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PROTOZOOLOGY - ORIGINAL PAPER

Outbreak of toxoplasmosis in a flock of domestic chickens (*Gallus gallus domesticus*) and guinea fowl (*Numida meleagris*)

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Abstract

Toxoplasmosis is a disease with a worldwide distribution that affects a wide variety of animal species, though with rare descriptions in chickens. We describe the clinical, epidemiological, pathological, and molecular aspects of a toxoplasmosis outbreak in domestic chickens and guinea fowl in southern Brazil. The flock was composed of 47 domestic chickens and 29 guinea fowl. Of these, 22 birds showed clinical signs of lethargy, anorexia, and neurological signs over a clinical course of 24–72 h, and 15 died. Epidemiological data were obtained through fieldwork performed at the chicken farm and necropsies of six birds. Gross lesions were absent at necropsy, and histopathological findings included inflammatory infiltrate of macrophages, lymphocytes, and plasma cells and necrosis in several tissues associated with intralesional *Toxoplasma gondii*. Immunohistochemistry for *T. gondii* was positive. Additionally, restriction fragment length polymorphism (RFLP) analysis with 11 markers (SAG1, SAG2 (5′3′SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico, and CS3) and microsatellite (MS) analysis with 15 markers (TUB2, W35, TgMA, B18, B17, M33, IV.1, XI.1, N60, N82, AA, N61, N83, M48, and M102) were performed. PCR-RFLP revealed *T. gondii* genotype ToxoDB-PCR-RFLP #280, and MS analysis also showed a unique genotype. This is the first description of this genotype in chickens and adds to the evidence suggesting considerable genotypic diversity of *T. gondii* in Brazil.

Keywords Diseases of poultry · Protozoal diseases · Genotyping · PCR-RFLP · Microsatellite · Pathology

Introduction

Toxoplasmosis is an important zoonotic disease caused by *Toxoplasma gondii*, an apicomplexan coccidia (Vesco et al. 2007) with a heterogeneous life cycle. The only known species of the genus *Toxoplasma*, *T. gondii* has three infectious stages: oocysts, tachyzoites, and bradyzoites (Dubey 2008). Domestic and wild cats are the definitive hosts that excrete oocysts in the environment, and other species of mammals, including humans and birds, serve as intermediate hosts harboring tissue cysts (Tenter et al. 2000). Infection in humans and animals occurs mainly through ingestion of oocysts in contaminated food or water, usually resulting in subclinical disease. Congenital toxoplasmosis can also occur in several species (Dubey and Jones 2008; Dubey 2008; Dubey and Lappin 2015).

Natural infections by *T. gondii* in free-range chickens are important because they serve as sentinels for detecting soil

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contamination with oocysts due to their alimentary habits (Dubey 2008). However, clinical toxoplasmosis in poultry is rare because they rarely develop clinical signs of the disease (Dubey 2010), and there are few reports describing characteristics and morphological alterations associated with toxoplasmosis in poultry due to acute infection with *T. gondii* (Jones et al. 2012). Nevertheless, genetic characterization of *T. gondii* in this species has been well reported (Dubey et al. 2002, Dubey et al. 2007a). Among the highest genotypic diversity of *T. gondii* occurs in Brazil, and chickens have been widely employed to isolate and characterize, both biologically and genotypically, the agent on a worldwide scale (Shwab et al. 2014).

This report describes the epidemiological, clinical, pathological, immunohistochemical, and molecular findings of an outbreak of toxoplasmosis in domestic chickens (*Gallus gallus domesticus*) and guinea fowl (*Numida meleagris*) of a flock in southern Brazil.

Materials and methods

Epidemiological and clinical investigation

An outbreak of toxoplasmosis occurred on a small chicken farm in a rural area of southern Brazil (30° 4′ 51″ S, 51° 1′ 22″ W) over a period of 60 days during June–July 2016. On the farm, there were 47 and 29 free-range domestic chickens and guinea fowl, respectively. Of these, 22 adult poultry (13 domestic chickens and nine guinea fowl) showed clinical signs, and 15 died (nine domestic chickens and six guinea fowl). The birds were obtained from different areas of southern Brazil and were fed corn grain and rice bran. The grain was dropped on the ground or placed in community troughs. Cats on the premises were in direct contact with the flock of chickens. Epidemiological and clinical data were obtained during an on-site visit to the affected farm and by several interviews with the owner. A total of 10 birds were evaluated using different diagnostic methods, the results of which are summarized in Table 1.

Pathological and immunohistochemical examination

Ten birds were evaluated in this study: a necropsy following natural death was performed on six (two helmeted guinea fowl [1, 2] and four domestic chickens [3–6]), and serology was performed for four animals which were not necropsied (domestic chickens [7–9] and guinea fowl [10]). Fragments of different organs (skeletal muscle, spleen, myocardium, kidney, bone marrow, small intestine, lung, liver, and central nervous system [CNS]) were sampled, stored in 10% buffered formalin, routinely processed for histology, and stained with hematoxylin and eosin. Fragments of these tissues were also stored at 4 °C for isolation and molecular analysis.

To detect *T. gondii*, immunohistochemical analysis was performed on selected sections of several tissues (bone marrow, brain, liver, lung, myocardium, small intestine, and spleen) obtained from all necropsied birds (1–6) and embedded in paraffin blocks. For antigen retrieval, the samples were incubated for 10 min with a polyclonal antibody (VRMD, Pullman, WA, USA) at a 1:1000 dilution with 0.1% trypsin. A modified avidin-biotin-peroxidase complex method (LSAB Universal kit, Dako Cytomation, Glostrup, Denmark) was employed using 3-amino-9-tilcarbazoln (AEC, K3469, Dako Cytomation, Glostrup, Denmark) as the chromogen. Histological sections from a dog positive for *T. gondii* were used as a positive control, as previously described by do Nascimento et al. (2017); for negative control, the primary antibody was replaced by phosphate-buffered saline solution (PBS).

Serodiagnosis, isolation, and genotyping of *T. gondii*

To investigate serum anti-*T. gondii* antibodies, blood from four domestic chickens (4, 7–9) and two guinea fowl (1 and 10) was examined using the modified agglutination test (MAT) with a 1:5 cutoff point, according to methods described in a previous publication (Dubey and Desmonds 1987). Domestic chickens 7–9 and guinea fowl 10 were birds on the farm that exhibited no apparent clinical signs; birds 1 and 4 were affected birds that were eventually subjected to necropsy.

Lungs from chickens 4–6 were macerated individually using a mortar and pestle, homogenized with 0.85% saline solution, and separately inoculated subcutaneously into two Swiss Webster mice for each lung homogenate. The brain, heart, and pectoral muscle from chicken 4 were pooled, homogenized in a blender with 0.85% saline, digested in acidic pepsin, and washed; the homogenate was inoculated subcutaneously into another three Swiss Webster mice (Dubey 1998). The inoculated mice showing ruffled fur and decreased activity were humanely euthanized (first sedated, intramuscularly, with a combination of ketamine—100 mg/kg and xylazine—10 mg/kg, and then euthanized in a closed chamber with isoflurane). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in the lungs and/or brain (Dubey 2010). Mouse lungs positive for *T. gondii* were individually macerated and kept at − 20 °C for molecular analysis. The protocols conducted in this study were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine (CEUA/FMVZ/207041215) of the University of São Paulo, Brazil.

For DNA extraction, chicken fragment tissues—CNS (5, 6); spleen, bone marrow, lungs, liver, kidney, skeletal muscle (4, 5, 6); and heart (5, 6)—and lungs from mice used in the

Table 1 Birds evaluated in the study and diagnostic methods performed for *Toxoplasma gondii*

Identification and species	Clinical signs	Necropsy and histopathology	IHC	MAT titers	PCR	Bioassay	RFLP*/MS genotyping
1—*Numida meleagris*	Present	Necrosis and inflammatory infiltrate of macrophages, lymphocytes, and plasma cell-associated parasitic structures	Positive	1280	N/D	N/D	N/D
2—*N. meleagris*	Present	Necrosis and inflammatory infiltrate of macrophages, lymphocytes, and plasma cell-associated parasitic structures	Positive	N/D	N/D	N/D	N/D
3—*Gallus gallus domesticus*	Present	Necrosis and inflammatory infiltrate of macrophages, lymphocytes, and plasma cell-associated parasitic structures	Positive	N/D	N/D	N/D	N/D
4—*G. gallus domesticus*	Present	Necrosis and inflammatory infiltrate of macrophages, lymphocytes, and plasma cells	Positive	2560	Negative	Positive	#280/atypical
5—*G. gallus domesticus*	Present	Necrosis and inflammatory infiltrate of macrophages, lymphocytes, and plasma cells	Positive	N/D	Positive	Positive	#280/atypical
6—*G. gallus domesticus*	Present	Necrosis and inflammatory infiltrate of macrophages, lymphocytes, and plasma cells	Positive	N/D	Negative	Negative	N/D
7—*G. gallus domesticus*	Absent	N/D	N/D	320	N/D	N/D	N/D
8—*G. gallus domesticus*	Absent	N/D	N/D	2560	N/D	N/D	N/D
9—*G. gallus domesticus*	Absent	N/D	N/D	2560	N/D	N/D	N/D
10—*N. meleagris*	Absent	N/D	N/D	10	N/D	N/D	N/D

IHC immunohistochemistry, *MAT* modified agglutination test, *PCR* polymerase chain reaction, *RFLP* restriction fragment length polymorphism, *MS* microsatellite analysis, *N/D* not done; *RFLP genotype number

bioassay were macerated in 0.85% saline solution. Aliquots of 250 μl were then washed in Tris-EDTA buffer (Tris-HCl 10 mM, EDTA 1 mM) by centrifugation at 12,000×*g* for 5 min, and the DNeasy® Blood & Tissue commercial kit (Qiagen) was used according to the manufacturer's protocol.

PCR targeting a repeated 529-bp fragment (REP-529 PCR) of the *T. gondii* genome was used for molecular detection following the protocol of Homan et al. (2000). The positive control consisted of DNA extracted from tachyzoites of the RH reference strain.

DNA extracted from mouse lung homogenates were used for genotyping. Multilocus nested PCR-RFLP genotyping was performed using the markers SAG1, SAG2 (5′3′SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico (Su et al. 2010), and CS3 (Pena et al. 2008). Reference strains RH (type I), PTG (type II), and CTG (type III) and non-archetypal strains TgCgCa1 (Cougar), MAS, and TgCatBr5 were used as positive controls in all reactions. Additionally, isolates were genotyped using 15 microsatellite (MS) markers (TUB2, W35, TgMA, B18, B17, M33, IV.1, XI.1, N60, N82, AA, N61, N83, M48, and M102), as previously described (Ajzenberg et al. 2010), and the results were analyzed using GeneMapper 4.1 software (Applied Biosystems). Reference strain PTG (type II) was used as the positive control.

Results

Clinical, pathological, and immunohistochemical findings

The first birds affected on this farm were guinea fowl, and in general, all showed nonspecific clinical signs, including prostration, apathy, isolation from the flock, and diarrhea. One guinea fowl also exhibited ataxia. The clinical course of the disease was 24–72 h.

A severely poor body condition was the only gross change found at necropsy in four domestic chickens (3–6) and two guinea fowl (1–2); the remaining birds (7–10) had a regular body condition and were unaffected clinically. Histologically, the lesions observed in all birds were similar, with varying degrees of severity. Necrosis and inflammatory infiltrate of macrophages, lymphocytes, and plasma cells were observed

predominantly in the air sacs, myocardium (Fig. 1a), brain, kidney, liver, lungs, small intestine, and spleen. In three birds (1–3), lesions in the brain, lungs, myocardium, and spleen were associated with numerous or isolated parasitic structures that were morphologically consistent with *T. gondii*.

These cysts were approximately 30–50 μm in diameter and filled with numerous elongated basophilic bradyzoites measuring approximately 0.5–1 μm in diameter (Fig. 1b); the diameters were obtained by measuring 10 cysts/bradyzoites from different tissues. *Toxoplasma gondii* cysts and infective stages (tachyzoites/bradyzoites) were observed both free in the parenchyma and within the cytoplasm of macrophages. Other common histological changes observed included fibrin deposition and fibrinoid necrosis of the walls of blood vessels in the affected organs. In addition, random multifocal neuronal necrosis with moderate microgliosis and vacuolization of the white and gray matter was observed in the CNS (Fig. 1c). In the lungs, varying degrees of proliferation of alveolar type II pneumocytes and hyaline membranes were found, and mild hyperplasia of hematopoietic cells associated with multifocal areas of necrosis was observed in the bone marrow of two birds.

Tissues of all six birds submitted for necropsy were positive according to immunohistochemical analysis with a polyclonal anti-*T. gondii* antibody. Variable intensity of immunostaining was found among tissues, including the bone marrow, lungs, brain (Fig. 1d), myocardium, liver, small intestine, and spleen, as well as in tissues from three birds (4–6) in which cysts were not observed on routinely stained histological preparations. *T. gondii* cysts, tachyzoites, and bradyzoites within the cytoplasm of macrophages or free amidst the inflammatory infiltrate were also positive.

Due to the suspicion and confirmation of a diagnosis of toxoplasmosis, the remaining birds were treated by the owner based on his own choice with a sulfa-based antibiotic in the water for 4 days. He reported that the deaths of the birds had ceased but did not provide more detailed information regarding the remaining birds.

Serodiagnosis, isolation, and genotyping of *T. gondii*

Anti-*T. gondii* antibodies were found both in the animals subjected to necropsy (guinea fowl 1 and domestic chicken 4) and in clinically unaffected birds (domestic chickens 7–9 and guinea fowl 10). Titers in the affected birds ranged from 1280 to 2560, and those in the unaffected birds ranged from negative (guinea fowl 10) to 2560. Additional information is provided in Table 1.

Tissues of chickens 4 and 6 were negative by REP-529 PCR, but *T. gondii* was detected in the CNS, skeletal muscle, lungs, liver, kidneys, and heart of chicken 5. Of the three bioassays performed with lung homogenates, *T. gondii* was isolated from chicken 5; only one mouse that became sick at 23 days postinoculation (dpi) was infected, and this isolate was named TgCkBrRS20. Another isolate, named TgCkBrRS21, from chicken 4 was also obtained from the tissue pool (CNS, skeletal muscle and heart), causing disease in the three mice at 15, 19, and 28 dpi.

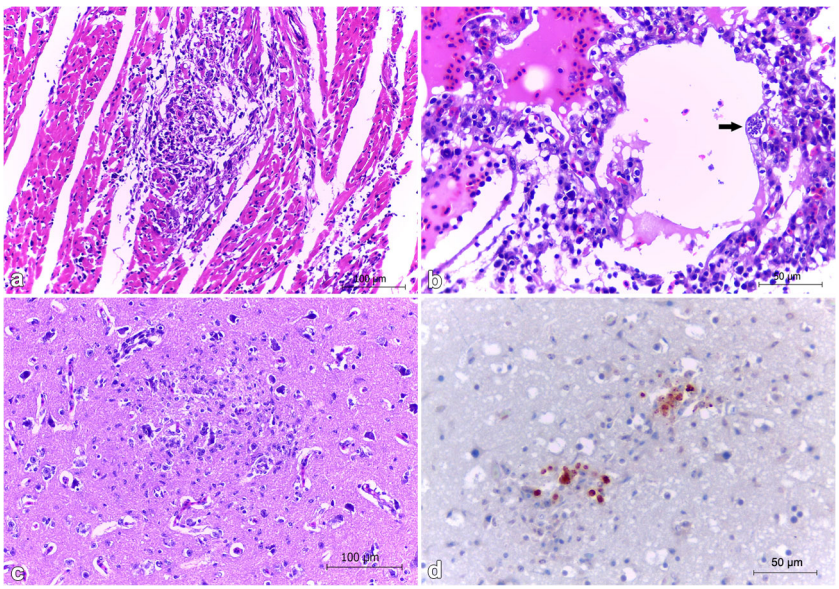

Fig. 1 Outbreak of toxoplasmosis in a flock of domestic chickens and guinea fowl. **a** *Numida meleagris*—heart: focal area with severe necrosis and cardiomyocytes associated with marked inflammatory infiltrate of macrophages, lymphocytes, and plasma cells. Hematoxylin-eosin (H&E) × 20. **b** *Gallus gallus domesticus*—lung: *Toxoplasma gondii* cyst measuring approximately 30 μm in diameter (arrow) located in the cytoplasm of an epithelial cell of a tertiary bronchiole. The cyst is filled with numerous bradyzoites. H&E × 40. **c** *N. meleagris*—brain: the neuropile displayed a focal area of gliosis. H&E × 20. **d** *N. meleagris*—brain: a closer image demonstrating immunostaining of bradyzoites and tachyzoites inside an area of a gliosis. Streptavidin-biotin-peroxidase (IHC) × 40

PCR-RFLP analysis identified an atypical Brazilian genotype (non-archetypal) (ToxoDB PCR-RFLP #280) for both isolates TgCkBrRS20 and TgCkBrRS21: SAG1 = I, SAG2 = I, alt SAG2 = I, SAG3 = I, BTUB = III, GRA6 = III, c22-8 = II, c29-2 = III, L358 = III, PK1 = I, Apico = III, and CS3 = I. To our knowledge, this is the first description of this PCR-RFLP genotype in Brazil and in the world, not only in isolates from chickens but considering all isolates investigated to date.

MS analysis indicated that both isolates have the same non-archetypal genotype with the following allele sequences: TUB2 = 291, W35 = 248, TgMA = 209, B18 = 160, B17 = 348, M33 = 165, IV.1 = 278, XI.1 = 356, N60 = 140, N82 = 111, AA = 265, N61 = 87, N83 = 306, M48 = 213, and M102 = 166. To our knowledge, this is the first description of this allele sequence for MS markers in Brazil or other countries.

Discussion

The diagnosis of toxoplasmosis in the birds in the current study was based on epidemiological, pathological, immunohistochemical, and molecular methods. The association of these data provides a valuable aid for suspecting and diagnosing toxoplasmosis (Dubey et al. 2007b; Jones et al. 2012).

Although clinical disease is uncommon in chickens, this is one of the most susceptible domestic species to *T. gondii* infection (Dubey et al. 2002). Contamination occurs through sporulated oocysts shed in the feces of cats, which consequently contaminate the environment (Ruiz and Frenkel 1980). Due to closer contact with contaminated soil, free-range chickens are more predisposed toward infection than are those raised intensively (Millar et al. 2012). It is possible that the birds in our study had point-source contact with a large number of oocysts, causing several birds to become sick within the same time period; this is not unlikely if one considers that a cat is capable of excreting millions of oocysts in the feces in a single day, which become infective between 1 to 5 days later and are also environmentally resistant (Dubey 2010).

Several factors may be responsible for the clinical manifestations and death of chickens, including the virulence of the strain and individual and environmental factors (Dubey 2009). Indeed, the *T. gondii* strain and dose of inoculum (oocysts) has already been reported as a cause of the development of clinical signs in experimentally infected naïve chickens. Dubey et al. (1993) observed anorexia and death in chickens that were orally inoculated with 100,000 oocysts of the GT1 strain (type I), but no clinical signs were observed when other groups of chickens were inoculated with the same dose of oocysts of the Me-49 strain (type II). Clinical expression of the disease can also occur when the exposed individual is immunosuppressed, and despite the lack of reports of stress causing acute toxoplasmosis in chickens, stress may have been a triggering factor in these cases because these chickens were acquired from different regions and kept as a sizeable crowded flock. Coinfection with the protozoan *Cryptosporidium baileyi* has been implicated in the clinical outcome of experimentally infected chickens (Meireles et al. 1995), but the presence of other parasites was not investigated in the present study.

Clinical signs of toxoplasmosis are rarely observed in Galliformes, and there are few reports of fatal disease caused by natural infection in animals of this order (Howerth and Rodenroth 1985; Quist et al. 1995; Jones et al. 2012; Casagrande et al. 2015). In addition to being rare, clinical manifestations in chickens are nonspecific and include diarrhea and lethargy 2–3 days before death (Biancifiori et al. 1986; Jones et al. 2012). In the current study, the birds showed clinical signs similar to those previously mentioned, and although these signs were nonspecific, they should be taken into account for differential diagnosis of toxoplasmosis. The neurological signs observed in one bird were related to encephalic lesions caused by *T. gondii* (Dubey et al. 2007b).

Aside from a poor body condition, no lesions were observed in the six birds necropsied in the current study. As described, gross lesions are uncommon (Dubey et al. 2007b; Jones et al. 2012), though microscopic findings are valuable for diagnosing the disease. Lesions consisting of necrosis and inflammatory infiltrate of macrophages, lymphocytes and plasma cells associated with intralesional stages of *T. gondii* are frequent changes in toxoplasmosis. The necrosis that occurs in toxoplasmosis is related to intracellular protozoal growth (Dubey and Lappin 2015). Moreover, the association of lesions with intralesional protozoal cysts is essential for definitive diagnosis and suggests an active infection (Casagrande et al. 2015; Dubey and Lappin 2015). As described in other reports (Howerth and Rodenroth 1985; Quist et al. 1995; Jones et al. 2012), we observed a predominance of pathological changes in the brain, liver, lungs, myocardium, small intestine, and spleen in this study, and it is suggested that these are organs of choice for sampling and evaluation for toxoplasmosis diagnosis. Bone marrow necrosis was also observed in this study but is considered an uncommon lesion in birds with toxoplasmosis (Jones et al. 2012).

Immunohistochemistry and histopathological examination are valuable tools for confirming the diagnosis of toxoplasmosis (Quist et al. 1995; Dubey et al. 2002; Jones et al. 2012). This technique is more relevant when cysts are not visualized on routine histopathological preparations, as occurred in half of the cases in this study. Although the polyclonal anti-*T. gondii* antibody has lower specificity than monoclonal antibodies, it has higher sensitivity and allows for identifying several *T. gondii* proteins, whereas monoclonal antibodies recognize only a single protein (Dubey et al. 2002). The *T. gondii* immunolabeling observed for the cases in this study varied

from weak to strong. In cases of weak labeling, careful and detailed evaluation should be performed because *T. gondii* tachyzoites and bradyzoites can occur inconspicuously in the cytoplasm of macrophages or free amidst the inflammatory infiltrate.

Toxoplasma gondii strains exhibit wide genetic diversity globally, particularly in South and Central America (Shwab et al. 2014), and fatal cases of acute toxoplasmosis in other species have been associated with the virulence of different strains (Santos et al. 2017).

The isolation of *T. gondii* from mice directly from the lung tissue of chicken 5 and the molecular detection of *T. gondii* in different tissues from this bird suggest that the animal was in the acute phase of the infection and circulating tachyzoites had reached different organs. We were able to isolate *T. gondii* from two sick chickens (4, 5) and genotyped both isolates by RFLP and MS, demonstrating that the same circulating isolate caused the death of the birds and confirming this outbreak. Because isolates with the same RFLP genotype can represent different strains when examined by MS (Feitosa et al. 2017), the latter was applied to evaluate eight typing markers and seven fingerprinting markers, offering increased genotyping resolution (Ajzenberg et al. 2010).

Before the current study, there were no reports of *T. gondii* isolation from tissues of chickens with clinical disease. There is increasing interest in studying correlation between *T. gondii* genotype and clinical presentation. For example, PCR-RFLP genotyping of 151 *T. gondii* isolates from healthy living hens has been reported (Dubey et al. 2008), and more recently, 48 (Beltrame et al. 2012) and 33 (Feitosa et al. 2017) *T. gondii* isolates were obtained from healthy free-living chickens. These isolates caused acute toxoplasmosis in most of the mice inoculated, similar to the isolates of the current study.

Brazilian *Toxoplasma gondii* isolates are characterized by high pathogenicity in mice, regardless of genotype (Dubey et al. 2002), and the various forms of human toxoplasmosis in South America, particularly in Brazil and Colombia, present with high frequency and severity. These more severe aspects may be related to the genetic diversity of *T. gondii* and to factors inherent to the host and rate of exposure (Khan et al. 2006; Gilbert et al. 2008).

In Brazil, more than 150 genotypes have already been described; in contrast, the predominant types in Europe and North America are clonal types II and III (Shwab et al. 2014). To our knowledge, the genotype identified by PCR-RFLP and MS analyses is herein described for the first time in the world. These findings add to the rapidly expanding pool of knowledge regarding *T. gondii* diversity.

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