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Original Article

Molecular characterization of *Toxoplasma gondii* isolates from free-range chickens reveals new genotypes in Goiânia, Goiás, Brazil

Caracterização molecular de *Toxoplasma gondii* isolados de galinhas caipiras revela novos genótipos em Goiânia, Goiás, Brasil

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Abstract

The aim of this study was to evaluate the genotypic characteristics of *Toxoplasma gondii* isolated from free-range chickens in the metropolitan area of Goiânia, Goiás, in Brazil's central-west region. The seroprevalence rate was found to be 96%, according to an indirect hemagglutination assay. Brain and heart samples were processed by peptic digestion for a mice bioassay. The tissues were homogenized and the resulting samples were subjected to polymerase chain reaction (PCR), which revealed that 64% of them contained the parasite's DNA. The mice bioassay revealed 15 isolates, 8 of them tachyzoites isolates from the peritoneal lavage and 7 from brain cysts. *T. gondii* genotypes were determined through PCR-RFLP, using the following markers: SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, alt. SAG2, Apico and CS3. Three genotypes were identified, inclued ToxoDB #65, and the other two are not yet described in the literature. Hence, we conclude that the isolates obtained from the metropolitan area of Goiânia showed relatively low genetic diversity.

Keywords: *Toxoplasma gondii,* molecular characterization, free-range chickens, bioassay, PCR-RFLP, genetic diversity.

Resumo

O objetivo deste estudo foi avaliar as características genotípicas de *Toxoplasma gondii* isolados de galinhas caipiras da Região Metropolitana de Goiânia, Goiás, Região Centro Oeste do Brasil. A soroprevalência foi de 96% dos animais, determinada por hemaglutinação indireta. As amostras de cérebro e coração foram processadas através da digestão péptica para o bioensaio em camundongos. Os tecidos foram homogeneizados, e as amostras resultantes foram analisadas por reação em cadeia da polimerase (PCR), que possibilitou a detecção do DNA do parasito em 64% deles. Por meio do bioensaio em camundongos, foi possível detectar 15 isolados, 8 deles apresentando taquizoítos na lavagem peritoneal e 7 apresentando cistos cerebrais. A determinação dos genótipos de *T. gondii* foi realizada por PCR-RFLP com os seguintes marcadores: SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, alt. SAG2, Apico e CS3. Foi possível definir 3 genótipos, incluindo o ToxoDB # 65 e dois deles ainda não foram descritos na literatura. Portanto, conclui-se que os isolados obtidos na região metropolitana de Goiânia apresentaram diversidade genética relativamente baixa.

Palavras-chave: *Toxoplasma gondii,* caracterização molecular, galinhas caipiras, bioensaio, PCR-RFLP, diversidade genética.

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Introduction

Free-range chickens (*Gallus gallus*) are considered indicators of environmental contamination by *Toxoplasma gondii* because they feed directly from the soil, which exposes them to oocysts (Dubey et al., 2003a; Zhu et al., 2008; Millar et al., 2012). Humans infected with *T. gondii* are usually asymptomatic. However, the infection may present clinically in congenitally infected children and in immunocompromised individuals (Dubey & Jones, 2008; Murat et al., 2013). The seroprevalence of toxoplasmosis among pregnant women in the municipality of Goiânia, state of Goiás, Brazil, was reported to be 51.85% (Avelar et al., 2015). It was also demonstrated that 13.6% of stray cats in the same region were shedding *T. gondii* oocysts in their feces. Both these rates are considered high when compared to those in other regions of the world (Rezende, 2015).

In Brazil, the seropositivity rate of anti-*T. gondii* antibodies in free-range chickens varies from 22.4.3% to 88.4% (Dubey et al., 2020). Prevalence in the eastern part of the state of Goiás was found to be 16.5% and in the metropolitan area of Goiânia it was 50% in chicken sold in the market (Alves, 2007), while free-range chicken sold on farms showed 38% prevalence (Silveira, 2013). These data indicate the high seroprevalence of toxoplasmosis in the state of Goiás among free-range chicken, which is directly attributable to environmental contamination, and this, in turn, leads to high seroprevalence rates in humans and other hosts (Dubey et al., 2003b; Millar et al., 2012).

Molecular characterization studies of *T. gondii* isolates in North America and Europe showed low variability, enabling them to be grouped into three clonal types (I, II, III) (Howe & Sibley, 1995). Type I isolates were classified as highly lethal in mice, regardless of the initial inoculum, while types II and III isolates showed lower lethality, since this characteristic was directly attributed to the initial inoculum (Sibley et al., 2009). *T. gondii* isolates from different regions in South American countries have shown high genetic diversity, revealed by studies of genetic polymorphisms of DNA fragments generated by restriction enzymes and polymerase chain reaction (polymerase chain reaction-restriction fragment length polymorphism – PCR-RFLP). The parasite has been shown to be highly genetically diversified and should therefore not be considered clonal, especially since recombinant and atypical strains have been detected (Brandão et al., 2006; Lehmann et al., 2006; Pena et al., 2008).

A genetic analysis of isolates from Brazil led to the identification of typically Brazilian clonal lineages, which were classified as BrI, BrII, BrIII and BrIV. These are different from the classic types according to their virulence. Therefore the Brazilian genotypes were classified as: BrI virulent, BrIII non-virulent, while genotypes BrII and BrIV present intermediate virulence. The level of virulence was classified based on a mouse mortality model, as virulent (100% mortality), intermediate (>30% mortality) and non-virulent (<30% mortality) (Pena et al., 2008).

Knowledge about the phenotypic and genotypic characteristics of *T. gondii* isolates is necessary in order to understand the complex host-parasite relationship (Saraf et al., 2017). However, pathogenicity depends on several factors, including host susceptibility, virulence of the isolate and time of infection (Dubremetz & Lebrun, 2012).

There are no studies that demonstrate the *T. gondii* genotypes circulating among different hosts in Goiás. Considering the role of free-range chickens as environmental bioindicators, the purpose of this study was the molecular characterization of *T. gondii* isolates from these animals, thus shedding light on the level of toxoplasmosis in the metropolitan area of Goiânia, Goiás, Brazil.

Material and Methods

This study was approved by the Ethics Committee on Animal Use of the Federal University of Goiás, Brazil, (CEUA/UFG), under Protocol no. 024/2016.

Characterization of the study area

The state of Goiás is located in the central west region of Brazil, and its capital is Goiânia. The metropolitan area of Goiânia (MAG) comprises 18 satellite towns and rural areas with highly developed farming activities, especially cattle, poultry, fruit and vegetable farming. The predominant climate in Goiás is the seasonal tropical, dry winter, with temperature ranging from 22 to 23 ° C (Casaroli et al., 2018).

Free-range chickens

Fifty free-range chickens were randomly selected from six farms within the metropolitan area of Goiânia, Goiás, Brazil. The 50 chickens came from the following municipalities: 10 from Abadia de Goiás; 17 from Aparecida de Goiânia; 5 from Goiânia; 10 from Hidrolândia, and 8 from Trindade (Figure 1).

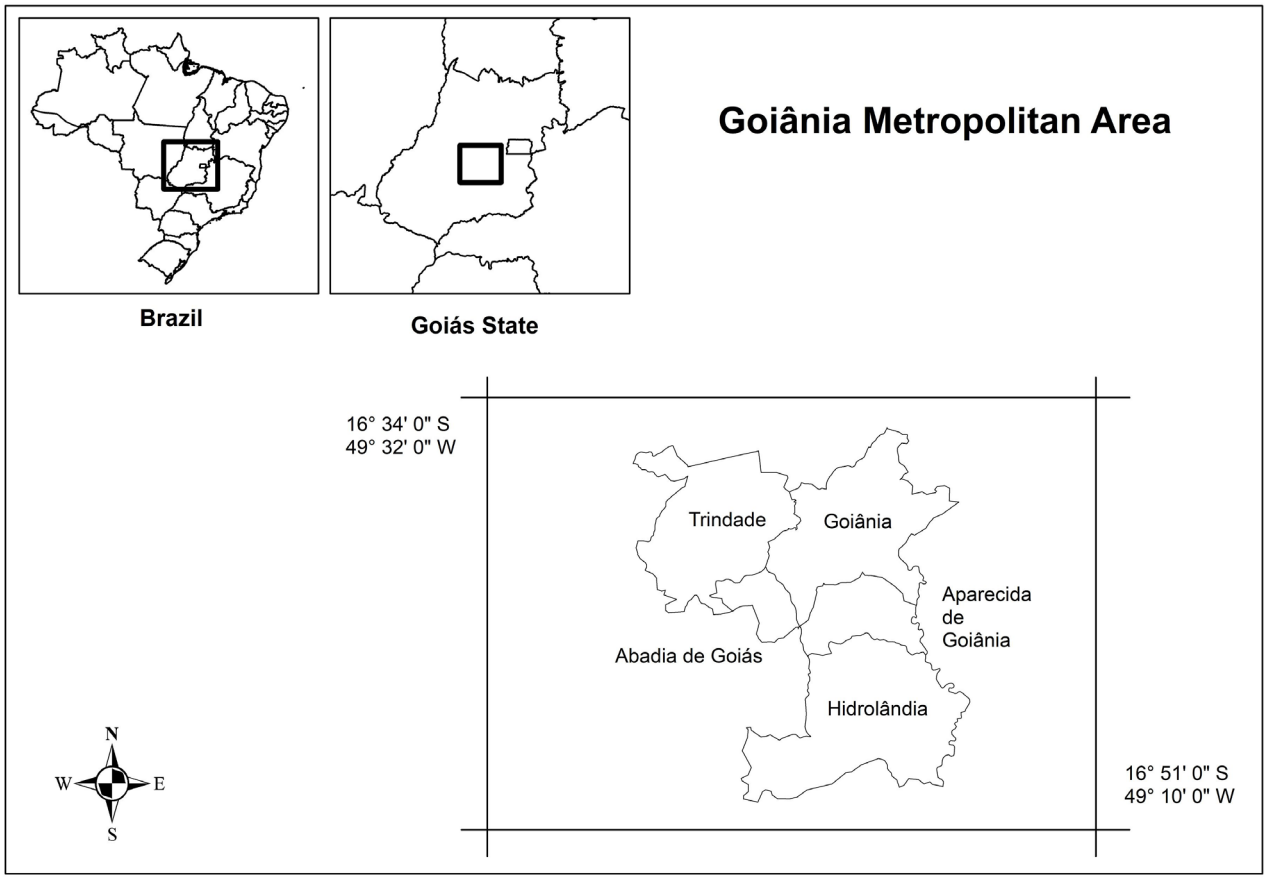

Figure 1. Metropolitan area of Goiânia, capital of the state of Goiás, in Brazil's central western region.

The chickens were euthanized on the farms, exsanguinated, and a blood sample was collected without anticoagulant. The animals were then decapitated, and their brains and hearts were removed. The samples were placed in a sterile container at 4ºC and taken to the Laboratory of Host-Parasite Relationship Studies at the Federal University of Goiás (LAERPH/UFG). The blood samples were centrifuged at 2,500 *g* for 15 min in order to separate the serum, which was stored at -20ºC until it underwent serological analysis.

Serological analysis

The serum samples were subjected to an indirect hemagglutination assay (IHA) using a Toxotest Wiener Lab® commercial kit. The samples were considered reactive when they presented a titer of ≥32. All the reactions were carried out with a positive and negative control, of the commercial kit itself, and the reactive samples were diluted down to a titer of ≥1024.

Bioassay in mice

The brains and hearts of seropositives chickens' in the IHA were weighed and then macerated together in a domestic food processor, totalizing about 50 grams of tissue, with 250 ml of 0.85% NaCl, followed by peptic digestion with acid pepsin (Dubey, 1998). The resulting homogenate was treated with 1,000 U of penicillin and 200 mg of streptomycin. After this, 1 ml of the homogenate was injected intraperitoneally into groups of three 30-day-old Swiss mice (male or female). The rest of the homogenate was stored at -80 C for subsequent DNA extraction.

The inoculated mice were monitored daily for a period of up to 60 days to identify the acute signs of toxoplasmosis. Symptomatic animals were euthanized, a peritoneal lavage was performed with 0.85% NaCl, and the tachyzoites were examined under an optical microscope. Positive samples from the peritoneal lavage and other organs of these mice were stored at -80ºC for subsequent DNA extraction. Part of the peritoneal lavage was inoculated in other mice to maintain the isolate.

After the 60 day period, the asymptomatic animals were euthanized and blood samples were collected by intracardiac puncture. The serum samples were examined under indirect immunofluorescence (IFI) (Camargo, 1964), using anti-mouse IgG conjugate (Sigma-Aldrich®). Mice were considered positive when they presented titers of >40. Their lungs and brains were removed and a fragment of these tissues was analyzed under a light microscope to look for *T. gondii* cysts (Dubey & Beattie, 1988). Samples of tissue that tested positive were macerated in sterile 0.85% NaCl and also stored at -80 C for later DNA extraction.

DNA extraction and *Toxoplasma gondii* detection by PCR

DNA was extracted directly from the chicken tissue homogenate, peritoneal lavages from mice with virulent isolates and from macerated brain/lungs from mice with non-virulent isolates. Tachyzoites from *Toxoplasma gondii* RH strain were used as positive control and blood from non-inoculated / non-infected mice as negative control. All the extractions were performed using commercial BIOPUR® *Kit Mini Spin Plus*.

DNA samples extracted from chicken tissue homogenate were subjected to PCR amplification of *T. gondii* B1 gene using the following *primers*: Toxo-B5 (5'-TGA AGA GAG GAA ACA GGT GGT CG-3'), Toxo-B6 (5'-CCG CCT CCT TCG TCC GTC GTA-3') (Santos et al., 1993). A final volume of 25 µl was used, containing 17.3 µl of sterilized Milli-Q water, 1.0 µL of $MgCl_2$, 2.5 µl of Buffer 10X (Invitrogen®), 0.2 µl of *Taq* DNA Polymerase (Invitrogen®), 0.5 mM of each deoxynucleotide (dATP/ dTTP/ dGTP/ dCTP, Sigma®), 50 pmol of each reaction initiator (Invitrogen®), and 2 µl of extracted DNA.

The amplification process consisted of an initial denaturation at 94ºC (5 min), 35 denaturation cycles at 94ºC (1 min), annealing at 62ºC (1 min) and an extension at 72ºC (1 min), followed by a final extension at 72º C for 10 min.

Molecular analysis

The PCR-RFLP procedure was performed on DNA samples from the isolates, using 12 different markers: SAG1, 5'-3' SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, APICO, and CS3 (Pena et al., 2008; Su et al., 2010).

Samples of clonal archetypes (Type I-GT1, Type II-PTG and Type III-CTG) and reference samples (TgCgCa1, MAS, TgCatBr5, TgCatBr64, and TgRsCr1) were used as control. The primers, reaction conditions and the respective restriction enzymes were the same as those described in the literature (Pena et al., 2008; Su et al., 2010).

The results of agarose gel electrophoresis were analyzed and the genotypes were classified using the ToxoDB platform (ToxoDB, 2018). The identified genotypes were combined with the control genotypes, using Splits Tree software version 5 (Huson & Bryant, 2006).

Results

Serology and detection of *Toxoplasma gondii* DNA in free-range chicken tissues

Of the total of 50 analyzed free-range chickens, 96% (48/50) were positive for anti-*T. gondii* IgG antibodies. Antibody titers varied from 32 to ≥1024. Forty-two percent (21/50) of the samples presented a titer equal to 64. *T. gondii* DNA was detected by PCR in 64% (32/50) of the samples of tissue homogenates, a summary of results are presented in Table 1.

Toxoplasma gondii isolated from tissues of free-range chickens

A total of 48 mice bioassays were performed from the positive chicken tissues, resulting in 15 isolates (31.3%). Of these, 8 presented tachyzoites from mice with acute toxoplasmosis symptoms, while 7 isolates were obtained from tissue cysts removed from asymptomatic mice.

Table 1. Results of serology by HAI, PCR of Gene B1 of tissue homogenate and result of bioassay of 50 free-range chickens in the metropolitan area of Goiânia, state of Goiás, in Brazil's central western region.

Chicken	Locality	HAI title	PCR the homogenate tissue	Mice positive in bioassay	Parasitic form	Nomenclature
01	Hidrolândia	32	+	-	-	-
02	Hidrolândia	128	+	1/3	Cyst	TgCkBrGO01
03	Hidrolândia	256	+	-	-	-
04	Hidrolândia	128	+	-	-	-
05	Hidrolândia	>1024	+	3/3	Tachyzoite	TgCkBrGO02
06	Hidrolândia	128	+	-	-	-
07	Hidrolândia	>1024	-	-	-	-
08	Hidrolândia	256	-	-	-	-
09	Hidrolândia	128	+	-	-	-
10	Hidrolândia	128	+	1/3	Cyst	TgCkBrGO03
11	Aparecida de Goiânia	128	+	-	-	-
12	Aparecida de Goiânia	128	+	3/3	Tachyzoite	TgCkBrGO04
13	Aparecida de Goiânia	64	+	3/3	Tachyzoite	TgCkBrGO05
14	Aparecida de Goiânia	256	+	3/3	Tachyzoite	TgCkBrGO06
15	Aparecida de Goiânia	64	+	2/3	Cyst	TgCkBrGO07
16	Aparecida de Goiânia	NR*	-	-	-	-
17	Aparecida de Goiânia	64	+	1/3	Tachyzoite	TgCkBrGO08
18	Aparecida de Goiânia	256	+	-	-	-
19	Aparecida de Goiânia	64	+	2/3	Tachyzoite	TgCkBrGO09
20	Aparecida de Goiânia	64	+	3/3	Tachyzoite	TgCkBrGO10
21	Aparecida de Goiânia	NR*	-	-	-	-
22	Aparecida de Goiânia	64	-	-	-	-
23	Aparecida de Goiânia	64	+	-	-	-
24	Aparecida de Goiânia	64	+	-	-	-
25	Aparecida de Goiânia	64	+	1/3	Cyst	TgCkBrGO11
26	Aparecida de Goiânia	64	-	-	-	-
27	Aparecida de Goiânia	64	-	-	-	-
28	Abadia de Goiás	32	+	-	-	-
29	Abadia de Goiás	128	-	-	-	-
30	Abadia de Goiás	64	-	-	-	-
31	Abadia de Goiás	32	-	-	-	-
32	Abadia de Goiás	64	-	-	-	-
33	Abadia de Goiás	64	-	-	-	-
34	Abadia de Goiás	32	+	-	-	-
35	Abadia de Goiás	64	+	-	-	-

*Non reagent.

Table 1. Continued...

Chicken	Locality	HAI title	PCR the homogenate tissue	Mice positive in bioassay	Parasitic form	Nomenclature
36	Abadia de Goiás	64	+	-	-	-
37	Abadia de Goiás	64	+	-	-	-
38	Trindade	>1024	+	-	-	-
39	Trindade	64	+	1/3	Cyst	TgCkBrGO12
40	Trindade	256	+	3/3	Tachyzoite	TgCkBrGO13
41	Trindade	256	-	-	-	-
42	Trindade	64	-	-	-	-
43	Trindade	128	+	-	-	-
44	Trindade	128	-	-	-	-
45	Trindade	64	+	1/3	Cyst	TgCkBrGO14
46	Goiânia	128	+	1/3	Cyst	TgCkBrGO15
47	Goiânia	128	-	-	-	-
48	Goiânia	64	-	-	-	-
49	Goiânia	32	+	-	-	-
50	Goiânia	128	-	-	-	-

*Non reagent.

Genotypic analysis of *Toxoplasma gondii* isolates

The genotypic characterization of the 15 isolates from the bioassays is described in Table 2. Only 9 isolates presented amplification of all the markers. Seven isolates pertained to genotype #65 from ToxoDB, and two isolates had not been previously described in the literature.

The phylogenetic tree computed using Splits Tree software shows the diversity of the isolates and the distance between the clonal archetypes (Figure 2).

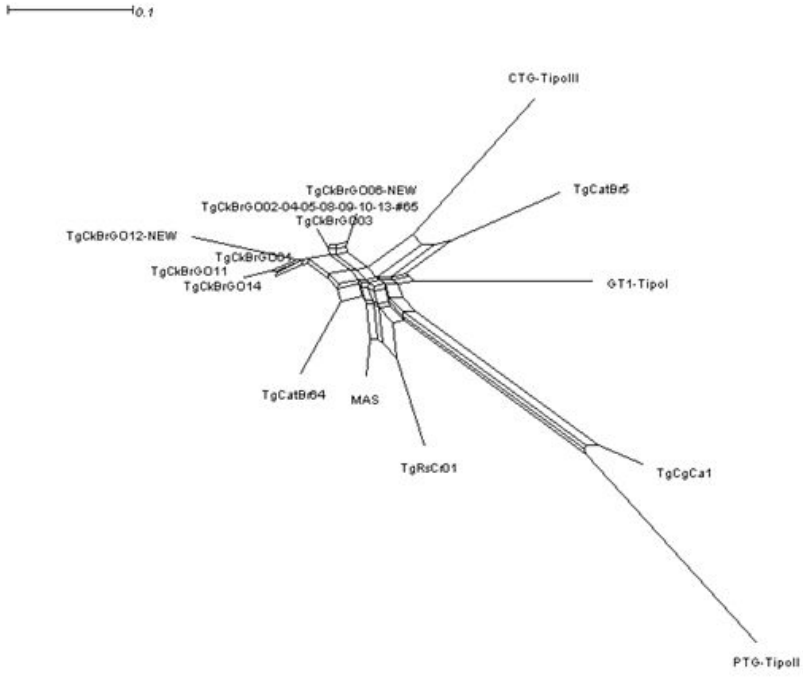

Figure 2. Phylogenetic tree of *Toxoplasma gondii* isolates from naturally infected free-range chickens in the metropolitan area of Goiânia, state of Goiás, in Brazil's central western region. The reference genotypes included here were GT1 = type I, PTG = type II, and CTG = type III, and the atypical genotypes were MAS, TgCatBr5, TgCatBr64, TgCgCa1 and TgRsCr1.

Table 2. Genotypic characterization of *Toxoplasma gondii* isolates obtained from naturally infected free-range chickens in the metropolitan area of Goiânia, state of Goiás, in Brazil's central western region.

Isolates	Parasitic form	MARKER												
		SAG1	5-3 SAG2	alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	CS3	Genotype ToxoDB
TgCkBrGO06	Tachyzoite	I	I	I	III	II	III	I	I	I	III	I	II	NEW1
TgCkBrGO12	Tachyzoite	I	I	II	II	I	III	u-1	u-1	III	I	I	III	NEW2
TgCkBrGO02		I	I	II	III	III	III	u1	I	I	III	I	II	#65
TgCkBrGO04														
TgCkBrGO05														
TgCkBrGO08	Tachyzoite													
TgCkBrGO09														
TgCkBrGO10														
TgCkBrGO13														
TgCkBrGO01	Cyst	I	I	UD*	III	UD	III	UD	UD	UD	III	I	UD	
TgCkBrGO03	Cyst	I	I	UD	III	UD	III	UD	UD	I	III	I	UD	Probably #65
TgCkBrGO07	Cyst	I	UD	UD	III	UD	UD	UD	UD	UD	UD	UD	II	
TgCkBrGO11	Cyst	I	I	III	III	UD	III	UD	u-1	UD	III	III	II	UD
TgCkBrGO14	Cyst	I	I	UD	III	UD	III	I	UD	II	III	UD	UD	UD
TgCkBrGO15	Cyst	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD

*UD: Undefined.

Discussion

This study evaluated the genotypic characteristics of *T. gondii* isolates identified in the state of Goiás. The frequency of seropositive free-range chickens (96%) observed in our study is higher than that described in earlier studies in Goiás, which reported prevalence rates ranging from 16.5% to 50% in free-range chickens (Alves, 2007; Silveira, 2013, Silveira et al., 2014). Mato Grosso do Sul, another state in Brazil's central western region, has a reported prevalence rate of 67.5% (Silveira, 2009). In the north of Brazil, a prevalence rate of 66% was reported in the state of Rondônia (Dubey et al., 2006), while 88.4% was reported on the island of Fernando de Noronha, in northeastern Brazil (Magalhães et al., 2016). Other reported prevalence rates in Brazil were 71.3% in the southeastern state of Minas Gerais, and 74.4% in the southern state of Rio Grande do Sul (Camillo et al., 2015). The differences in reported prevalence rates may be attributed to the level of environmental contamination by *T. gondii* oocysts, as well as the different techniques used in serological surveys, the farming systems, the influence of climate, etc. (Millar et al., 2012). Another important factor that deserves to be mentioned, albeit outside the scope of this study, was the visual confirmation of the close coexistence of cats and chickens on the farms of this study. This may explain the high seroprevalence found in the analyzed chickens. However, it should be noted that cats roam over a distance of up to five kilometers (Dubey & Beattie, 1988; Millar et al., 2012).

In this study, *T. gondii* DNA was amplified in 64% (32/50) of the samples of chicken tissues. The detection of DNA in these tissues is an important finding, even when the viability of the parasite is not confirmed in cysts (Gutierrez et al., 2010), because *T. gondii* may be transmitted by handling carcasses, or eating raw or undercooked chicken meat (Alvarado-Esquivel et al., 2009). Transmission may also occur through the consumption of undercooked chicken viscera, which is a very common habit in many regions of Brazil, including in the state of Goiás (Millar et al., 2012; Fernandes et al., 2016).

In this study, 31.3% of the bioassays were positive for *T. gondii* (15 isolates from 48 bioassays). Other studies have reported *T. gondii* positivity rates of 28.05% to 100% in isolates from chicken tissues (Dubey et al., 2007; Trevisani et al., 2017). The variations in these rates can probably be attributed to the tested tissues, given that some may show higher rates than others. In this study we used a heart and brain mixture, and it has been reported that the inoculation of a homogenate from a single organ into a single mouse increases the chance of isolation (Dubey et al., 2007; Beltrame et al., 2012).

Nine of the 15 isolates were successfully genotyped, and none of them were found to belong to the classic clonal types I, II or III, according to other Brazilian studies. The Brazilian isolates had a high reticulated phylogenetic relationship, suggesting that the genetic recombination plays an important role in the diversification of this species in Brazil (Pena et al., 2008). Meantime, our results illustrate the low genetic variability of the isolates under study, which was confirmed by their distance on the phylogenetic tree (Figure 2).In addition, to this day, there is no evidence of the other two genotypes in the literature or database, therefore, authors consider these as novel genotypes. Seven genotypes were identified as ToxoDB #65 (Table 2). Genotype #65 has been identified in chickens in Rio de Janeiro (Dubey et al., 2003a) and Espirito Santo (Pena et al., 2013), pigeons in Paraná (Barros et al., 2014), pigs in Pernambuco (Samico-Fernandes et al., 2015) and cats in São Paulo (Pena et al., 2006). In humans, this genotype was identified in São Paulo, where it was reported in chorioretinitis and in toxoplasmic encephalitis (Ferreira et al., 2011). These data demonstrate the wide circulation and geographic distribution of this genotype in several different hosts.

Conclusions

The high frequency of seropositive animals detected in this study should be examined carefully by public health authorities, since it is indicative of a highly contaminated environment. The *T. gondii* isolates obtained in this study in the metropolitan area of Goiânia showed low genetic variability and diversity, leading to the detection of two isolates not heretofore described in the literature. This study demonstrated the risk of *T. gondii* transmission to humans through the ingestion of free-range chicken, which is part of the region's traditional cuisine. Based on the results, preventive measures should be adopted, such as preventing cats from having access to chicken farms, environmental sanitation measures, introducing chicken-based food, and guidance on proper cooking and good feeding practices.

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