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Mariana Bernstein: Investigation; Methodology; Writing - Original Draft; Writing - Review & Editing

Lais L. Pardini: Investigation; Validation; Writing - Review & Editing

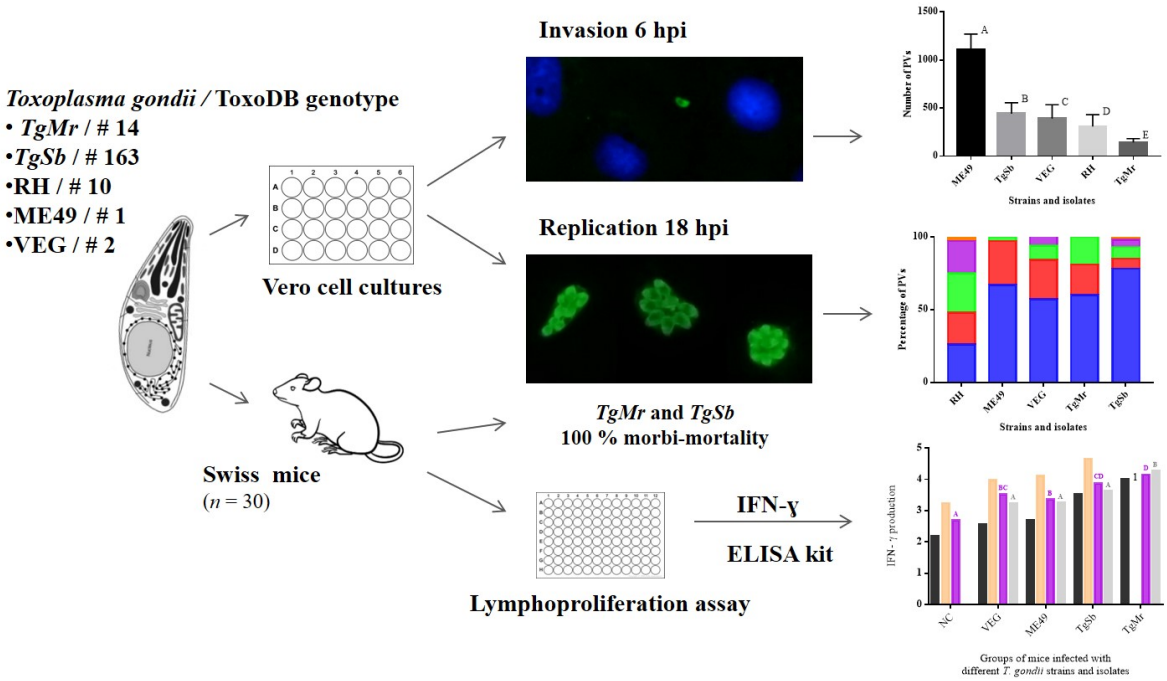
Lucía M. Campero: Investigation

M. Elisa Helman: Formal analysis

Juan M. Unzaga: Funding acquisition

María C. Venturini: Funding acquisition; Supervision

Gastón Moré: Conceptualization; Supervision; Writing - Review & Editing



Evaluation of biological behavior of *Toxoplasma gondii* atypical isolates # 14 and # 163

Mariana Bernstein[1, 2*], Lais L. Pardini[1, 2], Lucía M. Campero[1, 2], M. Elisa Helman[1, 2], Juan M. Unzaga[1], María C. Venturini[1], Gastón Moré[1, 2]

[1]*Laboratorio de Inmunoparasitología (LAINPA), FCV-UNLP, La Plata, Bs. As., Argentina*

[2] *Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina*

*Corresponding author:

Mariana Bernstein, Laboratorio de Inmunoparasitología, Universidad Nacional de La Plata, Facultad de Ciencias Veterinarias, calle 60 y 118 (1900) La Plata, Argentina.

E-mail address**:** mbernstein@fcv.unlp.edu.ar

ABSTRACT:

Toxoplasma gondii is an obligate intracellular protozoan parasite capable of infecting warm-blooded animals, including humans. A highly diverse genetic population has been reported in Central and South America, predominating mainly atypical genotypes. Different genotypes showed different biological behavior in mice. The aim of this study was to evaluate the biological behavior of *T. gondii* isolates obtained from *Macropus rufogriseus* (*TgMr*) and *Saimiri boliviensis* (*TgSb*) identified as atypical genotypes # 14 and # 163, respectively. Strains RH, ME49 and VEG were used as reference for clonal types I, II and III, respectively. *In vitro* invasion and replication capacity assays were analyzed at 6 and 18 hpi, respectively. *In vivo* assay was performed in Swiss mice (n = 30) using $1x10^2$ and $1x10^3$ parasites/mouse as infective doses (ME49, VEG, *TgMr*, *TgSb* and negative control). Morbi-mortality and tissues PCR were assessed. Lymphoproliferation assays were performed and gamma interferon was measured by ELISA. The ME49 strain showed the highest invasion, followed by *TgSb* and VEG, while RH and *TgMr* presented the lowest invasions. The RH strain and the *TgSb* isolate showed more endodyogeny events (fastest doubling times) than VEG and ME49 strains and the *TgMr* isolate. Both atypical isolates showed high virulence (100 % morbi-mortality, at 8-10 dpi) and parasite DNA was detected in all tissue samples. Splenocytes from mice inoculated with *TgMr* and *TgSb* registered the highest values of gamma interferon. An *in vitro* invasion-replication index was established which correlates inversely with virulence in mice. In conclusion, *T. gondii* atypical isolates # 14 and # 163 showed a different *in vitro* behavior than clonal strains, with low invasion-replication indexes but being highly virulent in mouse model.

KEYWORDS: *T. gondii*; atypical isolates; invasion; replication; mouse virulence.

1. INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite capable of infecting warm-blooded animals, including humans (Dubey, 2010). Felines are the definitive hosts and several species of mammals and birds act as intermediate hosts presenting different clinical signs (Dubey, 2010). Toxoplasmosis in humans is generally asymptomatic, however, it may cause a variety of neurological signs and multi-organic failure in immunocompromised patients and fetal lesions in the retina (ocular toxoplasmosis), central nervous system injuries and even abortion in primarily infected pregnant women (Weiss and Kim, 2014). In the northern hemisphere (Europe, USA and Canada) a predominance of clonal population of *T. gondii* has been reported, with clonal type II as dominant (Weiss and Kim, 2014). However, a highly diverse population has been reported in Central and South America (especially Colombia, Brazil and Argentina), being detected non-clonal or atypical genotypes with combinations of types I, II and III alleles, or even new alleles (Bernstein *et al*., 2018; Pena *et al*., 2008; Weiss and Kim, 2014). Different genotypes showed different biological behavior analyzed mainly in mice model (Dubremetz and Lebrun, 2012).

Toxoplasma gondii survival relies on its capacity of invasion of new host cells (Weiss and Kim, 2014). The protozoan multiplies asexually in virtually all nucleated host cells producing tachyzoites (fast division) or bradyzoites (slow division). The tachyzoites enter the host cell by an active invasion mechanism, releasing enzymes which produce the invagination of the host cell membrane. This invagination surrounds the tachyzoites and forms the parasitophorous vacuole (PV) (Weiss and Kim, 2014). After the formation of the

71 PV, the tachyzoites multiply by endodyogeny, in which two daughter cells are formed
72 inside the mother cell. The divisions continue resulting in a geometric expansion of the
73 parasites until the host cell is destroyed (Dubey *et al*., 1998). The first *in vitro* experiments
74 showed that the tachyzoites of the RH strain (clonal type I) have the highest multiplication
75 rate while the tachyzoites of the ME49 strain (clonal type II) have the highest invasion rate
76 (Cañedo-Solares *et al*., 2013; Contreras-Ochoa *et al*., 2012; Saadatnia *et al*., 2010). Some
77 authors suggest that strain virulence depends on replication capacity more than on invasion
78 capacity (Cañedo-Solares *et al*., 2013; Dubremetz and Lebrun, 2012). Similar studies have
79 been carried out with the protozoan *Neospora caninum*, closely related to *T. gondii*
80 (Dellarupe *et al*., 2014b). It has been shown that *in vitro* invasion and replication capacity
81 can be used as virulence traits in *N. caninum* and correlated with virulence in mice model
82 (Dellarupe *et al*., 2014a, b).
83 The *T. gondii* invasion and replication experiments are very heterogeneous and most of
84 them use clonal isolates (Alomar *et al*., 2013; Cañedo-Solares *et al*., 2013; Contreras-
85 Ochoa *et al*., 2012; Cuellar *et al*., 2012; Malkwitz *et al*., 2018; Saadatnia *et al*., 2010).
86 Therefore, a standard protocol is needed to make results comparable (Contreras-Ochoa *et*
87 *al*., 2012).
88 *Toxoplasma gondii* virulence has been defined as the mortality rate in different mice
89 models (Dubremetz and Lebrun, 2012; Saraf *et al*., 2017; Sibley and Boothroyd, 1992).
90 The clonal type I (ToxoDB # 10) is highly virulent in mice, with a LD_{100} = 1 parasite
91 (Sibley and Boothroyd, 1992). In contrast, clonal type II is considered as intermediate
92 virulence with a $LD_{50} \geq 10^3$ parasites (ToxoDB # 1), and clonal type III (ToxoDB # 2) is
93 considered non-virulent with a $LD_{50} \geq 10^5$ parasites (Sibley and Boothroyd, 1992). Until
94 now, non-clonal genotypes have shown different virulence in mouse model, leading to

difficulties on predict virulence based only on genotypes (Dubey, 2010; Dubremetz and Lebrun, 2012; Weiss and Kim, 2014). The morbidity rates, different degrees of histopathological lesions, the presence of *T. gondii* DNA and tissue cysts are associated with virulence in mouse models (Costa *et al*., 2018; Pereira *et al*., 2017; Pinheiro *et al*., 2015). Immune response in experimentally infected mice is characterized by the production of IFN-γ (gamma interferon), which induces the development of Th1 profile and macrophages activation helping to control the parasite proliferation, however paradoxically, its overproduction has been associated with acute virulence (Chen *et al*., 2016; Mordue *et al*,, 2001; Rodgers *et al*., 2005; Wang *et al*., 2016).

Toxoplama gondii genotype # 163 has been frequently detected in Brazil and considered of variable virulence in mice model (Gennari *et al*., 2015; Silva *et al*., 2014). Genotype # 14 has been reported in several South American countries (Argentina, Brazil, Colombia, Chile and Venezuela) and the USA showing a wider distribution than genotype # 163 (Pardini *et al*., 2019). In Brazil, this genotype generated 100 % mortality in mice model, indicating a highly virulent phenotype (Pena *et al*., 2008; Rajendran *et al*., 2012).

The biological behavior of non-clonal *T. gondii* genotypes *in vitro* model (expressed as invasion and replication) and its relation with the virulence in mouse model (expressed as mortality and morbidity) are still not clear (Dubey, 2010; Dubremetz and Lebrun, 2012; Weiss and Kim, 2014). It is necessary the establishment of accurate and reliable *in vitro* protocols which allow the parasites behavior evaluation and help to reduce, replace and refine the number of animals for *in vivo* assays.

The aim of this study was to evaluate the biological behavior of *T. gondii* isolates identified as atypical genotypes # 14 and # 163 by *in vitro* and *in vivo* studies. Additionally, this study aimed to establish a relation between the *in vitro* and *in vivo* assays results.

2. MATERIALS AND METHODS

Toxoplasma gondii isolates were obtained from fatal cases of toxoplasmosis in zoo animals *Saimiri boliviensis* (Bolivian squirrel monkey) and *Macropus rufogriseus* (Bennett´s wallaby) in Argentina (Basso *et al*., 2007; Pardini *et al*., 2015), named in this study as *TgSb* (genotype # 163) and *TgMr* (genotype # 14), respectively (Bernstein *et al*., 2018). Tachyzoites were maintained in liquid nitrogen and re-isolated in mice deficient for IFN-γ (GKO; C.129S7 (B6) -ifng<tm1Ts> / J, The Jackson Laboratory, UK) to reduce number of passages and adaptation to cell culture (Contreras-Ochoa *et al*., 2012; Dellarupe *et al*., 2014b). Strains RH, ME49 and VEG were used as reference for clonal types I, II and III, respectively.

2.1 In-vitro invasion and replication assay

This assay was designed based on previous studies from Alomar *et al*., 2013 and Dellarupe *et al*., 2014b.

2.1.1 VERO cell cultures

VERO cells were cultivated with RPMI-1640 medium with glutamine (Gibco, USA), supplemented with 10 % fetal bovine serum (FBS) (Natocor, Argentina) and 1 % of a solution of antibiotics and antimycotic (Gibco BRL, UK). All cultures were incubated at 37 ^{o}C with 5 % CO_2.

2.1.2 Preparation of tachyzoites

Tachyzoites were harvested when cell monolayer was 80 % infected and the parasites were inside the PVs. Cell monolayer was detached with a cell scraper (GBO, Germany), and the suspension was passed through the 22G, 25G and 27½ G needles, to release the parasites

142 inside the PVs. Tachyzoites were resuspended in the required infection dose to inoculate
143 the VERO cultures. All infections were made within 1 h post released of the parasites.

144 *2.1.3 In vitro assay in 24-well plates*

145 The invasion and the amount of produced tachyzoites (replication) were determined in a
146 24-well plate assay. Circular glass coverslips of 15 mm (MATSUNAMI, Micro Cover
147 Glass, USA) were placed in 24-well plates, and a concentration of $1x10^5$ VERO cells *per*
148 well was added with the conditions described in section 2.1.1. The cell cultures were left
149 overnight (16 to 18 h), to obtain an 80 % confluent monolayer, and the tachyzoites were
150 prepared as described in section 2.1.2 and seeded in an infection dose of $1x10^5$/well. Plates
151 were placed on ice for 10 min to synchronize cell invasion (Alomar *et al*., 2013). One hour
152 after infection, the culture medium was changed. Plates were washed with sterile phosphate
153 buffer solution (PBS) and fixed with cold methanol for 5 min at -20 °C at 6 and 18 hours
154 post infection (hpi). Fixed cultures were preserved with sterile PBS at 4 °C. Three
155 independent experiments were performed for each cut-off time, with 3 replicates each one.
156 Tachyzoites identification was performed by indirect fluorescent antibody test (IFAT). The
157 cultures were permeabilized with 0.25 % Triton X100 (Promega, USA) in PBS for 10 min.
158 Then, PBS with 5 % FBS was added as blocking solution for 30 min. As a primary
159 antibody a serum from a *T. gondii* naturally infected goat (with IFAT titer higher than 800),
160 diluted 1:100 in PBS-0.5 % Tween20 (Metraquímica s.r.l., Argentina) with 0.5 % bovine
161 serum albumin (BSA, Sigma, USA) was used (Gos et al ., 2017). As secondary antibody
162 anti-goat IgG conjugated with Alexa 488 fluorochrome (Life technologies, USA) was
163 diluted 1: 500 in PBS-0.5 % Tween20 and 0.5 % BSA and incubated in the dark. As nuclei
164 staining, DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride, Molecular Probes,
165 Invitrogen, USA) was used diluted 1: 2000 in PBS, incubated for 5 min at room

166 temperature. All antibodies incubations took place in humid chamber at 37 °C for 45 min
167 and 3 washes were made after incubations with sterile PBS. Finally, cultures were mounted
168 with a drop of ~10 µl of mounting fluid MOWIOL 4-88 (Sigma Aldrich, USA) inverting
169 each coverslip over the mounting fluid, air dried at room temperature overnight and stored
170 at -20 °C until microscope examination.
171 Fifty microscopic fields (40X objective) were randomly selected from each coverslip and
172 the PVs were counted by discriminating among PVs with 1, 2, 4, 8, and 16 tachyzoites. The
173 invasion was analyzed by the total number of PVs produced by each strain and isolate at 6
174 hpi. The replication was analyzed by calculating the total amount of parasites that showed
175 at least one event of endodiogeny (PVs with 2 or more tachyzoites) at 18 hpi for each strain
176 and isolate.
177 *2.2 Mice bioassay*
178 The experimental design was evaluated and approved by the CICUAL (Institutional
179 Committee for the Care and Use of Laboratory Animals), Faculty of Veterinary Sciences,
180 UNLP (protocol 42.6.14T). According their recommendations and to reduce the number of
181 animals, the RH strain was excluded from the mice bioassay due to its known LD_{100} = 1
182 parasite (Sibley and Boothroyd, 1992). The infective doses of isolates *TgMr* and *TgSb*
183 were defined by a previous trial were all inoculated mice with $1x10^3$ and $1x10^4$ tachyzoites
184 died with acute toxoplasmosis. A total of 30 specific pathogens free female Swiss mice, of
185 8 weeks age and 23-29 gr average weight, were used for the assay. Water and food were
186 provided *ad libitum* and animals were handled to minimize stress conditions throughout the
187 experiment.
188 Five groups (6 mice/group), were subcutaneously inoculated with the following infection
189 doses: $1x10^2$ tachyzoites (3 mice/group) and $1x10^3$ tachyzoites (3 mice/group) in each

group for *TgSb* (# 163), *TgMr* (# 14), ME49 and VEG, and with 0.5 ml PBS for the negative control group (NC). The parasites were maintained in VERO cell cultures as explained in sections 2.1.1. and 2.1.2.

The mice were monitored daily for 4 weeks, if compatible toxoplasmosis signs were observed were sacrificed according to the requirements established by the CICUAL. Autopsy was performed and samples of central nervous system (CNS) for histopathology and PCR, lung for PCR and spleen for lymphoproliferation assay were obtained as detailed below. Blood samples were obtained by cardiac puncture and sera were stored at -20 °C until used.

2.2.1 T. gondii DNA identification by PCR

Total DNA extraction was performed from CNS and lung samples with the Wizard® genomic DNA purification kit, according to the manufacturer's instructions (Promega, USA). PCR amplification was performed using TOX5-TOX8 primers as previously described (Pardini *et al*., 2015).

2.2.2 Histopathology

A portion of the CNS from each mouse was preserved in 10 % buffered formalin. Samples were processed routinely, stained with hematoxylin and eosin (H&E) and examined microscopically according to the routine protocols of LAPAVET "Dr. Bernardo Epstein ", FCV, UNLP. The histopathological lesions were evaluated with the following scoring scheme: grade 1: mild non-suppurative meningoencephalitis, scarce focal gliosis and scarce neuronal degeneration; grade 2: moderate non-suppurative meningoencephalitis, perivascular cuffs; grade 3: non-suppurative and multifocal necrotizing meningoencephalitis (Venturini *et al*., 1996).

2.2.3 Anti-T. gondii antibody detection

Mice sera were diluted in PBS from 1:25 in base 2 and IFAT was performed to final titer. Samples were considered positive until the last dilution where complete peripheral fluorescence was observed (Gos *et al*., 2017).

2.3 Lymphoproliferation assay

2.3.1 Splenocytes cultures

Splenocytes were obtained from a third of each spleen. The organ was smear under laminar flow and purified by removing the red blood cells using 1 ml of lysis buffer solution (0.83 % ammonium chloride). Cells were counted with 0.5 % trypan blue viability stain in the Neubauer chamber and seeded onto 96-well culture plates at a concentration of 2 x 10^5 viable cells *per* well in 200 µl of culture medium (Wang *et al*., 2016).

2.3.2 T. gondii total lysate antigen, stimulation and measure of IFN-γ

The total lysate antigen (TLA) was produced from the *T. gondii* RH, ME49 and VEG reference strains and from isolates *TgMr* and *TgSb*, maintained in cell cultures as described in sections 2.1.1 and 2.1.2. The tachyzoites of each strain and isolate were centrifuged (3000 g, 10 min) and resuspended in 500 µl of sterile PBS. The suspension was sonicated in 3 cycles, 1 min / cycle, at 45W/s (Omni International, USA). The protein content of each TLA was quantified with a commercial kit (BCA, Pierce, USA) according to the manufacturer's instructions.

The following treatments were performed in triplicate for each mouse splenocytes according previous studies (Chen *et al*., 2016; Wang *et al*., 2016):

A- Splenocytes seeded in medium with 10 % FBS as negative control. B- Stimulation with concanavalin A (5 µg / ml; Biorad, USA) as positive control. C- Stimulation with TLA from the RH strain (10 µg / ml). D- Stimulation with TLA from homologous tachyzoites (10 µg / ml) (Rodgers *et al*., 2005).

Cultures were maintained at 37 °C with 5 % CO_2 for 72 h, the supernatants were collected and stored at -20 °C until use. The IFN-γ was measured using a commercial ELISA kit (Mouse IFN-γ ELISA Kit, Catalog n°: BMS606; Thermo Fisher Scientific Inc., USA) according supplier's instructions, on the pool of the triplicates of each treatment *per* mouse splenocytes.

2.4 Statistical analysis

Invasion and replication assay: A Generalized Linear Mixed Model (GLMM) with log link function was applied. Fixed factor was defined as: strains and isolates, and the response variables were defined as: number of PVs at 6 hpi and total number of tachyzoites at and 18 hpi. The experiment effect was considered as a random parameter. When significant differences were detected, the LSD Fisher test was performed (Balzarini *et al* ., 2008). Percentages of PVs with 1, 2, 4, 8, and 16 tachyzoites were calculated for the different *T. gondii* strains and isolates at 18 hpi and were plotted as stacked column graphics.

Mice virulence assay: Morbi-mortality was calculated as: total of clinically ill and sacrificed animals / total of infected animals * 100. Murine IgG anti-*T. gondii* titers among the groups were analyzed by Kruskal-Wallis test.

Lymphoproliferation assay: the IFN-γ production values obtained by ELISA were log10 transformed and analyzed by ANOVA and subsequent LSD Fisher.

The Pearson correlation coefficient was used to evaluate the association between the parameters evaluated in the *in vitro* assay (invasion and replication) and the morbi-mortality in the *in vivo* assay.

The InfoStat software version 2018 (Di Rienzo *et al*., 2008) was used for all statistical analyzes. The degree of significance was established at $p < 0.05$ for all analyzes. All the

graphics were generated using the GraphPad Prism software version 7.00 (San Diego, USA).

3. RESULTS

3.1 In-vitro invasion and replication assay

Homogeneity among replicates was observed: no significant differences were found among the replicates at 6 and 18 hpi ($p > 0.05$).

Significant differences were found in the invasion capacity between the reference strains and the atypical isolates analyzed ($p < 0.0001$). The ME49 strain showed the highest invasion capacity ($p < 0.05$), while the *TgMr* isolate presented the lowest invasion capacity of all parasites (**Figure 1A**).

Significant differences were observed in parasite replication, where the RH strain showed the highest replication capacity (**Figure 1B**).

Significant differences were observed in the load and number of PVs at 18 hpi ($p < 0.0001$) according to different strains and isolates. Only the RH strain and the *TgSb* isolate produced PVs with 16 tachyzoites (**Figure 2**). In summary, the RH strain and the *TgSb* isolate showed high replication capacity (PVs of 16 tachyzoites = 4 endodyogeny events), while the VEG and ME49 strains, and the *TgMr* isolate showed low replication capacity (PVs of 8 tachyzoites = 3 endodyogeny events). The doubling times were on average ~ 4.5 h for RH and *TgSb* (18 h / 4 division events) and ~ 6 h for VEG, ME49 and *TgMr* (18 h / 3 division events).

All strains and isolates had PVs with parasites that did not replicate or had fewer events of endodyogeny. The RH strain showed less than 30 % of PVs with 1 tachyzoite and equal percentages of PVs with 2, 4 and 8 parasites demonstrating high replication capacity as

285 above-mentioned. Also, the *TgSb* isolate showed high replication capacity but very small
286 percentages of PVs with 2 to 16 parasites (~ 25 % of PVs). The VEG and ME49 strains,
287 and the *TgMr* isolate evidenced ~ 60 % of PVs without replication events (**Figure 2**).
288 An index between invasion and replication (invasion-replication index) was established for
289 each strain and isolate, using the following formula: (average invasion * 18 hpi average
290 replication) / 1000. The resulting index values were: ME49: 790; VEG: 260; *TgSb*: 237;
291 RH: 234; *TgMr*: 42.

292 *3.4 Mice bioassay*

293 Clinical signs compatible with toxoplasmosis infection (xiphosis, hirsute hair,
294 conjunctivitis, decay and tachypnea) were observed in all the inoculated mice with the
295 isolates *TgSb* (sacrificed at 10 dpi) and *TgMr* (sacrificed at 8 dpi - inoculated with 10^3
296 tachyzoites - and 9 dpi -inoculated with 10^2 tachyzoites -). Both atypical isolates showed
297 high virulence (100 % morbi-mortality) in mouse model. Reference strains ME49 and VEG
298 produced no morbidity, all mice were sacrificed at the end of the assay together with the
299 NC group.
300 No correlation was observed between *in vitro* invasion and mortality (Pearson coefficient
301 0.19, $p > 0.05$), nor between *in vitro* replication and mortality (Pearson coefficient 0.04, $p >$
302 0.05). The *in vitro* invasion-replication index was inversely related to the mortality
303 recorded in this assay: the lower value of the *in vitro* index (isolate *TgMr* < strain RH <
304 isolate *TgSb*) the higher mortality (higher virulence) in mice model.
305 Results from histopathological analysis and presence of *T. gondii* DNA in mice tissue
306 samples are showed in **Table 1**. Out of all CNS from *T. gondii* inoculated mice ($n = 24$): 9
307 presented grade 1 lesions (5 *TgSb*; 4 ME49), 3 grade 2 (1 ME 49; 2 VEG), 2 grade 3 (2
308 VEG) and 10 did not present lesions. Different tissue lesions grades are shown in **Figure 3**.

All the mice inoculated with *T. gondii* were seropositive. Antibody titers were higher in mice inoculated with ME49 (final titer 6400: *n* 4/6, and 3200, *n* 2/6) and VEG (final titer 12800, *n* 6/6), than mice inoculated with *TgMr* (final titer 50: *n* 4/6 and 25: *n* 2/6) and *TgSb* (final titer 100; *n* 4/6 and 50: *n* 2/6). Differences were significant ($p < 0.05$).

3.4 Lymphoproliferation assay

The IFN-γ values obtained from splenocytes of mice inoculated with the different strains and isolates, unstimulated, stimulated with Concanavalin A, stimulated with TLA of RH, stimulated with homologous TLA and its statistical comparison are shown in **Figure 4**. Significance differences were observed among the splenocytes stimulated with RH TLA, showing *TgMr* and *TgSb* the highest values ($p < 0.05$). Also, *TgMr* splenocytes showed the highest value ($p < 0.05$) when stimulated with homologous TLA. No significant differences were detected between homologous TLA and RH TLA stimulation, only *TgMr* showed an slightly higher IFN-γ value when challenged with homologous TLA than with the RH TLA. Results from all the assays are summarized in **Table 1**.

4. DISCUSSION

Most studies with *T. gondii* atypical genotypes biological behavior have focused the analysis on mice virulence. In this study, we evaluated the *in vitro* capacity of invasion and replication of *T. gondii* atypical isolates in correlation with an *in vivo* model. We adapted a methodology previously used in the biological characterization of reference genotypes of *T. gondii* and the related protozoan *N. caninum* (Alomar *et al* ., 2013; Dellarupe *et al*., 2014b). The clonal reference strains in this study showed an *in vitro* behavior very similar to that described by other researchers (Cañedo-Solares *et al*., 2013; Saadatnia *et al*., 2010). The clonal type I (RH = virulent) showed low invasion capacity but high replication capacity,

while the clonal type II (ME49 = intermediate virulence) showed high invasion capacity, but low replication capacity (Cañedo-Solares *et al*., 2013; Saadatnia *et al*., 2010). On the other hand, clonal type III (VEG = non virulent), presented intermediate invasion and low replication, similar to what was previously described (Malkwitz *et al*., 2018). The doubling time of the RH strain tachyzoites was slightly faster than that recorded in previous studies (Cañedo-Solares *et al*., 2013), which could be related to limited cell culture adaptation and strain re-isolation in mice (Contreras-Ochoa *et al*., 2012; Dellarupe *et al*., 2014b). Malkwitz *et al*., 2018, reported the ME49 replication values in Vero cells higher than NED strain (clonal type III) after 12 hpi., using qPCR. In our study, the ME49 strain showed an active behavior at the beginning of infection (high invasion at 6 hpi), but low and slow replication (most PVs only with 1 or 2 tachyzoites at 18 hpi), in contrast with the clonal type III (VEG strain) that showed low replication capacity but PVs even with 8 tachyzoites. The *in vitro* behavior we observed in the ME49 and VEG strains could be associated with its reported mice virulence, where a low and slow replication would allow the infected cells survival and the establishment of an effective immune response (Dubremetz and Lebrun, 2012; Mordue *et al*., 2001; Sibley and Boothroyd, 1992). The atypical isolate *TgMr* (# 14) had an *in vitro* behavior similar to the RH strain with respect to its low invasion capacity (even lower than RH), however, it had the lowest replication of all strains and isolates used in the assay. On the other hand, the atypical isolate *TgSb* (# 163) had an intermediate invasion capacity (similar to VEG strain) and a high replication (similar to RH strain). Despite the fact that the capacity and the intrinsic mechanism of invasion by reference strains (mainly RH) has been extensively evaluated (Contreras-Ochoa *et al*., 2012; Dubey *et al*., 1998; Dubremetz and Lebrun, 2012; Weiss and Kim, 2014), the results of the present study suggest it is negatively related to virulence.. In addition, considering our results, the

capacity of invasion and possibly its intrinsic mechanism, could not be fully extrapolated between different *T. gondii* strains.

It has been stated that the clonal strains virulence correlates with high replication capacity (Cañedo-Solares *et al*., 2013; Dubremetz and Lebrun, 2012; Malkwitz *et al*., 2018). Based on our results, the RH strain and *TgSb* isolate showed the highest speeds (or the lowest doubling times), the replication and the "speed" of division correlates positively with virulence. Invasion and replication capacity have been used as phenotypic virulence traits for *N. caninum* isolates (Dellarupe *et al*., 2014b) and were useful in this work to evaluate the behavior of clonal and atypical *T. gondii* isolates. Nevertheless, the total amount of intracellular parasites for *TgMr* and *TgSb* were lower than the three reference strains, suggesting that there may be other virulence factors not directly associated with invasion and replication. In summary, the atypical isolates of *T. gondii* obtained from *M. rufogriseus* and *S. boliviensis* have a different *in vitro* behavior than the clonal reference strains, showing invasion and replication characteristics similar to the virulent RH clonal type, respectively.

Both atypical isolates showed 100 % morbi-mortality, using doses as low as $1x10^2$ tachyzoites, similar as previously described using similar atypical isolates (Gennari *et al*., 2015; Pardini *et al*., 2019; Pena *et al*., 2008; Rajendran *et al*., 2012; Rego *et al*., 2017; Silva *et al*., 2014). Probably the use of a lower parasite dose could help to identify possible virulence differences, as suggested by other authors (Saraf *et al* ., 2017). On the other hand, mice infected with clonal strains (ME49 and VEG) had a 0 % morbi-mortality and were considered as non-virulent (Mordue *et al*., 2001; Sibley and Boothroyd, 1992).

Mice infected with atypical genotypes, showed absence (*TgMr*) or low severity (*TgSb*) of CNS lesions, possibly due to sacrifice in the acute stage of the infection (8-10 dpi) as

reported by others (Mordue *et al*., 2001; Pinheiro *et al*., 2015). Despite of this, *T. gondii* DNA was detected in all CNS and lung samples, confirming the presence of the parasites. Mice infected with ME49 and VEG strains showed no clinical signs, however, severe lesions in the CNS were detected, which could be related to a cell-mediated response controlling the excessive multiplication of protozoa (Weiss and Kim, 2014). These mice were considered chronically infected as confirmed by the tissue cysts observation in CNS (Dubey, 2010). In addition, 5/6 and 2/6 mice infected with ME49 resulted positive to *T. gondii* DNA in CNS and lung, respectively, possibly due to the preferential location in CNS of the chronic stages of the infection (Costa *et al*., 2018; Dubey, 2010). As expected for non-lethal clonal strains, tachyzoites multiplication was controlled by the host immune system and protozoa were confined to CNS cells as bradyzoites (Pinheiro *et al*., 2015). Additionally, the differences in the IgG titers obtained in this study could be associated with the time of infection (Dubey, 2010).

In our study, the isolate with the genotype # 163, was characterized as virulent, both in *in vitro* and in the *in vivo* assays, similar as reported for one of the isolates from Rego *et al*., (2017). It seems that the same genotype could present differences in virulence and therefore the biological behavior could not be 100 % predictable from the molecular markers used in this work (Rego *et al*., 2017). Similar to other studies, our results indicate that isolate with the genotype # 14, is characterized as virulent (Pardini *et al*., 2019; Pena *et al*., 2008; Rajendran *et al*., 2012).

As a final goal, mice cellular (IFN-γ) immune response was evaluated. In both cases, the isolates *TgMr* and *TgSb* induced a higher IFN-γ production than clonal strains ME49 and VEG, similar to what has been described by other authors (Chen *et al*., 2016; Wang *et al*., 2016). The extremely high IFN-γ production from infected mice splenocytes (especially

405 with *TgMr*), could indicate a greater number of effector lymphocytes. These results agree
406 with those reported by other researchers in which high levels of IFN-γ were detected in
407 lethal infections (RH strain), whereas moderate levels were detected in non-lethal infections
408 (Mordue *et al*., 2001). Therefore, the adaptive immune response generated by these atypical
409 isolates could contribute to the pathogenesis and the fatal outcome of toxoplasmosis
410 (Mordue *et al*., 2001; Weiss and Kim, 2014). It has been suggested that the *T. gondii* strain
411 used to prepare the TLA affects the cytokines produced by lymphocytes *in vitro* (Rodgers
412 *et al*., 2005). Our results indicate that all TLA stimulated splenocytes produced similar
413 amounts of INF-□. In general, splenocytes stimulated with TLA from RH, produced
414 slightly higher concentrations with the exception of those stimulated with homologue of
415 *TgMr*. It is probable that the *TgMr* isolate expresses different antigens recognized as
416 virulence factors, such as rhoptry proteins, which could induce an overproduction of IFN-γ
417 in infected animals (Dubremetz and Lebrun, 2012; Rodgers *et al*., 2005). Splenocytes were
418 collected at the time of sacrifice which differed between atypical isolates and clonal strains
419 (ME49 and VEG) leading to potential differences. Probably more accurate results could be
420 obtain performing this assay at fix time, however, it requires a higher number of infected
421 mice and the use of non-lethal dose or strains, which is not always possible with highly
422 virulent atypical strains. In summary, the overproduction of IFN-γ in mice infected with
423 *TgMr* and *TgSb* could be related to the expression / secretion of immune response
424 modulators by these protozoa and could be associated with the higher virulence detected in
425 the *in vivo* model (Dubremetz and Lebrun, 2012; Mordue *et al*., 2001; Rodgers *et al*.,
426 2005). On the other hand, the evaluation of the humoral response (IgG titers) did not allow
427 comparisons since blood sampling was conducted at different times. Futures studies aiming

humoral response evaluation should considerer sampling at fix time (Chen et al., 2016; Tao et al., 2013).

Finally, an *in vitro* invasion-replication index was established that could correlate with virulence and mice morbi-mortality inversely (**Table 1**). The RH strain and the atypical isolates, presented the lowest index values, mainly due to a low invasion. The lower invasion-replication index was associated with the higher virulence, although a larger number of strains and isolates should be evaluated to confirm this assumption. Other factors not measured in the *in vitro* assay may possibly be influencing virulence such as the expression and allelic profiles of ROP proteins (Dubremetz and Lebrun, 2012). In the same way, CS3 is a useful marker to predict the virulence of *T. gondii* in the "atypical" isolates from Brazil (Silva *et al*., 2014). It would be interesting to analyze the mentioned molecular markers in a higher number of isolates, including atypical isolates obtained from Argentina. It would be important to evaluate the *in vitro* behavior of other phylogenetically related *T. gondii* atypical isolates in order to confirm the usefulness of the assay presented here as complement / substitute to mouse model experiments. Also, performing the protocols described in this study with different cell lines, could allow the improvement of virulence prediction and the design of comparable studies (Contreras-Ochoa *et al*., 2012). The validation of an *in vitro* invasion-replication index could be useful to indirectly predict virulence in mice.

In conclusion, the *T. gondii* atypical isolates # 14 and # 163 resulted in a different *in vitro* behavior than clonal strains, with low invasion-replication indexes, but showing high virulence in mice model in association with high levels of INF-□.

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7. CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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Figure captions:

Figure 1. *In vitro* assay: Invasion values (number of parasitophorous vacuoles at 6 hpi, **A**) and replication values (number of tachyzoites at 18 hpi, **B**) for each *T. gondii* strain and isolate. Different letters indicate significant differences ($p < 0.05$, LSD Fisher). Reference: PVs = parasitophorous vacuoles.

Figure 2. *In vitro* assay: Comparison of the percentages of PVs of 1, 2, 4, 8 and 16 tachyzoites for each *T. gondii* strain and isolate at18 hpi. Reference: PVs = parasitophorous vacuoles.

Figure 3. *In vivo* assay: Representative photomicrographs of *T. gondii* infected mice CNS sections showing different grades of lesions (H&E staining). Grade 1: Neural necrosis

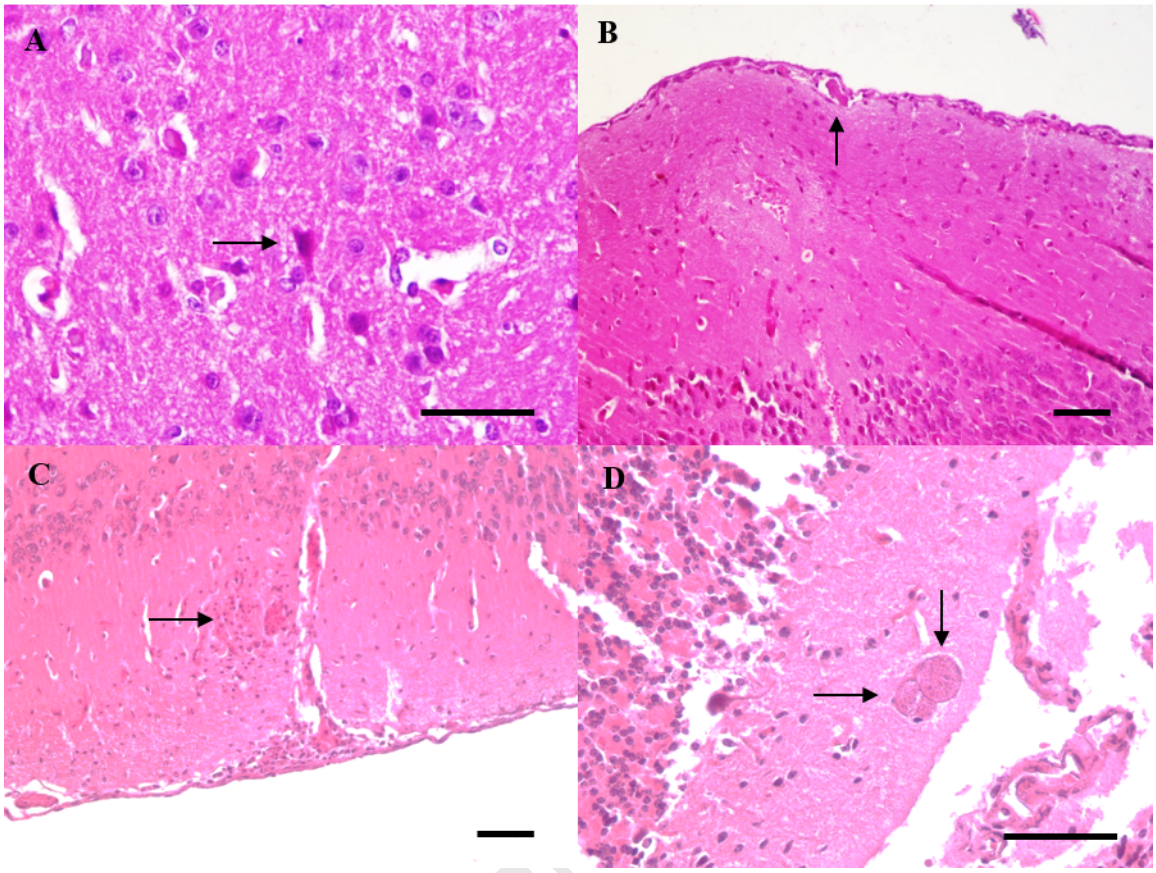
(arrow), 40x (**A**, mouse inoculated with *TgSb*). Grade 2: mild non-suppurative focal meningitis (arrow), 20x (**B**, mouse inoculated with VEG). Grade 3: numerous mononuclear cells, focal gliosis (arrow), 20x (**C**, mouse inoculated with VEG). Two large cysts in cerebellum without inflammatory reaction (arrow), 40x (**D**, mouse inoculated with VEG). Scale bars = 100 µm.

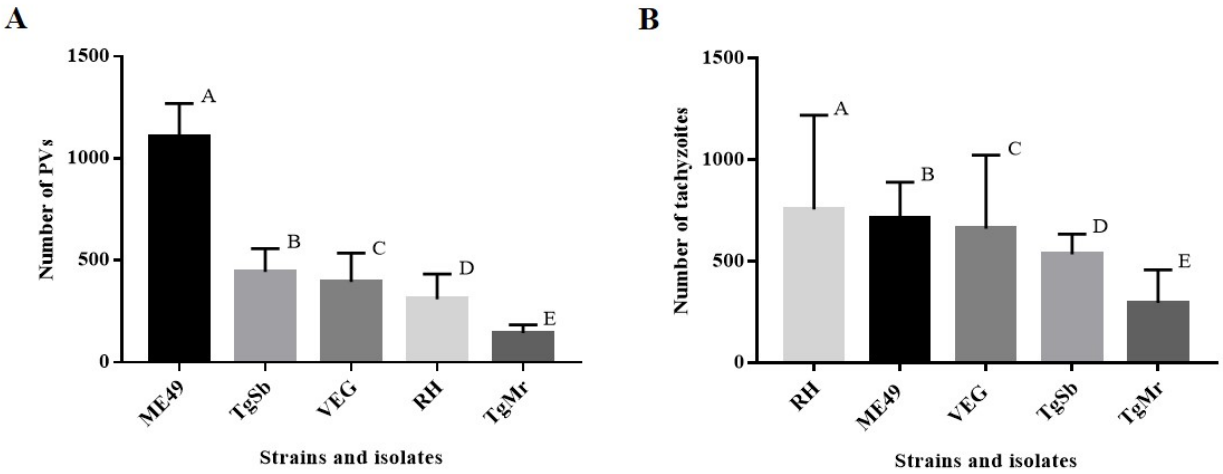
Figure 4. Lymphoproliferation assay: production values of IFN-γ by splenocytes of mice inoculated with the different *T. gondii* strains and isolates, unstimulated (A, black), stimulated with Concanavalin A (B, orange), stimulated with TLA of RH (C, purple) and homologous TLA (D, gray). Values obtained by ELISA (pg/ml) were log10 transformed to apply an ANOVA. Different letters indicate significant differences among the columns with the same color ($p < 0.05$). [1] Note: stimulation with Concanavalin A of splenocytes from mice infected with *TgMr* exceeded the IFN-γ detection values from ELISA kit.

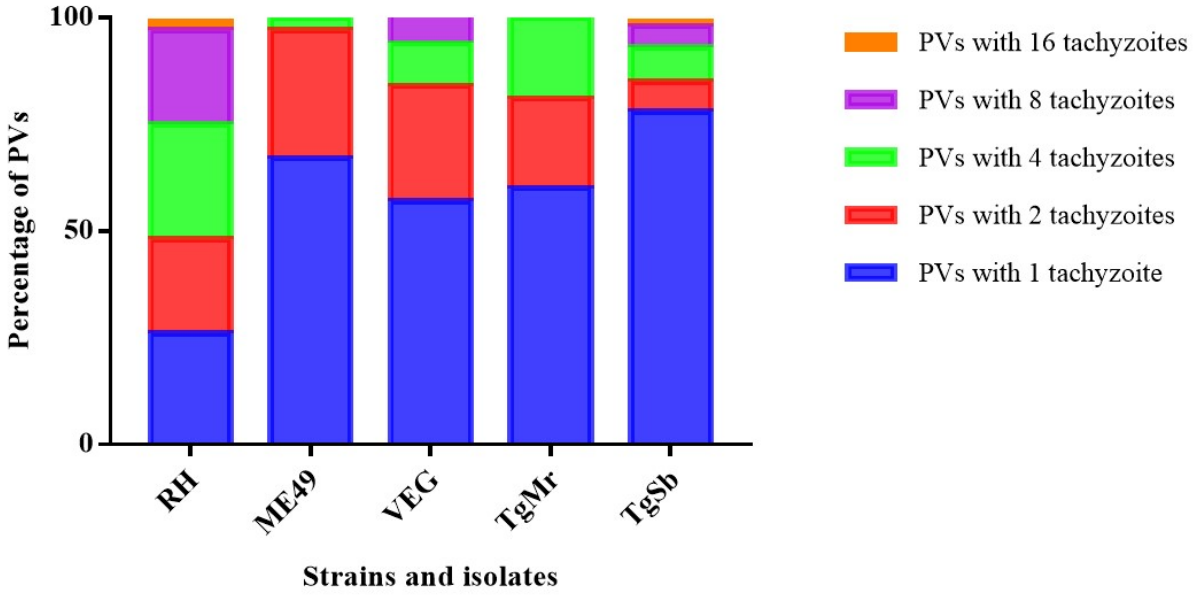
Table 1. Summary of the results from the *in vitro* and the *in vivo* assays with different *T. gondii* strains and isolates.

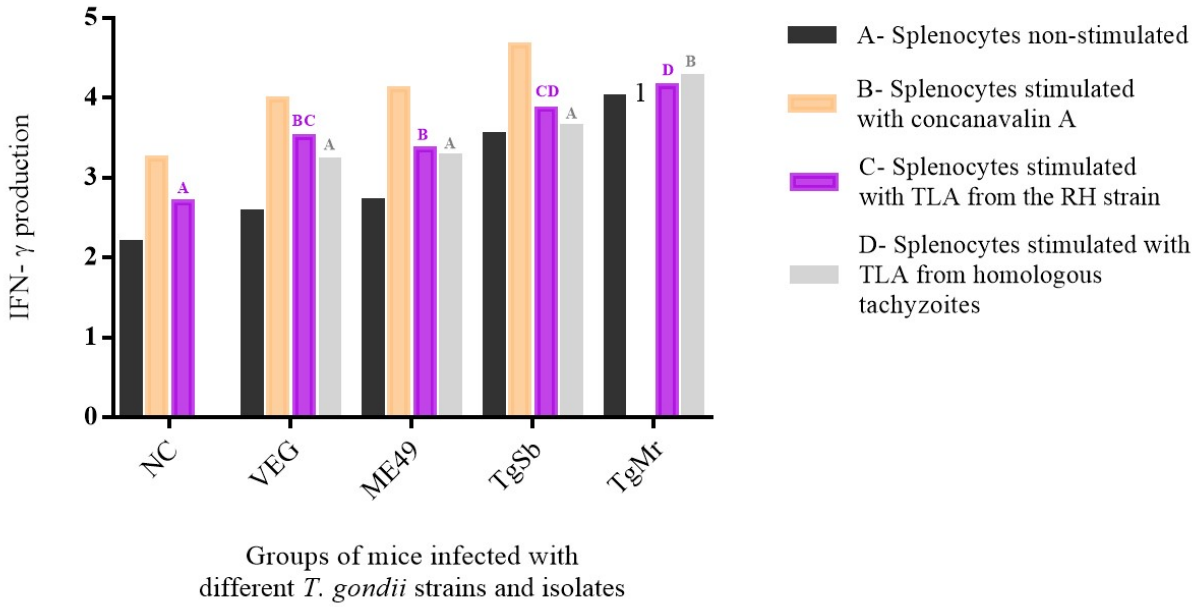
Isolates / Strains	Toxo DB-genotype	*In vitro* assay		Invasion-replication index [c]	*In vivo* assay							
		Invasion [a]	Replication [b]		Morbi-mortality [d] (%)	Sacrifice	Lesions [f] (%)	*T. gondii* cysts [g] (%)	*T. gondii* DNA in CNS [h] (%)	*T. gondii* DNA in lung [i] (%)	IFN-γ Production [j] (pg/ml)	IgG titers [k] (*n*)
1-*TgSb*	# 163	443	535	237	6/6 (100)	10 dpi	5/6 (83,3)	0/6 (0)	6/6 (100)	6/6 (100)	3.86/3.64 (12542/8005)	100/50 (4/6;2/6)
2-*TgMr*	# 14	143	295	42	6/6 (100)	8-9 dpi [e]	0/6 (0)	0/6 (0)	6/6 (100)	6/6 (100)	4.15/4.26 (17333/24288)	50/25 (4/6;2/6)
3-RH	# 10	310	757	234	ND	ND	ND	ND	ND	ND	ND	ND
4-ME49	# 3	1108	713	790	0/6 (0)	30 dpi	5/6 (83.3)	0/6 (0)	5/6 (83.3)	2/6 (33.3)	3.35/3.27 (2977/2227)	6400/3200 (4/6;2/6)
5-VEG	# 2	394	662	260	0/6 (0)	30 dpi	4/6 (66.6)	4/6 (66.6)	5/6 (83.3)	5/6 (83.3)	3.51/3.22 (3811/1741)	12800 (6/6)

References:
[a]: Invasion: average number of PVs at 6 hpi.
[b]: Replication: average number of the total amount of parasites that showed at least one event of endodiogeny (PVs with 2 or more tachyzoites) at 18 hpi.
[c]: Invasion-replication index: (average invasion 6 hpi * average replication 18 hpi) / 1000.
[d]: Morbi-mortality: total of clinically ill and sacrificed animals / total of infected animals * 100.
[e]: Mice inoculated with $1x10^3$ tachyzoites were sacrificed 8 dpi, while mice inoculated with $1x10^2$ tachyzoites were sacrificed 9 dpi.
[f]: Number of mice with CNS lesions / number of mice inoculated.
[g]: Number of mice with *T. gondii* cysts in CNS / number of mice inoculated.
[h]: Number of mice with *T. gondii* DNA detection in CNS / number of mice inoculated.
[i]: Number of mice with detection of *T. gondii* DNA in lung / number of mice inoculated.
[j]: Average values obtained from splenocytes stimulated with TLA from RH/and homologous TLA. Values were log10 transform for statistical analysis. Original average values obtained by ELISA (pg/ml) between brackets.
[k]: IFAT final titers: number of mice with IgG final titer / number of mice inoculated.
dpi: days post infection.
ND: no data.









HIGHLIGHTS

- *In vitro* and *in vivo* behavior of 2 *T. gondii* atypical isolates was evaluated.
- Atypical isolates showed similar *in vitro* behavior to the virulent type I strain.
- Atypical isolates showed 100 % morbi-mortality in mice with a 10^2 parasites.
- High virulence was associated with high IFN-□ in lymphoproliferation assay.
- Low values of invasion-replication index correlated with high virulence in mice.