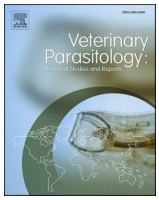
Veterinary Parasitology: Regional Studies and Reports 72 (2026) 101495

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr



Original Article

# Viability and genetic diversity of *Toxoplasma gondii* in retail pork from a Brazilian region known for waterborne toxoplasmosis

Amanda Lucía Jiménez-Sanz [a], Edwards Frazão-Teixeira [a,b,c,*], Francisco Carlos Rodrigues de Oliveira [a,c], Regiane de Fátima Ferreira [a,c], Samira Salim Mello Gallo [a,c]

[a] *Laboratório de Sanidade Animal, Centro de Ciências e Tecnologias Agropecuárias (CCTA), Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, RJ, Brazil*
[b] *Laboratório de Biologia Estrutural (LBE), Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, RJ, Brazil*
[c] *Programa de Pós-Graduação em Medicina Veterinária, Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Campos dos Goytacazes, RJ, Brazil*

ARTICLE INFO

*Keywords:*
*Toxoplasma gondii*
Retail pork
Mouse bioassay
Genetic diversity
Multilocus sequence typing
Brazil

ABSTRACT

Toxoplasmosis is a major public health concern in Brazil due to its high prevalence and associated clinical burden. The northern region of Rio de Janeiro State is particularly notable for elevated human seroprevalence and strong evidence of waterborne transmission as a major route of infection. In parallel, farm animals in this region also exhibit high levels of exposure, together with marked genetic diversity of circulating *Toxoplasma gondii* strains. This study investigated the presence of *T. gondii* in retail pork from a highly endemic municipality, its potential to expose humans through the consumption of viable parasites, and the genetic diversity of isolates. A total of 100 pork samples (500 g each) were obtained from mapped butcher shops and subjected to mouse bioassay, resulting in the isolation of three viable strains. Multilocus sequence typing (MLST) identified three distinct genotypes, and in silico PCR-RFLP classified isolate TgPgBr17 within a genotype previously reported in chickens, supporting circulation across host species. In addition, nested PCR targeting the single-copy P43 gene detected *T. gondii* DNA in 31.3% (10/32) of a subset of retail pork samples, indicating that exposure along the pork supply chain may be more frequent than suggested by parasite isolation alone. Together, the detection of viable and genetically diverse *T. gondii* in retail pork highlights the epidemiological importance of farm animals as sources of human exposure in this endemic region.

## 1. Introduction

*Toxoplasma gondii* is an intracellular parasite of warm-blooded animals, including humans, and is widely distributed worldwide (Dubey, 2022). Approximately one-third of the global human population is infected, mainly through ingestion of food or water contaminated with oocysts shed by felids, or through the consumption of raw or undercooked meat containing tissue cysts (Dubey, 2022). While many infections remain asymptomatic, severe clinical manifestations can occur, particularly in immunocompromised individuals (Dubey, 2022).

In Brazil, toxoplasmosis is a major public health concern due to its high prevalence in both humans and animals. The northern region of Rio de Janeiro State (RJ) presents some of the highest reported seroprevalence rates, reaching up to 82%, with strong evidence indicating waterborne transmission as a dominant route of infection (Bahia-Oliveira et al., 2003). Consistent with this scenario, the environment in this region is heavily contaminated with *T. gondii* oocysts, as evidenced not only by high infection rates in sentinel chickens but also in pigs, cattle, sheep, horses and ratites (da Silva et al., 2003; Frazão-Teixeira and de Oliveira, 2011; Frazão-Teixeira et al., 2011; Cosendey-Kezen-Leite et al., 2014; Venturi et al., 2017; Gallo et al., 2019).

Virulent and genetically diverse *T. gondii* strains have previously been isolated from fresh pork sold in the central public market of Campos dos Goytacazes (Campos), the most endemic municipality in this region (Frazão-Teixeira et al., 2011). The remarkable genetic diversity of *T. gondii* in Brazil—frequently characterized by non-archetypal genotypes that differ substantially from the classical clonal lineages I, II and III described in North America and Europe—has been consistently reported across South America (Brito et al., 2023) and has important implications for parasite pathogenicity, transmissibility and

* Corresponding author at: Edwards Frazão-Teixeira, Laboratório de Biologia Estrutural/IOC/Fiocruz, Av. Brasil 4365, CEP 21040-361 Rio de Janeiro, RJ, Brazil.
*E-mail address:* edwards.teixeira@ioc.fiocruz.br (E. Frazão-Teixeira).

https://doi.org/10.1016/j.vprsr.2026.101495
Received 9 January 2026; Received in revised form 25 February 2026; Accepted 30 April 2026
Available online 5 May 2026

epidemiology (Frazão-Teixeira et al., 2011; Maciel et al., 2014).

In this context, we expanded the investigation of retail pork sold throughout the municipality as a source of viable *T. gondii*, focusing on the virulence traits and genetic diversity of circulating isolates. Knowledge of the genotypes circulating in animals and in the environment is fundamental, as it allows comparison with genotypes detected in human infections and provides insights into transmission dynamics in this highly endemic setting.

## 2. Materials and methods

### 2.1. Ethics

All procedures involving animals and the collection of biological samples were conducted in accordance with the guidelines approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), under license number 108/2011. This study analyzed samples and isolates obtained during a field survey conducted between 2011 and 2012, while sequence analyses were completed in 2025.

### 2.2. Survey of butcher shops and sample collection

In Brazil, particularly in rural and peri-urban areas such as the northern region of Rio de Janeiro State, many meat-selling establishments operate without formal registration in governmental agricultural or sanitary oversight agencies. Consequently, between 2011 and 2012, an active, citywide survey was conducted to identify butcher shops selling fresh pork for human consumption. This survey involved systematically driving through the streets of all districts of the municipality of Campos dos Goytacazes (Campos) to locate and map these establishments.

A total of 84 butcher shops were identified and sampled across all 16 districts of the municipality. The number of butcher shops sampled per district was as follows: Central area (30), Guarús (14), Goitacazes (5), Ururaí (5), Morro do Coco (5), Farol de São Tomé (4), Travessão (4), Vila Nova (3), Santo Eduardo (3), Baixa Grande (3), Santa Maria (2), Saturnino Braga (2), Tocos (1), Ponta da Lama (1), Dores de Macabu (1), and Poço Gordo (1). Some establishments in the Central area were sampled more than once, resulting in a total of 100 pork samples collected: 60 refrigerated and 40 frozen samples (Table 1).

### 2.3. Pepsin digestion of collected tissues

From each butcher shop, 500 g of retail pork was purchased. Muscle cuts included loin, ham, and ribs, depending on availability. Immediately after collection, all samples were transported under refrigeration and processed at the Advanced Research Center for Parasitology (NUPAP), located within the Veterinary Hospital of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), which operates under the Laboratório de Sanidade Animal (LSA), Centro de Ciências e Tecnologias Agropecuárias (CCTA).

Tissue digestion was performed following Dubey (1998), with minor modifications. Each 500 g sample was trimmed and homogenized in a laboratory blender (Waring® Commercial Laboratory Blender) using a minimal volume of phosphate-buffered saline (PBS, pH 7.2) to facilitate processing. Blender cups were washed and autoclaved between samples to prevent cross-contamination. From each homogenized sample, 40 g were transferred to individually labeled 200 mL Erlenmeyer flasks and mixed with acidic pepsin solution (pH 1.1–1.2) to a final volume of 200 mL. Digestion was carried out in a thermostatic orbital shaker (Novatécnica®, NT 715) at 37 °C for 1 h.

The digest was filtered through double-layered gauze and aliquoted into four 50 mL conical tubes, which were centrifuged at 1300 ×*g* for 10 min. The supernatant was discarded, leaving a 2–3 mL pellet, which was neutralized with sodium bicarbonate solution containing phenol red (1.2%, pH 8.3). The contents of all four tubes were pooled into a single tube and centrifuged again at 1300 ×*g* for 20 min. After discarding the supernatant, 5–10 mL of antibiotic solution (100 µg/mL streptomycin and 1000 IU penicillin) was added, and the suspension was maintained at room temperature for 30 min before mouse inoculation.

**Table 1**
Type of cut and conservation status of pork samples submitted to bioassay in mice for *Toxoplasma gondii* isolation in the endemic area of Campos, Rio de Janeiro, Brazil.

| Type of cut | Conservation | | |
|---|---|---|---|
| | Refrigerated | Frozen | Total |
| Loin | 40 | 32 | 72 |
| Ham | 18 | 8 | 26 |
| Ribs | 2 | 0 | 2 |
| Total | 60 | 40 | 100 |

### 2.4. Bioassay in mice

For each sample, groups of three to five mice were subcutaneously inoculated with 1 mL of the digested tissue suspension and housed in individually labeled cages with food and water provided ad libitum. Animals were monitored daily for six weeks. Mice that died or showed signs of acute toxoplasmosis (e.g., lethargy, ruffled fur, half-closed eyes) were examined for the presence of *T. gondii* tachyzoites in lung smears under light microscopy (Nikon® Eclipse E100, 40× objective). Lung smears were prepared by pressing a 1–3 $mm^3$ tissue fragment onto a glass slide, adding one drop of saline solution (0.9% NaCl), and applying a coverslip for microscopic examination.

Mice that survived the observation period were bled and their sera tested for anti-*T. gondii* antibodies using the modified agglutination test (MAT). Seropositive mice were euthanized, and brain squash preparations were examined under light microscopy (10–20× objective) for tissue cysts. Tissues from infected mice were sub-inoculated into two additional mice for isolate amplification and cryopreservation. Mice showing clinical illness were treated with sulfadiazine (1 mg/mL) in drinking water until recovery. After euthanasia, brains were stored at −20 °C for subsequent molecular characterization.

### 2.5. Modified agglutination test (MAT)

Serum samples from inoculated mice were tested for anti-*T. gondii* antibodies using MAT as described by Dubey and Desmonts (1987). Assays were performed in U-bottom 96-well microplates (Greiner Bio-One®) with sera diluted in PBS (0.01 M, pH 7.2). The antigen consisted of inactivated tachyzoites of the *T. gondii* RH strain. Positive and negative control sera were included in each plate. Plates were incubated at 37 °C for 12 h, and reactions were read based on sedimentation patterns: complete or diffuse layers were considered positive, whereas a compact blue button was considered negative.

### 2.6. Multi-locus sequence typing (MLST)

DNA extracted from tissues of infected mice was subjected to nested PCR targeting 11 genetic markers: *SAG1*, *SAG2* (5′ + 3’ *SAG2*), *SAG3*, *BTUB*, *GRA6*, *c22–8*, *c29–2*, *L358*, *PK1*, *alt.SAG2*, and *Apico*, as previously described (Frazão-Teixeira et al., 2011). PCR products were separated by electrophoresis in 1.5% agarose gels stained with SYBR Safe and run at 100 V for 90 min. Amplicons were purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare®) according to the manufacturer's instructions and submitted for Sanger sequencing at the Genomic Analysis Facility of the Animal Experimentation Unit (UENF). Sequences were aligned and manually edited using BioEdit v7.2.5 to determine allelic profiles and genotypes.

### 2.7. In silico PCR-RFLP genotyping

In silico PCR-RFLP profiles were generated from the same MLST sequences following the method of Castro et al. (2020). Sequences for each locus were virtually digested using the restriction enzymes employed in conventional PCR-RFLP protocols. Restriction maps were predicted using NEBcutter 2.0 (New England Biolabs), and multilocus genotypes were inferred based on the presence or absence of restriction sites. Virtual electrophoretic patterns were generated using gel simulation tools (2.5–3% agarose) and compared with reference genotypes reported in public databases (e.g., GenBank, ToxoDB) and in previous studies.

### 2.8. Direct detection of Toxoplasma gondii DNA in pork samples

DNA was extracted from approximately 25 mg of 32 pork tissue samples using the DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Detection of *T. gondii* DNA was performed by nested PCR targeting the single-copy *P43* gene, using primers described by Grigg et al. (2001). The first amplification used 5 μL of extracted DNA as template, and 1 μL of the first-round product was used in the nested reaction. Each run included positive (*T. gondii* DNA) and negative (no-template) controls, as well as extraction controls. Amplification products were resolved in 2% agarose gels stained with GelRed and visualized under UV illumination.

## 3. Results

### 3.1. Bioassay

Viable *Toxoplasma gondii* strains were isolated from three of the 100 retail pork samples analyzed (3.0%), corresponding to three of the 84 butcher shops surveyed (3.6%). The isolates were designated TgPgBr17, TgPgBr18 and TgPgBr19 ("Tg" = *Toxoplasma gondii*; "Pg" = pig; "Br" = Brazil), following the isolate nomenclature proposed by Velmurugan et al. (2009). Data on mouse infectivity and mortality are summarized in Table 2. All three isolates showed high infectivity in mice, with 100% infection (3/3) observed for TgPgBr17 and TgPgBr19, whereas TgPgBr18 infected two of three inoculated mice, with one animal remaining seronegative by MAT and without clinical signs of infection.

All isolates were obtained from pork samples purchased from different butcher shops in Campos dos Goytacazes, RJ. Two isolates (TgPgBr17 and TgPgBr18) originated from butcher shops located in the central area of the city, whereas the third isolate (TgPgBr19) was obtained from the district of Goitacazes, approximately 10 km away. TgPgBr19 showed the highest virulence, with a mortality rate of 66.7% (2/3) in primary infected mice, whereas TgPgBr18 and TgPgBr17 showed mortality rates of 50% and 33.3%, respectively (Table 2).

For TgPgBr19, two of the three inoculated mice developed acute clinical signs and died at 17 days post-inoculation (dpi); lung smears from both animals confirmed the presence of tachyzoites (Table 2). The surviving mouse remained alive throughout the six-week observation period and was seropositive by MAT (titer ≥1:25).

For TgPgBr18, one of three inoculated mice died at 20 dpi, with tachyzoites detected in lung smears. Of the two surviving mice, one was seropositive by MAT and the other remained seronegative (Table 2).

For TgPgBr17, one of three inoculated mice died at 18 dpi, with tachyzoites observed in lung smears. The two surviving mice were seropositive by MAT (Table 2). Mice inoculated with the remaining 97 pork samples survived the six-week observation period and were all seronegative by MAT.

### 3.2. Multi-locus sequence typing (MLST)

Two independent sequencing attempts were required to reduce gaps in the 11-locus MLST profiles of the isolates. During sub-passaging in mice, isolates TgPgBr18 and TgPgBr19 were lost, probably due to inappropriate timing of sulfadiazine administration, resulting in insufficient parasite biomass for complete resequencing. In contrast, TgPgBr17 was successfully resequenced using DNA extracted from a more concentrated parasite stock, allowing full MLST resolution. Nevertheless, despite some missing loci, the partial MLST profiles of TgPgBr18 and TgPgBr19 were sufficient to establish them as novel genotypes (Table 3). Single-nucleotide polymorphisms (SNPs) defining the three genotypes are summarized in Fig. 1.

Isolate TgPgBr17 exhibited a recombinant MLST profile composed of archetypal alleles III ($n = 6$), I ($n = 1$), and unique alleles ($n = 4$) across the 11 loci analyzed. Unique alleles were detected at c22–8, c29–2, PK1 and GRA6, whereas allele I was identified only at SAG1.

For TgPgBr18, allele distribution across the 10 resolved loci (Apico excluded due to incomplete sequence resolution) included alleles III ($n = 2$), I ($n = 3$), II (n = 1), and unique alleles (n = 4). Unique alleles were observed at c22–8, L358, GRA6 and alt-SAG2, while the only allele II was identified at PK1.

Analysis of TgPgBr19, also based on 10 resolved loci, revealed a predominance of atypical alleles (n = 6) at c22–8, L358, SAG1, BTUB, GRA6 and alt-SAG2. Alleles III (n = 2) and I (n = 2) were detected at c29–2 and SAG3, and at PK1 and 5′ + 3' SAG2, respectively. The MLST allele profiles of these three new isolates, together with those of five previously described pig isolates from the same municipality, are presented in Table 3.

### 3.3. In silico PCR-RFLP genotypes

The in silico multilocus PCR-RFLP analysis yielded a complete genotype for TgPgBr17, which showed an identical restriction pattern to TgCkBr8, corresponding to ToxoDB genotype #125, as defined by Dubey et al. (2020). This genotype has previously been reported in chickens from São Paulo State. In contrast, isolates TgPgBr18 and TgPgBr19 lacked complete sequence information for several loci, resulting in missing restriction sites and preventing definitive PCR-RFLP genotype assignment (Table 3).

### 3.4. Molecular detection of T. gondii in pork samples

Nested PCR targeting the P43 gene detected *T. gondii* DNA in 10 of the 32 retail pork samples analyzed (31.3%). Among these, seven originated from loin cuts (36.8%) and three from ham (37.5%). Two samples were positive by both PCR and mouse bioassay, corresponding to isolates TgPgBr17 and TgPgBr19.

**Table 2**
Swiss Webster mice bioassay for *Toxoplasma gondii* isolation from retail pork purchased in Campos dos Goytacazes, RJ, Brazil.

| Isolate designation | Tissue type | Conservation at purchase | District | Infectivity | | Mortality | | DAI [1] |
|---|---|---|---|---|---|---|---|---|
| | | | | n | % | n | % | |
| TgPgBr17 | loin | refrigerated | Central area [2] | 3/3 | 100 | 1/3 | 33.3 | 18 |
| TgPgBr18 | ham | refrigerated | Central area | 2/3 | 66.7 | 1/2 | 50 | 20 |
| TgPgBr19 | ham | refrigerated | Goitacazes | 3/3 | 100 | 2/3 | 66.7 | 17 |

[1] Days mice died.
[2] Isolates TgPgBr17 and 18 were from different butcher shops in the Central area of the municipality.

**Table 3**
Multi-locus genotypes of *Toxoplasma gondii* isolates of pigs from Brazil.

| Method | Strain | Genotype | Genetic markers / alleles [1] | | | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | c22–8 | c29–2 | L358 | PK1 | SAG1 | 5′ + 3′ SAG2 | BTUB | GRA6 | SAG3 | Apico | Alt-SAG2 | |
| MLST | TgPgBr1,2 | # 1 | u-1 | I | I | u-1 | I | u-1 | I | u-1 | III | I | | Frazão-Teixeira et al. (2011) |
| | TgPgBr3 | # 2 | u-2 | u-1 | u-1 | u-1 | u-1 | u-2 | I | u-2 | III | I | | |
| | TgPgBr4 | #3 | III | III | III | u-2 | II/III | III | III | u-3 | III | III | | |
| | TgPgBr5 | # 4 | u-1 | u-1 | u-2 | u-3 | u-1 | u-2 | u-1 | II | III | III | | |
| | TgPgBr17 | #5 | u-1 | u-1 | III | u-1 | I | III | III | u-1 | III | III | III | This research |
| | TgPgBr18 | #6 | u-2 | III | u-1 | II | I | I | I | u-2 | III | | u-1 | |
| | TgPgBr19 | #7 | u-3 | III | u-2 | I | u-1 | I | u-1 | u-2 | III | | u-2 | |
| PCR-RFLP | TgPgBr1,2 | #1 | I | I | I | I | I | I | I | II | III | I | | Frazão-Teixeira et al. (2011) |
| | TgPgBr3 | #2 | II | I | I | I | u-1 | u-1 | I | II | III | I | | |
| | TgPgBr4 | Type III | III | III | III | III | II/III | III | III | III | III | III | | |
| | TgPgBr5 | #4 | I | I | III | III | u-1 | u-1 | III | II | III | III | | |
| | TgPgBr17, TgCkBr8 | ToxoDB #125 | II | III | III | u-1 | I | III | III | III | III | III | III | This research Dubey et al. (2020) |

[1] Blank alleles were not completely resolved or done.

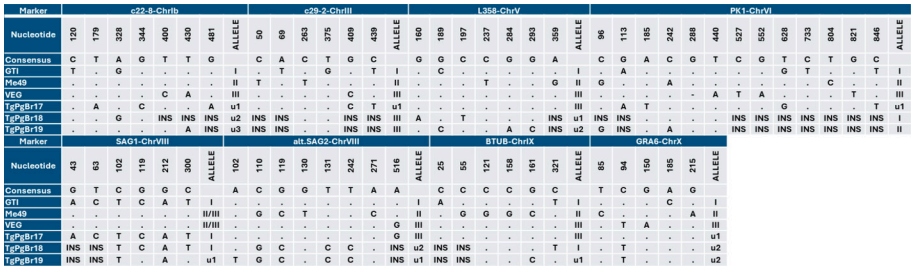



**Fig. 1.** Polymorphisms at 8 genetic markers identified by MLST in *Toxoplasma gondii* from retail pork in a Brazilian region known for waterborne toxoplasmosis. Consensus sequence is defined as the nucleotide shared by at least two of the three archetypal Types I, II and III strain alleles. "." indicates identity with consensus; "-" indicates an insertion/deletion. "u" indicates a nonarchetypal allele; I, II or III refers to an archetypal allelic sequence from a Type I, II or III strain. "INS" indicates insufficient sequence for analysis.

## 4. Discussion

The present study provides a citywide assessment of the occurrence of viable *Toxoplasma gondii* in retail pork intended for human consumption in Campos dos Goytacazes (Campos), a municipality recognized as highly endemic for both human and animal toxoplasmosis in northern Rio de Janeiro State, Brazil (Bahia-Oliveira et al., 2003; da Silva et al., 2003; Frazão-Teixeira and Oliveira, 2011; Frazão-Teixeira et al., 2011; Cosendey-KezenLeite et al., 2014; Venturi et al., 2017; Gallo et al., 2019). By sampling butcher shops located in the central urban area as well as in distant districts, we aimed to capture the potential exposure risk faced not only by local consumers but also by individuals from neighboring areas who purchase fresh pork in Campos. In addition, we provide phenotypic observations from mouse bioassay and high-resolution genotyping data that together refine the current understanding of the local *T. gondii* population structure and support future efforts to infer plausible routes of human infection in this endemic setting.

Although epidemiological evidence has consistently supported waterborne transmission as a major route of human infection in the region (Bahia-Oliveira et al., 2003), extensive environmental contamination with oocysts and infection of multiple intermediate hosts indicate that foodborne transmission through carnivorism warrants parallel consideration (da Silva et al., 2003; Frazão-Teixeira and Oliveira, 2011; Frazão-Teixeira et al., 2011; Cosendey-KezenLeite et al., 2014; Venturi et al., 2017; Gallo et al., 2019). Our group has progressively addressed this issue by documenting high seroprevalence in pigs and cattle (Frazão-Teixeira and Oliveira, 2011), demonstrating viable parasites in brains and hearts of slaughtered pigs sold for consumption (Frazão-Teixeira et al., 2011), and, in the present study, extending the investigation to retail pork cuts directly purchased from butcher shops across the municipality.

The prior detection of anti-*T. gondii* antibodies in pigs (Frazão-Teixeira and Oliveira, 2011) and the isolation of viable parasites from pig brains and hearts sold in the central popular market of Campos (Frazão-Teixeira et al., 2011) suggested that tissue cysts could also be present in edible skeletal muscle. Here, viable parasites were isolated from ham and loin cuts, emphasizing the tangible risk of infection associated with the consumption of raw or undercooked pork in Campos. Experimental studies support this interpretation: Dubey et al. (1984) demonstrated long-term persistence of tissue cysts in edible pig tissues, and subsequent work showed that seropositivity correlates with parasite recovery from multiple organs and commercial cuts (Dubey, 1988). Together, these data reinforce the public health relevance of pork in highly endemic areas where food handling practices may favor undercooking.

This study also reflects the local production and commercialization context. In Campos and similar rural-to-peri-urban settings in Brazil, pigs supplying small butcher shops often originate from small-scale family farms, mirroring the predominant local production system and potentially increasing heterogeneity in biosecurity and husbandry practices. Moreover, many retail vendors operate outside routine municipal sanitary oversight, limiting the availability of official registries. Accordingly, we used an active mapping approach, systematically driving through all districts to identify and georeference butcher shops, resulting in 84 establishments and 100 pork samples. We isolated three

strains and designated them TgPgBr17–19, continuing the sequential nomenclature following TgPgBr16 (Bezerra et al., 2012).

The infectivity and virulence patterns observed in these isolates provide biological context complementary to the detected genetic diversity. Although virulence was not quantified under controlled parasite doses, the primary bioassay outcomes indicate that these isolates can establish infection and induce acute disease in mice, consistent with a biologically active phenotype. This aligns with prior observations for porcine isolates from Campos (TgPgBr1–5), which were highly pathogenic to mice, including mortality at low inocula (Frazão-Teixeira et al., 2011). Similar patterns have been reported for genetically divergent porcine isolates from northeastern Brazil (TgPgBr6–16), where virulence was frequent despite marked genetic divergence from clonal lineages (Bezerra et al., 2012). Nevertheless, future studies should employ standardized inoculation routes, controlled parasite burdens, and dose–response designs to define virulence phenotypes more precisely and to disentangle the influence of genotype, inoculum size, and host factors.

Regarding genetic characterization, early studies of porcine isolates frequently relied on one or few loci (e.g., single-marker genotyping) and classified strains within the classical clonal types I, II, or III (Howe and Sibley, 1995; Belfort-Neto et al., 2007). Subsequent multi-locus approaches revealed extensive non-archetypal diversity in Brazil, including atypical and recombinant genotypes (Pena et al., 2008; Frazão-Teixeira et al., 2011; Bezerra et al., 2012; Brito et al., 2023). Multi-locus PCR-RFLP has become widely used due to practicality and comparability with ToxoDB-defined genotypes, but its resolution is limited in highly diverse settings because it relies on restriction site polymorphisms rather than nucleotide-level variation. In contrast, MLST provides higher resolution by capturing SNPs directly, improving inference of genetic relationships among circulating isolates and strengthening epidemiological interpretation in regions where extensive recombination occurs (Frazão-Teixeira et al., 2011; Rajendran et al., 2012).

Sequencing of 11 markers indicated that TgPgBr17–19 represent three distinct genotypes, different from each other and from prior pig isolates recovered in Campos (Frazão-Teixeira et al., 2011) and from porcine isolates reported in Bahia (Bezerra et al., 2012). When the eight pig isolates from Campos (TgPgBr1–5 and TgPgBr17–19) are considered together, the local population structure is characterized by extensive allelic mosaicism, with mixtures of archetypal type I/II/III alleles and multiple unique alleles across loci (Table 3; Fig. 1). This pattern is consistent with a recombining population and supports broader South American observations of high genetic diversity and recombinant structures (Rajendran et al., 2012; Brito et al., 2023). Notably, across these eight isolates, identical sequences were shared only at SAG3 (type III), whereas other loci showed substantial polymorphism, highlighting the importance of multi-locus sequence data for accurate inference in this setting.

The observed predominance of type III alleles at SAG3, including in the present isolates and earlier isolates from Campos, is noteworthy and may reflect regional clustering trends described in Brazil (Pena et al., 2008). Further work integrating human isolates from Campos is needed to determine whether the same genetic backgrounds occur in clinical cases and whether particular allelic combinations associate with disease severity, including ocular toxoplasmosis, which has been linked elsewhere to atypical genotypes (Grigg et al., 2001; Ajzenberg et al., 2010).

Because global comparative datasets are dominated by PCR-RFLP genotypes (and ToxoDB assignments), we complemented MLST with in silico conversion of sequence data to PCR-RFLP profiles following Castro et al. (2020). This approach improves comparability with existing literature while retaining the high-resolution MLST framework. Only TgPgBr17, which had complete sequence information, could be reliably converted; TgPgBr18 and TgPgBr19 were excluded due to missing loci preventing robust RFLP discrimination. Under PCR-RFLP, TgPgBr17 matched TgCkBr8 (ToxoDB genotype #125), previously reported in chickens from São Paulo (Dubey et al., 2020). This finding supports the circulation of at least some genotypes across different intermediate hosts and highlights how method resolution influences perceived "uniqueness": MLST can resolve diversity within PCR-RFLP-defined genotypes, whereas PCR-RFLP captures broader lineage relationships that facilitate global comparisons. The availability of MLST data for TgCkBr8 would be necessary to determine whether both isolates are identical at nucleotide level across loci.

From a One Health perspective, these genotypic findings should be interpreted within the well-documented epidemiological context of Campos, where untreated water consumption was identified as a major risk factor for human infection (Bahia-Oliveira et al., 2003) and environmental oocyst contamination is likely intense (da Silva et al., 2003). Free-range chickens are established sentinels of soil contamination, whereas pigs represent a direct link between environmental exposure and human infection via meat consumption. The detection of viable and genetically diverse *T. gondii* in retail pork indicates that foodborne exposure may occur alongside waterborne transmission in this endemic system. The recovery of isolates from butcher shops located ~10 km apart further supports widespread transmission across the municipality, potentially amplified by high densities of free-roaming cats and flooding events that can facilitate oocyst dissemination (Bahia-Oliveira et al., 2003).

To further examine parasite occurrence in pork, we used nested PCR targeting the single-copy P43 gene and detected *T. gondii* DNA in 31.3% of the tested retail pork subsample. Molecular detection alone, however, does not demonstrate viability or infectivity, particularly when freezing or other processing steps may inactivate tissue cysts (Kotula et al., 1991; Tenter et al., 2000). Conversely, mouse bioassay may underestimate infection when parasite density is low or patchily distributed, and when only a small portion of a larger sample is processed (Dubey et al., 2005). These complementary limitations likely explain the partial discordance between PCR and bioassay in our study. Similar discrepancies have been reported in experimental infections, where PCR and bioassay may detect infection in different tissues within the same animal, reflecting uneven parasite distribution and low cyst burdens (Yai et al., 2003; Tsutsui et al., 2007). Thus, combining molecular screening with bioassay provides a more informative estimate of potential consumer exposure than either method alone.

Overall, our findings reinforce that retail pork can harbor viable and genetically diverse *T. gondii* strains in a region where human toxoplasmosis is highly endemic. The combined evidence of (i) viable parasite isolation from commercial cuts, (ii) molecular detection in a substantial fraction of retail samples, and (iii) high-resolution genotypic diversity supports the need for continued vigilance regarding pork as a potential source of human infection in endemic settings. Future studies integrating isolates from animals and humans, employing standardized virulence phenotyping, and expanding high-resolution genotyping will be essential to clarify genotype–phenotype relationships and to better understand transmission routes in Brazil. Collectively, these data emphasize the relevance of integrated food safety and One Health approaches linking environmental, veterinary, and human health compartments in regions of intense *T. gondii* transmission.

## 5. Conclusions

This study demonstrates that retail pork marketed in Campos dos Goytacazes (Rio de Janeiro State, Brazil), a highly endemic setting for toxoplasmosis, can harbor viable *T. gondii* and genetically diverse strains. Mouse bioassay confirmed viable parasites in 3% (3/100) of pork samples, and multilocus sequence typing (MLST) identified three distinct genotypes among the isolates, including recombinant allele profiles consistent with the high genetic diversity reported for Brazil. In a subset of samples tested directly, P43-based nested PCR detected *T. gondii* DNA in 31.3% (10/32), suggesting that exposure along the pork supply chain may occur more frequently than indicated by parasite isolation alone; however, PCR positivity does not demonstrate parasite

viability. Collectively, these findings reinforce pork as a relevant potential route of human exposure in this endemic region and provide sequence-based baseline data to support future integrated investigations linking animal, environmental, and human isolates.

## CRediT authorship contribution statement

**Amanda Lucía Jiménez-Sanz:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Edwards Frazão-Teixeira:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francisco Carlos Rodrigues de Oliveira:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Regiane de Fátima Ferreira:** Writing – review & editing, Methodology. **Samira Salim Mello Gallo:** Writing – review & editing, Methodology.

## Ethical statement

All animal procedures were conducted in accordance with Brazilian regulations for animal experimentation and were approved by the Ethics Committee on Animal Use (CEUA) of Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) under license number **108/2011**. The study used biological samples and parasite isolates obtained during field and laboratory activities conducted between 2011 and 2012, with molecular analyses completed subsequently. All efforts were made to minimize animal suffering and to reduce the number of animals used.

## Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used generative artificial intelligence tools to assist with language editing, grammar, and stylistic refinement. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication. The use of AI did not affect the scientific content, data interpretation, or conclusions of this work.

## Funding

This research was supported by grants from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Finance Code 001, Brazil), FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro, Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil). The funding agencies had no role in the study design, data collection, analysis, interpretation of the data, or writing of the manuscript.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## References

Ajzenberg, D., Yera, H., Marty, P., Paris, L., Dalle, F., Menotti, J., et al., 2010. Genotype of 88 *Toxoplasma gondii* isolates associated with toxoplasmosis in immunocompromised patients and correlation with clinical findings. J. Infect. Dis. 199 (8), 1155–1167. https://doi.org/10.1086/597477.

Bahia-Oliveira, L.M.G., Jones, J.L., Azevedo-Silva, J., Alves, C.C., Oréfice, F., Addiss, D. G., 2003. Highly endemic, waterborne toxoplasmosis in North Rio de Janeiro state, Brazil. Emerg. Infect. Dis. 9 (1), 55–62. https://doi.org/10.3201/eid0901.020160.

Belfort-Neto, R., Nussenblatt, V., Rizzo, L., Muccioli, C., Silveira, C., Nussenblatt, R., Khan, A., Sibley, L.D., Belfort Jr., R., 2007. High prevalence of unusual genotypes of *Toxoplasma gondii* infection in pork meat samples from Erechim, Southern Brazil. An. Acad. Bras. Cienc. 79 (1), 111–114. https://doi.org/10.1590/s0001-37652007000100013.

Bezerra, R.A., Carvalho, F.S., Guimarães, L.A., Rocha, D.S., Maciel, B.M., Wenceslau, A. A., Lopes, C.W., Albuquerque, G.R., 2012. Genetic characterization of *Toxoplasma gondii* isolates from pigs intended for human consumption in Brazil. Vet. Parasitol. 189 (2–4), 153–161. https://doi.org/10.1016/j.vetpar.2012.04.036.

Brito, R.M.M., Bessa, G.L., Bastilho, A.L., Dantas-Torres, F., Andrade-Neto, V.F., Bueno, L.L., Fujiwara, R.T., Magalhães, L.M.D., 2023. Genetic diversity of *toxoplasma gondii* in South America: occurrence, immunity, and fate of infection. Parasit. Vectors 16, 461. https://doi.org/10.1186/s13071-023-06080-w.

Castro, B.B.P., Gennari, S.M., Lorenzi, H., Su, C., 2020. A simple method to generate PCR-RFLP typing profiles from DNA sequences in *toxoplasma gondii*. Infect. Genet. Evol. 85, 104590. https://doi.org/10.1016/j.meegid.2020.104590.

Cosendey-KezenLeite, R.I., de Oliveira, F.C., Frazão-Teixeira, E., Dubey, J.P., de Souza, G.N., Ferreira, A.M., Lilenbaum, W., 2014. Occurrence and risk factors associated to *Toxoplasma gondii* infection in sheep from Rio de Janeiro. Brazil. *Trop. Anim. Health Prod.* 46 (8), 1463–1466. https://doi.org/10.1007/s11250-014-0667-5.

da Silva, D.S., Bahia-Oliveira, L.M.G., Shen, S.K., Kwok, O.C.H., Lehman, T., Dubey, J.P., 2003. Prevalence of *Toxoplasma gondii* in chickens from an area in Southern Brazil highly endemic to humans. J. Parasitol. 89 (2), 394–396. https://doi.org/10.1645/0022-3395(2003)089[0394:POTGIC]2.0.CO;2.

Dubey, J.P., 1988. Long-term persistence of toxoplasma gondii in tissues of pigs inoculated with T. Gondii oocysts and effect of freezing on viability of tissue cysts in pork. Am. J. Vet. Res. 49, 910–913.

Dubey, J.P., 1998. Refinement of pepsin digestion method for isolation of *toxoplasma gondii* from infected tissues. Vet. Parasitol. 74 (1), 75–77. https://doi.org/10.1016/S0304-4017(97)00135-0.

Dubey, J.P., 2022. Toxoplasmosis of Animals and Humans, 3rd ed. CRC Press, Boca Raton. https://doi.org/10.1201/9781003199373.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *toxoplasma gondii* oocysts. Equine Vet. J. 19 (4), 337–339. https://doi.org/10.1111/j.2042-3306.1987.tb01429.x.

Dubey, J.P., Murrell, K.D., Fayer, R., 1984. Persistence of encysted *toxoplasma gondii* in tissues of pigs fed oocysts. Am. J. Vet. Res. 45 (10), 1941–1943.

Dubey, J.P., Hill, D.E., Jones, J.L., Hightower, A.W., Kirkland, E., Roberts, J.M., et al., 2005. Prevalence of viable *toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. J. Parasitol. 91 (5), 1082–1093. https://doi.org/10.1645/GE-683.1.

Frazão-Teixeira, E., de Oliveira, F.C.R., 2011. Anti-*toxoplasma gondii* antibodies in cattle and pigs in a highly endemic area for human toxoplasmosis in Brazil. J. Parasitol. 97 (1), 44–47. https://doi.org/10.1645/GE-2457.1.

Frazão-Teixeira, E., Sundar, N., Dubey, J.P., Grigg, M.E., de Oliveira, F.C.R., 2011. Multilocus DNA sequencing of *toxoplasma gondii* isolated from Brazilian pigs identifies genetically divergent strains. Vet. Parasitol. 175 (1–2), 33–39. https://doi.org/10.1016/j.vetpar.2010.09.030.

Gallo, S.S.M., Frazão-Teixeira, E., Ederli, N.B., Oliveira, F.C.R., 2019. Prevalence of anti-*toxoplasma gondii* antibodies in ratites from Brazil. J. Parasitol. 105 (5), 733–737. https://doi.org/10.1645/19-101.

Grigg, M.E., Ganatra, J., Boothroyd, J.C., Margolis, T.P., 2001. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. J. Infect. Dis. 184 (5), 633–639. https://doi.org/10.1086/322800.

Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172, 1561–1566. https://doi.org/10.1093/infdis/172.6.1561.

Kotula, A.W., Dubey, J.P., Sharar, A.K., Andrews, C.D., Shen, S.K., Lindsay, D.S., 1991. Effect of freezing on infectivity of *toxoplasma gondii* tissue cysts in pork. J. Food Prot. 54 (9), 687–690. https://doi.org/10.4315/0362-028X-54.9.687.

Maciel, B.M., Moura, R.L.S., Carvalho, F.S., Costa, E.A., Albuquerque, G.R., 2014. Identification and genetic characterization of a new Brazilian genotype of *toxoplasma gondii* from sheep intended for human consumption. Parasitol. Int. 63 (3), 567–570. https://doi.org/10.1016/j.parint.2014.03.001.

Pena, H.F.J., Gennari, S.M., Dubey, J.P., Su, C., 2008. Population structure and mouse virulence of *toxoplasma gondii* in Brazil. Int. J. Parasitol. 38 (5), 561–569. https://doi.org/10.1016/j.ijpara.2007.09.004.

Rajendran, C., Su, C., Dubey, J.P., 2012. Molecular genotyping of *toxoplasma gondii* from central and South America revealed high diversity within and between populations. Infect. Genet. Evol. 12, 359–368. https://doi.org/10.1016/j.meegid.2011.12.010.

Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30 (12−13), 1217–1258. https://doi.org/10.1016/s0020-7519(00)00124-7.

Tsutsui, V.S., Freire, R.L., Garcia, J.L., Gennari, S.M., Vieira, D.P., Marana, E.R.M., Prudêncio, L.B., Navarro, I.T., 2007. Detection of *toxoplasma gondii* by PCR and mouse bioassay in commercial cuts of pork from experimentally infected pigs. Arq. Bras. Med. Vet. Zootec. 59 (1), 30–34. https://doi.org/10.1590/S0102-09352007000100006.

Velmurugan, G.V., Su, C., Dubey, J.P., 2009. Isolate designation and characterization of *toxoplasma gondii* isolates from pigs in the United States. J. Parasitol. 95 (1), 95–99. https://doi.org/10.1645/GE-1746.1.

Venturi, S.S., da Silva, A.F., Frazão-Teixeira, E., de Oliveira, F.C.R., Consalter, A., Padilha, F.G.F., Fonseca, A.B.M., Reis Ferreira, A.M., 2017. Characterization of the zoonotic potential of *toxoplasma gondii* in horses from Rio de Janeiro state. Acta Trop. 171, 159–162. https://doi.org/10.1016/j.actatropica.2017.03.036.

Yai, L.E.O., Vianna, M.C.B., Soares, R.M., Cortez, A., Freire, R.L., Richtzenhain, L.J., Gennari, S.M., 2003. Avaliação da infecção experimental por *Toxoplasma gondii* em suínos por bioensaio em camundongos e PCR. Braz. J. Vet. Res. Anim. Sci. 40 (3), 227–234. https://doi.org/10.1590/S1413-95962003000300010.