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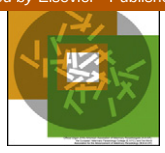

Genetic characterization of *Toxoplasma gondii* isolates from pigs intended for human consumption in Brazil

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ABSTRACT

This study genetically *Toxoplasma gondii* isolates obtained from pigs intended for human consumption in northeastern Brazil; multilocus PCR-RFLP and sequencing techniques were utilized. Bioassays were conducted using the brain and tongue of 20 pig heads purchased at butcher shops in the city of Ilheus, Bahia, Brazil. Overall, 11 *T. gondii* isolates designated TgPgBr06-16 were identified. Application of multilocus PCR-RFLP with seven molecular markers (SAG1, SAG2, SAG3, BTUB, C22-8, PK1 and Apico) identified six different genotypes. Isolates TgPgBr 06, 08, 11, 12, 14 and 15 were indistinguishable by this technique, forming a single genotype; the remaining isolates were characterized as distinct genotypes. However, when five genetic markers (SAG1, SAG2, SAG3, BTUB and c22-8) were employed in multilocus PCR-sequencing, all eleven strains of *T. gondii* were shown to be different. All isolates differed from Type I, II and III clonal genotypes using both genotyping techniques. These results demonstrate that the multilocus PCR-RFLP assay underestimated the true diversity of the *T. gondii* population in this study. Thus, DNA sequencing is the preferred technique to infer the genetic diversity and population structure of *T. gondii* strains from Brazil. Moreover, it is necessary to develop new molecular markers to group and characterize atypical *T. gondii* isolates from South America.

1. Introduction

Toxoplasma gondii is an apicomplexan parasite with great medical and veterinary importance; it is distributed worldwide and infects warm-blooded animals, including humans (Tenter et al., 2000). Its life cycle is complex and involves an asexual phase in a wide variety of intermediate hosts and a sexual phase that occurs exclusively in feline small intestine epithelial cells (Dubey, 2009).

Molecular studies using PCR-RFLP and microsatellite analysis of *T. gondii* isolates from Europe and North America ranked *T. gondii* strains into three genetic lineages, designated as Type I, Type II and Type III; these lineages are considered to be clonal genotypes that exhibit low genetic diversity (Howe and Sibley, 1995; Ajzenberg et al., 2002). However, the use of new molecular markers and the study of isolates from South America and Brazil, in particular, has demonstrated that *T. gondii* has a larger genetic variability (Lehmann et al., 2004, 2006; Su et al., 2006; Dubey et al., 2007a,b; Pena et al., 2008; Khan et al., 2009).

In Brazil, Type I and III *T. gondii* parasites have been identified; however, parasites with atypical genotypes alleles have also been observed (Dubey et al., 2007a,b; Pena et al., 2008). Recently, clonal Type II parasites were isolated in chickens from the island of Fernando de Noronha and in

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sheep from the state of São Paulo (Dubey et al., 2010; Da Silva et al., 2011).

Virulence differences have been observed in experimental animal models (Da Silva et al., 2005). Because of this correlation, the improvement of genetic characterization methods to monitor and properly treat different cases of infection is justified (Howe and Sibley, 1995; Ajzenberg et al., 2005). Thus, it is necessary to analyze a larger number of *T. gondii* isolates from multiple sources of infection to evaluate associations between parasite genotype and disease severity in humans and animals from different regions of the world (Mondragon et al., 1998; Owen and Trees, 1999).

Pigs are an important source of *T. gondii* infection in human populations (Tenter et al., 2000; Dubey, 2009). However, in Brazil, there are few studies concerning the genetic characterization of *T. gondii* isolates from this animal; moreover, all of the available studies were conducted in the southern and southeastern regions of the country (Dos Santos et al., 2005; Ferreira et al., 2006; Belfort-Neto et al., 2007; Frazão-Teixeira et al., 2011). Therefore, it is necessary to perform additional studies in other regions to generate data that will demonstrate the importance of this etiological agent for public health.

Thus, the objective of this study was to perform the genetic characterization of *T. gondii* isolates from pigs intended for human consumption from southern Bahia, in northeastern Brazil; the techniques of multilocus PCR-RFLP and multilocus PCR-sequencing were employed.

2. Materials and methods

2.1. Toxoplasma gondii isolated from naturally infected pigs

Experimental samples were obtained from 20 pig heads purchased at butcher shops that traded fresh pork for human consumption at the wholesale produce market of Ilheus, Bahia, Brazil, from September 2009 to February 2010. The heads were individually placed in refrigerated containers and taken to the Veterinary Parasitology Laboratory of State University of Santa Cruz, Brazil; the brains were then removed.

2.2. Bioassay

Peptic digestion of samples was performed according to the protocol established by Dubey (1998) with several modifications. Briefly, each brain was ground in a blender. While grinding, a minimum volume of PBS was added to facilitate the procedure. The jar of the device was properly washed with a solution of 2.5% sodium hypochlorite and neutral detergent between each organ to prevent contamination between samples. For each sample, 40 g of homogenate was removed and placed in a 250 mL Erlenmeyer flask; next, an acid pepsin solution (pH 1.1–1.2) was added until a final volume of 200 mL was reached. Homogenate digestions were incubated in an orbital shaker at a temperature of 37 °C for 1 h. The digested materials were then strained in a sieve with double cheesecloth and centrifuged twice at 1200 × *g* for 10 min. Supernatants were discarded, and the sediments were resuspended in a neutralizing solution of 1.2% sodium bicarbonate (pH 8.3) and centrifuged at 1200 × *g* for 20 min. Supernatants were again discarded, and the sediments were resuspended in 5 mL of an antibiotic solution that contained 1000 IU of penicillin and 100 μg streptomycin per mL of PBS.

This product was subcutaneously inoculated in three Swiss Webster mice (25–35 g) at a dose of 1 mL per mouse; mice were given a second identical injection 24 h after the first inoculation. For each group, an additional mouse was inoculated with sterile PBS as control. The mice were observed for 42 days and sacrificed at the end of this period for brain retrieval. The virulence analysis of the samples was realized according to previous report (Bezerra et al., 2012).

2.3. Molecular diagnosis and genetic characterization of Toxoplasma gondii

2.3.1. DNA extraction

Following brain removal, 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 Protocol 3 of the manufacturer.

2.3.2. Diagnosis by PCR

PCR amplifications were performed using two sets of primers that amplified a 529 bp fragment: Tox4 Forward, CGCTGCAGGGAGGAAGACGAAAGTTG and Tox5 Reverse, CGCTGCAGACACAGTGCATCTGGATT (Homan et al., 2000). Each 50 μL PCR mixture contained 10 μL of sample DNA, 0.2 mM of sense and antisense primers, 100 mM dNTPs (Invitrogen), 60 mM Tris–HCl (pH 9.0), 2.5 mM $MgCl_2$ and 2 U of Taq DNA polymerase (Invitrogen). The amplification 37 cycle consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C with a final extension step of 10 min at 72 °C. To visualize the PCR products, 5 μL of each reaction mixture was loaded and run on 2% agarose gels in 1X TBE buffer, stained with ethidium bromide and examined under UV light. As a positive control, DNA was extracted using an Easy-DNA™ Kit (Invitrogen) from RH strain tachyzoites diluted to 10^7 parasites per mL. The negative control consisted of DNA extracted from brain samples of mice not inoculated. Positive and negative controls were used in all tests.

2.3.3. Multilocus PCR-RFLP

T. gondii isolate genotypes were determined using multilocus PCR-RFLP with seven genetic markers: SAG1, SAG2, SAG3, BTUB, C22-8, PK1 and Apico (Table 1). The amplification reactions were performed in a final volume of 50 μL; each reaction mixture contained 0.2 mM of each primer, 100 mM dNTPs (Invitrogen), 60 mM Tris–HCl (pH 9.0), 2.5 mM $MgCl_2$, 2 U of Taq DNA polymerase (Invitrogen) and 3 μL of isolate DNA. In the second amplification, each reaction utilized 1 μL of the product of the first PCR. Each amplification process consisted of an initial denaturation step of 5 min at 94 °C, followed by 33 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C with a final extension step of 10 min at 72 °C. For the restriction enzyme

Table 1
Molecular markers used for PCR-RFLP typing of *Toxoplasma gondii*.

Markers		Primers	Nested PCR (pb)	Restriction enzymes	Digestion[a]	Reference
SAG1	External	F:GTTCTAACCACGCACCCTGAG	390	Sau96I	NEB4, BSA	Grigg and
		R:AAGAGTGGGAGGCTCTGTGA		HaeII	37 °C 60 min	Boothroyd (2001)
	Internal	F:CAATGTGCACCTGTAGGAAGC			2.5% gel	
		R:GTGGTTCTCCGTCGGTGTGAG				
SAG2	External	F:GGAACGCGAACAATGAGTTT	546	Hinf I	NEB2, BSA	Khan et al. (2005);
		R:GCACTGTTGTCCAGGGTTTT		Taq I	37 °C 50 min, 65 °C 50 min	Su et al. (2006)
	Internal	F:ACCCATCTGCGAAGAAAACG			2.5% gel	
		R:ATTTCGACCAGCGGGAGCAC				
SAG3	External	F:CAACTCTCACCATTCCACCC	225	Nci I	NEB4, BSA	Su et al. (2006)
		R:GCGCGTTGTTAGACAAGACA			37 °C 60 min	
	Internal	F:TCTTGTCGGGTGTTCACTCA			2.5% gel	
		R:CACAAGGAGACCGAGAAGGA				
BTUB	External	F:TCCAAAATGAGAGAAATCGT	411	BsiEI	NEB4, BSA	Khan et al. (2005);
		R:AAATTGAAATGACGGAAGAA		Taq I	37 °C 60 min	Su et al. (2006)
	Internal	F:GAGGTCATCTCGGACGAACA			2.5% gel	
		R:TTGTAGGAACACCCGGACGC				
c22-8	External	F:TGATGCATCCATGCGTTTAT	521	BsmAI	NEB4, BSA	Khan et al. (2005);
		R:CCTCCACTTCGGTCTCA		Mbo II	37 °C50 min, 60 °C 50 min	Su et al. (2006)
	Internal	F:TCTCTCTACGTGGACGCC			2.5% gel	
		R:AGGTGCTTGGATATTCGC				
PK1	External	F:GAAAGCTGTCCACCCTGAAA	903	Ava I	NEB4, BSA	Khan et al. (2005);
		R:AGAAAGCTCCGTGCAGTGAT		Rsa I	37 °C 60 min	Su et al. (2006)
	Internal	F:CGCAAAGGGAGACAATCAGT			2.5% gel	
		R:TCATCGCTGAATCTCATTGC				
Apico	External	F:TGGTTTTAACCCTAGATTGTGG	640	Afl II	NEB4, BSA	Su et al. (2006)
		R:AAACGGAATTAATGAGATTTGAA		Dde I	37 °C 60 min	
	Internal	F:TGCAAATTCTTGAATTCTCAGTT			3.0% gel	
		R:GGGATTCGAACCCTTGATA				

(F) forward primer; (R) reverse primer.
[a] The digestion protocols were performed with some modifications of the reference protocols.

digestions, 5 mL of the nested-PCR products was used; restriction enzymes, alone or in combination, according to the markers, were added, and digestions were incubated at their respective cleavage temperatures (Table 1). All products were analyzed by agarose gel electrophoresis (2.5 or 3% depending on the marker), stained with ethidium bromide and examined under UV light. The DNA banding patterns of the isolates were compared with genotypes deposited in ToxoDB (http://toxodb.org/toxo/).

2.3.4. DNA sequencing

Nested-PCR products were purified by using the Wizard® SV Gel and PCR Clean-up System (Promega) and sequenced for five genetic markers (SAG1, SAG2, SAG3, BTUB and C22-8) using the *ABI-PRISM 3100 Genetic Analyzer* automatic sequencer (Applied Biosystems). Sequences amplified using the genetic markers PK1 and APICO did not present good quality in the DNA chromatograms and they were discarded for the sequencing analysis. DNA samples (45 ng) were sequenced using 3.2 pmol of the respective primers according to each marker and 2 μL of the *BigDye Terminator v 3.1 Cycle Sequencing RR-100* reagent (Applied Biosystems); the final volume of each reaction was 10 μL. The amplifications were performed in a *GeneAmp PCR System 9700* (Applied Biosystems) thermocycler with an initial denaturation phase of 96 °C for 3 min, followed by 25 cycles of 96 °C for 10 s for denaturation, 55 °C for 5 s for annealing and 60 °C for 4 min for extension. Nucleotide sequences determined in this study were mounted using DNA STAR SeqMan application. The chromatograms were analyzed by Phred program at Núcleo de Biotecnologia Computacional e Gestão de Informações Biotecnológicas (NBCGIB/UESC). SAG1, SAG2, SAG3, BTUB and C22-8 gene sequences from the 11 *T. gondii* isolates of this study were aligned with ClustalW (version 1.83; Thompson et al., 1994) and corrected using BioEdit Sequence Alignment Editor. For additional comparison, sequences from *T. gondii* GT1 strain (BioProject accession no. PRJNA16727), *T. gondii* ME49 strain (BioProject accession no. PRJNA28893) and *T. gondii* VEG strain (BioProject accession no. PRJNA19097) available in the NCBI database (http://www.ncbi.nlm.nih.gov/bioproject/9535isolates) were also inputted. All sequences were also compared with sequences available in the GenBank database using the BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for validation.

2.3.5. Phylogenetic analyses

A distance matrix was constructed using the banding PCR-RFLP pattern obtained for the seven genetic markers tested (SAG1, SAG2, SAG3, BTUB, C22-8, PK1 and APICO). The 11 *T. gondii* pig isolates and Type I, II and III strains were analyzed. A phylogenetic tree was constructed using the nearest neighbor method; branch distances were computed using the Euclidian method.

2.3.6. Statistical analysis

Tajima's test of neutrality (Tajima, 1989) was used to compare the number of segregating sites per site with the nucleotide diversity of the DNA sequences. This test

Table 2
Multi-locus genotypes of *Toxoplasma gondii* by PCR-RFLP.

Isolates	Genotype	Genetic markers							Reference
		SAG1	SAG2	SAG3	BTUB	C22-8	PK1	APICO	
TgPgBr 06,08, 11, 12, 14, 15	5	I	I	III	III	I	I	III	This study
TgPgBr 7	6	I	I	III	III	u-1	ND	III	
TgPgBr 9	7	I	I	III	II	I	I	III	
TgPgBr 10	8	u-1	I	III	III	III	I	III	
TgPgBr 13	9	I	I	III	III	I	u-1	III	
TgPgBr 16	10	I	I	III	I	I	I	III	
RH88	Type I	I	I	I	I	I	I	I	Dubey et al. (2008)
PTG	Type II	II/III	II	II	II	II	II	II	
CTG	Type III	II/III	III	III	III	III	III	III	
TgCatBr 42, 47, 53, 54, 55, 62, 71, 75 TgCkBr 123, 124, 55, 79, 86, 87, 10, 98, 101, 102, 104, 144	BR I	I	I	III	I	u-1	I	I	Pena et al. (2008) Dubey et al. (2008)
TgCatBr 39, 51, 52, 56, 61, 68, 77, 78 TgCkBr 57, 64, 97	BR II	I	II	III	III	I	II	III	Pena et al. (2008) Dubey et al. (2008)
TgCatBr 58, 59, 60, 73, 74 TgCkBr I 7, 11, 17, 131, 132, 133, 134	BR III	I	III	III	III	II	III	III	Pena et al. (2008) Dubey et al. (2008)
TgCkBr 81, 147, 148, 151, 154, 160, 162, 163	BR IV	u-1	II	III	III	u-1	III	I	Dubey et al. (2008)
TgPgBr 1, 2	#1	I	I	III	I	I	I	I	Frazão-Teixeira et al. (2011)
TgPgBr 3	#2	u-1	u-1	III	I	II	I	I	
TgPgBr 4	Type III	II/III	III	III	III	III	III	III	
TgPgBr 5	#4	u-1	u-1	III	III	I	III	III	

(u-1) Atypical alleles; (ND) Not determined.

computes a standardized measure 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. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

3. Results

3.1. Genetic characterization by multilocus PCR-RFLP

In total, 11 strains of *T. gondii* were isolated from the 20 pig heads analyzed; the strains were designated as TgPgBr 06-16. Application of PCR-RFLP with seven genetic markers (SAG1, SAG2, SAG 3, BTUB, c22-8, PK1 and APICO) revealed six different genotypes that were combinations of type I, II, III and u-1 alleles (Table 2). Isolates TgPgBr06, 08, 11, 12, 14 and 15 were indistinguishable by this technique, representing a single genotype. After comparison with the genotypes deposited in ToxoDB, these samples were similar to TgCkBr156 isolated from chicken in the State of Rio Grande do Sul, Brazil (Dubey et al., 2007b). The remaining isolates were characterized as distinct genotypes. None of the isolates in this study were classified into Type I, II or III clonal genotypes (Fig. 1). Furthermore, none of the isolates was classified as any of the main Brazilian clonal genotypes (BrI, BrII, BrIII and BrIV) defined by Dubey et al. (2008) and Pena et al. (2008) (Table 2). Isolates were also genetically distinct from *T. gondii* genotypes previously isolated from pigs in Brazil, as described by Frazão-Teixeira et al. (2011).

A cluster analysis of the PCR-RFLP band profiles showed that isolates TgPgBr06, 08, 11, 12, 14 and 15 formed a single group. Isolates TgPgBr07, 09 and 10 exhibited the same Euclidean distance. All isolates were closer to clonal Type I (Fig. 1).

3.2. Genetic characterization by PCR-sequencing

DNA sequencing of the 11 *T. gondii* strains (TgPgBr06-16) using five genetic markers (SAG1, SAG2, SAG3, BTUB and c22-8) revealed a total of 1,623 bases that could be aligned with Type I (Tg GT1), Type II (Tg ME49) and Type III (Tg VEG) sequences available in the GenBank database. Alignments revealed that the samples were different at 28 nucleotide positions (1.7% of segregation sites) that were distributed along the different genetic markers; isolates also differed from Type I, II and III clonal genotypes (Fig. 2). A total of 73 DNA polymorphisms (deletion, transition or transversion) were found at the 28 segregation sites (Table 3). Tajima's *D* test showed a negative result (−1.468), which is indicative of an excess of low frequency polymorphism. The sequences amplified with the markers SAG3 and c22-8 were the most polymorphic, representing almost 96% of the total polymorphism (Table 3, Fig. 2). In these markers, the PCR-sequencing could discern the isolates between each other and from the clonal types, while the PCR-RFLP grouped the samples at Type III in SAG3 marker, and at Type I or III in c22-8 marker (Fig. 2). In contrast, regions amplified with markers SAG1 and SAG2 were more conserved and similar to Tg clonal Type I in both methodology (Fig. 2, Table 3). As depicted in Fig. 2, the isolate TgPgBr15 was the most polymorphic.

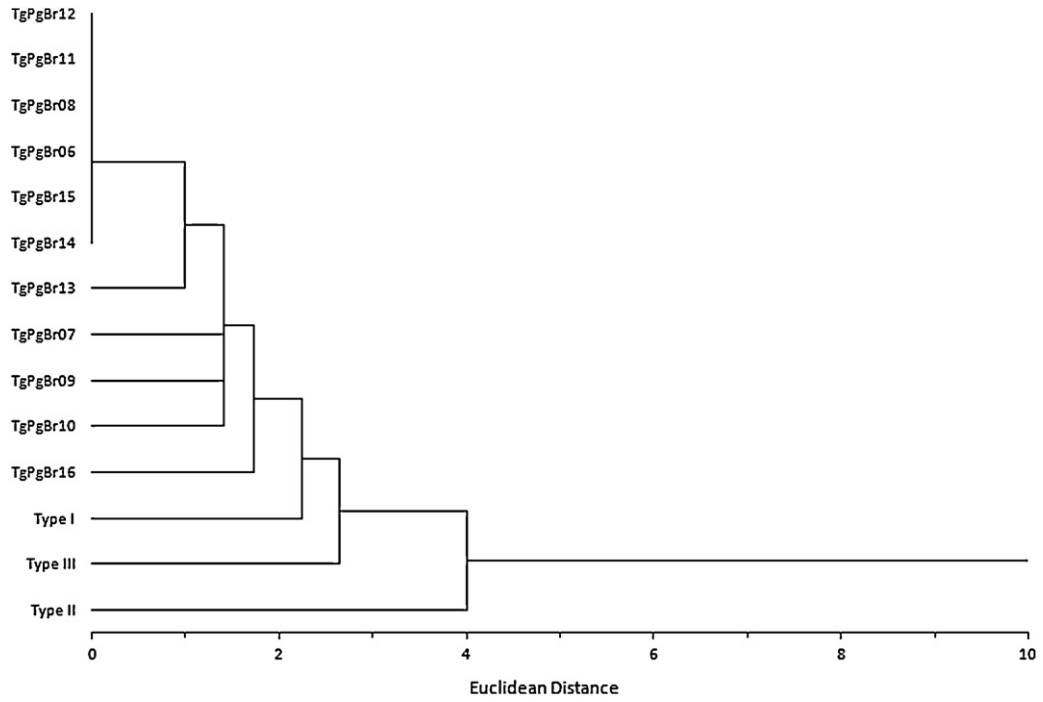

Fig. 1. Dendrogram of *Toxoplasma gondii* strains isolated from pigs from Ilhéus, BA, Brazil, as determined by banding pattern of PCR-RFLP using seven genetic markers (SAG1, SAG2, SAG3, BTUB, C22-8, PK1 and APICO). The tree was constructed using the Nearest Neighbor method. The distances were computed using the Euclidian method.

4. Discussion

The genetic characterization of *T. gondii* isolates from pigs from the state of Bahia in northeastern Brazil was performed to investigate whether these isolates exhibited similarity to Type I, II or III clonal genotypes or other Brazilian genotypes (Pena et al., 2008; Dubey et al., 2008).

From the 20 pig brains analyzed, 11 distinct *T. gondii* isolates were obtained (Table 2, Fig. 2). Isolate genetic characterization performed using multilocus PCR-RFLP and DNA sequencing techniques suggested a high level of parasite genetic diversity in pigs of the region (Table 2; Figs. 1 and 2). In Brazil, high levels of genetic diversity have been previously observed in *T. gondii* isolates from cats and dogs (Pena et al., 2008). However, studies with a larger variety of vertebrate hosts are still necessary to understand the molecular diversity and population structure of *T. gondii* in Brazil (Dubey et al., 2008). With the data currently available, when the genotypes of different hosts and geographical locations are compared, clear clustering is generally not observed (Pena et al., 2008).

Multilocus PCR-RFLP analyses performed by Dubey et al. (2008) and Pena et al. (2008) in *T. gondii* isolates obtained from birds, cats and dogs identified four main clonal genotypes in the Brazilian states sampled; these were termed types BrI, BrII, BrIII and BrIV. Frazão-Teixeira et al. (2011) identified an additional three distinct genotypes of isolates from pigs in Brazil, called #1, #2, and #4. However, none of the isolates characterized in this study through PCR-RFLP grouped with any of the *T. gondii* genotypes previously described in Brazil, or even with Types I, II or III clonal genotypes. Although the isolates in this study exhibited

Table 3
Number of polymorphisms in different genetic marker detected by PCR-sequencing of Toxoplasma gondii TgPgBr 06-16 isolates.

Polymorphism	Genetic markers[a]					Total
	SAG1	SAG2	SAG3	BTUB	c22-8	
Insertion	0	0	0	0	0	0
Deletion	0	0	1 (8.3%)	0	11 (91.7%)	12
Transition	0	0	8 (47.1%)	3 (17.6%)	6 (35.3%)	17
Transversion	0	0	16 (36.4%)	0	28 (63.6%)	44
Total	0	0	25 (34.2%)	3 (4.1%)	45 (61.6%)	73
Tajima' *D* (statistic test)		−1.468				

Note: A negative Tajima's *D* signifies an excess of low frequency polymorphisms.
[a] Total of 1623 bases aligned with 28 segregating sites.

Marker - SAG1 (340 bp) - chromossome VIII												
Nucleotide	8	26	61	78	171	259	No. Polymorphisms					Genotype
Consensus	G	T	C	G	G	C	In	Del	Ts	Tv	Total (%)	PCR-RFLP
Tg GT1	A	C	T	C	A	T						I
Tg ME49	.	.	.	.	.	.						II
Tg VEG	.	.	.	.	.	.						III
TgPgBr06	A	C	T	.	A	T	0	0	0	0	0	I
TgPgBr07	A	C	T	.	A	T	0	0	0	0	0	I
TgPgBr08	A	C	T	.	A	T	0	0	0	0	0	I
TgPgBr09	A	C	T	.	A	T	0	0	0	0	0	I
TgPgBr10	A	C	T	.	A	T	0	0	0	0	0	u-1
TgPgBr11	A	C	T	.	A	T	0	0	0	0	0	I
TgPgBr12			T	.	A	T	0	0	0	0	0	I
TgPgBr13			T	.	A	T	0	0	0	0	0	I
TgPgBr14			T	.	A	T	0	0	0	0	0	I
TgPgBr15	A	C	T	.	A	T	0	0	0	0	0	I
TgPgBr16	A	C	T	.	A	T	0	0	0	0	0	I
						AVERAGE:	0	0	0	0	0	

Marker - SAG2 (450 bp) - chromossome VIII											
Nucleotide	2	245	385	396	405	No. Polymorphisms					Genotype
Consensus	T	T	C	C	G	In	Del	Ts	Tv	Total (%)	PCR-RFLP
Tg GT1	.	.	.	.	.						I
Tg ME49	.	G	A	G	C						II
Tg VEG	C	.	.	.	.						III
TgPgBr06	C	.	.	.	.	0	0	0	0	0	I
TgPgBr07	C	.	.	.	.	0	0	0	0	0	I
TgPgBr08	C	.	.	.	.	0	0	0	0	0	I
TgPgBr09						0	0	0	0	0	I
TgPgBr10	C	.	.	.	.	0	0	0	0	0	I
TgPgBr11		.	.	.	.	0	0	0	0	0	I
TgPgBr12		.	.	.	.	0	0	0	0	0	I
TgPgBr13		.	.	.	.	0	0	0	0	0	I
TgPgBr14	C	.	.	.	.	0	0	0	0	0	I
TgPgBr15	C	.	.	.	.	0	0	0	0	0	I
TgPgBr16	C	.	.	.	.	0	0	0	0	0	I
					AVERAGE:	0	0	0	0	0	

Marker - SAG3 (179 bp) - chromossome XII																																		
Nucleotide	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	22	30	37	50	54	55	86	93	95	102	110	125	154	No. Polymorphisms					Genotype
Consensus	C	C	T	T	C	T	G	C	A	A	C	G	T	C	A	A	C	C	G	A	G	T	G	G	C	C	A	G	In	Del	Ts	Tv	Total (%)	PCR-RFLP
Tg GT1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.						I
Tg ME49	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	G	.	.	A	.	.	.	G	.						II
Tg VEG	.	T	.	C	T	G	C	A	.	-	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.						III
TgPgBr06	.	.	.	.	.	.	.	.	.	-	A	C	G	T	C	.	.	.	.	.	.	C	.	A	.	.	.	.	0	0	1	4	2.8	III
TgPgBr07	.	.	.	.	.	.	.	.	.	-	-	C	G	T	C	.	.	.	.	.	.	C	.	A	.	.	.	.	0	1	1	3	2.8	III
TgPgBr08																.	.	.	.	.	.	C	.	A	.	.	.	.	0	0	0	0	0	III
TgPgBr09	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	C	.	T	.	.	A	C	.	A	.	.	.	.	0	0	2	1	1.7	III
TgPgBr10																.	.	.	.	.	.	C	.	A	.	.	.	.	0	0	0	0	0	III
TgPgBr11																.	.	.	.	.	.	C	.	A	.	.	.	.	0	0	0	0	0	III
TgPgBr12	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	0	0	0	0	0	III
TgPgBr13																	.	.	.	.	.	C	.	A	.	.	.	A	0	0	1	0	0.6	III
TgPgBr14	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	0	0	0	0	0	III
TgPgBr15	G	.	C	.	T	C	T	G	C	-	A	C	G	T	C	.	.	.	.	.	.	C	.	A	.	.	.	.	0	0	3	8	6.1	III
TgPgBr16																			.	.	.	C	.	A	.	.	.	.	0	0	0	0	0	III
																												AVERAGE:	0	0.1	0.7	1.5	1.3	

Fig. 2. Polymorphisms at five genetic markers (SAG1, SAG2, SAG3, BTUB and C22-8) by direct PCR-DNA sequencing of *Toxoplasma gondii* isolates from pig from Ilhéus, BA, Brazil after multiple alignment analysis (see Section 2). The size of amplicon means the number of base pair that matched in all samples after the multiple alignment. Numerical position of nucleotides refers to sites after multiple alignment and excision of unequal extremities. Consensus sequence is defined as the nucleotide shared by at least two of the three alleles from Type I, II and III reference strains. Sequences of reference strains were obtained from the following Genome Project (National Center for Biotechnology Information): Tg GT1strain, Type I (Project no. PRJNA 16727), Tg ME49 strain, Type II (Project no. PRJNA 28893) and Tg VEG, Type III (Project no. PRJNA 19097). In each marker, sites demarcated by (.) indicate identity with

Marker - BTUB (339 bp) - chromossome IX

Nucleotide	133	261	275	291	No. Polymorphisms					Genotype
Consensus	T	T	A	T	In	Del	Ts	Tv	Total (%)	PCR-RFLP
Tg GT1	.	.	.	.						I
Tg ME49	.	.	.	.						II
Tg VEG	.	C	.	.						III
TgPgBr06	.	C	.	.	0	0	0	0	0	III
TgPgBr07	.	C	.	.	0	0	0	0	0	III
TgPgBr08	.	C	.	.	0	0	0	0	0	III
TgPgBr09	C	C	.	C	0	0	2	0	0.6	II
TgPgBr10	.	C	.	.	0	0	0	0	0	III
TgPgBr11	.	C	.	.	0	0	0	0	0	III
TgPgBr12	.	C	.	.	0	0	0	0	0	III
TgPgBr13	.	C	.	.	0	0	0	0	0	III
TgPgBr14	.	C	.	.	0	0	0	0	0	III
TgPgBr15	.	C	G	.	0	0	1	0	0.3	III
TgPgBr16	.	.	.	.	0	0	0	0	0	I
		AVERAGE:			0	0	0.3	0	0.1	

Marker - c22-8 (315 bp) - chromossome Ib

Nucleotide	3	19	48	52	82	90	115	138	153	154	242	267	303	No. Polymorphisms					Genotype
Consensus	C	A	G	A	A	T	T	C	A	T	G	G	A	In	Del	Ts	Tv	Total (%)	PCR-RFLP
Tg GT1	.	.	.	.	.	.	.	.	.	C	.	.	.						I
Tg ME49	.	.	.	.	.	.	.	.	.	.	.	.	.						II
Tg VEG	.	.	.	T	G	.	.	.	.	.	.	.	.						III
TgPgBr06	.	-	C	.	.	.	.	G	.	C	.	.	T	0	1	0	3	1.3	I
TgPgBr07	T	-	.	T	G	.	.	.	.	C	.	.	.	0	1	1	0	0.6	u-1
TgPgBr08	.	-	C	.	.	.	.	G	.	C	A	.	T	0	1	1	3	1.6	I
TgPgBr09	.	-	C	.	.	C	.	G	.	C	.	.	T	0	1	1	3	1.6	I
TgPgBr10	.	-	C	.	.	.	.	G	G	C	.	.	T	0	1	1	3	1.6	III
TgPgBr11	.	-	C	.	.	.	C	G	.	C	.			0	1	1	2	1.3	I
TgPgBr12	.	-	C	.	.	.	.	G	.	C	.	.	T	0	1	0	3	1.3	I
TgPgBr13	.	-	C	.	.	.	.	G	.	C	.			0	1	0	2	0.9	I
TgPgBr14	.	-	C	.	.	.	.	G	.	C	.	.	T	0	1	0	3	1.3	I
TgPgBr15	.	-	C	.	.	.	.	G	.	C	.	.	T	0	1	0	3	1.3	I
TgPgBr16	.	-	C	.	.	.	.	G	.	C	.	A	T	0	1	1	3	1.6	I
											AVERAGE:			0	1	0.5	2.5	1.3	

Fig. 2. (*continued*)

many of the alleles present in other Brazilian genotypes, the alleles presented a different segregation between the loci examined; thus, they were characterized as new genotypes (Table 2).

Previously, *T. gondii* populations were thought to be strictly clonal (Ferreira et al., 2006). However, an analysis of isolates from South America confirmed high genetic diversity, making the phylogenetic relationship between

consensus; (-) indicate a deletion. Gray boxes indicate nucleotide polymorphisms not shared by alleles from Type I, II and III. The number of insertions (In), deletions (Del), transitions (Ts) and transversions (Tv) were calculated comparing the sequence of each sample with the pattern obtained from Type I, II or III reference strains. Doted lines indicate conserved regions presented only in the isolates of this study. The results of PCR-RFLP genotyping are indicated at the end of each sequence analysis.

PCR-RFLP data from isolates from this geographical location and those from North America and Europe unclear. The characterization of isolates using PCR-RFLP is known to produce consistent results when applied in locations with low parasite genetic diversity. However, in regions such as South America where the genetic diversity of the parasite is high, the PCR-RFLP technique does not accurately describe the genetic variation of the samples being analyzed (Pena et al., 2008).

To improve the genetic characterization of atypical isolates, Khan et al. (2006) and Frazão-Teixeira et al. (2011) used DNA sequencing. Following the comparison of sequencing and PCR-RFLP results, these authors concluded that the exclusive use of multilocus PCR-RFLP may underestimate the real diversity of the *T. gondii* population. Thus, DNA sequencing is the technique of choice to infer the real genetic diversity and population structure of *T. gondii* strains found in Brazil. In this study, PCR-RFLP analysis grouped six isolates in a single genotype (Table 2, Fig. 1), while sequencing analysis differentiated all isolates (Figs. 2). Therefore, sequencing analysis generates more accurate information compared with PCR-RFLP analysis.

Tajima's *D* test was utilized to analyze sequencing results and presented a negative value (−1.468) (Table 3). This result indicates the occurrence of low frequency polymorphisms that may characterize an expanding population of *T. gondii*. Overall, these findings are consistent with Pena et al. (2008), who suggested that Brazilian genotypes (BrI, BrII, BrIII and BrIV) exhibit multiple isolates and are therefore expanding.

Diversity of the regions amplified with markers SAG3 and c22-8 was observed (Table 3, Fig. 2). These results were different from the PCR-RFLP data. Although these regions are considered to be efficient in differentiating clonal genotypes I, II and III, they make the grouping of Brazilian isolates more difficult. To continue the use of the PCR-RFLP to characterize the isolates of South America, the development of new molecular markers becomes primordial to better group these atypical isolates.

Conflict of interest statement

None of the authors of this study has a conflict of interest.

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