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Genetic diversity of *Toxoplasma gondii* in goats and sheep from the Northeast Region of Brazil destined for human consumption

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ABSTRACT

This study aimed to genotype isolates of *Toxoplasma gondii* obtained from samples of brain, diaphragm and heart of goats and sheep intended for human consumption in the State of Paraíba, Brazil. Tissue samples from 14 animals, goats ($n = 5$) and lambs ($n = 9$), were sourced from public slaughterhouses in seven cities and bioassayed in mice. The brains of the mice were utilized for DNA extraction. Genotyping was carried out by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) using 10 markers (SAG1, SAG2, SAG3, BTUB, c22-8, PK1, GRA6, L358, c-29-2 and Apico). A total of 10 isolates were fully genotyped (i.e. at all loci), three from goats and seven from sheep, revealing five distinct genotypes: #13 ($n = 4$); #48 ($n = 3$); #57 ($n = 1$); #273 ($n = 1$); and one new genotype that had not been previously described. Genotype #13 is frequently found in the Northeast of Brazil and represents a clonal lineage circulating in this region and was the most prevalent genotype identified ($n = 4$). Moreover, in the present study genotypes #13, #48, #57, and #273 were documented for the first time in sheep from Brazil, and the novel genotype was isolated from a goat. Our findings align with previous studies on *T. gondii* from Brazil, where new genotypes are continuously being identified, highlighting a high level of genetic diversity of *T. gondii* isolates in the country.

1. Introduction

Toxoplasma gondii is a protozoan that infects virtually all warm-blooded animals, including humans, domestic animals, terrestrial and marine wildlife (Dubey et al., 2020). Hosts can be infected with *T. gondii* through the following primary routes: transplacental transmission, ingestion of undercooked meat containing infectious cysts, as well as ingestion of water and food contaminated with sporulated oocysts (from cat faeces) (Dubey, 2009).

In small ruminants (goats and sheep), *T. gondii* has been identified as a major cause of outbreaks of reproductive problems, leading to economic losses in the production chain (Dubey et al., 2020; Nayeri et al., 2021). Small ruminants can become infected with *T. gondii* through the ingestion of oocysts and/or the vertical transmission of tachyzoites across the placenta during pregnancy, which may result in abortion at any stage of gestation (Castaño et al., 2016). In addition to the economic losses caused by toxoplasmosis in small ruminant farming, there are concerns regarding human health, as goats and sheep can act as a potential source of infection for humans through the consumption of undercooked meat containing infectious cysts, resulting in significant health risks. Furthermore, goats and sheep can serve as sources of infection for cats when they consume raw tissues from infected animals (Rani et al., 2020; Dubey, 2022).

In humans, toxoplasmosis is usually subclinical in immunocompetent adults; however, infection can lead to encephalitis and even death in immunosuppressed individuals (Zhou et al., 2011; Fang et al., 2021). Toxoplasmosis is particularly significant in pregnant women, as there can be transplacental transmission of the parasite from the mother to the foetus, potentially leading to congenital toxoplasmosis, which is often characterized by chorioretinitis, intracranial calcifications, hydrocephalus, and even abortion (McAuley, 2014; Melo et al., 2020a).

Toxoplasmosis is a zoonosis of significant concern across all of Brazil, as the country demonstrates high levels of human seroprevalence of toxoplasmosis. Brazil also experiences more frequent outbreaks and

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more severe congenital forms of the disease compared to Europe. Additionally, Brazil also exhibits high incidence levels of severe ocular toxoplasmosis, contributing to one in every four diagnosed uveitis cases (Gonzalez-Fernandez et al., 2017). Severe systemic forms of the disease are also routinely identified in immunocompetent individuals, which are rarely observed in other parts of the world (Gilbert et al., 2008; Pappas et al., 2009; Dubey, 2021; Pena et al., 2021). The largest human toxoplasmosis outbreak in the world was reported in Brazil, occurring in 2018 in the city of Santa Maria, Rio Grande do Sul. The outbreak affected more than 1100 individuals including 35 pregnant women, resulting in two foetal deaths and two abortions. This 2018 Santa Maria outbreak underscores the magnitude of the challenge faced by Brazilian health authorities (Arquilla et al., 2019; Dal Ponte et al., 2019).

The pathogenicity and virulence of *T. gondii* can vary significantly depending on many factors including on the infected host species. Studies carried out on virulence strains in mice cannot be directly extrapolated to humans. Some reports have demonstrated that some lineages are lethal for mice, while these same lineages are not pathogenic in humans (Gazzinelli et al., 2014; Galal et al., 2018).

Toxoplasma gondii exhibits extensive genetic variability. Genotypic characterization studies based on multiplex nested PCR-restriction fragment length polymorphism (mnPCR-RFLP) techniques have revealed clear differences in pathogenicity when comparing classical clonal types I (virulent), II, and III (low virulent or avirulent, respectively), which are prevalent in Europe and the USA. In Brazil, some lineages are considered as "Brazilian clonal", designated as BrI (highly virulent), BrII and BrIV (medium virulent), and BrIII (avirulent); however, most genotypes present are atypical, which means that they do not belong to I or BrI, II or BrII, III or BrIII or IV or BrIV lineages, and they are usually virulent (Pena et al., 2008; Shwab et al., 2014; Su and Dubey, 2020; Witter et al., 2022).

Given the importance of toxoplasmosis in the production of goats and sheep, as well as in human health in Brazil, this study aimed to genotype *T. gondii* isolates obtained from goats and sheep intended for human consumption, enhancing our understanding of the genetic diversity of this parasite in Brazil.

2. Materials and methods

2.1. Sample collection and storage

Fourteen *T. gondii* isolates from slaughterhouses in seven cities from Paraíba State, were used, five from goats and nine from sheep. The isolates were obtained through mouse bioassays as previously described in detail by Silva et al. (2021) and Batista et al. (2022). DNA was extracted from the brains of the infected mice using a commercial extraction kit (PureLink™ Genomic DNA Mini Kit, cat K182002-Invitrogen, Carlsbad, CA, USA) and stored at −20 °C until analysed.

2.2. Multi-locus genotyping using PCR-RFLP

To confirm the presence of *T. gondii*, DNA samples from the brains of the mice experimentally infected with the isolates were tested by PCR for the ITS1 region of *T. gondii* as previously described by Burrells et al. (2013). Subsequently, only the samples that tested positive for *T. gondii* ITS1 were further analysed by genotyping using mnPCR-RFLP targeting 10 genetic markers: SAG1, SAG2 (5′3′ SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico as outlined by Su and Dubey (2020). The isolates S48 (Type I), M4 (Type II variant), and NED (Type III) were used as positive controls to ensure complete digestion of each marker for all samples.

For mnPCR protocols, we followed Su and Dubey (2020) with the following modifications. The target DNA sequences were initially amplified by multiplex PCR using external primers for all markers. Each reaction consisted of 50 μl, including 5 μl of 10× PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM $(NH_4)2SO_4$, 4.5 mM $MgCl_2$, 4.4 μM EDTA, 113 μg/ml BSA, 1 mM each of the four deoxyribonucleotide triphosphates (dNTPs), 2 U of Taq polymerase (Bioline BioTaq), 0.15 μM of forward and reverse primers for all markers (except for 5′-SAG2 because this is covered by the external primers of alt. SAG2), 3 μl of each DNA sample reactions were made to a final volume of 50 μl with DNase/RNase-free water. Primary PCR products were then further diluted (1:1), i.e. by adding 50 μl of nuclease-free water. The secondary nested PCR reactions were carried out individually for each marker in 20 μl reactions including 2 μl of 10× PCR buffer (as described previously), 0.75 U of Taq, 0.3 μM of each forward and reverse primer, 1.5 μl of diluted primary PCR amplicon and water to bring the total volume to 20 μl.

Nested PCR products were digested with the restriction enzymes previously described (Su and Dubey, 2020). In the present study, we used the combinations described in Supplementary Table S1. The digested products were then resolved by agarose gel electrophoresis and the bands were visualized under UV light (GBox).

2.3. Genetic diversity of T. gondii

After genotyping by mnPCR-RFLP, the isolates were compared and classified based on the genotypes available in the ToxoDB database (http://toxodb.org/toxo/and http://web.utk.edu/~csu1/Toxoplasma.html) and recently published studies. The phylogenetic relationships of *T. gondii* isolates in the present study along with other genotypes previously isolated from the same region and reference strains were examined using SplitsTree4 software, through a reticulated network analysis applying the neighbor-joining method (Huson and Bryant, 2006).

3. Results

Out of the 14 isolates analysed using ITS1 PCR, 10 tested positive and were further analysed by mnPCR-RFLP (Table 1). The most frequent genotype identified was #13 ($n = 4$), followed by #57 ($n = 3$), #48 ($n = 1$), #273 ($n = 1$), and a unique new genotype ($n = 1$), which has not been previously described. The Brazilian clonal lineages (BrI, BrII, BrIII and BrIV), as well as the clonal archetypal lineages I, II, and III, were not detected by RFLP in any of the samples. Mixed infections, commonly found in Brazil, were also not identified.

The overall mortality rate in mice infected with the genotyped isolates was 21.4% (6/28). The most frequently identified genotype, genotype #13 (Caribbean 1), caused clinical disease in 27.3% (3/11) of infected mice, while genotype #273 resulted in the death of 3 out of 5 infected mice. Genotypes #57, #48, and the new genotype did not induce clinical disease in the infected mice (Table 1).

Genotype #13 exhibited a widespread distribution across the State of Paraiba, being found in both Atlantic rainforest areas and semiarid climates. On the other hand, genotypes #48, #57, #273, and the new genotype were restricted to the semiarid regions of the state (Fig. 1).

The phylogenetic network analysis of the isolates is depicted in Fig. 2. The genotypes identified in this study were separated by significant genetic distances and were scattered throughout the network. The genotypes identified during this study were also clearly distinct from the Type II, BrII, and BrIV genotypes. The new genotype identified in this study was closely related to Type III and BrIII. Genotypes #13, #48, #57 and #273 were closely related to type I, BrI, type III and BrIII genotypes, respectively.

4. Discussion

Four of the genotypes (#13, #48, #57 and #273) identified in sheep during this study had previously only been described in samples from chickens, pigs and goats in the Northeast Region of Brazil (Feitosa et al., 2017a, b; Rêgo et al., 2017). One of the genotypes discovered in a goat is considered novel, never having been described in the literature before.

Table 1
PCR-RFLP genotyping of *Toxoplasma gondii* isolates from goats and sheep destined for human consumption in Paraíba State, Northeast Region of Brazil, and lethality in mice.

DIsolate ID	Location	Mouse bioassay *N/n*	PCR-RFLP markers											TOXO DB-RFLP genotype[a]
			SAG1	5′3′ SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	
TgGtPB5	Sousa	1/3[b]	I	I	I	I	I	III	II	III	III	I	III	#13
TgGtPB9	Patos	0/1[b]	I	I	I	I	I	III	II	III	III	I	III	#13
TgShBrPB11	Campina Grande	0/2[c]	I	I	I	I	I	III	II	III	III	I	III	#13
TgShBrPB12	Campina Grande	2/5[c]	I	I	I	I	I	III	II	III	III	I	III	#13
TgShBrPB2	Aparecida	0/3[c]	I	III	III	III	III	III	III	III	III	III	III	#48
TgShBrPB1	Aparecida	0/5[c]	I	I	I	III	I	II	u-1	I	III	II	III	#57
TgShBrPB29	Vieiropolis	0/2[c]	I	I	I	III	I	II	u-1	I	III	II	III	#57
TgShBrPB19	Malta	0/1[c]	I	I	I	III	I	II	u-1	I	III	II	III	#57
TgShBrPB7	Cacimba de Areia	3/5[c]	I	I	I	III	I	II	u-1	III	III	I	III	#273
TgGtPB2	Cacimba de Areia	0/1[b]	I	III	III	I	I	III	III	III	III	I	III	New

Note: *N/n*, no. sick/no. infected.
[a] TOXO-DB (http://toxodb.org/toxo/) and http://web.utk.edu/~csu1/Toxoplasma.html.
[b] Isolate obtained from goats.
[c] Isolate obtained from sheep.

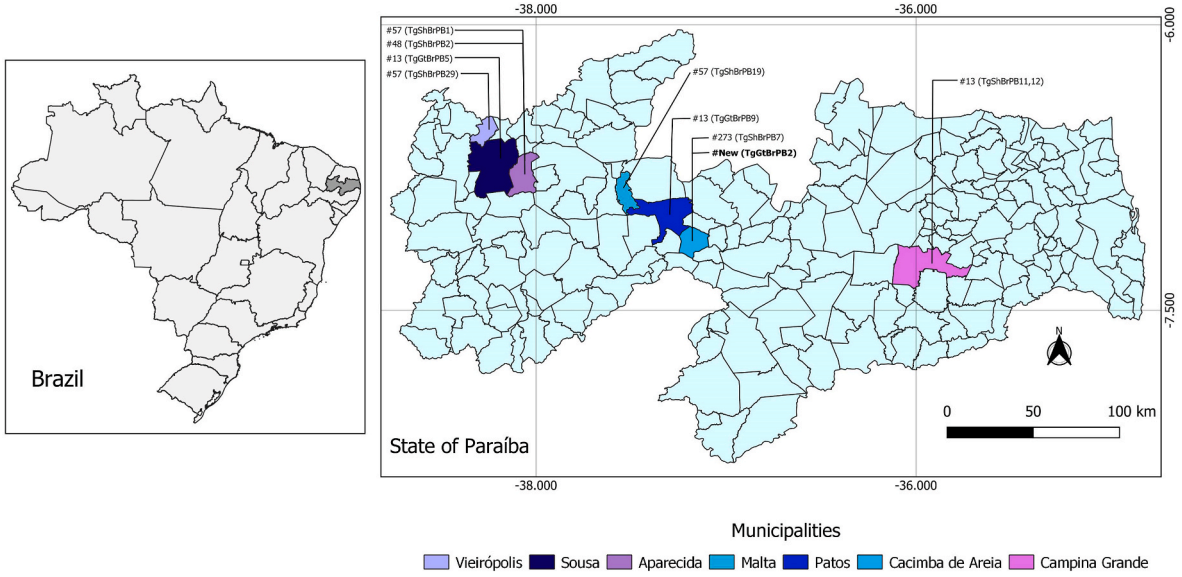

Fig. 1. Map showing the municipalities in the Paraíba State, Northeast Region of Brazil, from which goat and sheep samples were collected, along with their respective identified genotypes.

According to Silva et al. (2023), many domestic cats in the Northeast Region of Brazil have unrestricted outdoor access and frequently hunt small rodents and birds. Furthermore, neutering animals is not a government policy, and this sustains a stray cat population with a significant number of young cats continuously being born (Timóteo et al., 2020). The young cats serve as the primary source of oocysts following their initial infection (Zulpo et al., 2018). New genotypes are formed when a feline is infected simultaneously with different strains, through hunting superinfected intermediate hosts, or successively in a very short time (Dubey, 2008; Sibley and Ajioka, 2008; Jensen et al., 2015). If a cat eats two infected intermediate hosts on the same day in Brazil, due to the high genetic diversity of *T. gondii*, there is a high probability that these intermediate hosts have different genotypes, which may result in new a genotype being produced by recombination. Otherwise, in other parts of the world, where there is not as high genetic diversity of *T. gondii*, if a cat eats two intermediate hosts on the same day, the genotypes are likely to be of the same type, and the recombination will result in the same genotype.

The Brazilian clonal lineages (BrI, BrII, BrIII and BrIV) were not found in this study. However, in other studies conducted in the Northeast Region of Brazil, it has been demonstrated that genotypes BrII and BrIII are frequently encountered from production animals, while the clonal lineages BrI and BrIV are more commonly found further south in Brazil (Carlos et al., 2009; Soares et al., 2011; Feitosa et al., 2017b; Witter et al., 2022).

Genotype #13 proved to be quite common among goats and sheep from slaughterhouses in the State of Paraíba. This genotype is frequently reported in studies conducted in the Northeast of Brazil and has been previously described in chickens, pigs, and goats intended for human consumption (Clementino Andrade et al., 2013; Feitosa et al., 2017a, b), suggesting it is prevalent among the various animal species used in food production in the Northeast Region (Ragozo et al., 2010; Rêgo et al.,

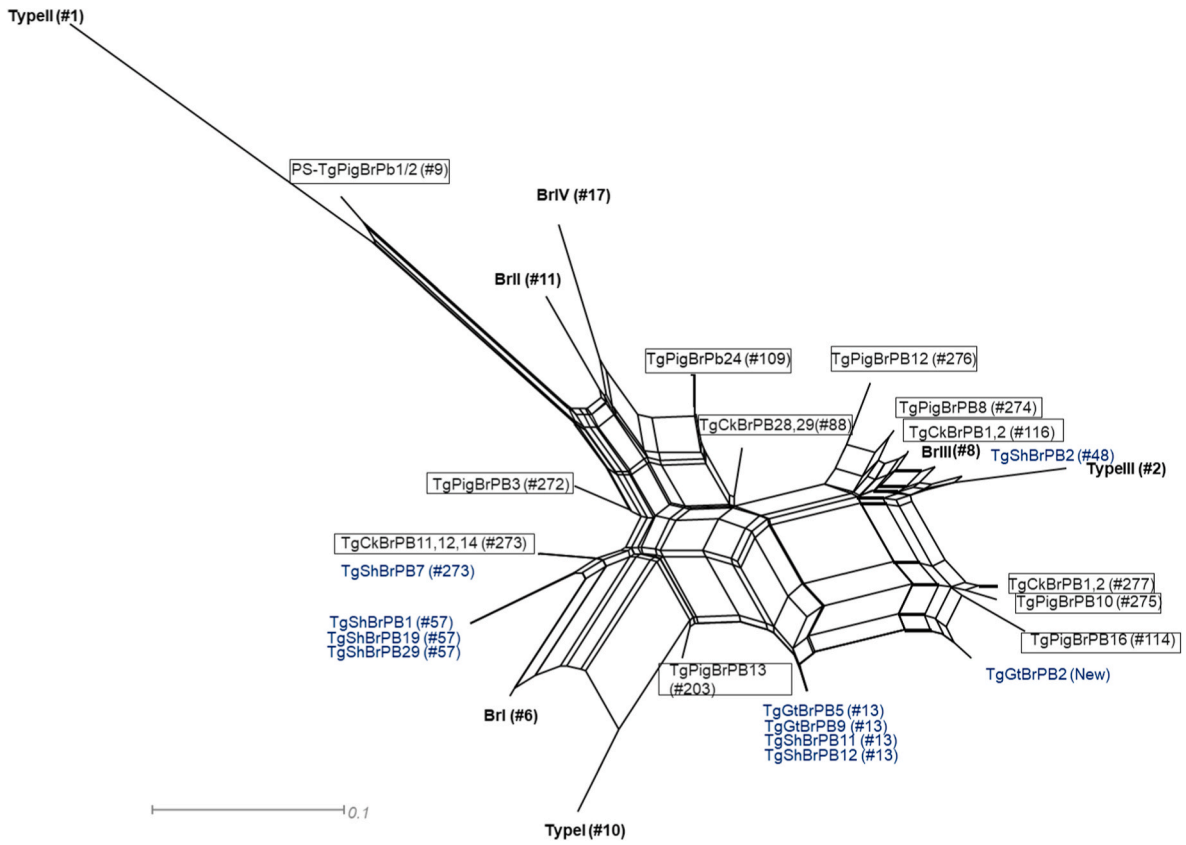

Fig. 2. Phylogenetic network analysis of *Toxoplasma gondii* in goats and sheep from the Northeast Region of Brazil destined for human consumption, using PCR-RFLP data. Genotype ID and representative strain with TOXODB number in parentheses are listed for each taxonomic branch. Reference strains are represented in black, the strains from this study are indicated in blue, and strains previously described in the region but not covered in this paper are enclosed in boxes.

2017). Hamilton et al. (2019) also identified this genotype on St Kitts, the Caribbean, and classified it as virulent, as during a bioassay genotype #13 killed 100% of the infected mice inoculated with as few as 200 tachyzoites intraperitoneally within 16 days post-inoculation. Furthermore, genotype #13 has been previously reported to cause toxoplasmic encephalitis and pulmonary toxoplasmosis in immunocompromised patients from the French West Indies (Ajzenberg et al., 2009) and in patients from French Guyana (Mercier et al., 2011). Genotype #13 has also been identified as a source of an abortion storm in goats in the Northeast of Brazil (Vilela et al., 2023 in press).

Genotype #48 has previously been described in chickens in the Northeast of Brazil by Feitosa et al. (2017b) and has also been isolated from rabbits, chickens, and rats from Argentina and Guyana by Bernstein et al. (2018) and Dubey et al. (2007). Genotype #57 has previously been described in pigs, goats, and chickens from Piauí and Maranhão States in the Northeast of Brazil (De Oliveira et al., 2009; Rêgo et al., 2017). Finally, genotype #273 has been described in pigs and chickens from the State of Paraíba, Brazil (Feitosa et al., 2017a, b).

The phylogenetic network analysis showed that the genotypes described in this study have proximity to the reference strains TypeI, BrI, TypeIII, and BrIII. When the genotypes obtained from the present study were compared with other genotypes from the Northeast Region of Brazil, it became evident that the northeastern genotypes are distributed throughout the phylogenetic tree, lacking a closely related pattern to any specific reference strain. Our findings are in agreement with Galal et al. (2022) who conducted research on *T. gondii* evolution and the mechanisms of global dispersal, observing that most Brazilian strains are hybrids of native South American strains and recently introduced clonal lineages from Europe and Africa. Genotypes related to various classical clonal types and variants are described, except for Type II and Type II variants, which are not commonly reported in Brazil, except on Fernando de Noronha Island, Paraiba and Sao Paulo, where these genotypes were described previously (Silva et al., 2017; Feitosa et al., 2017a, b; Silva et al., 2018; Melo et al., 2020b).

5. Conclusion

In conclusion, even with the increasing number of studies on the genotypic profiles of *T. gondii* isolates, each new research effort reveals new genotypes. We also found that atypical genotypes are circulating among different hosts in various regions of Brazil and that the mouse-virulent genotype #13 (Caribbean 1) is prevalent among goats and sheep.

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Ethical approval

The experiment conducted in this study was approved and carried out following the recommendations of the Ethics Committee on Animal

Use (CEUA) - IFPB, under protocol number: 23000.001679.2019-78.

CRediT authorship contribution statement

Thais Ferreira Feitosa: Conceptualization, Data curation, Writing – original draft, Visualization. **Vinícius Longo Ribeiro Vilela:** Methodology, Validation, Writing – review & editing. **Samira Pereira Batista:** Formal analysis, Investigation. **Samara Santos Silva:** Formal analysis, Investigation. **Rinaldo Aparecido Mota:** Conceptualization, Writing – review & editing. **Frank Katzer:** Methodology, Writing – review & editing, Funding acquisition, Project administration. **Paul M. Bartley:** Methodology, Writing – review & editing, Supervision, All authors read and approved the final manuscript.

Declaration of competing interests

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. Given their role as Co-Editor, Frank Katzer had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Professor Aneta Kostadinova (Editor-in-Chief).

Data availability

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2023.100163.

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