Short communication

Isolation and genetic characterisation of Toxoplasma gondii from a red-handed howler monkey (Alouatta belzebul), a jaguarundi (Puma yagouaroundi), and a black-eared opossum (Didelphis aurita) from Brazil


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ABSTRACT

Toxoplasma gondii isolates are highly diverse in domestic animals from Brazil. However, little is known about the genetics of this parasite from wild mammals in the same region. Reveal genetic similarity or difference of T. gondii among different animal populations is necessary for us to understand transmission of this parasite. Here we reported isolation and genetic characterisation of three T. gondii isolates from wild animals in Brazil. The parasite was isolated by bioassay in mice from tissues of a young male red handed howler monkey (Alouatta belzebul), an adult male jaguarundi (Puma yagouaroundi), and an adult female black-eared opossum (Didelphis aurita). The monkey and the jaguarundi had inhabited the Zoo of Parque Estadual Dois Irmãos, Pernambuco State, Northeastern Brazil, for 1 year and 8 years, respectively. The wild black-eared opossum was captured in São Paulo State, Southeastern Brazil, and euthanised for this study because it was seropositive for T. gondii (titre 1:100 by the modified agglutination test, MAT). Ten PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) markers, SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico, were used to genotype the isolates. T. gondii was isolated from the brain and heart homogenate of the monkey, the muscle homogenate of the jaguarundi, and the heart homogenate of the black-eared opossum. This was the first isolation of T. gondii from a neotropical felid from Brazil. The isolate from the monkey (TgRhHmBr1) was not virulent in mice, whereas the isolates from the jaguarundi (TgJagBr1) and the black-eared opossum (TgOpBr1) were virulent in mice. The genotype of the isolate from the monkey has been identified in isolates from a goat and ten chickens in the same region of Brazil, suggesting that it may be a common lineage circulating in this region. The genotypes of the isolates from the jaguarundi and the black-eared opossum have not been previously reported. Although there are already 88 genotypes identified from a variety of animal hosts in Brazil, new genotypes are continuously being identified from different animal species, indicating an extremely high diversity of T. gondii in the population.

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1. Introduction

*Toxoplasma gondii* is a globally distributed protozoan parasite. Domestic cats and other felids are the definitive hosts (Frenkel et al., 1970), and virtually all warm-blooded animals, including humans, are the intermediate hosts (Dubey and Beattie, 1988). Animals and humans can be infected by ingesting food or water contaminated with *T. gondii* oocysts or consuming *T. gondii* tissue cysts from undercooked meat (Dubey and Beattie, 1988). Only a small percentage of exposed adult humans or animals develop clinical signs of toxoplasmosis. Several factors can be related to the severity of toxoplasmosis in immunocompetent hosts, including parasite strain, host variability and genetic variability of the parasite.

Most *T. gondii* isolates from humans and animals in Europe and North America have been classified into one of the three clonal lineages named Types I, II and III (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002). However, recent studies have reported that the parasite isolates in Brazil are biologically and genetically different (Dubey et al., 2002, 2007a,b; Lehmann et al., 2006). Pena et al. (2008) identified 48 RFLP (Restriction Fragment Length Polymorphism) genotypes in 125 isolates from chickens, dogs and cats; four of these isolates are considered to be common clonal lineages in Brazil, designated as Types Br1, BrII, BrIII and BrIV.

Little is known about the genotypes of *T. gondii* circulating in wild animals in Brazil. Brazil is considered to be the country with the greatest biodiversity on the planet, accounting for the highest numbers of both terrestrial vertebrates and invertebrates in the world (Lambertini, 2000). Recently, Yai et al. (2009) have identified 16 genotypes among 36 *T. gondii* isolates from capybaras (*Hydrochaeris hydrochaeris*) in Brazil, corroborating the previous finding that this parasite population is highly diverse in this region. In the present study, we described the genetic and biological characteristics of *T. gondii* isolates from a red-handed howler monkey (*Aloatta belzebul*), a jaguarundi (*Puma yagouaroundi*), and a black-eared opossum (*Didelphis aurita*) from two Brazilian regions.

2. Materials and methods

A young male red-handed howler monkey (*A. belzebul*) had inhabited the Zoo of Parque Estadual Dois Irmãos, in the municipality of Recife, Pernambuco State, Northeastern Brazil, since January 2008. It was fed on fruits and leaves and died 5 days after showing prostration, diarrhoea and hyperthermia in July 2009, suspected with toxoplasmosis. Several factors can be related to the severity of toxoplasmosis in immunocompetent hosts, including parasite strain, host variability and genetic variability of the parasite.

An adult male jaguarundi (*P. yagouaroundi*) had inhabited the same Zoo since 2001. It was fed on pre-frozen beef, chicken and viscera. This feline died of trauma in July 2009. Its heart, brain and muscles were collected for the study.

A wild adult female black-eared opossum (*D. aurita*) was captured alive in Sorocaba municipality, São Paulo State, Southeastern Brazil. Serologic examination using the modified agglutination test (MAT) (Dubey and Desmonts, 1987) was performed soon after the capture; the result showed that this female black-eared opossum carried antibodies to *T. gondii* (MAT titre 1:100). This animal was euthanised, and its tissues (brain, heart and diaphragm) were collected for bioassay analysis. This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of the University of São Paulo (project no. 855/2006) and by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA – protocol no. 12.285-1).

For the bioassay analysis in mice, tissues of each animal were homogenised in a blender with 0.85% NaCl (saline). The brain and heart of the monkey were homogenised together. The brain, heart and skeletal muscles of the jaguarundi were homogenised separately. The brain, heart and diaphragm of the black-eared opossum were also homogenised separately. The homogenates were digested with an acidic pepsin solution, neutralised and washed (Dubey and Beattie, 1988), after which the homogenates were subcutaneously inoculated into five outbred female Swiss mice (1 ml per mouse). Aliquots of the tissues and tissue homogenates were kept at −70 °C for DNA extraction.

Imprints of lungs and brains of the mice died after inoculation were examined for *T. gondii* tachyzoites and tissue cysts as previously described (Dubey and Beattie, 1988). The surviving mice were bled 6-week post-inoculation (p.i.), and a 1:25 dilution of the serum of each mouse was tested for *T. gondii* antibodies by the MAT. The mice were kept 2-month p.i. and their brains were examined for *T. gondii* tissue cysts. Parasite virulence is defined based on the mortality of positively infected mice within 4-week p.i.; virulent is defined as 100% mortality of infected mice within 4 weeks, intermediately virulent is greater than 30% and less than 100% of infected mice, and non-virulent is less than or equal to 30% mortality (Pena et al., 2008). Aliquots of positive mouse tissues (lungs and brains) were kept at −70 °C for DNA extraction.

*T. gondii* DNA was extracted from tissues of the wild animals (primary samples) or from tissues of the *T. gondii*-positive mice (isolates) using a phenol–chloroform protocol as described in detail by Pena et al. (2006). Molecular detection was performed with a 155-bp fragment of the B1 gene (Burg et al., 1989). Strain typing was performed using a multilocus PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) genotyping assay with the genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico as previously described (Su et al., 2006; Dubey et al., 2007a,b).

3. Results

*T. gondii* was isolated from the three examined wild animals. The parasite was isolated from the tissue homogenate (heart and brain) of the howler monkey (*TgrHmBr1*), one mouse was infected (1/5) and survived. The parasite was isolated from muscle homogenate of the jaguarundi (*TjagBr1*), five mice died of toxoplasmosis (5/5) 17–20 days p.i. *T. gondii* was isolated from the heart homogenate of the black-eared opossum (*TgOpBr1*), only one mouse was infected (1/5) and died of toxoplasmosis 40 days p.i.
Using a 155-bp fragment of the B1 gene as the target, *T. gondii* was detected in the heart, brain, diaphragm and tissue homogenate (brain and heart) from the howler monkey. The parasite was not detected in heart, muscle or brain homogenates from the jaguarundi. The black-eared opossum tissues could not be examined using this assay, because there was no material left.

*T. gondii* was detected in tissues (lung or brains) from positive mice for each of the isolates.

Genotyping results of the isolates from the three wild animals at all the markers are shown in Table 1. Genotyping was also performed at all these markers with all the tested primary samples from the howler monkey and was successful. Three genotypes were detected. The genotypes from the jaguarundi and the black-eared opossum isolates were detected for the first time in Brazil. The genotype from the red-handed howler monkey isolate has been previously described in an isolate from a goat in Rio Grande do Norte State and in isolates from 10 chickens in seven states of Northeastern Brazil.

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>County – state</th>
<th>PCR-BFLP genotype</th>
<th>Identity with other isolates from Brazil (state)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgRhHmBr1</td>
<td>Recife – PE</td>
<td>I</td>
<td>SAG1 5 to SAG2*, 5′ SAG2a, SAG2b, SAG3, BTUB, GRA6, C22-8, C29-2, L538, PK1, Apico</td>
</tr>
<tr>
<td>TgJagBr1</td>
<td>Recife – PE</td>
<td>III</td>
<td>c22-8, L538, PK1, Apico</td>
</tr>
<tr>
<td>TgOpBr1</td>
<td>Sorocaba – SP</td>
<td>III</td>
<td>New genotype</td>
</tr>
</tbody>
</table>

4. Discussion

Most *T. gondii* isolates genotyped in Brazil are from domestic animals, including free-range chickens, cats, dogs, sheep and goat; little is known about the genetics of *T. gondii* isolates from wild mammals in Brazil. Yai et al. (2009) genotyped isolates from capybaras (*H. hydrochaeris*), the largest rodent in the world, widely present in tropical America; among the 16 genotypes identified from the 36 studied isolates, seven genotypes, corresponding to 10 isolates, were described for the first time and eight of the isolates were grouped into the common clonal lineages in Brazil, designated as Types BrI, BrII and BrIII (Pena et al., 2008).

In the present study, we isolated and genotyped *T. gondii* from three different species of wild mammals in Brazil. These animals were chosen because of convenience. The red-handed howler monkey (*A. belzebul*) and the jaguarundi (*P. yagouaroundi*) were captive animals, inhabiting the same zoo in a state of Northeastern Brazil. Many species of wild animals in Brazil are kept in zoos or by animal breeders as part of conservation programs. Serological studies showed a high prevalence of anti-*T. gondii* antibodies in zoo animals (Silva et al., 2001; Spencer et al., 2003).

Brazil is the richest country in the world in terms of primate species. Red-handed howler monkeys, fed on leaves, fruits and insects, are endemic to Brazil and inhabit the northern and northeastern regions. Currently, there are no reports regarding the seroprevalence of *T. gondii* antibodies in this species. Garcia et al. (2005) observed a seroprevalence of 17.6% (3/17) in captured wild *Alouatta caraya* (black and golden howler monkeys) in the southern region. In the present study, we isolated *T. gondii* from a red-handed howler monkey. It is the first isolation of *T. gondii* in this species. This animal was suspected of dying of toxoplasmosis. Neotropical primates are one of the most susceptible groups to clinical and fatal toxoplasmosis (Dubey and Beattie, 1988; Garrel, 1999). It was not feasible to perform another examination to confirm the infection in this
study. However, it is noteworthy that the parasite could be molecularly detected in all the examined primary samples using not only the B1 gene but also all genetic markers as the targets. Multilocus PCR-RFLP was primarily designed to genotype *T. gondii* isolated in mice (Su et al., 2006) carrying a great amount of the parasite; very poor results were observed among the genetic markers when primary samples from cats were used to genotype the parasite (H.F.J. Pena, Personal communication). This result suggests that a great amount of the parasite might be circulating in the monkey's body, which would be compatible with an acute toxoplasmosis infection.

The howler monkey had previously inhabited Maranhão State in Northern Brazil before it was brought to the Zoo in Pernambuco State, where it remained for a year. Therefore, we are not certain about the origin of this isolate. However, the genotype detected in the howler monkey had already been described in isolates from ten chickens (Dubey et al., 2008) and one goat (Ragozo et al., 2010), all in the same region of Brazil, including one isolate from a chicken in Pernambuco State. This finding suggests that there is a common lineage circulating in animals in Northeastern Brazil, including wild animals. These 12 isolates were non-virulent in mice, indicating that it is a mouse non-virulent genotype. More isolates tested for a given genotype, more confidence we have on the identification of the mouse virulence phenotype (Pena et al., 2008). Also, this genotype has not been identified among the 15 isolates from chickens in Pará (Dubey et al., 2007a,b) or in the two isolates from chickens in Maranhão State (Dubey et al., 2008) that represented 12 different genotypes. Both states are in Northern Brazil.

Not only domestic cats but also virtually all wild felid species are likely to excrete *T. gondii* oocysts in the feces (Dubey and Beattie, 1988). Eight of 10 species of neotropical cats inhabit Brazil (Oliveira, 1994). Silva et al. (2001) reported a seroprevalence of 45.9% (45/99) in captive jaguarundis (*P. yagouaroundi*) in different regions of Brazil. In the present study, *T. gondii* was isolated from a captive jaguarundi. This was the first isolation of *T. gondii* from a captive neotropical felid in Brazil. The parasite was isolated only from the skeletal muscle homogenate, consistent with the previous report of Dubey et al. (2004) demonstrating that the density of *T. gondii* in cat muscles is higher than that in the brain. This was also the first time that the genotype identified in this isolate was described in Brazil.

Opossums are wild mammals with a very broad diet and synanthropic habits; thus, they live in both wild and domestic environments, which may represent a potential zoonotic factor. The southern black-eared opossum (*M. aurita*) is a common omnivorous marsupial species in Eastern Brazil. There are no extensive studies on *T. gondii* in opossums in Brazil. Yai et al. (2003) reported a *T. gondii* seroprevalence of 20.4% (82/396) in the black-eared opossums in São Paulo city, São Paulo State. In the present study, *T. gondii* was for the first time isolated from this species. The genotype identified from this isolate was also for the first time described in Brazil.

Although there are already 88 genotypes identified (Su et al., 2006; Pena et al., 2008; Dubey et al., 2008; Yai et al., 2009; Ragozo et al., 2010) from a variety of animal hosts in Brazil, new genotypes are continuously being identified from different animal species, indicating an extremely high diversity of *T. gondii* in the population.

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