PCR-RFLP genotyping of *Toxoplasma gondii* from chickens from Espírito Santo state, Southeast region, Brazil: New genotypes and a new SAG3 marker allele


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**A B S T R A C T**

Brazil is one of the regions with the highest prevalences of *Toxoplasma gondii* in humans and animals. Because free-range chickens become infected by feeding from ground contaminated with oocysts, the prevalence of *T. gondii* in this host has been widely used as an indicator of the strains prevalent in the environment. The genetic variability among *T. gondii* isolates from different healthy and sick hosts all over the world has been recently studied. Three clonal genetic lineages (Types I, II and III) were initially recognised as predominant in Western Europe and the United States. *T. gondii* strains are genetically diverse in South America. In Brazil, recombination plays an important role in strain diversification. The objective of this study was to genetically characterise *T. gondii* isolates from free-range chickens from Espírito Santo state, Southeast region, Brazil, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). A total of 44 isolates among 47 previously described isolates (TgCkBr234-281) from free-range chickens were included in this study. Strain typing was performed using 12 PCR-RFLP markers: SAG1, SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3. Eleven genotypes were identified. Ten isolates (23%) were grouped into four novel genotypes. Four isolates, distributed in four counties, corresponded to the Type BrI lineage, the genotype found most frequently in Brazil. No clonal Types I, II or III lineages were found. Two novel genotypes were represented by single isolates. Unique alleles were identified for the markers SAG1, c22-8 and CS3, and for the first time, a unique allele was found for the marker SAG3. Although a large number of *T. gondii* genotypes have already been identified from a variety of animal hosts in Brazil, new genotypes are continuously identified from different animal species. This study confirmed the diversity of *T. gondii* in Brazil and demonstrates clonal Type I, II and III lineages are rare in this country.

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1. **Introduction**

*Toxoplasma gondii* can infect almost all homoeothermic animals, including humans (Dubey and Beattie, 1988).

Brazil is one of the regions with the highest prevalences of *T. gondii* in humans and animals (Bahia-Oliveira et al., 2003; Sobral et al., 2005; Cavalcante et al., 2006). Because free-range chickens become infected by feeding from ground contaminated with oocysts, the prevalence of *T. gondii* in this host has been widely used as an indicator of the strains prevalent in the environment (Ruiz and Frenkel, 1980; Dubey et al., 2007a). The genetic variability among
T. gondii isolates from different healthy and sick hosts all over the world has been recently studied. Three clonal genetic lineages (Types I, II and III) were initially recognised as predominant in Western Europe and the United States (Howe and Sibley, 1995; Ajzenberg et al., 2002); however, a re-evaluation of the population of T. gondii in wildlife in North America revealed a new clonal lineage referred to haplogroup 12 (Khan et al., 2011).

In Brazil, a highly reticulated phylogenetic relationship was identified when several genetic markers were used to analyse Brazilian isolates, suggesting that recombination plays an important role in strain diversification in this country (Pena et al., 2008; Soares et al., 2011). The objective of this study was to genetically characterise T. gondii isolates from free-range chickens from Espírito Santo (ES) state, Southeast region, Brazil, using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) to provide new information on the distribution of T. gondii genotypes and on T. gondii diversity in this country.

2. Materials and methods

2.1. Multilocus PCR-RFLP strain typing

A total of 44 isolates from 47 previously described T. gondii isolates (TgCkBr234-281) (Beltrame et al., 2012) from free-range chickens in six counties in ES state (Colatina, Guarápari, Linhares, Marechal Floriano, Serra and Vila Velha), Southeast region, Brazil, were included in this study. These counties were located 50–110 km apart from each other. For each isolate, peritoneum exudates positive for tachyzoites from two infected mice were used to extract DNA using a standard phenol–chloroform protocol, as described in detail by Pena et al. (2006).

Strain typing was performed using 12 PCR-restriction fragment length polymorphism (PCR-RFLP) markers: SAG1, SAG2, alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico (Su et al., 2006; Dubey et al., 2007a) and CS3 (Pena et al., 2008). Reference strains, including Type I (RH), Type II (PTG), Type III (CTG) and other strains (TgCgCa1, MAS, TgCatBr5), were used in the genotyping assays as positive controls. Briefly, the target DNA sequences were first amplified by two rounds of multiplex PCR using external primers, followed by nested PCR using internal primers for individual markers as described previously (Su et al., 2006; Dubey et al., 2007a; Pena et al., 2008). Each multiplex PCR reaction was carried out in 25 μl containing 1 × PCR buffer, 2 mM MgCl₂, 200 μM each of the dNTPs, 0.10 μM each of the forward and reverse primers, 0.5 units of Platinum® Taq DNA Polymerase (Invitrogen) and 1.5 μl of DNA extract. The reaction mixture was incubated at 95 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min. The multiplex products were 1:1 diluted in water and then used for a second round of amplification (nested PCR) with internal primers for each marker separately. The nested PCR was carried out in 25 μl containing 1 × PCR buffer, 2 mM MgCl₂, 200 μM each of the dNTPs, 0.30 μM each of the forward and reverse primers, 0.5 units of Platinum® Taq DNA Polymerase (Invitrogen) and 1.5 μl of diluted multiplex PCR products. The reaction mixture was treated at 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min. The nested PCR products were treated with restriction enzymes and resolved in agarose gels by electrophoresis to reveal the RFLP patterns of the isolates.

Mouse virulence data for these T. gondii isolates previously reported by Beltrame et al. (2012) were used to determine if the CS3 marker can predict parasite virulence in mice.

2.2. SAG3 marker sequencing and genetic diversity of T. gondii

The nested PCR products for the isolate TgCkBr274, with a unique allele for the marker SAG3 revealed by PCR-RFLP, were sequenced along with the nested products for the strains used as positive controls (Type I, Type II and Type III). To purify these PCR products, a clean-up system (COSTAR® Spin-X®, Corning, NY, USA) was used. The purified PCR products were sequenced in both directions using the internal primers (P43S2 and P43AS2) and the ABI PRISM Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, USA). The sequencing products were analysed on an ABI377 automated sequencer. Derived sequences from TgckBr274, RH, PTG and CTG were deposited in GenBank (accession numbers JX218224, JX218225, JX218226, JX218227, respectively) and were aligned against each other using ClustalX (Thompson et al., 1997).

The evolutionary history was inferred using a reticulated network (Su et al., 2006; Dubey et al., 2008; Soares et al., 2011), combining the RFLP genotype results from this paper and the representative RFLP genotypes results described to date in Brazil. Using Simpson’s Diversity Index (D) (Simpson, 1949), the genetic diversity of T. gondii was calculated and compared with values obtained in other studies. This index represents the probability that two individuals randomly selected from a sample will belong to different species. This value is calculated as follows:

\[ D = 1 - \left( \frac{1}{N(N-1)} \right) \sum_{n=1}^{s} \frac{n_i(n_j - 1)}{N^2} \]

3. Results

3.1. Multilocus PCR-RFLP strain typing

The genotyping results for the 44 chicken T. gondii isolates for all PCR-RFLP markers are shown in Table 1. Eleven PCR-RFLP genotypes were detected. No clonal Types I, II or III lineages were found. Chicken isolates from Marechal Floriano and Colatina counties accounted for four genotypes each; Vila Velha and Linhares accounted for three, Guarápari for two, and Serra for one. Most of the analysed isolates from chickens from ES state had genotypes previously described in other states (São Paulo, Rio de Janeiro and Paraná) in the same region of Brazil.

The Type Brl genotype (ToxoDB RFLP genotype #6) was found in four isolates, each from a different county, indicating that this genotype is well distributed in the state of ES. This genotype has already been found in five other
Table 1
Multilocus genotyping of *Toxoplasma gondii* isolates from free-range chickens from Espírito Santo state, Southeast region of Brazil, by PCR-RFLP analysis and identity with other isolates from Brazil.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>County</th>
<th>PCR-RFLP genotype</th>
<th>Identity with other isolates from Brazil</th>
<th>ToxoDB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SAG1 5′ SAG2a</td>
<td>SAG2b</td>
<td>SAG3 PCR-RFLP</td>
</tr>
<tr>
<td>TgChBr</td>
<td>Linhares, M. Floriano, Serra, Vila Velha</td>
<td>I I I I I I I I I I I I I I</td>
<td>TgCatBr2, 12, 17, 21, 30 – (PR)</td>
<td>#6</td>
</tr>
<tr>
<td>265, 273, 277, 281</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TgChBr</td>
<td>M. Floriano</td>
<td>I I I I I I I I I I I I I I</td>
<td>TgCatBr2, 42, 47, 53, 54, 55, 62, 71, 75 – (PR)</td>
<td>Type BR1</td>
</tr>
<tr>
<td>267, 268, 269, 270, 271</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TgChBr</td>
<td>Colatina</td>
<td>I I I I I I I I I I I I I I</td>
<td>TgCatBr177 – (CE)</td>
<td>#109</td>
</tr>
<tr>
<td>249, 250, 252</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TgChBr</td>
<td>Colatina</td>
<td>I I I I I I I I I I I I I I</td>
<td>TgCatBr15 – (SP)</td>
<td>#14</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>TgChBr</td>
<td>M. Floriano</td>
<td>u-1 I I I I I I I I I I I I I I</td>
<td>TgCatBr82 – (SP)</td>
<td>#65</td>
</tr>
<tr>
<td>272</td>
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<tr>
<td>TgChBr</td>
<td>Vila Velha</td>
<td>I I I I I I I I I I I I I I</td>
<td>TgCatBr89 – (RJ)</td>
<td>#108</td>
</tr>
<tr>
<td>280</td>
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<td></td>
</tr>
<tr>
<td>TgChBr</td>
<td>Colatina, Linhares</td>
<td>I I I I I I I I I I I I I I</td>
<td>TgCatBr57 – (SP)</td>
<td>#206</td>
</tr>
<tr>
<td>TgChBr</td>
<td>Colatina, Vila Velha</td>
<td>u-1 I I I I I I I I I I I I I I</td>
<td>New genotype</td>
<td>#213</td>
</tr>
<tr>
<td>244, 245, 246, 278, 279</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TgChBr</td>
<td>Guarapari</td>
<td>I I I I I I I I I I I I I I</td>
<td>New genotype</td>
<td>#214</td>
</tr>
<tr>
<td>258, 259, 260</td>
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<td></td>
<td></td>
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<tr>
<td>TgChBr</td>
<td>Guarapari</td>
<td>u-1 I I I I I I I I I I I I I I</td>
<td>New genotype</td>
<td>#215</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TgChBr</td>
<td>M. Floriano</td>
<td>u-1 I I u-1 I I I I I I I I u-2</td>
<td>New genotype</td>
<td></td>
</tr>
<tr>
<td>274</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a SAG2 marker based on 5′- and 3′-ends of the gene sequence (Howe et al., 1997).
b A new SAG2 marker based on the 5′-end of the gene sequence (Su et al., 2006).
c TgCatBr – (PR): isolates from cats from Paraná state genotyped by Su et al. (2006).
d TgCatBr – (SP): isolates from cats from São Paulo state genotyped by Pena et al. (2008).
e TgDgBr – (SP): isolates from dogs from São Paulo state genotyped by Dubey et al. (2007b).
f TgCkBr – (SP), (RJ), (PR), (RO), (RN), (BA), (CE), (SE) and (AL): isolates from chickens from the following states: São Paulo, Rio de Janeiro, Paraná, Rondônia, Rio Grande do Norte, Bahia, Ceará, Sergipe and Alagoas, respectively, genotyped by Dubey et al. (2008).
g TgCkBr – (PA), (RS): isolates from chickens from Pará and Rio Grande do Sul states genotyped by Dubey et al. (2007a).
h TgCkBr – (MS): isolates from chickens from Mato Grosso do Sul states genotyped by Soares et al. (2011).
i TgCpBr – (SP): isolates from capybaras from São Paulo state genotyped by Yai et al. (2009).
j TgShBr, TgGtBr – (SP): isolates from sheep and goat, respectively, genotyped by Ragozo et al. (2010).
k CS3 marker was only included in the genotyping studies of cats, sheep, goats and capybaras isolates from São Paulo (SP) and of chickens from Mato Grosso do Sul (MS).
hosts (dogs, cats, capybaras, sheep and goats) and in chickens from Brazil. Type Brl has been described not only in isolates from Brazilian states in the same region as ES (São Paulo, Rio de Janeiro and Paraná states) but also in isolates from the Northeast region (Pará and Rondônia states) and Central-West region (Mato Grosso do Sul state).

Chicken isolates TgCkBr267-271 from Marechal Floriano county had RFLP genotype #162, which has been previously described only in a capybara from São Paulo state (same region). Chicken isolates TgCkBr249, 250 and 252 from Colatina county had RFLP genotype #109, which has been previously found in a chicken isolate, but this chicken isolate was from Ceará state, located in the Northeast region. Chicken isolates 236, 237 and 241, from Colatina county, had RFLP genotype #14, which has been previously reported in a cat, a dog and two chickens also in the Southeast region and in a chicken in the South region (Rio Grande do Sul state). RFLP genotype #108 corresponded to 17 chicken isolates from two counties in this study and has been reported before only in a cat from São Paulo (TgCatBr57). This genotype is most likely a common genotype circulating in the state of ES.

In the present study, ten isolates (23%) distributed in four counties were grouped into four novel RFLP genotypes based on the 12 markers analysed. The data were uploaded to ToxoDB.org and designated as genotypes #206, #213, #214 and #215.

The new genotypes identified had unique alleles for the markers SAG1 (genotypes #206, #214 and #215), c22-8 (genotype #213) and CS3 (genotype #215), and for the first time, a unique allele, denoted u-1, was found for the marker SAG3 (genotype #215).

The enzyme NciI cut the SAG3 fragment from RH into three smaller segments of 65, 62 and 99 bp, respectively, whereas the homologous PCR products from CTG were cut into two fragments of 65 and 161 bp. The SAG3 fragment from PTG was not cleaved by NciI. The RFLP analysis of the SAG3 fragment from TgCkBr274 (genotype #215) revealed a pattern different from those of the three archetypes. In this case, the SAG3 fragment from TgCkBr274 was cleaved into three segments of 65, 83 and 99 bp (Fig. 1).

No difference was observed between alleles I and II of the marker CS3 when mouse mortality was compared. Both alleles were strongly associated with virulence in mice, as 100% and 98.7% of mice harbouring strains with alleles I and II died within 4 weeks post inoculation, respectively, according to Beltrame et al. (2012). No allele III at CS3 was found among the genotypes.

3.2. SAG3 marker sequencing and genetic diversity of T. gondii

After sequence analysis, the nested PCR product for the marker SAG3 from TgCkBr274 was shown to have an insertion of 21 nucleotides between positions 1003 and 1004 from the start of the gene (numbering based on a homologous sequence of T. gondii registered in GenBank with the accession number JF312642). This insertion corresponds to the insertion of seven amino acids in the putative protein. In addition to the 21 bp insertion, other single-nucleotide polymorphisms were detected in the new sequence. This sequence differed from RH at three nucleotide positions (1027, 1053 and 1061), from CTG at three nucleotide positions (1027, 1037 and 1046), and from PTG at six nucleotide positions (981, 1001, 1005 1027, 1044 and 1076), as shown in Fig. 2.

The evolutionary reconstruction revealed a population with high genetic diversity, with taxa positioned in the typical star-like network (Fig. 3). The value of D for the whole population surveyed in this study was 0.86.

4. Discussion

Free-range chickens from different regions have been extensively surveyed for T. gondii isolation and genotyping in Brazil (Dubey et al., 2008).

In the present study, only non-archetypal genotypes were found, supporting other findings that archetypal lineages are rare in Brazil. Seven atypical genotypes, corresponding to 34 chicken isolates from five counties, had already been reported for isolates from different hosts, mostly from the Southeast region of Brazil. The presence
Fig. 2. Alignment of sequences of nested PCR products from SAG3 locus from *T. gondii* Types I, II, and III and genotype #215. Shaded boxes at the ends of the sequences correspond to hybridisation sites of primers. The shaded box in the middle of the alignment corresponds to insertion sequence found in the genotype #215. Unshaded boxes correspond to the cleavage sites for the enzyme NciI.

Fig. 3. Neighbour-Net phylogenetic network with representatives of *T. gondii* genotypes available in ToxoDB. Genotypes in ES state are marked in red, and clonal genotypes are marked in blue.
of multiple isolates for individual genotypes is most likely a consequence of the epidemic clonality that is characteristic of the population structure of *T. gondii* in South America (Pená et al., 2008; Soares et al., 2011).

The Type Brl lineage was also found for chicken isolates in the present study, confirming that this lineage is the most common and widespread in different regions and hosts in Brazil. As far as we know, there have been 40 Type Brl isolates previously reported among 363 isolates studied (Dubey et al., 2012).

The marker CS3, located on chromosome Vlla of *T. gondii*, was previously shown to be linked with the acute virulence of *T. gondii* (Khan et al., 2005). The results from the present study corroborates the previous findings of Pená et al. (2008), Yai et al. (2009) and Dubey et al. (2010) that alleles I and II at the CS3 locus are strongly associated with mortality in infected mice.

Even though the markers used in the multilocus PCR-RFLP were developed based on sequence polymorphisms in the clonal Type I, II and III lineages (Su et al., 2006), non-clonal alleles (unique alleles) have already been revealed for SAG1, alt. SAG2, c22-8, c29-2, PK1 and CS3 in different studies. In this current study, for the first time, a non-clonal allele was found for the SAG3 marker (new genotype #215). This is the first non-clonal allele for this marker, not only among Brazilian isolates but, to the best of our knowledge, also among all isolates from other countries ever genotyped using PCR-RFLP. This result suggests that many *T. gondii* isolates are highly divergent from the clonal Type I, II and III lineages at the DNA sequence level (Pená et al., 2008).

The magnitude of genetic diversity of *T. gondii* isolates from chickens in ES sampled in this study was slightly lower than those revealed found in other studies in Brazil (Soares et al., 2011). The value of Simpson’s Diversity Index for the population surveyed in this study was 0.86, whereas a value of 0.90 was found in a population of 40 chickens sampled in the state of Mato Grosso do Sul (Soares et al., 2011), in the Central-West region of Brazil. Simpson’s Diversity Index is a measurement of diversity that is used to quantify the biodiversity of a habitat by taking into account the number of species present and the abundance of each species. Nevertheless, the degree of genetic diversity between each genotype separated the genotypes into divergent clusters, as revealed by the genealogical analysis depicted in Fig. 3.

The genetic structure found in the isolates from chickens from ES state in this study corroborates the findings of previous studies that *T. gondii* has a high level of diversity in Brazil.

Conflict of interest statement

There is no conflict of interest.

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References


