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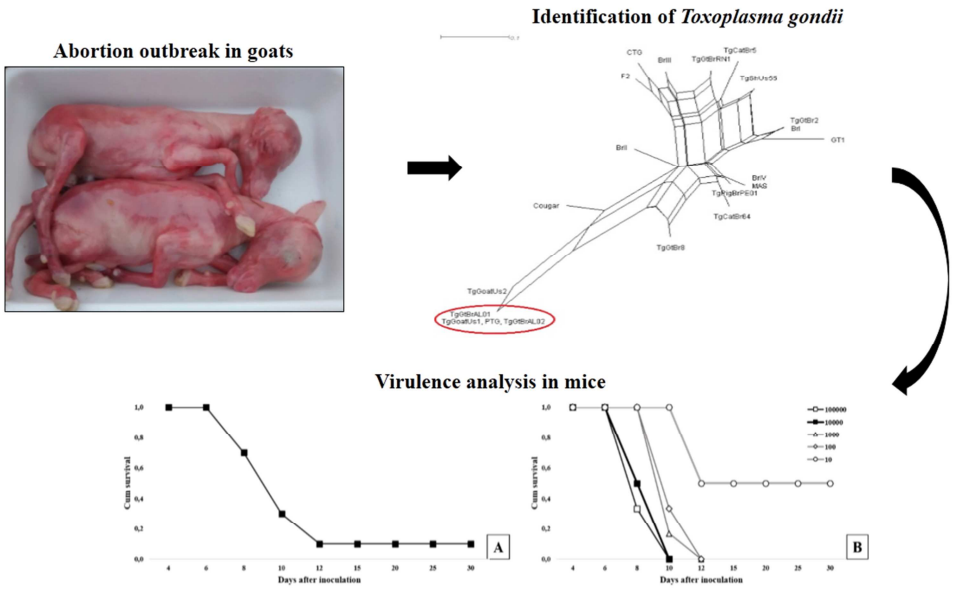

First description of clonal lineage type II (genotype #1) of *Toxoplasma gondii* in abortion outbreak in goats

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Abstract

The purpose of this study was to perform genotypic characterization and to evaluate the virulence of *Toxoplasma gondii* obtained from aborted fetuses in an abortion outbreak in goats from northeastern Brazil. Brain samples from 32 fetuses were submitted to mouse bioassay for *T. gondii* isolation. Two isolates were obtained and subjected to genotypic characterization. Isolate virulence was evaluated using murine model in different doses (from 10^5 to 10^1 tachyzoites/mL). In genotyping, both isolates were classified as clonal lineage type II (genotype #1 ToxoDB) and showed to be virulent for mice. This is the first description of genotype #1 in cases of goat abortion, showing the circulation of virulent *T. gondii* isolate producing reproductive disorders in pregnant goat.

Keywords: Goat, reproductive disorders, toxoplasmosis, virulence, genotyping.

1. Introduction

Toxoplasmosis is caused by the tissue cyst forming coccidian *Toxoplasma gondii* (Tenter et al., 2000). *T. gondii* infection is an important cause of reproductive disorders in goats (Caldeira et al., 2011; Unzaga et al., 2014) and it is considered a risk to public health (Dubey et al., 2011).

Toxoplasma gondii presents three different clonal lineages classified as I, II and III (Howe and Sibley, 1995), which are common in Europe and North America, however, atypical strains are the most frequently/commonly found in South America (Dubey et al., 2012). In Europe and United States, the genotypes more frequently in goats are type II and III (Dubey et al., 2011), although, in Brazil, predominate atypical genotypes (Ragozo et al., 2010). There is a lack of information concerning *T. gondii*

genotypes involved in goat abortion cases, but there are reports of atypical strains in Argentina (Unzaga et al., 2014) and type III in Brazil (Silva Filho et al., 2008).

The purpose of this study was to perform genotypic characterization and to evaluate the virulence of *Toxoplasma gondii* isolates obtained from aborted fetuses in an abortion outbreak in goats.

2. Materials and methods

2.1. Ethics aspects

This study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at Universidade Federal Rural de Pernambuco under the license number 122/2015and was conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation (CONCEA, 2013).

2.2. Study design and sampling

During an abortion outbreak that occurred between September 2014 and October 2015 in a flock of goats, 32 fetuses at different stages of pregnancy were collected. Goats were raised in intensive system receiving water and mineral supplementation *ad libitum*. Feral and domestic cats had access to the facilities, food storage and water supply.

The fetuses were necropsied within 24 hours after abortion to collect the brain for bioassay. Blood samples from the aborted goats were collected for the detection of anti-*Toxoplasma gondii* antibodies using the enzyme-linked immunosorbent assay protocol (ELISA) adapted from Álvarez-García et al. (2003).

2.3. Mouse bioassay

Brain samples from fetuses were macerated with PBS (pH 7,2), filtered on gauze and centrifuged at 700g for 10 min. The supernatant was carefully discarded and the pellet resuspended in PBS and centrifuged again. The supernatant was discarded and the final product was resuspended in PBS containing antibiotic (1.000 IU of penicillin and 100 μg of streptomycin per mL) and inoculated intraperitoneally in two Swiss Webster (SW) mice. Mice were observed daily and those who not died were euthanized 45 days post-inoculation (d.p.i).

Tissue samples (brain, liver, lungs, heart and peritoneal lavage) of mice were collected and stored for DNA extraction. Imprints of the brain and peritoneal lavage were examined for *T. gondii* cysts and tachyzoites, respectively. Positive samples for presence of *T. gondii* were inoculated in MARC-145 cells (Regidor-Cerrillo et al., 2008).

2.4. DNA extraction and PCR

Samples from aborted fetuses (brain) and mice (peritoneal lavage, brain and pooled tissues containing liver, lungs and heart) were submitted to DNA extraction using the commercial kit Wizard Genomic DNA Purification System (Promega®, Madison, WI, USA), according to the manufacturer's protocol. The concentration of DNA for all samples was verified using spectrophotometry and adjusted to 100 ng/μL. *T. gondii* DNA detection was performed by single tube nested PCR, using the external primers TgNN1-TgNN2 and internal primers TgNP1-TgNP2, amplifying a fragment of 227 bp of the ITS1 region of the parasite (Hurtado et al., 2001)

A suspension of *T. gondii* tachyzoites (RH strain, 10^4 tachyzoites/mL) and ultrapure water were used as positive and negative controls, respectively. The amplified

PCR products were subjected to electrophoresis on 1.5% agarose gel stained with BlueGreen (LGC® Biotecnologia, Cotia, São Paulo, Brasil), and visualized under UV light.

2.5. Multilocus PCR-RFLP and phylogenetic analysis

Potencial *T. gondii* isolates obtained in the mice bioassay were characterized by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) using 12 molecular markers (SAG1, 3'-SAG2, 5'-SAG2, Alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) as previously described (Su et al., 2006), to determine the genetic diversity of *T. gondii* circulating in the fetuses. The PCR products were visualized by agarose gel electrophoresis at 2.5%, stained with Sybr Safe DNA Gel Stain (Invitrogen®, USA) and visualized using Safe Imager TM (Invitrogen®, USA). The results were identified, compared and classified according to genotypes present in ToxoDB (http://toxodb.org/toxo/).

For phylogenetic analysis, the electrophoresis banding patterns obtained by PCR-RFLP were transformed into binary data and tabulated. The SplitsTree software (Huson and Bryant, 2006) was used for phylogenetic reconstruction between the genotypes obtained in the present study and others previously isolated in Brazil and in the world.

2.6. Virulence analysis in mice

For virulence assessment, the isolate of *T. gondii* was inoculated into a monolayer culture of African green monkey kidney cells MARC-145 and incubated at 37°C in a 5% CO_2. The medium was changed after 24h. Blind passages of isolation cultures were made at 4 to 7-day intervals until the parasite was observed

122 microscopically in order to keep the total amount of cultured cells to a minimum. The
123 amount of tachyzoites was determined by Trypan blue exclusion, followed by direct
124 counting in a Neubauer chamber (Regidor-Cerrillo et al., 2008) and serial dilutions were
125 performed starting from the concentration from 10^5 to 10^1 tachyzoites. Each dilution
126 was inoculated intraperitoneally into six mice that were observed daily for four weeks.
127 At the end of this period, mice that did not die were euthanized and samples of blood
128 and brain were collected. The serum samples were submitted to the Modified
129 Agglutination Technique (MAT) for the detection of anti-*T. gondii* IgG antibodies
130 (Desmonts and Remington, 1980), considering the cut-off 1:20. The brain was studied
131 for *T. gondii* cysts. Virulence interpretation was performed according to Pena et al.
132 (2008).
133
134 2.7. *Statistical analysis*
135 Incubation period and survival were estimated using the Kaplan-Meier curve
136 (Goel et al., 2010). The IBM SPSS Statistics 23.0 software was used to perform the
137 statistical calculations and the level of significance adopted was 5.0%.
138
139 **3. Results**
140 The two goats that aborted were positive for anti-*T. gondii* antibodies by ELISA
141 with IRPC values of 57.767 and 79.759, respectively. The first fetus was aborted at 124
142 days of pregnancy and showed congestive encephalic vessels, being obtained isolate
143 TgGtBrAL01. The another one was aborted at 101 days of pregnancy and was in the
144 process of autolysis, being obtained isolateTgGtBrAL02.
145 Tachyzoites were recovered from the peritoneal lavage of inoculated mice in
146 bioassay but no tissue cyst was observed in brain. *T. gondii* isolation was also confirmed

147 by PCR in the brain samples of the two fetuses and in the peritoneal lavage of the mice
148 inoculated with these samples. Two isolates (TgGtBrAL01 and TgGtBrAL02) were
149 obtained, representing an isolation rate of 6.2% (2/32). In genotyping, the both isolates
150 were classified as clonal lineage type II (genotype #1 ToxoDB). The results of
151 phylogenetic analysis are shown in Figure 1.

152 As the result of genotyping was the same for both isolates, one was chosen for
153 virulence analysis (TgGtBrAL01). The clinical signs observed are shown in Table 1.
154 Tachyzoites were recovered from peritoneal lavage of all mice that died, in addition to
155 the presence of activated macrophages. Mice that survived did not present antibodies
156 anti-*T. gondii* nor cysts in brain or tachyzoites in peritoneal lavage.

157 The Incubation Period (IP) of isolate in the mice was 9.3 d.p.i. (I.C. 95% = 6.8-
158 11.9 d.p.i.). The mice inoculated with the highest concentrations of tachyzoites (10^5)
159 began to present clinical signs between the 4^{th} and 5^{th} d.p.i. Regarding the Survival
160 Period (SP) of inoculated mice, the mean SP was 11.3 d.p.i. (I.C. 95% = 9.1-13.6 d.p.i.)
161 Mouse deaths occurred according to the concentration of inoculated tachyzoites, with
162 the highest doses causing a faster death (Figure 2). The highest concentrations (10^5 and
163 10^4) caused the appearance of clinical signs in 100.0% (12/12) of inoculated mice
164 between the 4^{th} and 6^{th} d.p.i. and resulted in mouse deaths between 7^{th} and 9^{th} d.p.i. The
165 lowest concentration (10^1) caused the appearance of clinical signs in 50.0% (3/6) of the
166 mice on the 9^{th} d.p.i. and death on the 11^{th} d.p.i. This strain caused clinical signs and
167 death in 90.0% (27/30) of inoculated animals.

168

169 **4. Discussion**

170 *Toxoplasma gondii* infection is considered an important cause of reproductive
171 disorders in goats (Caldeira et al., 2011; Unzaga et al., 2014). The involvement of *T.*

172 *gondii* in abortion cases in that species has been described in different regions of the
173 world such as Spain, where the presence of the DNA of this protozoan was detected in
174 3.8% (1/26) of the aborted goat fetuses (Moreno et al., 2012). In Argentina, 24.0%
175 (6/25) of analyzed fetuses were positive for *T. gondii* by PCR (Unzaga et al., 2014). In
176 Brazil, a study reported that 100.0% (7/7) of fetuses from a goat herd with history of
177 reproductive disorders were positive for *T. gondii* by PCR (Caldeira et al., 2011).
178 This is the first report of *T. gondii* isolation in abortion outbreak in goats from
179 Brazil. Until the present moment, for that species, the isolates obtained in Brazil were
180 from tissue samples of chronically infected animals from commercial slaughterhouses
181 with an isolation rate of 8.4% (12/143) (Ragozo et al., 2009). It is known that the dose
182 of the parasite may influence the isolation rate, based on this, in most cases, there is no
183 way to control the parasitic load in tissue samples intended for isolation (Pena et al.,
184 2008). Thus, small concentrations of the protozoan in inoculated tissues may have
185 negatively influenced on the isolation result.
186 Regarding the genotypic characterization of *T. gondii* strains, there is a lack of
187 studies related to the detection of the genotypes involved in abortion cases in naturally
188 infected goats worldwide. In South America, there are reports of *T. gondii* genotypes
189 classified as atypical obtained from aborted goat fetuses in Argentina (Unzaga et al.,
190 2014). In Brazil, the genetic diversity of *T. gondii* isolates in small ruminants is high
191 (Ragozo et al., 2010), whereas the presence of classic clonal lineages is considered rare,
192 especially type II (Pena et al., 2008).
193 This is the first description of clonal lineage type II (genotype #1 ToxoDB) in
194 goat abortion cases. This clonal lineage was previously identified in sheep abortion
195 cases (Jungersen et al., 2002). This genotype was first described in goats slaughtered for
196 human consumption in the United States (Dubey et al., 2011). In Brazil, there are

197 records of this genotype in felines (Pena et al., 2008), and swine (Andrade et al., 2013).
198 This clonal lineage is frequently reported in Africa, Europe and North America,
199 however, it is considered rare in South America (Dubey et al., 2012).

200 The isolate obtained was considered intermediate virulent, corroborating with
201 those of other studies where genotype #1 was also virulent for mice (Pena et al., 2008).
202 All mice inoculated with TgGtBrAL01 tachyzoites died except at the concentration of
203 10^1 where the mortality rate was 50.0%. The surviving mice did not present tissue cysts
204 and anti-*T. gondii* antibodies, indicating that this dose was not able to induce infection
205 in all mice as previously described in other studies (Jungersen et al., 2002). Regarding
206 to the three mice negative, it is important to consider the possibility of *T. gondii* cysts in
207 other tissues that were not analyzed by this study. Furthermore, the low quantity of
208 tachyzoites inoculated (10^1) may have stimulated a slow immune response with
209 antibody titers below the cut-off to be classified as positive by the technique. Howe et
210 al. (1996) described that isolates of clonal type II are virulent to mice from a dose of 10^2
211 tachyzoites. Due to this pathogenicity difference, is interesting to perform other
212 virulence assay considering virulence markers such as the genotyping of ROP 5 and
213 ROP18 genes (Schwab et al., 2014).

214 Mice inoculated with *T. gondii* isolated from goats destinated for human
215 consumption died within three weeks p.i and those which survived did not present
216 specific anti-*T.gondii* antibodies (Dubey et al., 2011), corroborating with the data
217 obtained by our study. Nevertheless, according to the same authors, mice inoculated by
218 oral route with oocysts of clonal lineage type II isolate (TgGoatUS2) died between 7
219 and 13 d.p.i. Those inoculated with the lowest dilutions (1-100 oocysts) survived and
220 cysts were identified in the brains of mice.

The virulence of *T. gondii* may be influenced by different factors, such as the lineage of mice used in the bioassay, parasite life stage, inoculation route and number of passages (Dubey et al., 2011; Ragozo et al., 2009). These factors may probably be related to the difference between the virulence results previously mentioned.

5. Conclusion

This is the first isolation of clonal lineage type II (genotype #1 ToxoDB) in abortion goat cases. The obtained isolate showed intermediate virulence in the murine model, however, further studies are necessary to better understand the molecular epidemiology and the abortion pathogenesis of this isolate.

Competing interests

None.

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Figure 1. Phylogenetic analysis of the *Toxoplasma gondii* isolates obtained (circle), with the following strains used as references: GT1, PTG, CTG, MAS, TgCatBr5, TgCatBr64, Cougar, BrI, BrII, BrIII, BrIV.

Figure 2. Survival curve to determine the virulence of *Toxoplasma gondii* TgGtBrAL01 isolate in murine model (Swiss Webster). A – Survival curve for mice challenged with *Toxoplasma gondii* TgGtBrAL01 isolate. B – Survival curve for mice challenged with different doses of TgGtBrAL01 isolate.

332

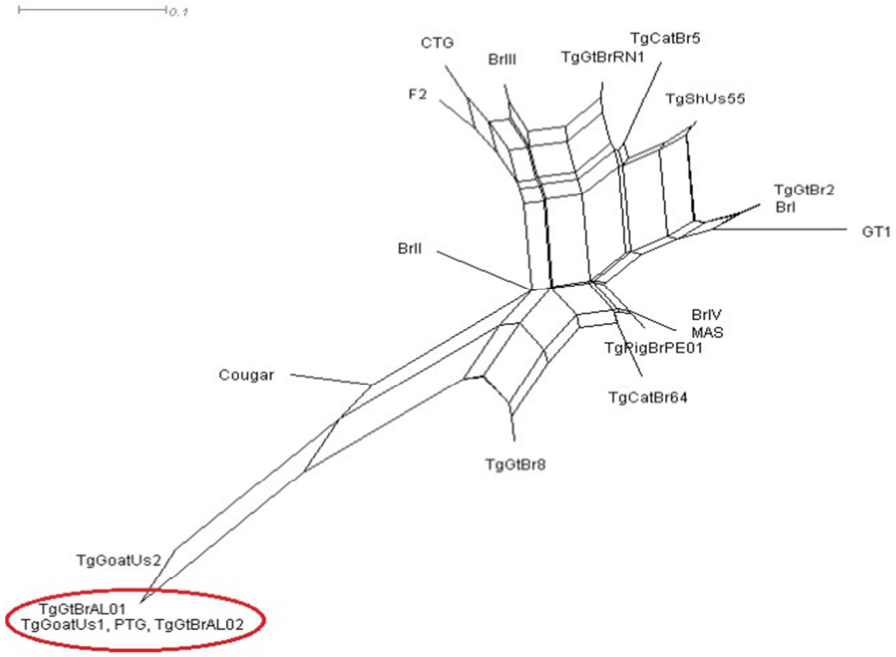
Isolate		Bristly hair	Abdominal pain	Dyspnea	Diarrhea	Ascites	Conjunctivitis
	%	100,0%	63,0%	44,4%	25,9%	0,0%	3,7%
TgGtBrAL01	n	27	17	12	7	0	1
	N	27	27	27	27	27	27

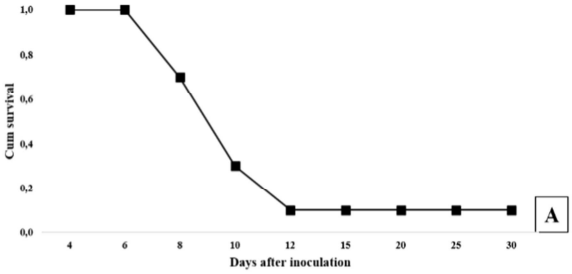
333 N – Total number of mice that showed clinical signs; n – number of mice that showed

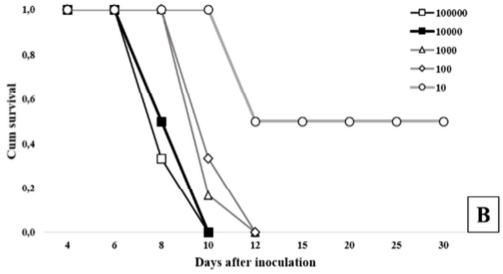
334 the specific alteration.

335 **Table 1.** Clinical signs observed in inoculated mice (Swiss Webster) with 10^5 to 10^1

336 tachyzoites of *Toxoplasma gondii* TgGtBrAL01 isolate.







Highlights

- Occurrence of *Toxoplasma gondii* in an outbreak of goat abortion
- First description of the clonal type II clone in aborted goat fetuses
- Evaluation of the virulence of the *T. gondii* strain obtained in the murine model