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Short communication

Atypical *Toxoplasma gondii* genotype in feral cats from the Fernando de Noronha Island, northeastern Brazil

R.P.B. Melo[a], J.C. Almeida[a], D.C.V. Lima[a], C.M. Pedrosa[a], F.J.R. Magalhães[a], A.M. Alcântara[a], L.D. Barros[b], R.F.C. Vieira[c], J.L. Garcia[b], R.A. Mota[a,*]

[a] *Department of Veterinary Medicine, Laboratory of Infectious-Contagious Diseases of Domestic Animals, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros, 52171-900, Recife, PE, Brazil*
[b] *Department of Veterinary Medicine, Laboratory of Animal Protozoology, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445 Km 380, 86057-970, Londrina, PR, Brazil*
[c] *Department of Veterinary Medicine, Laboratory of Zoonosis and Molecular Epidemiology, Universidade Federal do Paraná, Rua dos Funcionários 1540, 80035-050, Curitiba, PR, Brazil*

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ABSTRACT

Toxoplasma gondii isolates from Brazil have a different phenotypic and genotypic pattern, with predominance of virulent isolates and recombinant genotypes, compared to the North Hemisphere. Considering that a new *T. gondii* genotype, non-pathogenic to mice, was previously identified from free-range chickens from the Fernando de Noronha Island, Brazil, this study aimed to identify genotypes of this parasite in tissue samples of feral cats (*Felis catus*) from this Brazilian Island. Anti-*T. gondii* IgG antibodies were detected in 18/31 (58%) feral cats. Two non-virulent *T. gondii* isolates were obtained by mouse bioassay. Genotyping was performed by PCR-RFLP using 10 genetic markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, PK1, L358 and Apico) and an atypical strain of *T. gondii* (ToxoDB #146) was identified. This is the first report of this genotype in feral cats.

1. Introduction

Toxoplasmosis is a zoonotic disease, caused by the obligate intracellular coccidian parasite, tissue cyst forming, *Toxoplasma gondii* and represents an important public health burden worldwide. Felids are important in the disease epidemiology since they may shed oocysts in the environment (Dubey, 2010). It has been established that the phenotypic and genotypic profile of *T. gondii* isolates from Brazil differ from other countries; while in the North Hemisphere the clonal lineages (Type I, II, III) are more prevalent, atypical strains predominate in Brazil (Lehmann et al., 2006; Pena et al., 2008). However, *T. gondii* isolates from chickens from the Fernando de Noronha Island, northeastern Brazil, consist of unique genotypes as well as clonal genotypes that are dominant in Europe and North America (Dubey et al., 2010).

Considering that, domestic and wild animals from the Fernando de Noronha Island have showed highly prevalent to anti-*T. gondii* antibodies (Dubey et al., 2010; Costa et al., 2012), and that *T. gondii* isolates from the local definitive host have never been obtained, this study aimed to identify *T. gondii* genotypes in tissues samples of feral cats (*Felis catus*) from this Brazilian Island.

2. Materials and methods

The study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at Universidade Federal Rural de Pernambuco (UFRPE) (protocol number 104/2015) and was conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation.

2.1. Area

Fernando de Noronha Archipelago (03°45′-57′S, 032°19-41′W) is a special municipality of Pernambuco State, northeastern Brazil, located in the Atlantic Ocean 354 km offshore from the Brazilian coast. The area is composed of a main island and 21 islets of volcanic origin, covering a total area of 26 km^2 (Schulz-Neto, 2004).

The region presents a tropical climate with two well-defined seasons of rainfall, with an average temperature of 28 °C (INMET, 2015). Due to the wide biodiversity and protection programs for endangered species, UNESCO has declared the Archipelago as a World Heritage Site.

* Corresponding author.
E-mail address: rinaldo.mota@hotmail.com (R.A. Mota).

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2.2. Study design and sampling

A cross-sectional study was performed during a one-year period. The Center of Animal Surveillance of the Island captured weak feral cats from different locations of the island. Blood samples were taken from 31 feral cats under specific chemical restraint (ketamine hydrochloride 10% and xylazine hydrochloride 1%). All samples were collected in tubes without anti-coagulant and kept at room temperature (25 °C) until visible clot retraction, centrifuged at 500*g* for 10 min, and the serum was separated and kept at −20 °C until processing.

During the necropsy fragments of brain, heart, lung, diaphragm, and livers were collected, stored at +4 °C, and subsequently forwarded to analysis within 24 h. Eleven samples could not be sent within 24 h, become inappropriate to be submitted to mouse bioassay. Thus, these samples were frozen at −20 °C and subsequently subjected to PCR.

2.3. Indirect immunofluorescent assay (IFA) and mouse bioassay

Feral cat serum samples (n = 31) were evaluated by indirect immunofluorescence assay (IFA) for the detection of anti-*T. gondii* IgG antibodies, as previously described (Camargo, 1964). Tissue samples from animals with an antibody titer ≥16 were submitted to the pepsin digestion method (Dubey, 1998). Two Swiss Webster (SW) mice (25–30 g) were inoculated subcutaneously with 1 mL of the final product. Mice were observed daily for 45 days and euthanized.

Mouse blood and tissue samples (brain, heart, lungs and liver) were collected. The blood was centrifuged at 500*g* for 10 min, and the serum was separated and kept at −20 °C until processing. Mouse serum samples were evaluated by IFA (cut-off 1:16) for the detection of anti-*T. gondii* IgG antibodies, as previously described (Camargo, 1964). Imprints of the brain and lungs were examined for *T. gondii* cysts and tachyzoites, respectively.

2.4. DNA extraction and PCR

The pooled tissues (heart, lungs and liver) and the brain of the mice were subjected to DNA extraction using a commercial kit (Wizard SV Genomic DNA Purification System, Promega®, Madison, WI, USA), according to the manufacturer's protocol. Different fragments of tissue samples from 11 feral cats that could not be shipped in time for bioassay were individually submitted to DNA extraction, after the detection of anti-*T. gondii* IgG antibodies by IFA.

For detection of *T. gondii* DNA single tube nested PCR was performed using two pairs of previously described primers and PCR protocols able to amplify a region of 227 bp of the ITS1 region of the parasite (Hurtado et al., 2001). A suspension of *T. gondii* tachyzoites (ME49 strain, 10^4 tachyzoites/mL) and ultrapure water were used as positive and negative controls, respectively. The amplified PCR products were subjected to gel electrophoresis on 1.5% agarose gels stained with BlueGreen (LGC® Biotecnologia, Cotia, São Paulo, Brasil), and visualized under UV light.

2.5. Multilocus PCR-RFLP and phylogenetic analysis

Genotyping was performed by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) using 10 molecular markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) as previously described by Su et al. (2010). All products were visualized by agarose gel electrophoresis at 2.5%, stained with Sybr Safe DNA Gel Stain (Invitrogen®, USA) and visualized using Safe Imager TM (Invitrogen®, USA). The results were identified, compared, and classified according to genotypes present in ToxoDB (http://toxodb.org/toxo/).

For phylogenetic analysis, the electrophoresis banding patterns (genotypic data of restriction polymorphism) obtained by PCR-RFLP were transformed into binary data and tabulated. The SplitsTree software (Huson and Bryant, 2006) was used for phylogenetic reconstruction between the genotype obtained in the present study and others previously isolated in Brazil and in the world.

Table 1
anti-*T. gondii* IgG antibodies titers by Immunofluorescence Antibody Assay (IFA) in feral cats from Fernando de Noronha Island, Brazil, and result of mouse bioassay.

Sample	IgG antibodies titers	Mouse bioassay
Feline 11	16	Negative
Feline 21	16	Negative
Feline 32	256	Negative
Feline 42	64	Negative
Feline 52	256	Negative
Feline 62	256	Positive
Feline 92	256	Positive

Table 2
Molecular detection of *T. gondii* DNA (ITS1 region) in tissue sample of feral cats from Fernando de Noronha Island, Brazil.

Sample	Tissue	PCR Result	Genotyping
G01	Brain	Negative	–
	Heart	Positive	Not performed[a]
	Diaphragm	Positive	#146
F252	Brain	Positive	Not performed[a]
	Heart	Positive	Not performed[a]
	Diaphragm	Positive	Not performed[a]

[a] DNA quantity lower than necessary.

3. Results

Anti-*T. gondii* antibodies were found in 18/31 (58%) feral cats. Antibody titers ranged from 16 to 256, with frequency of 33.3% (6/18) for 16, 11.1% (2/18) for 64, 22.2% (4/18) for 128, 33.3% (6/18) for 256.

From 18 seropositive cats, seven mouse bioassays were performed and two *T. gondii* isolates were obtained (isolation rate of 28.5%). The parasite was isolated from two cats with anti-*T. gondii* antibody titer of 256 (Table 1). All mice inoculated with feral cats tissues remained asymptomatic. Positive mice in isolation had titer of 1:256 and the brain and pooled tissues were positive for *T. gondii* by PCR. The two isolates obtained by mouse bioassay were designated as TgCatBrPE01 and TgCatBrPE02.

Tissue samples from 11 cats that could not be sent <24 h were submitted only to PCR. Tissue samples from two feral cats were positive by PCR (Table 2) and nine were negative.

Strain typing with multilocus PCR-RFLP markers revealed the genotype ToxoDB #146, an atypical strain that was different from all genotypes so far reported in cats worldwide.

4. Discussion

In the present study, *T. gondii* genotype ToxoDB #146 was isolated from feral cats tissues. This genotype was already isolated from free-range chickens and cattle egret (*Bubulcus ibis*) from the Fernando de Noronha Island (Dubey et al., 2010; Vitaliano et al., 2014). Until the present moment, this atypical genotype was solely found on this Brazilian Island. Genotype #146 have been considered non-pathogenic for mice. Previous studies have found that most isolates from Brazilian mainland are virulent for mouse (Dubey et al., 2007,2012; Pena et al., 2008). Further studies should be con-