monkeys, good sanitary management practices, such as taking preventive measures against different pathogens, are of paramount importance to enable the conservation and maintenance of these animals in captivity. Despite the high genetic diversity of *T. gondii* strains in Brazil, reports on its isolation from neotropical primates remain scarce. The aim of the current report was to describe *T. gondii* isolation in mice bioassays, as well as the genotypic strain characterization of this protozoan in a captive howler monkey from Rio de Janeiro State, Brazil.

2. Methods

2.1. Sample collection and T. gondii isolation

Samples were collected from a 10-year-old male black-and-gold howler monkey (*Alouatta caraya*) kept at the Primatology Center of Rio de Janeiro State, Brazil. The aforementioned primate presented fever, prostration, inappetence, abdominal distension and pain, intestinal hypomotility and weight loss, and it was under medical treatment for suspected toxoplasmosis. It was euthanized after 36 days of treatment, when it did not show significant clinical improvement. All descriptions comprising symptoms, anti-*T. gondii* serology, treatment, disease evolution and post-mortem macroscopic and microscopic lesions were previously reported by Moreira et al. (2022). *T. gondii* isolation attempts were performed based on using 33g of liver, 19.8g of heart, 13.6g of lungs, 2g of axillary lymph nodes and 1.7g of cerebellum, due to suspected toxoplasmosis in the herein investigated primate. Tissue samples were digested in acid pepsin solution, according to Dubey (1998). Digested samples of each tissue, in separate, and a pooled tissue homogenate (comprising all tissues mentioned above) were subcutaneously inoculated in fourteen female Swiss Webster mice in the age group 25-to-30 days, who were divided into six different groups, as follows: cerebellum and heart (3 mice, each); and liver, lungs, lymph nodes and pooled tissues (2 mice, each). Animals showing clinical signs compatible to acute toxoplasmosis (ruffled coat, hunched behavior, ascites and inactivity) were euthanized for peritoneal washing in order to investigate the presence of tachyzoites. Isolate's virulence profile was defined based on Pena et al. (2008). Asymptomatic mice were euthanized 60 days after inoculation in order to investigate the presence of cysts in brain macerates. All procedures involving the animals used in the herein described bioassay were approved by the Ethics Committee on the Use of Animals, IOC/Fiocruz, under license L-041/2019.

2.2. Genetic characterization

Toxoplasma gondii DNA was extracted from peritoneal exudates deriving from mice, based on using Dneasy® Blood & Tissue commercial kit (Qiagen® Inc., USA), by following the manufacturer's protocols. Then, PCR amplification was performed in *T. gondii,* as described by Homan et al. (2000), based on using the 529-bp repeat element (REP529) fragment as target; DNA from *T. gondii* RH reference strain was used as positive control. The amplified DNA was visualized through electrophoresis on 2% agarose gels stained in SYBR® Safe DNA gel stain (Invitrogen™, USA).

T. gondii isolate genotyping was achieved first based on using multilocus PCR-Restriction Fragment Length Polymorphism (RFLP); then, it was compared to, and classified based on, other previously characterized Brazilian *T. gondii* strains available in the ToxoDB database (http://toxodb.org/toxo/) and in recent publications.

PCR-RFLP was performed as described by Su et al. (2010), based on using the following genetic markers: SAG1, SAG2 (3′5′SAG2 e alt. SAG2), SAG3, BTUB, GRA6, C22–8, C29–2, L358, PK1, Apico and CS3 (Pena et al., 2008). Reference archetypal strains, such as RH (Type I), PTG (Type II) and CTG (Type III), as well as non-archetypal *T. gondii* strains (TgCgCa1, MAS and TgCatBr5), were used as positive controls in all reactions.

The isolate was also genotyping by microsatellite analysis (MS) with eight typing markers (TUB2, W35, TgMA, B18, B17, M33, IV.1 and XI.1) and seven fingerprinting markers (N60, n82, AA, N61, N83, M48 and M102), as previously described (Ajzenberg et al., 2010). Results were analyzed in GeneMapper 4.1 software (Applied Biosystems). The PTG reference strain (Type II) was used as positive control.

3. Results

Toxoplasma gondii was isolated from heart, liver and tissue pool homogenate samples. The new isolate was named TgBgHmBrRJ1 (Tg = *T. gondii*; Bg = Black-and-gold; Hm = Howler monkey; Br=Brazil; RJ1 = first isolate of this species in Rio de Janeiro State). All seven mice inoculated with the aforementioned samples were infected with *T. gondii* and died of acute toxoplasmosis, a fact that indicated *T. gondii*'s high virulence. Clinical signs, such as ascites, ruffled coat (moderate to severe) and inactivity, were identified from the 7th (to the 12th post-inoculation day p.i.d), when the sick mice were followed up and, subsequently, euthanized in compliance with animal welfare guidelines. The remaining mice survived until the 60th p.i.d.; however, no cysts were observed in brain macerates of mice inoculated with digested lung, lymph node and cerebellum samples.

A non-archetypal genotype (ToxoDB PCR-RFLP #206) was obtained through PCR-RFLP. This isolate was also classified as non-archetypal, based on MS genotyping, and it presented genotypic identity close to that previously identified in isolates deriving from four free-range asymptomatic chickens (TgCkBr244, 245, 278, 279) in Espírito Santo State (Beltrame et al., 2012; HFJP, personal communication) (Table 1).

4. Discussion

Toxoplasma gondii isolation was performed based on using digested heart, liver and pooled tissue samples from a black-and-gold howler monkey. However, there are only four *T. gondii* isolates from neotropical NHP reported in the literature (Dubey et al., 2021). Pena et al. (2011) have isolated this parasite from tissue homogenate (heart and brain) from a captive red-handed howler monkey (*Alouatta belzebul*) who died of toxoplasmosis in Brazil. This protozoan was also isolated from squirrel monkeys in Argentina, China and Japan (Pardini et al., 2015; Huang et al., 2018; Nishimura et al., 2019)

Moreira et al. (2022) had previously reported clinical disease features, such as lesions in the liver, lungs, lymph nodes and spleen, in *A. caraya* - the same individual the herein described isolate was collected from. Acute presentation of toxoplasmosis in neotropical NHPs may be followed by systemic protozoan dissemination in animals' tissues. Therefore, it is recommended applying *T. gondii* isolation methods in suspected fatal cases of toxoplasmosis in Neotropical NHPs, based on using tissues deriving from these animals' necropsy, mainly heart tissues. It is essential isolating this protozoan to enable the subsequent assessment of factors, such as parasite's virulence and genotypic profile, as shown in the current study.

Table 1
Microsatellite genotyping of *Toxoplasma gondii* from a black-and-gold howler monkey (*A. caraya*) from Rio de Janeiro state, Brazil, which died with acute toxoplasmosis and comparison with other genotypically close *T. gondii* isolates.

Host	Strain designation*	MS Type	Microsatellite markers**												
			TUB2	W35	TgM-A	B18	B17	M33	IV.1	XI.1	*M48*	*M102*	*N60*	*N82*	*AA*	*N61*	*N83*
Black-and-gold howler monkey (*A. caraya*)	TgBgHmBrRJ1 (this study)	Non- archetypal	291	242	207	162	342	165	278	358	243	164	172	107	297	93	308
Chicken	TgCkBr244	Non- archetypal	291	242	207	162	342	165	278	358	229	164	168	107	297	93	308
Chicken	TgCkBr245	Non- archetypal	291	242	207	162	342	165	278	358	231	164	164	107	295	97	308
Chicken	TgCkBr278	Non- archetypal	291	242	207	162	342	165	278	358	231	164	164	107	295	93	308
Chicken	TgCkBr279	Non- archetypal	291	242	207	162	342	165	278	358	231	164	178	107	299	95	308
Reference	GT1	Type I	291	248	209	160	342	169	274	358	209	166	145	119	267	87	306
Reference	ME49	Type II	289	242	207	158	336	169	274	356	215	174	142	111	265	91	310
Reference	NED	Type III	289	242	205	160	336	165	278	356	209	190	147	111	267	91	312

A non-archetypal genotype (ToxoDB PCR-RFLP #206) was obtained through PCR-RFLP. This previously described genotype is characterized by the combination of typical alleles I, II and III to a unique allele (u-1) at SAG1 marker. In total, 12 *T. gondii* isolates were already characterized with this same genotype in Brazil: seven from free-range asymptomatic chickens (Pena et al., 2013; Ferreira et al., 2018; Silva et al., 2014); one, from an ostrich (*Struthio camelus*) in a slaughterhouse (da Silva and Langoni, 2016), and four, from humans affected by congenital toxoplasmosis (Carneiro et al., 2013; Silva et al., 2014). Similar to the present study, all these cases were reported in Brazilian Southeastern States; this finding indicates that this genotype may have high circulation in this region. Moreover, the non-virulent isolate deriving from the ostrich appears to be a variant strain, since it carries allele u-1 at the CS3 marker, whereas the other isolates carry allele II. All isolates #206 with allele II at the CS3 marker were referred to as virulent (as in the present study) or as having intermediate virulence in mice. Alleles I and II at the CS3 marker appeared to be linked to virulence in mice (Pena et al., 2008).

MS-based genotyping analysis of *T. gondii* strains is not often adopted in Brazil. The MS-based analysis of approximately 300 Brazilian strains (HFJP, personal communication) enabled finding that the TgBgHmBrRJ1 isolate is genotypically close to four isolates deriving from free-range asymptomatic chickens (TgCkBr244, 245, 278, 279) in Espírito Santo State (Beltrame et al., 2012). All five isolates have the same typing markers, but they are independent isolates, rather than clones, as indicated by their fingerprinting markers, a fact that corroborates the significant diversity of this parasite in Brazil.

The present study has contributed to the range of hosts with *T. gondii* infections associated with RFLP genotype #206. It was the first time a parasite strain with this genotype was associated with clinical disease in a neotropical NHP.

Declaration of competing interest

I declare that the authors of the article have no conflict of interest vis-a-vis the participants or any other direct or indirect contributor, in the development of the article hereby submitted “lsolation and genetic characterization of *Toxoplasma gondii* from captive black-and-gold howler monkey (*Alouatta caraya* Humboldt, 1812) in Brazil”, whose researchers involved are: “Maria Regina Reis Amendoeira, lgor Falco Arruda, Silvia Bahadian Moreira, Daniel Guimarães Ubiali, Alynne da Silva Barbosa, Hilda Fátima Jesus Pena, Asheley Henrique Barbosa Pereira, Clarissa Nascimento da Silveira, Thamires Francisco Bonifácio, Yara Souza Clemes, Thalita de Abreu Pissinatti, André Felipe Andrade dos Santos, Alcides Pissinatti".

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