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PROTOZOOLOGY - ORIGINAL PAPER

Genetic and histopathological characterization of *Toxoplasma gondii* genotypes isolated from free-range chickens reared in the metropolitan region of Rio de Janeiro state, Brazil

Luciana Casartelli-Alves[1] · Sandro Antonio Pereira[1] · Luiz Cláudio Ferreira[1] · Rodrigo de Macedo Couto[1] · Tânia Maria Pacheco Schubach[1] · Maria Regina Reis Amendoeira[2] · Rodrigo Costa da Silva[3] · Hélio Langoni[4] · Patrícia Riddell Millar[5] · Rodrigo Caldas Menezes[1]

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Abstract

This study aimed to genetically characterize *Toxoplasma gondii* isolates obtained from free-range chickens reared in the metropolitan region of the state of Rio de Janeiro, Brazil, and to evaluate the morbidity and histological changes associated with these isolates in mice. A mouse bioassay was used to isolate *T. gondii* from a pool of tissue samples (brain, heart, and thigh muscles) collected from 163 chickens. The 36 isolates obtained were genetically characterized by restriction fragment polymorphism (PCR-RFLP) analysis of the SAG1, 5′-3′SAG2, aSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico, and CS3 genomic regions. Seventeen atypical genotypes were identified and nine of them were reported for the first time. All identified genotypes caused clinical signs and histological changes in mice, with the majority being associated with high cumulative morbidity (65%) and severe or very severe histological changes (76%). The exclusive identification of atypical genotypes, with a predominance of new genotypes, indicates great genetic diversity of *T. gondii* in the region studied. In addition, the finding that all identified genotypes caused clinical signs and often severe histological changes in mice suggests potentially relevant virulence of these strains.

Keywords Bioassay · Chickens · Genotyping · Histopathology · *Toxoplasma gondii* · Morbidity

Introduction

Toxoplasmosis is a globally distributed zoonosis of great public health importance, which is caused by the protozoan *Toxoplasma gondii* (*T. gondii*) (Hill and Dubey 2016). In Brazil, the infection is highly prevalent among humans and animals (Dubey et al. 2012; Millar et al. 2014). Domestic chickens are very resistant hosts to the development of clinical toxoplasmosis; however, free-range chickens are good indicators of environmental contamination with *T. gondii* oocysts present in the area where the animals are raised (Dubey et al. 2010; Casartelli-Alves et al. 2014). Considered a foodborne disease, the consumption and handling of raw or undercooked chicken meat pose a risk of *T. gondii* infection for humans and other animals (Hill and Dubey 2013, 2016).

Analysis of isolates of *T. gondii* obtained from free-range chickens in different parts of the world has led to the discovery of a great genetic variety of this parasite (Dubey et al. 2008, 2012; Soares et al. 2011; Rajendran et al. 2012; Pena et al. 2013; Silva et al. 2014; Vieira et al. 2018; Dubey et al. 2020).

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✉ Patrícia Riddell Millar
patriciariddell@id.uff.br

[1] Instituto Nacional de Infectologia Evandro Chagas, Fundação Oswaldo Cruz (Fiocruz), Avenida Brasil, 4365, Rio de Janeiro, RJ, Brazil

[2] Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (Fiocruz), Avenida Brasil, 4365, Rio de Janeiro, RJ, Brazil

[3] Faculdade de Ciências Agrárias, Universidade do Oeste Paulista, Presidente Prudente, SP, Brazil

[4] Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista Júlio de Mesquita Filho, Botucatu, São Paulo, Brazil

[5] Instituto Biomédico, Universidade Federal Fluminense, Rua Prof. Hernani Pires de Melo, 101, Niterói, RJ, Brazil

Previously, only the clonal lineage types I, II, and III had been described in studies carried out in North America and Europe (Aubert et al. 2010). Hundreds of different genotypes, known as atypical genotypes, have now been identified. The highest genotypic diversity is found in South America, particularly in Brazil, where 108 different genotypes have been detected in chickens (Dubey et al. 2012; Pena et al. 2013; Dubey et al. 2020). The vast majority of these genotypes found in Brazil are atypical, while clonal types I, II, and III, which dominate in Europe, North America, and Africa, are rare (Pena et al. 2008; Soares et al. 2011; Dubey et al. 2012; Rajendran et al. 2012; Pena et al. 2013; Silva et al. 2014; Vieira et al. 2018; Pena et al. 2018; Vielmo et al. 2019). This clonal diversity observed in South America may be related to the high frequency of severe ocular and congenital toxoplasmosis in humans in this region, especially Brazil and Argentina (Gilbert et al. 2008; Rudzinski et al. 2016; Hamilton et al. 2019).

The importance of transmission of toxoplasmosis through oocysts is demonstrated by the presence of the same genotype in different hosts, as different genotypes may be associated with greater virulence and transmissibility to humans (Khan et al. 2006; Lindsay and Dubey 2011; Rajendran et al. 2012; Robert-Gangneux and Dardé 2012; Cunha et al. 2016). Atypical *T. gondii* genotypes have been associated with severe forms of toxoplasmosis, even in immunocompetent individuals (Ajzenberg et al. 2004; Demar et al. 2007; Nunura et al. 2010). In addition, women seropositive for typical genotypes may develop severe congenital toxoplasmosis when they become infected with atypical genotypes during the third trimester of gestation (Elbez-Rubinstein et al. 2009; Delhaes et al. 2010; Lindsay and Dubey 2011).

The municipalities of Itaboraí, Maricá, and Rio Bonito are located in the metropolitan region of Rio de Janeiro state, Brazil; the latter two are endemic for toxoplasmosis (Casartelli-Alves et al. 2014). However, the *T. gondii* genotypes circulating in these locations are unknown. In Maricá, an atypical human toxoplasmosis case was reported in an immunocompetent patient, which was characterized by meningoencephalitis and pneumonia (Neves Ede et al. 2011), but the involved genotype was not identified.

Therefore, this study aimed to genetically characterize *T. gondii* isolates obtained from free-range chickens reared in the metropolitan region of the state of Rio de Janeiro, Brazil, and to evaluate the morbidity and histological changes associated with these isolates in mice.

Methods

Sampling

One hundred and sixty-three adult chickens (*Gallus domesticus*) were sampled in the metropolitan region of Rio de Janeiro state, Brazil, between April 2009 and July 2011. Of these, 117 animals were obtained from 41 rural farms in the municipality of Maricá, 43 from 15 farms in the municipality of Rio Bonito, and three from a farm in the municipality of Itaboraí. All chickens were reared free-range for subsistence use and were sometimes sold in the neighborhood. One to three chickens were sampled per farm according to availability.

Ten batches of chickens were investigated at different times, with four batches of 15 chickens, four batches of 20 chickens, one batch of 10 chickens, and one batch of 13 chickens. The chickens were euthanized by cervical dislocation and necropsied. At necropsy, samples of the brain (brain, cerebellum, and brainstem), heart, and thigh muscles were collected for the mouse bioassays.

Mouse bioassay

The assay was performed using a previously described protocol (Casartelli-Alves et al. 2014). Briefly, a pool of 20 g of tissue samples (brain, heart, and thigh muscles) collected from each chicken was triturated, homogenized, digested with an acid pepsin solution, and inoculated into five specific pathogen-free female Swiss Webster mice. The control group, consisting of one mouse per chicken (total of 163 mice), was inoculated intraperitoneally with 1 mL of 0.9% sterile saline. After inoculation, these mice were monitored daily over a period of 45–50 days. Animals showing clinical signs indicative of toxoplasmosis such as piloerection, prostration, abdominal distention, dyspnea, and motor incoordination and those that were still alive at the end of the observation period were euthanized by intraperitoneal injection of an overdose of thiopental sodium. The bioassay was defined as positive when *T. gondii* was observed in mouse tissues (brain, lung, heart, spleen, or liver) or peritoneal exudate, or when anti–*T. gondii* antibody titers were detected in mouse serum by an indirect hemagglutination test. Brain and lung samples from positive mice were tested using molecular techniques. The number of mice with clinical signs indicative of toxoplasmosis, such as piloerection, prostration, abdominal distention, dyspnea, and motor incoordination, and with *T. gondii* infection, confirmed by the bioassay, was used to estimate morbidity (number of sick animals divided by the number of inoculated animals). The cumulative morbidity caused by each identified genotype was calculated by summing all mice that fell ill due to *T. gondii* infection (confirmed by the bioassay) after inoculation divided by the number of inoculated mice. The cumulative morbidity index of each genotype was classified as high when it reached 100%, moderate when it reached 33 to 84%, and low when it reached up to 20%. It is important to point out that the inoculated dosage was not calculated; hence, this is not a study of virulence but a descriptive study of the

histological changes and morbidity found after the inoculation of each genotype.

Genotyping

Brain and lung samples from positive mice were investigated for *T. gondii* DNA using the polymerase chain reaction (PCR), according to Brandão et al. (2006). The parasite DNA was extracted using the Illustra Tissue and Cells Genomic Prep Mini Spin kit (GE Healthcare Life Sciences do Brasil Ltda®, Brazil), following the manufacturer instructions. The DNA amplification was run using the TOX4 (5′ CGCTGCAGGGAGGAAGACGAAAGTTG3′) and TOX5 (5′CGCTGCAGACACGTGCATCTGGATT3′) primers, which amplify a 529-base pair (bp) region with approximately 300 copies in the parasite genome (Homan et al. 2000). The amplification products were homogenized with a bromophenol blue solution and visualized after running on a 1.5% agarose gel stained with ethidium bromide. The gel images were recorded with a digital photodocumentation system (GelDoc-It®, UVP, USA) and analyzed with the VisionWorks software (UVP, USA) under ultraviolet light and on a specific computer.

PCR-positive samples were amplified at various loci using multiplex PCR, nested PCR, and restriction fragment length polymorphism-PCR (RFLP-PCR) analysis of 12 genetic markers: SAG1, 5′-3′SAG2, aSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico, and CS3 (Su et al. 2006; Ferreira et al. 2006; Pena et al. 2008). Reference samples (GT1, PTG, CTG, TgCgCa1, MAS, and TgCatBr5) were used as controls of the restrictions.

After the final nested PCR products were obtained, RFLP-PCR was performed. Amplification and digestion of the 18S rRNA gene were used for the differential diagnosis of *Neospora caninum*, *Hammondia hammondi*, and *Sarcocystis* spp. (Silva et al. 2009). Next, the RFLP-PCR product was homogenized with a bromophenol blue solution and visualized after running on a 2.5% agarose gel (3% for the Apico marker) stained with ethidium bromide. The gel images were recorded with a digital photodocumentation system (GelDoc-It®, UVP, USA) under ultraviolet light and on a specific computer. The cut profiles of the samples were compared with those of the reference strains. The identified new genotypes were deposited in the ToxoDB virtual database (http://toxodb.org) and released on 8 May 2014.

Histopathology and immunohistochemistry

For the evaluation of histological changes in mice associated with infection by each identified *T. gondii* genotype, samples of the brain, heart, lungs, spleen, and liver were collected. These samples were fixed in 10% neutral buffered formalin, processed routinely for embedding in paraffin (Carson and Cappellano 2015), and subsequently submitted to histopathology (HE) and immunohistochemistry (IHC) techniques. For the HE technique, 5-μm-thick sections were cut from paraffin blocks and stained with hematoxylin-eosin (Carson and Cappellano 2015).

For IHC, 5-μm-thick sections of the same paraffin blocks were mounted on silanized slides and processed according to the protocol described by Casartelli-Alves et al. (2014). According to this protocol, the slides were incubated with the primary polyclonal anti–*T. gondii* antibody (rabbit) (Cell Marque, USA) diluted 1:50, overnight at 4 °C. This antibody detects cysts, pseudocysts, and tachyzoites of *T. gondii*. The Novo Link Max Polymer Detection System® (Novocastra, UK) was used for the detection of *T. gondii* according to manufacturer instructions.

The detection of *T. gondii* cysts and tachyzoites by IHC was described together with the histological changes observed in the HE technique. Ten negative control mice, corresponding to each of the 10 chicken batches examined in different periods, and 36 positive mice with identified *T. gondii* genotypes, corresponding to each chicken isolate, had their organs evaluated by the HE and IHC techniques. The inflammatory infiltrate in tissues was classified as follows: granulomatous, predominance of cells of the monocyte-macrophage system (activated macrophages, epithelioid macrophages, or multinucleate giant cells), non-granulomatous, predominance of other types of inflammatory cells (lymphocytes, plasma cells, and neutrophils). Based on the results of HE and IHC in the 5 examined organs of each mouse, the following severity classification of histological changes was used for the studied genotypes: mild, a positive organ for *T. gondii* and with inflammation, without necrosis; moderate, two positive organs for *T. gondii* and with inflammation, without necrosis; severe, three positive organs for *T. gondii* and with inflammation and/or necrosis; very severe, four to five positive organs for *T. gondii* and with inflammation and/or necrosis.

An exploratory approach was used for analysis of the data, describing the frequencies of the circulating *T. gondii* genotypes isolated by municipality and farm, in addition to the frequencies of histological changes for the studied genotypes.

Results

Seventeen different genotypes were identified among the 36 *T. gondii* isolates obtained, all of them atypical and nine described for the first time (Tables 1 and 2). A single genotype was obtained per isolate. The new identified genotypes had u-1 alleles for markers SAG1 (TgCkBrRj3, TgCkBrRj4, TgCkBrRj15, and TgCkBrRj22 TgCkBrRj28), C22-8 (TgCkBrRj15 and TgCkBrRj28), and CS3 (TgCkBrRj2, TgCkBrRj3, TgCkBrRj4, TgCkBrRj15, TgCkBrRj21, and TgCkBrRj22).

The 36 chicken isolates of *T. gondii* were obtained from 22 (39%) of the 57 rural farms investigated (Table 3). The distribution of genotypes by farm and by municipality is shown in Table 3 and Online Resource 1. Table 3 also shows the number of isolates and cumulative morbidity. For all genotypes found, with the exception of one isolate, the mice were euthanized between 8 and 17 days after infection (Online Resource 1). In 31/36 isolates (86%), euthanasia was performed within 14 days post-infection. This euthanasia before the preestablished end of the observation period (45–50 days post-infection) was necessary because at least one mouse in the same box had clinical signs: piloerection, prostration, abdominal distension, dyspnea, or motor incoordination.

The cumulative morbidity indices for each *T. gondii* genotype found are described in Table 3. The cumulative morbidity index was high for the TgCkBrRj2, TgCkBrRj4, TgCkBrRj9, TgCkBrRj9, TgCkBrRj15, TgCkBrRj21, TgCkBrRj22, TgCkBrRj28, TgCatBr1, TgCkBr13, TgCkBr37, and TgCkBr89 genotypes; moderate for TgCkBrRj3, TgCkBrRj6, TgCkBr11, and TgCkBr59; and low for TgDgBr6 (N15-N19) and TgCkBr10.

All genotypes found were able to cause necrosis and/or inflammation in mice in, at least, one of the organs examined (Fig. 1). The inflammation type observed in parasitized organs was granulomatous. The histological changes and their frequencies in the different organs of mice associated with each *T. gondii* genotype identified are described in Tables 4 and 5. The histological changes associated with *T. gondii* genotypes in mice were mild for the TgCkBr59 genotype; moderate for the TgCkBrRj15, TgCkBr11, and TgCkBr13 genotypes; severe for the TgCkBrRj2, TgCkBrRj6, TgCkBrRj21, TgCkBrRj22, TgCkBrRj28, TgCkBr37, TgCatBr1, TgCkBr89, and TgCkBr10 genotypes; and very severe for the TgCkBrRj3, TgCkBrRj4, TgCkBrRj9, and TgDgBr6 (N15-N19) genotypes. The mice in the control group showed no histological changes and were negative for *T. gondii* tissue cysts and tachyzoites in the HP and IHC techniques.

Regarding the CS3 marker, the u-1 allele was observed in 10/17 (59%) genotypes, allele I in 6/17 (35%) genotypes, and allele III in 1/17 (6%) genotype, and allele II was not observed. The distribution of the CS3 marker allele types according to cumulative morbidity and the severity of histological changes is shown in Table 6.

Discussion

The most frequent genotype observed in this study, with 10 isolates, was TgCkBrRj3, a new genotype, which also had the widest geographic distribution. This genotype is very similar to the ToxoDB #206 genotype. The latter genotype was commonly reported in chickens and newborn humans with congenital toxoplasmosis in Minas Gerais and Espírito Santo states (southeastern Brazil) (Carneiro et al. 2013; Pena et al. 2013; Ferreira et al. 2018) and was recently detected in pigs in northern Paraná state, southern Brazil (Miura et al. 2019). These two genotypes only differ in the CS3 marker, in which the ToxoDB #206 genotype has the II allele and TgCkBrRj3 has the u-1 allele. Regarding virulence in mice, the ToxoDB #206 genotype is virulent or intermediate virulent (Carneiro et al. 2013; Ferreira et al. 2018). Although virulence was not calculated in the present study, the TgCkBrRj3 genotype was associated with a moderate cumulative morbidity index and caused very severe histological changes in mice, which demonstrates that it is potentially virulent for these animals. The TgDgBr6 (N15-N19) genotype (ToxoDB #51), previously reported in chickens and pigs in São Paulo state (Dubey et al. 2007; Dubey et al. 2012), was the second most frequent and was also found in more than one farm and municipality,

Table 1 New genotypes of *Toxoplasma gondii* obtained from chicken isolates identified by the RFLP-PCR technique, from the municipalities of Maricá, Rio Bonito, and Itaboraí, Rio de Janeiro state, Brazil (2009 to 2011)

Genotypes	*N* of isolates	PCR-RFLP markers											
		SAG1	5′ + 3′ SAG2	aSAG2	SAG3	Btub	GRA6	C22-8	C29-2	L358	PK1	Apico	CS3
TgCkBrRj3	10	u-1	I	II	III	III	III	II	III	I	III	I	u-1
TgCkBrRj6	3	I	I	I	III	I	III	II	I	I	I	I	I
TgCkBrRj4	2	u-1	I	II	III	III	II	III	I	I	II	I	u-1
TgCkBrRj2	1	I	III	III	III	I	II	I	I	I	I	I	u-1
TgCkBrRj9	1	I	I	II	III	I	II	I	I	I	I	I	I
TgCkBrRj15	1	u-1	I	II	III	III	III	u-1	I	I	I	I	u-1
TgCkBrRj21	1	I	III	III	III	III	III	I	I	I	III	I	u-1
TgCkBrRj22	1	u-1	I	II	III	III	III	II	III	III	III	I	u-1
TgCkBrRj28	1	u-1	I	II	III	III	I	u-1	I	I	I	I	I

Table 2 *Toxoplasma gondii* genotypes identified by RFLP-PCR that were isolated from chickens in the present study and previously described in different hosts in Brazil and in different parts of the world. *N* number

Genotype	ToxoDB PCR-RFLP genotype no.	*N* of isolates	Hosts	Geographic distribution	References for isolates
TgDgBr6	51	5	Dogs, chickens	Brazil (states: SP, RJ)	Dubey et al. (2007); Dubey et al. (2012); Dubey et al. (2020).
TgCatBr1 (type BrII)	11	2	Dogs, chickens, rabbits, cats, capybaras, wood rats, maned wolves, sheeps, wild birds, humans	Brazil (states: MG, RJ, PR, SP, RS), Ghana, Argentina, USA, Germany	Vitaliano et al. (2014); Silva et al. (2014); Dubey et al. (2007, 2008, 2011); Pena et al. (2008); Yai et al. (2009); Silva et al. (2011); Carneiro et al. (2013); Ayi et al. (2016); Rêgo et al. (2018); Maksimov et al. (2013); Dubey et al. (2020); ToxoDB
TgCkBr59	36	2	Chickens, humans	Brazil (states: ES, RJ, MG, BA)	Dubey et al. (2008); Ferreira et al. (2018); Carneiro et al. (2013); Dubey et al. (2020); ToxoDB
TgCkBr11 (type BrIII)	8	2	Chickens, cats, dogs, capybaras, sheeps, wood fox, wild birds, humans, opossum, pigs	Brazil (states: SP, PR, RO, MS, MG, MT, BA), Venezuela, USA	Yai et al. (2009); Pena et al. (2008); Su et al. (2006); Dubey et al. (2008); Ragozo et al. (2008); Soares et al. (2011); Carneiro et al. (2013); Rêgo et al. (2018); Witter et al. (2020); Dubey et al. (2020); ToxoDB
TgCkBr10 (type BrI)	6	1	Chickens, humans, cats, dogs, sheeps, goats, capybaras, armadillos, eared doves	Brazil (states: ES, SP, MA, MG, RJ, PR, PA, RO, MS, MT), Belgium, France, Gabon, Cameroon, Turkey	Ferreira et al. (2011); Silva et al. (2014); Dubey et al. (2007, 2008); Pena et al. (2008); Su et al. (2006); Ragozo et al. (2008); Soares et al. (2011); Pena et al. (2013); Yai et al. (2009); Barros et al. (2014); Vitaliano et al. (2014); Sousa et al. (2016); Vieira et al. (2018); Ferreira et al. (2018); Witter et al. (2020); Dubey et al. (2020); ToxoDB
TgCkBr13	63	1	Chickens	Brazil (state: SP)	Dubey et al. (2008); Dubey et al. (2020).
TgCkBr37	107	1	Chickens	Brazil (state: RJ)	Dubey et al. (2008); Dubey et al. (2020)
TgCkBr89	65	1	Chickens, cats, humans, pigs, eared doves	Brazil (States: SP, RJ, PE, ES, PR)	Dubey et al. (2008); Pena et al. (2008); Ferreira et al. (2011); Pena et al. (2013); Barros et al. (2014); Samico-Fernandes et al. (2015); Dubey et al. (2020)

Table 3 Distribution and cumulative morbidity of *Toxoplasma gondii* genotypes in 57 farms in the municipalities of Maricá, Rio Bonito, and Itaboraí, Rio de Janeiro state, Brazil (2009–2011)

T. gondii genotypes	Municipality	Farms (total = 57), *n* (%)	*T. gondii* isolates (total = 36), *n* (%)	Cumulative morbidity (%)
TgCkBrRj3	A, B, C	8 (14%)	10 (27%)	42/50 (84%)
TgDgBr6 (N15-N19)	A, B	4 (7%)	5 (14%)	5/25 (20%)
TgCatBr1	B	2 (4%)	2 (5%)	10/10 (100%)
TgCkBrRj2	A	1 (2%)	1 (2%)	5/5 (100%)
TgCkBrRj6	B	1 (2%)	3 (8%)	5/15 (33%)
TgCkBrRj4	B	1 (2%)	2 (5%)	10/10 (100%)
TgCkBr59 (B29)	B	1 (2%)	2 (5%)	5/10 (50%)
TgCkBr11	B	1 (2%)	2 (5%)	5/10 (50%)
TgCkBrRj9	B	1 (2%)	1 (3%)	5/5 (100%)
TgCkBrRj15	B	1 (2%)	1 (3%)	5/5 (100%)
TgCkBrRj21	B	1 (2%)	1 (3%)	5/5 (100%)
TgCkBrRj22	B	1 (2%)	1 (3%)	5/5 (100%)
TgCkBrRj28	B	1 (2%)	1 (3%)	5/5 (100%)
TgCkBr10	B	1 (2%)	1 (3%)	1/5 (20%)
TgCkBr13 (C1)	B	1 (2%)	1 (3%)	5/5 (100%)
TgCkBr37 (B10)	B	1 (2%)	1 (3%)	5/5 (100%)
TgCkBr89 (B50)	B	1 (2%)	1 (3%)	5/5 (100%)

[A] Rio Bonito; [B] Maricá; [C] Itaboraí. *Total*, total number; *n*, number

demonstrating that it is common in the region studied and that it circulates in the southeast of Brazil.

Rio de Janeiro state has one of the largest numbers of *T. gondii* isolates reported in Brazil, although only chickens and pigs from Campos dos Goytacazes municipality, one of the 92 municipalities existing in the state, were investigated for these circulating genotypes of the parasite (Silva et al. 2003; Dubey et al. 2008; Frazão-Teixeira et al. 2011; Dubey et al. 2012). In the different geographic regions evaluated in the present study, the detection of nine new genotypes and the first description in the state of the already known genotypes TgCkBr11 (ToxoDB #8) and TgCkBr13 (ToxoDB #63) confirm that the genetic variability of *T. gondii* in Rio de Janeiro state is high and is still little known.

Considering this knowledge gap and the fact that the metropolitan region is the most populous in the state and one of the most populous in Brazil, new studies are needed to evaluate the genotypes that circulate among humans. It is also important that such studies assess the relationship of these human genotypes with those that circulate in animals, as well as their association with the clinical forms of human toxoplasmosis in the region. Silva et al. (2014) found overlapping *T. gondii* genotypes that affect humans and animals, suggesting the existence of a common source for both.

All genotypes found in the present study were atypical and some of them have already been described by other authors, including genotypes type BrI (TgCkBr10), type BrII (TgCatBr1), type BrIII (TgCkBr11), and type BrIV (TgDgBr6, TgCkBr13, TgCkBr37, TgCkBr59, TgCkBr89) (Pena et al. 2008; Dubey et al. 2012). The findings differ from other studies carried out in Brazil, in which most of the genotypes found in chickens were the BrI type, followed by the BrII and BrIII genotypes, and confirm the high genetic variability and low prevalence of clonal types I, II, and III in the country (Dubey et al. 2012; Pena et al. 2013; Ferreira et al. 2018). Of the genotypes described that are previously known, all have been found in chicken isolates, including genotypes TgCatBr1 (ToxoDB #11), TgCkBr10 (ToxoDB #6), TgCkBr11 (ToxoDB #8), TgDgBr6 (N15-N19) (ToxoDB #51), and TgCkBr89 (ToxoDB #65) already described in humans as shown in Table 2.

The results agree with those of Sibley et al. (2009) who observed similar genotypes in men and animals in Brazil, suggesting that genetic diversity is much more related to geographical differences than to differences between hosts. However, another factor that must be considered is that recent studies have shown a correlation between the geographic distribution of isolates and the presence of more severe clinical signs in immunocompetent individuals (Hamilton et al. 2019). For example, cases of severe systemic toxoplasmosis that resulted in the death of immunocompetent hosts infected with atypical strains have been reported in South America (Carme et al. 2009). There is a predominance of virulent strains in Brazil compared to Europe, where clonal genotypes predominate. In addition, the severity of ocular toxoplasmosis cases is greater in Brazil than in Europe, which suggests that observations of experimental virulence of atypical strains in mice could be extrapolated to humans (Hamilton et al. 2019).

Table 4 Positivity for *Toxoplasma gondii* new genotypes obtained in the present study and their histological changes in different mice organs submitted to the bioassay and examined by histopathology and immunohistochemistry technique

Genotypes	No. of examined mice	Histological changes	Examined organs				
			Brain	Lung	Heart	Spleen	Liver
TgCkBrRj2	1	Positive	0	1 (100%)	0	0	1 (100%)
		Necrosis	0	1 (100%)	0	1 (100%)	1 (100%)
		Inflammation	0	1 (100%)	0	0	1 (100%)
TgCkBrRj3	10	Positive	1 (10%)	8 (80%)	1 (10%)	8 (80%)	7 (70%)
		Necrosis	0	0	2 (20%)	7 (70%)	3 (30%)
		Inflammation	0	10 (100%)	4 (40%)	10 (100%)	8 (80%)
TgCkBrRj4	2	Positive	0	1 (50%)	1 (50%)	2 (100%)	1 (50%)
		Necrosis	0	0	1 (50%)	1 (50%)	1 (50%)
		Inflammation	0	2 (100%)	1 (50%)	2 (100%)	2 (100%)
TgCkBrRj6	3	Positive	2 (66%)	3 (100%)	0	3 (100%)	3 (100%)
		Necrosis	0	1 (33%)	0	3 (100%)	2 (66%)
		Inflammation	0	3 (100%)	0	3 (100%)	3 (100%)
TgCkBrRj9	1	Positive	0	1 (100%)	1 (100%)	1 (100%)	1 (100%)
		Necrosis	0	0	1 (100%)	1 (100%)	0
		Inflammation	0	1 (100%)	1 (100%)	1 (100%)	1 (100%)
TgCkBrRj15	1	Positive	0	1 (100%)	0	1 (100%)	1 (100%)
		Necrosis	0	0	0	0	0
		Inflammation	0	0	0	1 (100%)	1 (100%)
TgCkBrRj21	1	Positive	0	1 (100%)	0	1 (100%)	1 (100%)
		Necrosis	0	0	0	1 (100%)	0
		Inflammation	0	1 (100%)	0	1 (100%)	1 (100%)
TgCkBrRj22	1	Positive	0	1 (100%)	1(100%)	1 (100%)	1 (100%)
		Necrosis	0	0	1 (100%)	0	1 (100%)
		Inflammation	0	1 (100%)	1 (100%)	0	1 (100%)
TgCkBrRj28	1	Positive	0	1 (100%)	1 (100%)	1 (100%)	1 (100%)
		Necrosis	0	0	1 (100%)	0	1 (100%)
		Inflammation	0	0	1 (100%)	1 (100%)	1 (100%)

Positive, presence of *T. gondii* cysts or tachyzoites

In previous studies, a case of atypical human toxoplasmosis was detected in Maricá, and high environmental contamination with *T. gondii* was observed in Rio Bonito and Maricá, two of the municipalities investigated in the present study. In these cases, cats were present on 70.6% of the farms studied, 66.7% were located near water sources, and 5% were located in or near dense vegetation. In addition, the human population in the region studied consumed untreated water on 41.2% of the farms and the water supplied to the animals was also untreated (Casartelli-Alves et al. 2015; Luciano et al. 2011; Neves Ede et al. 2011). The presence of cats contaminating the environment together with the action of environmental factors such as wind and rain can promote the dispersion of *T. gondii* oocysts. Therefore, the oocysts of this parasite may contaminate water sources in the region that are used for consumption by the population without any treatment, causing the dispersion of genotypes among the hosts and contributing to the genetic diversity of the protozoan (Casartelli-Alves et al. 2015). Consequently, the genotypes found in the present study in chickens may be circulating among humans and other animals in the region, which needs to be confirmed in future studies.

Although this study described the presence of atypical genotypes in chickens and it is unclear whether a given *T. gondii* isolate exhibits the same virulent or avirulent behavior in humans as observed in mice, the observation of human toxoplasmosis in the same location where the presence of atypical genotypes is described in animals should be interpreted with caution. According to Ferreira et al. (2018), atypical strains can develop new mechanisms of pathogenicity. In addition, the fact that undercooked chicken hearts are frequently consumed at barbecues in several Brazilian states, including Rio de Janeiro state, increases the probability that these animals are acting as

Table 5 Positivity for *Toxoplasma gondii* genotypes previously described by other authors and obtained in the present study and their histological changes in different mice organs submitted to the bioassay and examined by histopathology and immunohistochemistry technique

Genotypes	*N* of examined mice	Histological changes	Examined organs				
			Brain	Lung	Heart	Spleen	Liver
TgDgBr6 (N15-N19)	5	Positive	1 (20%)	2 (40%)	1 (20%)	5 (100%)	5 (100%)
		Necrosis	0	0	1 (20%)	2 (40%)	3 (60%)
		Inflammation	0	3 (60%)	0	3 (60%)	5 (100%)
TgCkBr37	1	Positive	0	1 (100%)	0	1 (100%)	1 (100%)
		Necrosis	0	0	0	1 (100%)	0
		Inflammation	0	1 (100%)	0	1 (100%)	1 (100%)
TgCatBr1	2	Positive	0	2 (100%)	0	2 (100%)	1 (50%)
		Necrosis	0	0	0	2 (100%)	1 (50%)
		Inflammation	0	2 (100%)	0	2 (100%)	1 (50%)
TgCkBr89	1	Positive	0	1 (100%)	0	1 (100%)	1 (100%)
		Necrosis	0	0	0	1 (100%)	1 (100%)
		Inflammation	0	1 (100%)	0	1 (100%)	1 (100%)
TgCkBr11	2	Positive	0	1 (50%)	0	1 (50%)	1 (50%)
		Necrosis	0	0	0	0	0
		Inflammation	0	2 (100%)	0	0	2 (100%)
TgCkBr10	1	Positive	0	1 (100%)	0	1 (100%)	1 (100%)
		Necrosis	0	1 (100%)	0	1 (100%)	1 (100%)
		Inflammation	0	1 (100%)	0	1 (100%)	1 (100%)
TgCkBr59 (B29)	2	Positive	0	2 (100%)	1 (50%)	2 (100%)	2 (100%)
		Necrosis	0	1 (50%)	0	2 (100%)	1 (50%)
		Inflammation	0	2 (100%)	0	2 (100%)	1 (50%)
TgCkBr13 (C1)	1	Positive	1 (100%)	1 (100%)	0	1 (100%)	0
		Necrosis	0	0	0	0	0
		Inflammation	0	1 (100%)	1 (100%)	1 (100%)	1 (100%)

N, number; *Positive*, presence of *T. gondii* cysts or tachyzoites

carriers of highly pathogenic strains for humans (Ferreira et al. 2018).

All genotypes in the present study caused clinical signs and histological changes in mice, with the majority being associated with a high cumulative morbidity index (65%) and severe or very severe histological changes (76%), demonstrating that they are potentially virulent for these animals. These results corroborate other studies that also found the following genotypes to be virulent for mice: TgCatBr1 (ToxoDB#11), TgCkBr10 (ToxoDB #6), TgCkBr89 (ToxoDB #65), TgCkBr59 (ToxoDB #36), and TgDgBr6 (N15-N19) (ToxoDB #51) (Dubey et al. 2007; Pena et al. 2008; Silva et al. 2014; Pinheiro et al. 2015; Ferreira et al. 2018; Hamilton et al. 2019). Virulence of the TgCkBr13 (ToxoDB #63) and TgCkBr 37 (ToxoDB #107) genotypes for mice has not been reported. However, the TgCkBr11 genotype (ToxoDB #8), which is considered avirulent for mice (Pena et al. 2008; Silva et al. 2014), was potentially virulent for these animals in the present study.

In a virulence study, Pinheiro et al. (2015) also found that the ToxoDB #8 genotype is not always avirulent for mice and may exhibit intermediate virulence related to biological differences within the same genotype. The dose of the parasite inoculum may also have contributed to the virulence of the ToxoDB #8 genotype in this study. However, the present study has limitations since it was not a virulence study and the parasite inoculum dose was not calculated. In addition, few mice were submitted to histopathological examination.

The clinical signs observed in the mice of the present study agree with those reported by Sudan et al. (2015). Regarding histopathology, the presence of necrosis and an inflammatory infiltrate in different organs of experimentally infected mice associated with *T. gondii* has been reported previously, corroborating the results found (Sudan et al. 2015; Pinheiro et al. 2015; Hamilton et al. 2019). In the present study, the largest number of histological changes was observed in the spleen, followed by the liver and lungs, results similar to that reported by Pinheiro et al. (2015).

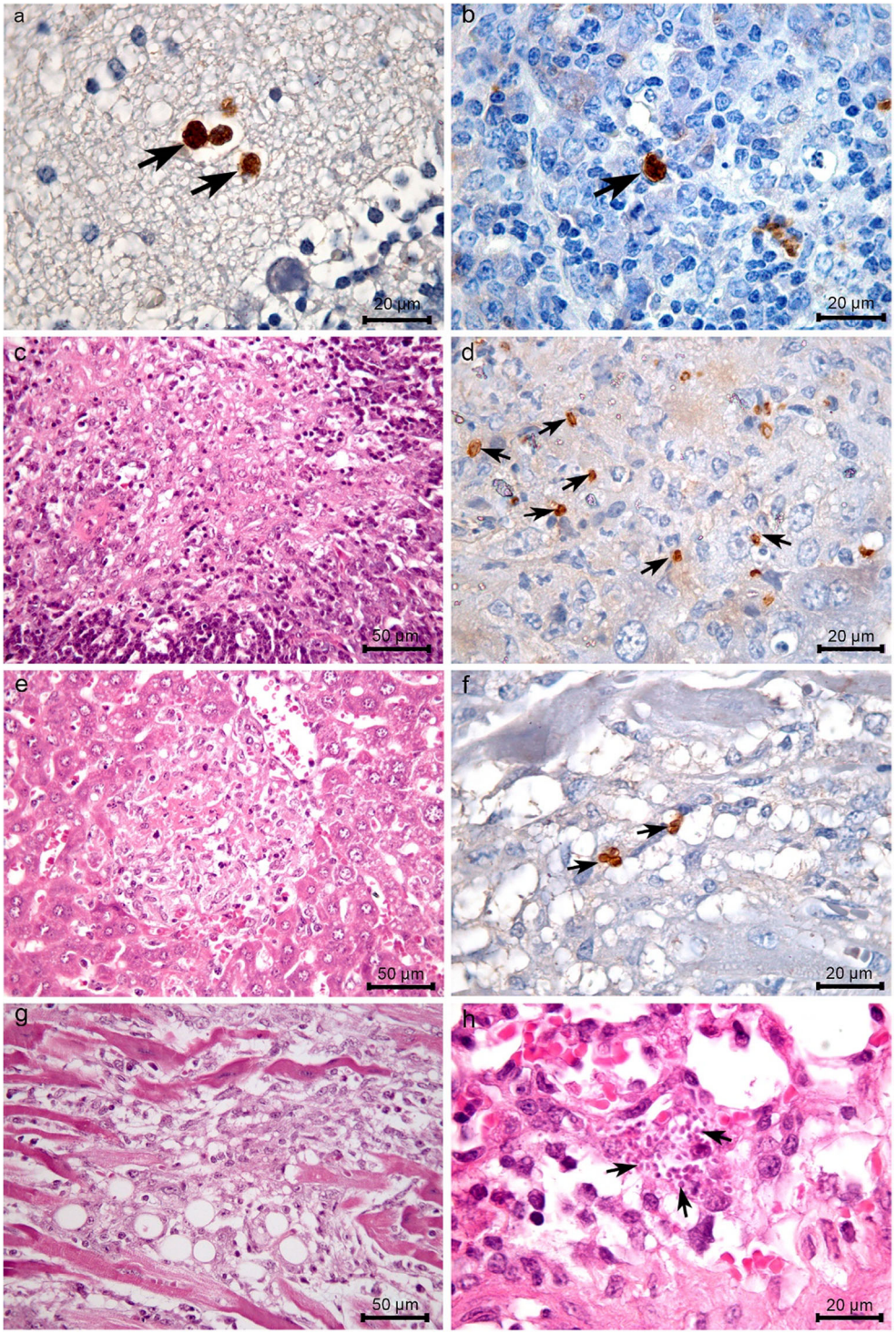

Fig. 1 Mice histological changes associated with *Toxoplasma gondii* isolates obtained from free-range chickens reared in the metropolitan region of Rio de Janeiro state, Brazil (2009–2011). **a** *T. gondii* cysts (TgCkBrRj3 genotype) marked in brown (arrows) in the cerebellum, 16 days p.i. **b** *T. gondii* pseudocyst (genotype TgCkBrRj3) marked in brown (arrows) on the spleen, 16 days p.i. **c** Focus of necrosis and granulomatous inflammation in the spleen associated with the TgCkBrRj6 genotype, 8 days p.i. **d** *T. gondii* tachyzoites (genotype TgCkBrRj6) marked in brown (arrows) on the liver, 8 days p.i. **e** Focus of necrosis and granulomatous reaction in the liver associated with the TgCkBrRj6 genotype, 8 days p.i. **f** *T. gondii* tachyzoites (TgCkBrRj6 genotype) marked in brown (arrows) in the heart, 12 days p.i. **g** Focus of necrosis and granulomatous reaction in the heart associated with the TgCkBrRj6 genotype, 12 days p.i. **h** Several *T. gondii* tachyzoites (TgCkBr37 genotype) in the cytoplasm of macrophages (arrows) associated with granulomatous inflammation, 11 days p.i. Hematoxylin-eosin staining (**c**, **e**, **g**, **h**); immunohistochemistry (**a**, **b**, **d**, **f**)

Silva et al. (2008) experimentally infecting mouse brains with *T. gondii* using blood, amniotic fluid, and cerebrospinal fluid samples obtained from newborns and women with clinical and laboratory signs of toxoplasmosis observed edema, an inflammatory infiltrate, and necrosis in the brains of these animals. However, in the present study, mouse brain was the tissue with the lowest frequency of parasitism by *T. gondii* and no histological changes were observed, in agreement with Pinheiro et al. (2015) and Hamilton et al. (2019). We believe that the absence of histological changes in this organ was possibly caused by a low parasite load related to the early euthanasia of the animals. According to Dubey et al. (2010), *T. gondii* begins to disappear from visceral organs and becomes more

Table 6 Allele type distribution of CS3 marker of *Toxoplasma gondii* isolates, obtained from chickens in the state of Rio de Janeiro metropolitan region, Brazil, in relation to the cumulative morbidity and severity of mice histological changes

Mice	Classification	Allele type in marker CS3				Total
		I	II	III	u-1	
Morbidity	100%	3	0	0	8	11
	33 to 84%	2	0	1	1	4
	20%	1	0	0	1	2
Histological alteration	Mild	1	0	0	0	1
	Moderate	1	0	1	1	3
	Severe	3	0	0	6	9
	Very severe	1	0	0	3	4

common in brain associated with lesions over 14 days post-infection.

Experimental studies on *T. gondii* in mice reported that the CS3 marker is related to virulence and mortality when associated with the I and u-1 alleles, while it is related to survival when associated with the III allele (Pena et al. 2008; Yai et al. 2009; Dubey et al. 2010). Similarly, in the present study, the genotypes carrying the I and u-1 alleles of the CS3 marker were potentially virulent and exhibited high morbidity and severe histological changes in most mice. However, in contrast to the literature, the TgCKBr11 genotype (ToxoDB #8), which carries the III allele of the CS3 marker, was potentially virulent and exhibited moderate morbidity and moderate histological changes. The virulence of *T. gondii* in mice with unknown parasite load is commonly classified according to the frequency of mortality (Pena et al. 2008). However, it was not possible to calculate mortality in this study since the animals were euthanized. The divergences between the present study and the literature may be related to the inoculation route, the infectious parasite load, and the fact that morbidity and histological changes were analyzed in the infected mice; these last three factors were not considered in the classification proposed by Pena et al. (2008).

Conclusion

The exclusive identification of atypical genotypes, with a predominance of new genotypes, indicates great genetic diversity of *T. gondii* in the region studied. In addition, all identified genotypes caused clinical signs and histological changes in mice that were often severe, suggesting relevant potential virulence of the strains. Further studies are needed to identify new strains and to investigate their virulence potential for other animal species and for humans in the region.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information file.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This project was approved by the Ethics Committee on Animal Use of the Oswaldo Cruz Foundation (CEUA/Fiocruz) under license number L-012/09.

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

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