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Genetic characterization of *Toxoplasma gondii* isolates from eared doves (*Zenaida auriculata*) in Brazil

Caracterização genética de isolados de *Toxoplasma gondii* de pombos (*Zenaida auriculata*) no Brasil

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Abstract

Eared doves (*Zenaida auriculata*), which are common in urban, rural and wild areas in many regions of Brazil, are frequently prey for domestic cats. Therefore *Toxoplasma gondii* isolates obtained from doves may reflect greater environmental diversity than those from other hosts. The aim of the present study was to evaluate *T. gondii* seroprevalence, isolate and genotype strains from *Z. auriculata*. Serum and tissue samples were collected from 206 doves for use in the modified agglutination test (MAT) and mouse bioassay. The prevalence of *T. gondii* antibodies in the doves was 22.3% (46/206), with titers ranging from 16 to 4096, and *T. gondii* strains were isolated from 12 of these doves. Five genotypes were detected by means of PCR-RFLP, including ToxoDB genotypes #1, #6, #17 and #65, and one genotype that had not previously been described (ToxoDB#182). This was the first report on isolation of *T. gondii* from *Z. auriculata*. This study confirmed the genetic diversity of *T. gondii* isolates and the existence of clonal type II (ToxoDB genotype #1) in Brazil.

Keywords: *Toxoplasma gondii*, eared doves, genotyping, PCR-RFLP, MAT, Biossay.

Resumo

Pombos silvestres (*Zenaida auriculata*), comuns em áreas urbanas, rurais e selvagens em muitas regiões do Brasil, são frequentemente predados por gatos domésticos. Sendo assim, os isolados de *T. gondii* obtidos de pombos podem refletir uma maior diversidade ambiental do que os outros hospedeiros. O objetivo do presente estudo foi avaliar a soroprevalência, isolar e genotipar *T. gondii* de *Z. auriculata*. Amostras de soro e tecido foram coletadas de 206 pombos para o teste de aglutinação modificado (MAT) e o bioensaio em camundongos. A prevalência de anticorpos contra *T. gondii* em pombos foi 22,3% (46/206), com títulos variando de 16 a 4096, e *T. gondii* foi isolado de 12 pombos. Cinco genótipos foram detectados por PCR-RFLP, incluindo os genótipos ToxoDB #1, #6, #17, #65 e um genótipo não descrito anteriormente (ToxoDB#182). Esse é o primeiro relato de isolamento de *T. gondii* de *Z. auriculata*. Este estudo também confirmou a diversidade dos isolados de *T. gondii* e a presença de tipo clonal II (ToxoDB #1) no Brasil.

Palavras-chave: *Toxoplasma gondii*, pombos, genotipagem, PCR-RFLP, MAT, Bioensaio.

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Introduction

Toxoplasma gondii is an intracellular parasite that is distributed worldwide and can infect all warm-blooded animals, including mammals and birds (DUBEY et al., 2012). Human infections occur either from consumption of raw or undercooked meat containing tissue cysts or ingestion of food or water that is contaminated with sporulated oocysts (DUBEY & JONES, 2008). Because it is difficult to estimate the oocyst contamination in the environment, free-range chickens have been used as indicators of soil contamination.

Other domestic and wild animals have also been used to isolate and genotype *Toxoplasma* strains in many parts of the world, including Brazil (YAI et al., 2009; DUBEY et al., 2011; PENA et al., 2011; CABRAL et al., 2013; MACIEL et al., 2014; SILVA et al., 2014). These studies have shown that the Brazilian *T. gondii* strains are highly diverse, in comparison with those from other parts of the world.

The eared dove (*Zenaida auriculata*, Des Murs, 1847) occurs from the Antilles to Tierra del Fuego. In Brazil, it is considered to be an important pest that causes significant agricultural losses, but it is also an important source of food for humans in some regions (TARODA et al., 2013). Because the feeding habits of eared doves are similar to those of chickens, we believe that these birds could be an important source for evaluating environmental contamination because of their abundance in many regions of Brazil and because they are a source of food for stray cats. Another reason is that this species of bird is endemic in South America, where they were living for a long time before the introduction of chickens to Brazil. *T. gondii* has been described in pigeons and other birds since the beginning of the last century, when Carini in São Paulo, Brazil, observed the parasite in liver and spleen smears from a rock dove (*Columba livia*) (DUBEY, 2002).

The aim of the present study was to evaluate the prevalence of anti-*T. gondii* antibodies and genetically characterize the isolates obtained from eared doves (*Z. auriculata*) in Brazil.

Materials and Methods

Sample collection and study area

Two hundred and six free-ranging eared doves (*Z. auriculata*), including 97 males and 109 females, were caught in traps in Londrina (23°08'47" to 23°55'46"S/50°52'23" to 51°19'11"W), state of Paraná, southern Brazil, between January 2010 and June 2011. These birds were collected from three different areas: 140 birds from a soybean seed plant, 50 from the campus of the State University of Londrina and 16 from a dairy cattle farm. The present experiment was approved by the Animal Ethics Committee of the State University of Londrina (CEEA no. 70/08) and the Brazilian Institute of the Environment (IBAMA; SISBIO no. 16.428-1).

Blood and tissue samples

The birds were sacrificed in a CO_2 chamber. They were then bled by means of cardiac puncture and the resultant serum samples were stored at –20°C until further analysis. A pool of tissues of around 30 g, comprising lung, liver, heart, pectoral muscle and brain tissues, was collected for bioassay in Swiss-Webster albino mice weighing approximately 20-25 g.

Serological examination

The RH strain was used for antigen production in our laboratory, for serological tests by means of the indirect immunofluorescent assay (IFA) and the modified agglutination test (MAT). The prevalence of anti-*T. gondii* antibodies in the doves was obtained from MAT, in accordance with the technique previously described by Desmonts and Remington (1980) and titers ≥16 were considered to be positive.

Mouse bioassay

The pooled tissue from each bird, as described in section 2, was used to evaluate the presence of *T. gondii* cysts as described previously (DUBEY, 1998). Briefly, 30 g of brain, lung, liver, heart and pectoral muscle tissue were homogenized in a blender, in 150 ml of saline solution (0.14M NaCl), for 30 seconds. After homogenization, 150 ml of pepsin solution (0.78 g pepsin of 1:10000 biological activity, 1.5 g of NaCl and 2.1 ml of HCl, at pH 1.2) was added and incubated at 37 °C for 1 hour. The homogenate was filtered through two layers of gauze and centrifuged at 1180 xg for 10 min. The supernatant was discarded and the sediment was suspended in 20 ml of PBS (pH 7.2); 9 ml of 1.2% sodium bicarbonate (pH 8.3) was then added and the mixture was centrifuged at 1180 xg for 10 min. The supernatant was discarded and the sediment was suspended in 3 ml of antibiotic saline solution (1,000 U of penicillin and 100 µL of streptomycin/ml of saline solution) and subcutaneously inoculated into three mice (1 ml/mouse).

Examination of mice

Impression smears from the lungs of the mice that died were fixed in methanol, stained with Giemsa and examined under a microscope. Blood samples were drawn from the mice that survived for 45 days after inoculation, and the brain of each mouse was examined under a microscope for *T. gondii* tissue cysts by squashing a portion of brain between a coverslip and a glass slide. Serum from each mouse was diluted at 1:16 and 1:64 and examined for *T. gondii* antibodies, using IFA, in accordance with the technique previously described (GARCIA et al., 2006). Mice with titers ≥ 16 were considered to be positive.

Genetic characterization of the T. gondii strains isolated

Brain and lung tissue and peritoneal fluid from the bioassayed mice (i.e. from those in which tissue cysts and tachyzoites were observed) were used to extract DNA. DNA extraction was performed using a commercial kit (PureLink™ genomic DNA kit, Invitrogen®, USA) in accordance with the manufacturer's

instructions, and DNA was collected in a final volume of 50 µL. Strain genotyping was performed using multilocus PCR-RFLP with 11 genetic markers (SAG1, 5' and 3'SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) as previously described (SU et al., 2006). DNA from eight reference strains (GT1, PTG, CTG, TgCgCa1, MAS, TgCatBr5, TgCatBr64 and TgRsCr1) was used as positive controls. Genotyping was performed following the method described previously (SU et al., 2010). The results were compared, identified and classified in accordance with the genotypes present in ToxoDB at http://toxodb.org/toxo/.

Statistical analysis

All the variables were analyzed by means of the chi-square test (c^2) or Fisher's exact test, using the Epi Info software, version 6.04b (DEAN et al., 1994). P-values ≤ 0.05 were considered to be significant.

Results

Antibodies against *T. gondii* were detected in 46 (22.3%) of the 206 doves. The most frequent titer was 16 (13.5%), followed by 64 (3.3%), 256 (3.0%), 1024 (2.0%) and 4096 (0.5%). In relation to the sex of the doves, 18.5% (18/97) of the males and 25.6% (28/109) of the females were seropositive, and there was no statistically significant difference between the groups ($p > 0.05$). However, when the capture sites were compared, there was a statistically significant difference ($p < 0.05$), such that there was a higher rate of positivity among the doves from the university campus (56%), compared with those from the soybean seed plant (12.1%) or the dairy cattle farm (6.2%) (Table 1).

Using the mouse bioassay, *T. gondii* was isolated from 12 doves. The strains were named TgDoveBr1 to TgDoveBr12, according to the order of isolation (Table 2). The genotyping results for all of the markers are shown in Table 3. Five different genotypes were detected; four matched with the ToxoDB PCR-RFLP genotypes #1, #6, #17 and #65, and one genotype had not been described previously. Three of the isolates did not amplify all of the markers, so it was not possible to determine their genotypes.

Discussion

In the present study, we showed that the *T. gondii* seroprevalence in *Z. auriculata* was 22.3%. Other studies in Brazil that used *C. livia* observed lower seroprevalence, which ranged from 0 to 5% (GODOI et al., 2010; SOUSA et al., 2010; LIMA et al., 2011). There was a statistically significant difference in the present study regarding the capture sites. Although the eared dove is a bird with the capacity to fly long distances and it has been shown to be able to fly daily distances of 117 km from breeding colonies (BUCHER & BOCCO, 2009), the birds caught on the university campus had higher incidence of antibodies directed against *T. gondii* (56%) than did those caught at the soybean seed plant (12.1%) and the dairy cattle farm (6.2%). Our hypothesis for this difference in occurrence between the capture sites is that some areas have high concentrations of cats, which could contribute towards environmental contamination. However, further studies should be conducted to confirm this hypothesis. Salant et al. (2009) found higher seropositivity in *C. livia* caught in rural areas, while Alvarado-Esquivel et al. (2011) reported greater seroprevalence in *C. livia* caught in areas near a zoo. This difference could be due to larger numbers of host animals, such as cats, rodents and birds, which may contribute towards parasite transmission.

When the sexes of the seropositive animals in our study were compared, there was no statistically significant difference. This was similar to the results observed previously for *C. livia* (TSAI et al., 2006; KARATEPE et al., 2011).

In the present study, two of the 12 *T. gondii* strains were isolated from seronegative animals (MAT < 16), which possibly can be explained by previous findings of absence of detectable antibodies in chronically infected birds (MINEO et al., 2009). Although some isolates caused death in mice, we could not determine the virulence, because the dose for infection in mouse bioassays is unknown.

Genotypic characterization of the isolates obtained in the present study revealed the presence of four previously reported genotypes and one new genotype that had not previously been described, which has now been classified as ToxoDB#182. In Brazil, genotypes #6, #17 and #65 had previously been isolated from chickens, dogs and cats (DUBEY et al., 2002, 2003, 2007; PENA et al., 2006). The overlap in the genotypes between

Table 1. Presence of antibodies against *Toxoplasma gondii* in eared doves (*Zenaida auriculata*) using the modified agglutination test (MAT) and associations with sex and with capture site, in Londrina, Paraná State, Brazil.

Variables	Positive[a] (%)	Negative (%)	Total	P-value
Sex				
Male	18 (18.5)	79 (81.5)	97	0.28
Female	28 (25.6)	81 (74.4)	109	
	46 (22.3)	160 (77.7)	206	
Capture site				
University campus	28 (56.0)	22 (44.0)	50	< 0.001
Soybean seed plant	17 (12.1)	123 (87.9)	140	
Dairy cattle farm	1 (6.2)	15 (93.8)	16	
	46 (22.3)	160 (77.7)	206	

[a]Cut-off value: 16.

Table 2. Isolation of *Toxoplasma gondii* from eared doves (*Zenaida auriculata*) according to mouse bioassay.

Dove no.	Capture site[a]	Isolate name	MAT titers	Infectivity of *T. gondii* isolates to mice (n = 3)			
				Mortality (%)	Positive mice[b] (%)	Tachyzoite	Tissue cyst
1	UC	TgDoveBr1	64	2 (66.6)	2 (66.6)	+	+c
6	UC	TgDoveBr2	16	1 (33.3)	2 (66.6)	+	-
12	UC	TgDoveBr3	16	3 (100)	3 (100)	+	-
13	UC	TgDoveBr4	16	3 (100)	3 (100)	+	-
14	UC	TgDoveBr5	16	1 (33.3)	1 (33.3)	+	-
36	UC	TgDoveBr6	256	3 (100)	3 (100)	+	+c
150	SBP	TgDoveBr7	1024	2 (66.6)	3 (100)	+	+
153	SBP	TgDoveBr8	< 16	0	1 (33.3)	-	+
155	SBP	TgDoveBr9	< 16	0	1 (33.3)	-	+
164	SBP	TgDoveBr10	256	3 (100)	3 (100)	+	-
185	SBP	TgDoveBr11	1024	3 (100)	3 (100)	+	-
200	DCF	TgDoveBr12	1024	1 (33.3)	1 (33.3)	+	-

[a]UC: university campus of State University of Londrina, SBP: soybean seed plant, DCF: dairy cattle farm; [b]mice were positive either through parasite detection in their tissues or through positive IFA test; [c]mice re-inoculated with peritoneal fluid and lung homogenates that developed tissue cysts in the brain.

Table 3. PCR-RFLP genotypic profile from *Toxoplasma gondii* strains isolated from eared doves (*Zenaida auriculata*) from Londrina, Paraná State, Brazil.

Isolate	Markers											Genotype
	SAG1	SAG2	alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	
TgDoveBr1	II/III	II	II	II	II	II	II	II	II	II	II	ToxoDB #1
TgDoveBr2	II/III	II	II	II	II	II	II	II	II	II	II	ToxoDB #1
TgDoveBr3	I	nd	nd	III	II	II	II	III	II	II	I	nd
TgDoveBr4	nd	nd	nd	III	nd	III	nd	nd	nd	nd	nd	nd
TgDoveBr5	I	I	II	III	nd	III	u-1	nd	II	nd	II	nd
TgDoveBr6	I	III	III	III	III	III	III	I	I	I	III	ToxoDB #182 – This study
TgDoveBr7	I	I	I	III	I	II	u-1	I	I	I	I	ToxoDB #6
TgDoveBr8	II/III	II	II	II	II	II	II	II	II	II	II	ToxoDB #1
TgDoveBr9	II/III	II	II	II	II	II	II	II	II	II	II	ToxoDB #1
TgDoveBr10	I	III	III	III	III	III	III	I	I	I	III	ToxoDB #182 – This study
TgDoveBr11	u-1	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #17
TgDoveBr12	I	I	II	III	III	III	u-1	I	I	III	I	ToxoDB #65

nd: not determined.

isolates from cats in the state of São Paulo and doves in the state of Paraná demonstrates that *T. gondii* genotypes are widespread across Brazil. Genotype #1 is the clonal type II and is rare in Brazil but common in North America and Europe. The presence of type II has been reported in guinea fowl and cattle in Brazil (DUBEY et al., 2011; MACEDO et al., 2012). Previous studies on free-living pigeons (*C. livia*) in Brazil did not obtain *T. gondii* isolates (GODOI et al., 2010; LIMA et al., 2011). However, using the PCR-RFLP method, Alvarado-Esquivel et al. (2011) classified the isolate from the pigeon *C. livia* in Mexico as atypical, thus confirming the genotypic variability of non-European and American isolates.

Conclusions

To the authors' knowledge, this is the first study in which *T. gondii* was isolated and genetically characterized from *Z. auriculata*, a native wild dove that can be found in urban, rural and wild areas in many regions of Brazil. In this study, we identified a unique genotype and also clonal type II, thus showing that the genotypic composition of *T. gondii* strains in Brazil is highly diverse. Our data indicate that *T. gondii* genotypes are spread across the country and that doves can contribute towards disseminating them.

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