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Short communication

Free-range chickens from Santa Catarina state, southern Brazil, as asymptomatic intermediate hosts for *Toxoplasma gondii* clonal type I and typical Brazilian genotypes

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ABSTRACT

Chickens are a host that is very resistant to the development of clinical toxoplasmosis. Free-range chickens have been used to indirectly track environmental contamination with *Toxoplasma gondii* oocysts because they feed on the ground. This study evaluated the genetic diversity of *T. gondii* isolates from free-range chickens from Florianópolis island in Santa Catarina state, southern Brazil. Sera from 21 chickens were tested for IgG anti-*T. gondii* antibodies using the modified agglutination test (MAT). Tissue homogenates from the 11 seropositive birds (MAT titres ≥5) were bioassayed in mice. The four obtained isolates (TgCkBrSC1-4) were genotyped using 11 PCR-RFLP markers and 15 microsatellite markers (MS). Four genotypes were identified, three of which are typical Brazilian genotypes (ToxoDB-RFLP #26 and #53 were previously reported and #278 is new), and the other is the rare clonal type I genotype. This type I isolate was considered a variant according to MS analysis, with two atypical alleles, which emphasizes the genetic diversity of the parasite in Brazil. The genetic variability of *T. gondii* in South America may be related to the high occurrence of severe ocular and congenital toxoplasmosis in humans in this region. High human seroprevalence and frequency of ocular toxoplasmosis are reported in southern Brazil, but there is limited information on the *T. gondii* strains that are circulating in this region, so more studies should be conducted to identify the strains in different hosts and in human toxoplasmosis cases.

1. Introduction

Felids are the only definitive hosts that can excrete oocysts of the zoonotic protozoan *Toxoplasma gondii* into the environment. Schares et al. (2005) reported that intact and still viable oocysts can pass through dog faeces if dogs eat cat faeces containing sporulated oocysts. Ingestion of sporulated oocysts is an important mode of transmission of this parasite to intermediate hosts, including humans and virtually all other mammals and birds (Dubey, 2010a). Chickens are a host that is very resistant to the development of clinical toxoplasmosis (Dubey et al., 1993). Free-range chickens have been used to indirectly track environmental contamination with *T. gondii* oocysts because they feed on the ground (Dubey et al., 1993). This type of tracking involves studying the prevalence of anti-*T. gondii* antibodies and genotypically characterizing the isolated strains to understand the population structure of the parasite in different regions of the world and their potentially associated biological traits (Shwab et al., 2014).

In contrast to *T. gondii* strains in Europe and North America, *T. gondii* strains in South America are highly diverse, and this region is considered a hotspot for *T. gondii* genetic variability (Bertranpetit et al., 2017). These diverse South American strains may be related to the high occurrence of severe ocular and congenital toxoplasmosis in humans in this region, particularly in Brazil, Argentina and Colombia (Gilbert et al., 2008; de-la-Torre et al., 2008; Rudzinski et al., 2016).

The diversity of *T. gondii* in a large set of isolates from chickens from Brazil was previously investigated (Dubey et al., 2008), but few samples from the southern region were included (30/151). Erechim municipality, in the southern state of Rio Grande do Sul, has an unusually high

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prevalence of ocular toxoplasmosis (Glasner et al., 1992). The aim of the present study was to contribute to our understanding of the circulating *T. gondii* genotypes in the southern Brazil and the geographic distribution of this parasite.

2. Materials and methods

Twenty-one free-range chickens (*Gallus Gallus domesticus*) were acquired by convenience from small farms in Florianópolis island, Santa Catarina state, southern Brazil. All chickens were from small farms with 20–30 chickens per property. Their owners used to consume eggs and meat and sometimes these products were marketed, traded or donated to people living nearby.

Sera of chickens were tested for IgG anti-*T. gondii* antibodies using the modified agglutination test (MAT) as described by Dubey and Desmond (1987). The brains and hearts of all seropositive chickens (MAT titres ≥5) were bioassayed individually in Swiss Webster mice for *T. gondii* infection. Tissues were homogenized, digested in acidic pepsin, neutralized with sodium bicarbonate, and the homogenate was inoculated subcutaneously into three mice (Dubey, 1998). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in the lungs and/or brain (Dubey, 2010a).

For DNA extraction, lungs or brain from positive mice (two mice per isolate, when there was at least two infected mice) were macerated in a 0.85% saline solution, and 250-μl aliquots were washed in Tris-EDTA buffer (Tris-HCl 10 mM, EDTA 1 mM) by centrifugation at 12,000 ×*g* for 5 min. Then, the DNeasy® Blood & Tissue commercial kit (Qiagen® Inc., USA) was used according the manufacturer's protocol.

Multilocus nested-PCR-RFLP genotyping was performed using markers SAG1, SAG2 (5′3′SAG2 and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico (Su et al., 2010) and CS3 (Pena et al., 2008). The reference strains RH (type I), PTG (type II) and CTG (type III) and the non-archetypal strains TgCgCa1 (Cougar), MAS and TgCatBr5 were the positive controls in all reactions. Additionally, isolates were genotyped using eight microsatellite typing markers (TUB2, W35, TgMA, B18, B17, M33, IV.1 and XI.1) and seven fingerprinting markers (N60, N82, AA, N61, N83, M48 and M102) as previously described (Ajzenberg et al., 2010), and the results were analysed using the GeneMapper 4.1 software program (Applied Biosystems). The reference strain PTG (type II) was used as the positive control.

A neighbour-joining tree was reconstructed from microsatellite data as they have a higher resolution than PCR-RFLP data. For this step, the results from the present study and from other *T. gondii* chicken isolates originating from the same and other Brazilian regions (Supplementary material) were used. The unrooted tree was reconstructed with Populations 1.2.32 (Langella, 1999) based on Cavalli-Sforza and Edwards chord-distance estimator (Cavalli-Sforza and Edwards, 1967) and generated with MEGA 7 (Kumar et al., 2016).

The Animal Ethics Committee from USP approved the protocols used in this study (CEUA 2955160516).

3. Results

Of the 21 chickens, 52.4% (11/21) were seropositive for *T. gondii*. The range of titres varied from 1:5 (three chickens) to 1:2560 (one chicken). Four isolates were obtained from the 11 bioassays, which corresponded to seropositive chickens with titres of 1:160 (TgCkBrSC1), 1:640 (TgCkBrSC3 and TgCkBrSC4) and 1:2560 (TgCkBrSC2). Negative bioassays were from seropositive chickens with titres of 1:5, 1:20, 1:40, 1:80 and 1:320.

Four ToxoDB-RFLP genotypes were identified (Table 1): #10, corresponding to the clonal type I (TgCkBrSC1), #26 (TgCkBrSC2), #53 (TgCkBrSC3) and a new genotype, #278 (TgCkBrSC4). The archetypal clonal type I was previously isolated from a chicken (TgCkBr146) in southern Brazil (Dubey et al., 2007) and, more recently, it was detected in a cat from the same state (Pena et al., 2017). Genotype #26 was also recently identified in chickens from the same state (Trevisani et al., 2017) and in cats (Pena et al., 2008) and chickens (Dubey et al., 2007) in southern and southeastern Brazil, respectively. Similarly, genotype #53 was already found circulating in chickens from southern Brazil and in dogs and capybaras from southeastern Brazil (Table 1). For this latter genotype, marker CS3 was able to differentiate isolates, as three alleles were identified (Table 1). No publications (until now) have shown the endonuclease digestion patterns for unique alleles identified for marker CS3, so an electrophoresis agarose gel image was included for future reference (Fig. 1).

Results obtained with microsatellite (MS) analysis can be seen in Table 2. Four genotypes were identified from the four isolates. The isolate TgCkBrSC1 was considered a type I variant, as it has two atypical MS alleles for typing markers B17 and M33. The same atypical alleles were found for the cat sample PS-TgCatBrSC1, which was also identified as a type I variant using MS (Pena et al., 2017). Nonetheless, they are not circulating clones, as they have different alleles for fingerprinting MS markers N82 and AA.

The result of the phylogenetic relationship study of the isolates from chickens from this present study and previously reported chicken isolates is presented in Fig. 2. TgCkBrSC1 clustered with TgCkBr146 in the type I group. TgCkBrSC2 and TgCkBrSC4 are close to each other, but divergent from TgCkBrSC3 and the other reported isolates from chickens from southern Brazil. Except for TgCkBrSC1 (type I variant), the other isolates found in the present study are divergent from the clonal archetypal genotypes I, II and III.

4. Discussion

The occurrence of anti-*T. gondii* antibodies in chickens from Florianópolis island was high (52.4%), corroborating recent results of Trevisani et al. (2017), who found a seroprevalence of 51.7% (15/29) for *T. gondii* in chickens on the same island, but the number of chickens analysed was small in both studies, so comparisons with other studies are difficult. More studies with larger samples are needed to evaluate the environmental contamination with oocysts on the island based on chicken seroprevalence. The seroprevalence is high (63.2%) considering the Santa Catarina state (Trevisani et al., 2017) and another southern state, Rio Grande do Sul, (74.4%) (Camillo et al., 2015). Oliveira et al. (2009) also reported high *T. gondii* seroprevalence in chickens from northeastern Brazilian states (53.3%). In the United States, a wider range was observed (14–100%) (Dubey, 2010b).

Dozens of papers have already been published on *T. gondii* genotyping from different hosts and regions of Brazil since 2006, but new genotypes are continuously being described. In the present paper, only four isolates were found, and a new PCR-RFLP genotype was identified for the TgCkBrSC4 isolate (ToxoDB-RFLP #278). Each newly identified genotype emphasizes the great genetic diversity of the *T. gondii* parasite in this continental country.

It was noteworthy to identify a clonal type I genotype (TgCkBrSC1). Archetypal clonal types I, II and III are rare in Brazil, particularly type I (Shwab et al., 2014). Clonal type I had been only isolated in pork sausages for human consumption (S11 isolate) and in a free-range chicken (TgCkBr146) in southern Brazil (Martins et al., 1990; Dubey et al., 2007). Recently, clonal type I was also identified in an immunosuppressed cat that died with acute toxoplasmosis in the same Brazilian state (Pena et al., 2017). In this case report, it was not possible to determine how the cat was infected, but the report of clonal type I with the same atypical microsatellite alleles for markers B18 and B17 in the TgCkBrSC1 isolate indirectly confirms environmental contamination with this parasite genotype and its circulation in the definitive host and the intermediate host. The identification of clonal type I isolates in food, asymptomatic and immunosuppressed animals in the same Brazilian region suggests a geographic pattern that should be better investigated.

The findings of this study are of great relevance, demonstrating the