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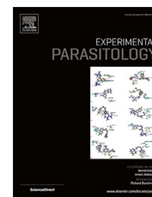

Short communication

Toxoplasma gondii isolates from chickens in an area with human toxoplasmic retinochoroiditis

Lais Pardini [a, b, *], Gastón Moré [a, b], Marcelo Rudzinski [c, d], María L. Gos [a, b], Lucía M. Campero [a, b], Alejandro Meyer [e], Mariana Bernstein [a, b], Juan M. Unzaga [a], María C. Venturini [a]

[a] Laboratorio de Inmunoparasitología, FCV-UNLP, La Plata, Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina
[c] Centro de uveítis y glaucoma, Oberá, Misiones, Argentina
[d] Facultad de Ciencias de la Salud Universidad Católica de las Misiones, Posadas, Misiones, Argentina
[e] Laboratorio de análisis clínicos Dr Meyer, 25 de Mayo, Misiones, Argentina

HIGHLIGHTS

- Chicken samples belonging to patients with toxoplasmic retinochoroiditis were obtained.
- *Toxoplasma gondii* isolates were obtained by mice bioassay in 27.7% (5/18) of inoculated chicken brain samples.
- The five isolates and 2 original chicken samples were genotyped using 9 markers.
- Two new genotypes were first described.

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ABSTRACT

The aim of this study was to detect, isolate and genetically characterize *Toxoplasma gondii* from tissues obtained from free range chickens which were breed in farms from patients with toxoplasmic retinochoroiditis in Misiones, Argentina. Thirty three samples of head (refrigerated = 18 and frozen = 15) from free range chickens were processed. Refrigerated (n = 18) chicken central nervous systems (CNS) were bioassay in mice. DNA was obtained from all samples (n = 33) and PCR was performed using TOX5-TOX8 *T. gondii* specific primers. Positive PCR samples were characterized by nested-PCR and restriction fragment length polymorphism using the markers SAG2, BTUB, GRA6, SAG3, PK1, L358, C22-8, C29-2 and Apico. *T. gondii* DNA was amplified in 30.3% (10/33) of CNS samples. Isolates were obtained in 27.7% (5/18) of inoculated CNS samples (TgCk11-9Arg, TgCk13-5Arg, TgCk14-5Arg, TgCk14-6Arg and TgCk14-7Arg). Seven samples showed a restriction pattern to all markers and were identified as atypical with several alleles type III. Genotyping of *T. gondii* from samples of patients with retinochoroiditis in the same area could improve the understanding of the epidemiology of toxoplasmosis in the region.
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1. Introduction

Toxoplasma gondii is an apicomplexa parasite that can affect human beings and a broad range of warm-blood animals. Toxoplasmosis is commonly asymptomatic but according to host susceptibility and biological parasite behavior can produce clinical signs and variable lesions. Some animal species are particularly susceptible to *T. gondii* infection suffering sudden death, multi-organic failure, encephalitis and neurological disorders (Dubey, 2010b).

Different parasite isolates have been associated with different virulence and behavior, especially in a mice model. Most isolates of *T. gondii* obtained from animals and humans in North America and Europe correspond with clonal types, overrepresented by the clonal type II (Weiss and Kim, 2014). The mentioned genotype showed low virulence in a mice model (Dubey, 2010b). In South America the

* Corresponding author. Laboratorio de Inmunoparasitología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, calle 60 y 118 (1900) La Plata, Argentina.
E-mail address: laispardini@fcv.unlp.edu.ar (L. Pardini).

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presence of recombinant or non-clonal isolates of *T. gondii* with high virulence to mice has been detected (Pena et al., 2008). Moreover, in south Brazil a high rate of human ocular toxoplasmosis (OT) has been reported and is suggested to be associated with these recombinant isolates of *T. gondii* (Grigg et al., 2015).

Bordering the south of Brazil, in the central-east region of Misiones province, Argentina, toxoplasmic retinochoroidal lesions are found in up to 20% of the patients attending the ophthalmic office (Rudzinski and Meyer, 2011). Reactivation of toxoplasmic retinochoroiditis on those patients were recently shown to occur more frequently during intense rainfall periods (Rudzinski et al., 2013). Unfortunately, obtaining isolates from humans is difficult as well as to obtain ocular fluid with enough amount of *T. gondii* DNA to perform a complete genotyping (Weiss and Kim, 2014). Until now, only two isolates of *T. gondii* from humans have been performed and genotyped in Argentina (Pardini et al., 2014, 2015). The knowledge of *T. gondii* genotypes circulating in Misiones is important because it may partially explain the reason why ocular toxoplasmosis is so frequent in this area of Argentina. As *T. gondii* has a wide hosts range, it is possible to correlate the epidemiology of toxoplasmosis in a determined environment by analyzing sentinel animals. It has been demonstrated that free range chickens are an excellent epidemiological sentinel for *T. gondii* environment contamination (Moré et al., 2012).

The aim of this study was to detect, isolate and genetically characterize *T. gondii* from tissues obtained from free range chickens which were breed in farms from patients with toxoplasmic retinochoroiditis in Misiones, Argentina.

2. Materials and methods

2.1. Samples and geographic location

Thirty-three heads of chickens were obtained from 17 farms, from 7 towns (*Alba Posse*, *25 de Mayo*, *Campo Ramón*, *Canal Torto*, *Colonia Aurora*, *El Soberbio* and *San Vicente*) located in center-east Misiones province (26°55′S 54°31′W), Argentina (Fig. 1), where their owners had been diagnosed of recurrent toxoplasmic retinochoroiditis. This province is located in northeast Argentina with river borderlines with Brazil, at east and north, and Paraguay, at west (Fig. 1). Climate conditions are humid sub-tropical with an annual average of 21 °C and annual precipitations ranging from 1500 to 2000 mm. Approximately 45% of Misiones province is covered by the remnants of the Atlantic (Paranaense) rainforest. Misiones has a population of 1.175.000 inhabitants.

Chicken samples were provided voluntarily by the farmers (1–6 samples per farm) and submitted to La Plata University, refrigerated at 4 °C (n = 18/33 samples) or frozen at −20 °C (n = 15/33 samples). Once arrived at laboratory, samples of central nervous system (CNS) were obtained and preserved to mice bioassay (only the 18 refrigerated samples) and DNA extraction (n = 33).

2.2. Isolation of *T. gondii* in mice bioassay and cell culture

The refrigerated CNS samples (n = 18) were homogenized separately with saline solution (NaCl 0.85%). Homogenates of each sample were inoculated subcutaneously, into 2 Balb-c GKO gamma interferon mice as previously described (Moré et al., 2012). Mice were daily controlled and out the offset of clinical sings were humanely euthanized. Brain and peritoneal wash samples were obtained. Brain samples were used for mice passages and peritoneal fluids with saline solution were inoculated in VERO cell culture as previously reported (Moré et al., 2012). Animal procedures used in this study were in accordance with standards established by the IACUC (Institutional Committee for the Care and Use of Laboratory Animals-FCV-UNLP). Additionally, chicken and mice CNS homogenates were microscopically examined in order to determine presence of *T. gondii* tissue cysts.

2.3. DNA isolation, PCR and genotyping of isolates

The DNA was obtained from all chickens CNS samples (n = 33),

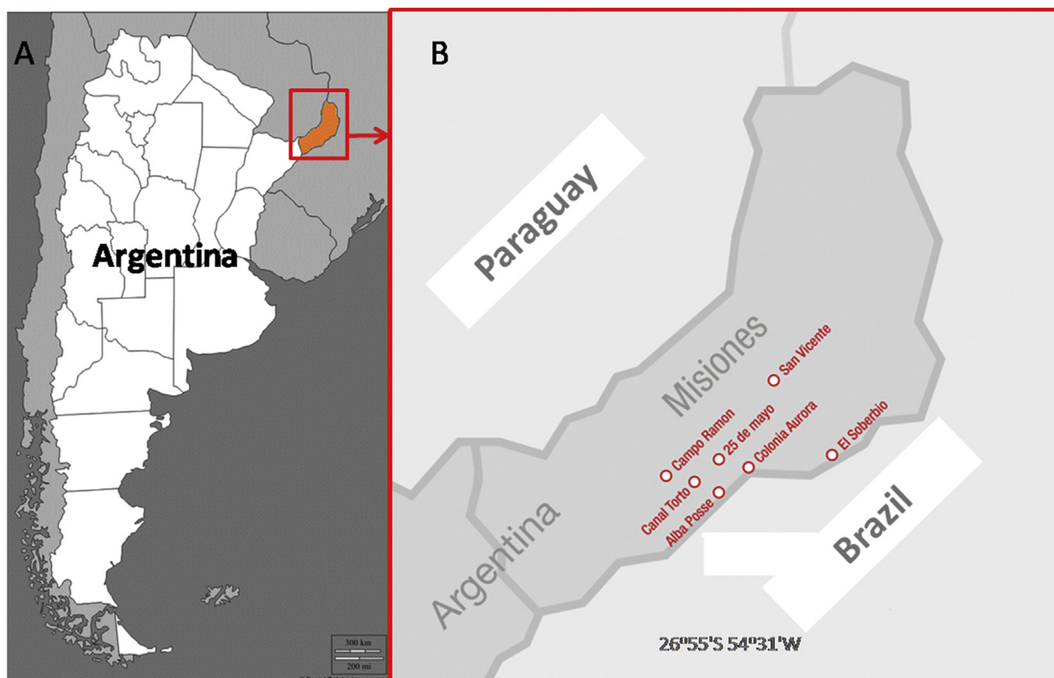

Fig. 1. A: Map of Argentina with Misiones province colored, B: Map with higher magnification of Misiones province showing the villages and towns where samples were obtained.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as well as from the mice brain samples and cell culture isolates, with a commercial kit (Promega Wizard Genomic DNA Purification Kit) according to manufacturer's recommendations. For each extraction routine, a control process sample (without tissue sample) was performed. As diagnosis of *T. gondii* DNA presence, a polymerase chain reaction (PCR) was performed using TOX5-TOX8 specific primers in a thermocycler (PCR Sprint Thermo Electron Corporation) as previously described (Moré et al., 2010). Each amplification routine was conducted with a positive control (DNA of *T. gondii* RH strain), a negative control (control process sample DNA) and a no template control (NTC).

Positive samples were characterized by nested-PCR and restriction fragment length polymorphism (nPCR-RFLP) using nine markers: SAG2, BTUB, GRA6, SAG3, PK1, L358, C22-8, C29-2 and Apico, proceeding essentially as previously (Basso et al., 2009; Su et al., 2006). Samples of DNA from RH, Me49 and NED *T. gondii* strains were used as Type I, II and III controls, respectively. Additionally, a negative control and a NTC were included.

The PCR and n-PCR/RFLP products were visualized in 1.5% and 2.5% (except for Apico [3%]) agarose gels respectively, and stained with SYBRsafe (Invitrogen, USA) using 100 bp standard (Cien Marker, Biodynamics) (Moré et al., 2012).

3. Results

By mouse bioassay *T. gondii* isolates were obtained from 27.7% (5/18) of chickens CNS, tachyzoites were observed in 10 (5 pairs) of the 36 inoculated mice, which were identified as TgCk11-9Arg, TgCk13-5Arg, TgCk14-5Arg, TgCk14-6Arg and TgCk14-7Arg. The isolates were obtained from 4 different farms (TgCk14-6Arg and TgCk14-7Arg belong to the same farm). Isolates were maintained by mice passages and cell culture. Mice were euthanized between 11 and 15 days post inoculation (dpi) for all isolates due to the presence of compatible toxoplasmosis signs, with the exception of mice inoculated with TgCk13-5Arg: one died suddenly at 19 dpi and the other was euthanized at 80 dpi.

Specific *T. gondii* DNA was amplified in 30.3% (10/33) of chicken CNS samples. The 10 positive samples belong to 9 farms (Chicken samples 14-6 and 14-7 belong to the same farm). All sampled towns showed at least 1 positive farm (2 farms from *25 de Mayo* and *San Vicente* respectively) (Fig. 1). Complete genotyping results by nPCR-RFLP of the chicken CNS positive samples (7/10) are shown in Table 1. Three positive samples at the screening PCR (Chicken samples 11-7 [*25 de Mayo*], 13-1B [*San Vicente*] and 13-4 [*Alba Posse*]) evidenced restriction pattern for 4 markers or less.

The *T. gondii* genotype identified in mice brain samples and cell culture derived tachyzoites were identical to the original chicken CNS sample (Table 1). Tissue cysts were observed in 9% (3/33) of chicken CNS homogenates, and also in 3 CNS of mice where *T. gondii* isolation was achieved (TgCk11-9Arg, TgCk14-5Arg, TgCk14-7Arg). *T. gondii* tissue cysts were also observed in brain of mice inoculated with TgCk13-5Arg CNS after average 20 dpi.

4. Discussion

Despite of detecting serologically positive animals or humans worldwide, the achievement of *T. gondii* isolates remains as defiance for researchers (Dubey, 2010b). Moreover, obtaining *T. gondii* isolates from tissue samples from seropositive humans in South America is infrequent and only two isolates have been reported in Argentina (Pardini et al., 2014, 2015; Weiss and Kim, 2014). However, *T. gondii* isolates from human patients from Brazil have been obtained and genotyped (Carneiro et al., 2011, 2013; Cunha et al., 2016; Silva et al., 2014; Weiss and Kim, 2014) and also in French Guiana where fatal cases in immunocompetent human population were related to neotropical forest cycle ("Amazonian toxoplasmosis") (Demar et al., 2012). The present study was conducted in an area with a high proportion of human OT (Rudzinski and Meyer, 2011) and samples from chickens owned by the patient's family were used as monitor for *T. gondii* contamination in the mentioned region.

In the present study, a proportion of around 30% of the sampled animals (10/33) result positives in a screening PCR to specifically detect *T. gondii* DNA. This proportion is particularly high in comparison to other studies (Dubey, 2010a; Moré et al., 2012). Moreover, such detection levels as well as the isolation efficiency (5/18) are comparable for studies performed with samples from seropositive animals (Moré et al., 2012). In the present study serum samples were not available and therefore, serological comparisons were not plausible. Despite of the high proportion of positive chicken brain samples detected by PCR, the amount of *T. gondii* DNA present in 3 samples was not enough to perform a complete genotyping. It is generally assumed that in order to obtain a complete genotyping, both by nPCR-RFLP or by microsatellite analysis, a high amount of DNA is required (Weiss and Kim, 2014). Such level of *T. gondii* DNA generally responds to mice or cell culture isolates and only occasionally this amount is present in tissue samples. The results of the present study suggest that the brain positive samples were heavily infected with *T. gondii*, allowing the complete nPCR-RFLP genotyping in 7/10 samples. This idea was also supported by the detection of *T. gondii* tissue cysts in 3 chicken brain samples which is not frequently reported (Dubey, 2010b).

T. gondii populations from South America showed a higher genetic variability than the reported in North America and Europe. In contrast to the wide distribution of genotype II in the last mentioned continents, the genotype III as well as atypical and/or non-canonical *T. gondii* genotypes are overrepresented in South America (Pena et al., 2008; Rajendran et al., 2012). In the present study, the 7 samples with restriction pattern results for all markers were identified as atypical or non-canonicals with alleles type III and I, similar to isolates from other Argentinean provinces recorded in *T. gondii* Data Base (Toxo-DB; www.toxodb.org/) (Rajendran et al.,

Table 1
Toxoplasma gondii genotyping results from chickens isolates in mouse and genotyping direct form brain.

Sample/Markers	SAG2	BTUB	GRA6	SAG3	PK1	L358	C22-8	C29-2	Apico	TOXO-DB	Location
TgCk11-9Arg	III	III	III	III	u1	I	I	I	I	#19	Colonia Aurora
TgCk13-5Arg	III	I	III	III	III	III	II	III	III	#116	Campo Ramón
TgCk14-5Arg	III	III	III	III	III	III	III	I	III	#14/138	Canal Torto
TgCk14-6Arg	II	III	II	III	I	III	I	I	I	nd	25 de Mayo
TgCk14-7Arg	II	III	II	III	I	III	I	I	I	nd	25 de Mayo
Chicken 11-12[a]	III	III	III	III	u1	I	I	I	I	#19	San Vicente
Chicken 13-3[a]	II	III	III	III	III	III	I	I	III	nd	El Soberbio

nd = genotype not previously described in Toxo-DB (www.toxodb.org).
# = identity number in Toxo-DB.
[a] Genotyping direct of the brain; TgCk corresponds to *T. gondii* genotyping from mice and cell culture isolates.

2012). However, none of the genotypes reported in the present study showed the same allele combination to the previous registered in the Toxo-DB from animals in Argentina (Rajendran et al., 2012). The *T. gondii* genotypes from Misiones contrast with the reported from chickens in Buenos Aires province where different allele combinations and/or clonal type II were detected (Moré et al., 2012). Additionally, since we have not conducted a genotyping of other PCR-RFLP markers as SAG1 and CS3, a complete comparison with other reported isolates was not plausible (Pena et al., 2008). Despite of this, 2 different allele combinations reported here (Table 1) which have not matched with others reported at Toxo-DB are potentially new genotypes or allele combinations circulating in the studied area.

In particular the genotype of the isolate named TgCk11-9Arg and the one detected in the original brain sample of Chicken 11-12 were identical (Table 1); both presented an allele combination similar to genotype #19 however the samples were collected from different farms and 70 km distant between them. The genotype denominated as #19 at Toxo-DB has been reported in cats, cattle, chickens, capybaras, rabbit and bats from Brazil indicating wide distribution within this country (Weiss and Kim, 2014). Genotype #19 is considered one of the ten most frequent genotypes in Central and South America and besides has demonstrated high virulence in mice (Pena et al., 2008; Shwab et al., 2014). In our study mice should be euthanized early after inoculation because mice developed compatible toxoplasmosis signs indicating high virulence of this isolate.

Isolate TgCk14-5Arg presented an allele combination similar to genotype #14 which was described previously in chickens (Brazil, Chile, Colombia and Venezuela), coyote (USA), dogs (Brazil and Colombia) and cats (Brazil and Colombia) indicating a wide distribution of this genotype in North and South America (Toxo-DB). Isolate TgCk14-5Arg had also similar allele combination to genotype #138 which was described in chickens from Brazil (Dubey et al., 2008).

Genotype for TgCk13-5Arg isolate showed an allele combination similar to genotype #116 which was previously found only in chickens from Brazil, Peru and Venezuela (Dubey et al., 2006; Rajendran et al., 2012). Since most studies of *T. gondii* isolation in South America were conducted with chicken samples, additional studies in other animals species are needed in order to suggests if this particular genotype is specially adapted to South American chickens.

Isolates identified as TgCk14-6Arg, TgCk14-7Arg and *T. gondii* DNA from chicken CNS 13-3 showed genotypes which are not recorded at Toxo-DB. The 2 isolates belong to the same farm and showed an identical *T. gondii* genotype. Presence of compatible toxoplasmosis signs at around 15 dpi indicated a potentially high virulence of these new isolates (TgCk14-6Arg and TgCk14-7Arg). Further studies will be conducted in order to determine the biological behavior of the *T. gondii* isolates reported here, both *in vivo* and *in vitro* models. Additionally, virulence markers as ROP 18 and genotyping using microsatellites could also be applied in order to obtain higher resolution about genetic characteristics of the *T. gondii* isolates from the present study (Shwab et al., 2014; Taylor et al., 2006).

According to Toxo-DB, none of the genotypes reported in the present study have been identified in human infections worldwide, but it could be related to the less opportunity to isolate or characterize *T. gondii* DNA from human samples (Carneiro et al., 2013).

Potentially, these "atypical" *T. gondii* isolates could be involved in the severe cases of human OT reported in Misiones, Argentina (Rudzinski and Meyer, 2011) as was informed in Brazil (Grigg et al., 2015). However, it would also be important, genotyping of *T. gondii* from samples of patients with retinochoroiditis in the same area in future studies. New findings would enhance the understanding of the epidemiology of toxoplasmosis in the region and the improvement of preventive measures.

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