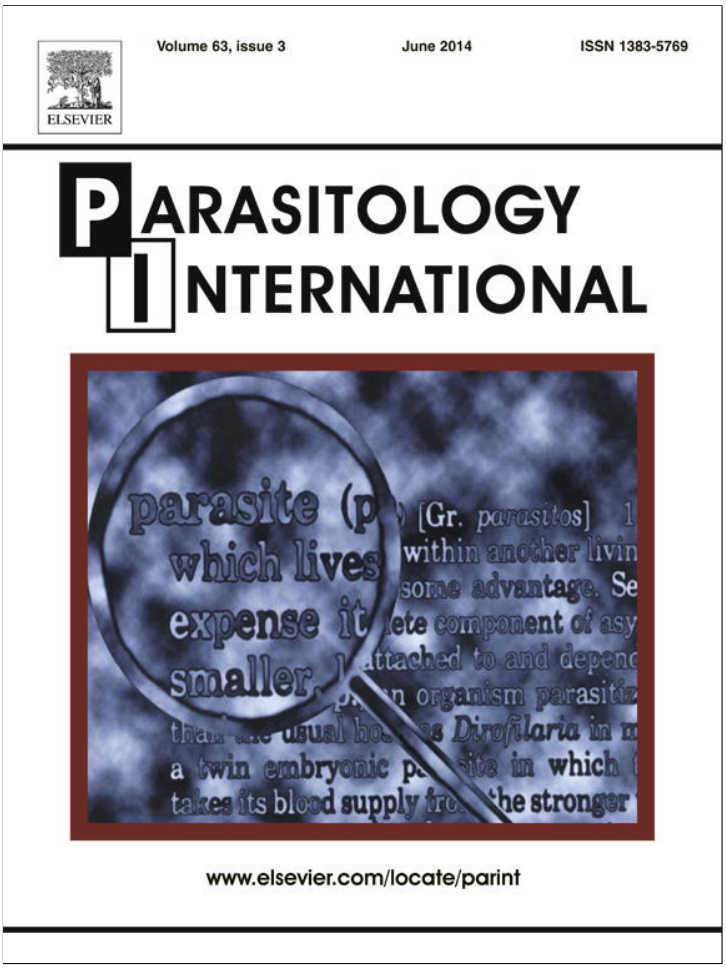

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Short communication

Identification and genetic characterization of a new Brazilian genotype of *Toxoplasma gondii* from sheep intended for human consumption

B.M. Maciel [a], R.L.S. Moura [b], F.S. Carvalho [b], E.A. Costa [c], G.R. Albuquerque [b,*]

[a] Universidade Estadual de Santa Cruz (UESC), Brazil
[b] Animal Science Graduate Program, Universidade Estadual de Santa Cruz (UESC), Brazil
[c] Center for Computational Biology and Biotechnology Information Management (NBCGIB), Universidade Estadual de Santa Cruz (UESC), Brazil

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ABSTRACT

Recent studies have demonstrated that strains of *Toxoplasma gondii* in Brazil are frequently different from those detected in other countries, thus making an accurate phylogenetic analysis difficult. The aim of this study was to genetically characterize *T. gondii* samples from sheep raised in southern Bahia and intended for human consumption, by means of PCR–RFLP and sequencing techniques. Experimental samples were obtained from 200 sheep brains purchased at butcher's shops in Itabuna, Bahia, Brazil. In total, three samples (#54, #124 and #127) were *T. gondii*-positive. The application of multilocus PCR–RFLP using ten molecular markers (SAG1, SAG2, SAG3, BTUB, c22-8, PK1, GRA6, L358, c-29-2 and Apico) revealed a single genotype common to all samples of this study, which differed from any other published *T. gondii* genotypes. An atypical allele was detected in the L358 genetic marker; this has not previously been shown in any other South American *T. gondii* isolates. Phylogenetic analysis on the sequences from multilocus PCR sequencing revealed that these three samples were classified into the same lineage. Extensive indel regions were detected in the Apico genetic marker. Together, our findings revealed a new Brazilian *T. gondii* genotype. Further research should be conducted to enrich the database of Brazilian *T. gondii* genotypes from different regions. This will make it possible to understand the phylogenetic relationship between isolates.

The strains of *Toxoplasma gondii* in Brazil are frequently different from those detected in other countries (see review [1]) and, so far, studies have identified only a small portion of the overall diversity of the parasite. This points towards the continuing expansion of the population of *T. gondii* and, therefore, is an indication of the need for extensive research to detect any other genotypes from different hosts. This is especially relevant in relation to Brazil, in order to better understand the phylogenetic relationships between Brazilian isolates, since there is a high prevalence of *T. gondii* in humans, with 56% seropositivity [1]. Among domestic animals, sheep are the ones most susceptible to clinical toxoplasmosis, which causes abortions in many countries [2,3]. From a public health perspective, these animals are considered to be potential transmitters because they are persistent hosts, with the cysts remaining in their tissues for long periods [4]. Thus, the objective of this study was to genetically characterize *T. gondii* samples from sheep intended for human consumption in southern Bahia, in the northeastern region of Brazil. For this, multilocus PCR–RFLP and multilocus PCR sequencing techniques were used and the results were compared to *T. gondii* reference strains and with strains from the same geographical origin.

Experimental samples were obtained from 200 sheep brains purchased at butcher's shops in Itabuna, state of Bahia, Brazil. The animals came from fourteen municipalities that neighbor Itabuna, in southern Bahia, which comprise a total area of 8263.9 km^2. The brains were then removed for an immediate DNA extraction procedure. The entire brain was mixed using a tissue homogenizer (Novatecnica) for 5 min, and 100 mg fragments were frozen in liquid nitrogen and macerated using a mortar and pestle. DNA extraction was performed using Easy-DNA® kits (Invitrogen) according to the manufacturer's "Protocol 3". *T. gondii* was detected by means of the polymerase chain reaction (PCR) using the two sets of primers that amplified a 529 bp fragment: Tox4 Forward, CGCTGCAGGGAGGAAGACGAAAGTTG; and Tox5 Reverse, CGCTGCAGACACAGTGCATCTGGATT [5]. The reactions were performed in accordance with a previous study [6]. As a positive control, DNA was extracted using an Easy-DNA™ kit (Invitrogen) from RH-strain tachyzoites diluted to 10^7 parasites per mL.

T. gondii genotypes were determined using multilocus nested PCR–RFLP with 10 genetic markers: SAG1, SAG2, SAG3, BTUB, c22-8, PK1, GRA6, L358, c29-2 and Apico [7,8]. The amplification reactions and restriction enzyme digestions were performed based on reference studies

* Corresponding author at: Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz—UESC, Ilhéus, Bahia, Brazil.
E-mail address: gralbu@uesc.br (G.R. Albuquerque).

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[9]. The DNA banding patterns of the samples were compared to genotypes deposited in ToxoDB (http://toxodb.org/toxo/).

The products from nested PCR were purified by using the PureLink™ (Invitrogen) and were sequenced for nine genetic markers (SAG1, SAG2, SAG3, BTUB, c22-8, PK1, GRA6, L358 and Apico) at Ludwig Biotec (Alvorada, RS, Brazil). Sequences amplified using the genetic marker c29-2 did not present good quality in the DNA chromatograms and therefore were discarded from the sequencing analysis. The sequencing was performed in both directions (forward and reverse); the forward amplification was conducted using two independent nested PCR amplification products for each locus from each brain sample and the consensus sequence was used for the final analyses. As positive controls, nested-PCR products from the RH (Type I), PTG (Type II) and CTG (Type III) strains were sequenced once in the forward direction. These sequences were used in accordance with the data available in public databases relating to the GT1, Me49 and VEG strains, respectively.

The chromatograms were analyzed by means of the Phred software, using the parameters "-trim_cutoff 0.01" (which supports the bases with a range of 99% confidence) and "-trim_out" (which does the trims using the modified Mott algorithm, which locates the high-quality region of each read defined by the "trim_cutoff" quality parameter). These analyses were conducted with the help of the Center for Computational Biology and Biotechnology Information Management, Universidade Estadual de Santa Cruz (NBCGIB/UESC).

The nucleotide sequences determined in this study were assembled in contigs using the CAP3 application. The sequences from the *T. gondii* samples of this study were aligned with the ClustalW (version 1.83), manually corrected using the BioEdit Sequence Alignment Editor and compared with the sequences of 10 *T. gondii* reference strains available in the NCBI database (http://www.ncbi.nlm.nih.gov/bioproject/), such as: GT1 strain (BioProject accession no. PRJNA16727), ME49 strain (BioProject accession no. PRJNA28893), VEG strain (BioProject accession no. PRJNA19097), FOU strain (BioProject accession no. PRJNA61561), MAS strain (BioProject accession no. PRJNA61545), VAND strain (BioProject accession no. PRJNA60839), RUB strain (BioProject accession no. PRJNA61119), p89 strain (BioProject accession no. PRJNA61547), TgCATBr5 (BioProject accession no. PRJNA61551) and TgCATBr9 strain (BioProject accession no. PRJNA61549). For the apicoplast (Apico), the sequences were aligned with the *T. gondii* apicoplast complete genome (U87145.2). All sequences were also compared with sequences available in the ToxoDB database, using the BLASTn program for validation.

Phylogenetic analysis was performed using MEGA version 5 by means of the neighbor-joining algorithm, and the distances were computed using the Tajima–Nei method. The stability of branches was assessed after bootstrapping with 500 replicates. To check the distance between samples from the same geographical origin, eight *T. gondii* isolates from pigs in southern Bahia, Brazil (TgPgBr06, TgPgBr08, TgPgBr09, TgPgBr11, TgPgBr12, TgPgBr13, TgPgBr15 and TgPgBr16) [6] were also included.

The equality of evolutionary rate between the sequences from the isolates of this study was calculated by means of Tajima's relative rate test [10], in which *P*-values less than 0.05 are often used to reject the null hypothesis of equal rates between lineages. All positions containing gaps and missing data were eliminated. The Tajima's *D* test of neutrality [11] was used to calculate the number of segregating sites, comparing the DNA sequences from the reference strains and from the samples of this study. This test computes a standardized measurement of the total number of segregating sites (polymorphic sites) and the average number of mutations between pairs in the sequence samples. All positions with less than 95% site coverage were eliminated. Evolutionary analyses were conducted in MEGA version 5.

In total, the three strains of *T. gondii* (1.5%) were detected from the 200 sheep brains analyzed; the samples were designated as #54, #124 and #127. Similar results were found by Berger-Schocha et al. [12], who detected *T. gondii* in 2% of their sheep samples in Switzerland. However, Asgari et al. [13] found a prevalence of 37.5% in southern Iran, using 1 g of tissue for DNA extraction. In Brazil, significant results were published by Ragozo et al. [14] and Da Silva et al. [15], who isolated viable *T. gondii* from seropositive sheep, in 19.5% and in 30.3%, respectively, by means of bioassays.

Genetic characterization by means of PCR–RFLP detected a single genotype in all three strains (Table 1), which were indistinguishable by this technique. The PK1, L358 and c29-2 genetic markers displayed an atypical allele. Rajendran et al. [16] summarized the typing results from PCR–RFLP on 164 isolates from South America and none of them showed an atypical allele in the L358 genetic marker. Thus, the three strains of the present study could not be grouped with any of the *T. gondii* genotypes from sheep previously described in Brazil [1,6,7,15,17], or even with any genotypes deposited in ToxoDB, including Types I, II and III or BRI, II, III and IV genotypes. This is not a surprise, since the PCR–RFLP technique has not produced consistent results that describe the genetic variation of samples when applied in locations with

Table 1
Genetic characterization by PCR–RFLP and multilocus sequencing of *Toxoplasma gondii* from sheep.

PCR–RFLP genetic markers										
Sample	SAG1	SAG2	SAG3	BTUB	c22-8	PK1	GRA6	L358	c29-2	Apico
#54	I	I	I	III	III	u-1	III	u-1	u-1	III
#124	I	I	I	III	III	u-1	III	u-1	u-1	III
#127	I	I	I	III	III	u-1	III	u-1	u-1	III

Total of polymorphisms in nine different genetic markers detected by PCR-sequencing of *T. gondii*						
Sample	Total of polymorphisms[a]				Tajima's relative rate test[b]	Tajima's *D* neutrality test[c]
	Indel	Ts	Tv	Total		
#54	61	23	33	117 (3.0%)	ud = 10	
#124	62	25	36	123 (3.6%)	ud = 15	
#127	63	22	39	124 (3.2%)	ud = 10	
Average between samples	62	23.3	36.0	121.3 (3.3%)	$P = 0.31731$	$D = -0.886015$

(u-1) Atypical alleles.

[a] The number of insertions and deletions (Indel), transitions (Ts) and transversions (Tv) were calculated comparing the sequence of each isolate with the pattern obtained from GT1, ME49, VEG, TgCATBr5, TgCATBr9, FOU, RUB, VAND, p89 and MAS reference strains.

[b] The equality of evolutionary rate between the sequences 54, 124 and 127. "ud" means unique differences in each sequence. All positions containing gaps and missing data were eliminated. *P*-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages.

[c] The analysis involved 13 multi-locus nucleotide sequences (GT1, ME49, VEG, TgCATBr5, TgCATBr9, FOU, RUB, VAND, p89, MAS, #54, #124, #127). All positions containing gaps and missing data were eliminated. There were a total of 2774 bases aligned with 105 segregating sites. A negative Tajima's *D* indicates an excess of low frequency polymorphisms. Evolutionary analyses were conducted in MEGA version 5.

a high parasite genetic diversity, such as in Brazil [7]. In these regions, clear clustering is generally not observed when the genotypes of different hosts and geographical locations are compared [7].

Through multilocus PCR sequencing, 2774 bases could be aligned showing 105 segregating sites. All positions containing gaps and missing data were discarded. The sequences amplified with the markers SAG3, GRA6, L358 and Apico represented 96% of the total number of polymorphisms. In contrast, regions amplified by the markers SAG1, SAG2 and BTUB were better conserved (Supplementary data). The three samples differed from the 10 *T. gondii* strains and from the apicoplast genome, showing an average of 121.3 DNA polymorphisms (3.3%), including insertion, deletion, transition or transversion (Table 1), distributed across the different genetic markers (Supplementary data).

Although the multilocus PCR sequencing analysis indicated that the three samples from sheep were different, the genetic variability between the samples was not sufficient to classify them as different genotypes in the phylogenetic analysis (Fig. 1). This was indicated through Tajima's relative rate test (Table 1), which calculated the equality of the evolutionary rate among the concatenated sequences generated by multilocus PCR sequencing. Through this test, the null hypothesis of equal rates between lineages was not rejected ($P > 0.05$) and the three samples can be considered to be from the same lineage, thus forming a single genotype. In the phylogram, they were clustered together, in a sister-group of the TgCATBr5 and TgCATBr9 strains, which is monophyletic to genotypes from the same geographical origin, i.e. southern Bahia, Brazil (Fig. 1). Through using more sequences (11 multilocus RFLP markers plus one marker for apicoplasts and four intron sequences), Su et al. [18] showed that the TgCATBr5 and TgCATBr9 strains did not belong to the same clade. Thus, the use of more sequences in the phylogenetic analysis will probably enable more detailed results in further studies.

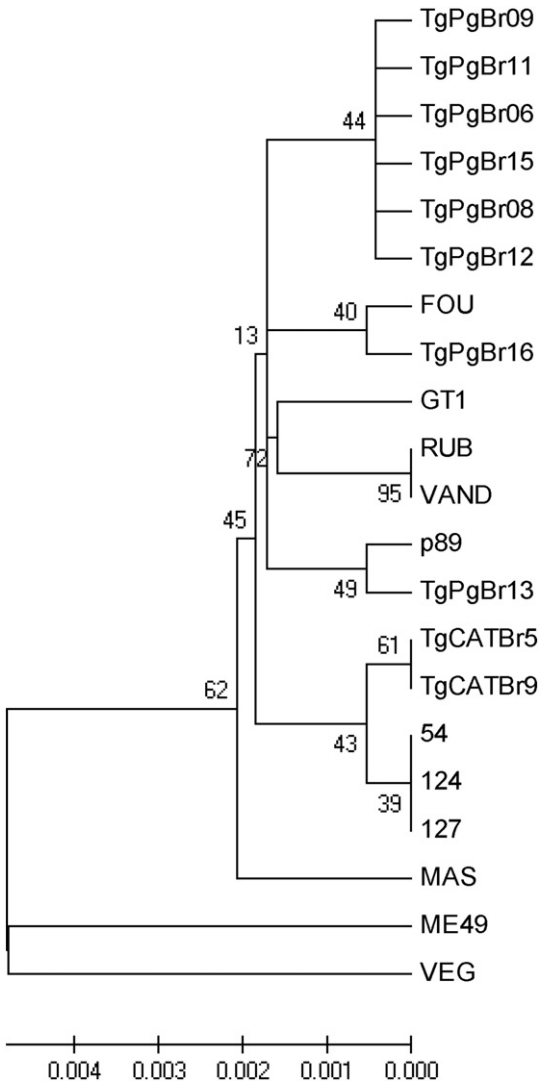

Fig. 1. Phylogram of *Toxoplasma gondii* strains as determined by sequence analysis of the eight genomic genetic markers (SAG1, SAG2, SAG3, BTUB, c22-8, PK1, GRA6 and L358). The tree was constructed using the Neighbor-joining method after bootstrapping with 500 repetitions. The distances were computed using the Tajim–Nei method.

The analysis using the Tajima's *D* neutrality test revealed a negative result (−0.89), which was indicative of an excess of low-frequency polymorphism between all the *T. gondii* strains used in this analysis. Similar results were found in the previous studies using Brazilian isolates [6,7]. This means that although there is a high degree of polymorphism in Brazilian isolates, the frequency of polymorphisms in the same locus is low, in comparison with different strains from different geographical origin. However, research on a larger variety of vertebrate hosts is still necessary to understand the molecular diversity and population structure of *T. gondii* in Brazil [1].

The most polymorphic sequences were detected in the Apico genetic marker, which presented extensive indel regions, including four insertion sites totaling 43 base-pair insertions and one deletion site of 15 base pairs. The alignment presented only 87% identicalness with the apicoplast genome (Supplementary data). Despite this high level of polymorphism, the samples were grouped into the Type III clonal genotype by means of PCR–RFLP (Table 1). Apicoplasts are chloroplast-like organelles that are essential to most apicomplexan parasites, including *Toxoplasma*. Structural and functional evaluations on apicoplast genes are a validated target for drug therapy [19] and, therefore, studies that characterize these genes in Brazilian isolates may be relevant to this approach, since the prevalence of *T. gondii* in the human population in Brazil is higher than 50% and prophylactic and therapeutic control need to be better adapted for these parasite samples.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2014.03.001.

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