

## Characterization of the Virulence of three Chronic Strains of *Toxoplasma gondii* according to Survival, Histopathology Detection Studies

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### Abstract

The objective of this work is to characterize the virulence of three chronic *Toxoplasma gondii* strains, isolated in Costa Rica and characterized molecularly by PCR-RFLP previously, according to survival and histopathology in tissues of Swiss CD-1 mice. Ten (10) mice (5 female and 5 male) were infected orally with serial dilutions of brain tissue cysts from previously infected mice. Three chronic strains of *T. gondii*, previously molecularly characterized, came from the domestic cat (*Felis catus*, TFC-1; A), to “caucel” (popular name) (*Leopardus wiedii*, TLW-1; B) and from white mice, (*Bos taurus*, TBT-1;D) fed beef. Mice were euthanized on day 30 of the experiment. Virulence based on survival was recorded and the organs of the mice were histologically studied. The fixed tissues were processed by routine methods and stained with Harry’s hematoxylin-eosin (H and E). The areas of consolidation (CA), the presence of tachyzoites (Tq) or tissue cysts (Q) were estimated by mm<sup>3</sup> of tissue analyzed. Strain D, characterized as a variant of Type I, was the most virulent. Strain A, less virulent, characterized as a type I variant, but different from strain D, was the one that manifested a greater number of cysts in the brain in the lowest dilution of the inoculum, despite having manifested a very high tissue inflammatory response in the lung, observed by a higher proportion of AC/mm<sup>3</sup> and Tq/mm<sup>3</sup> present in this tissue. The highest mortality was observed in female mice compared to males infected with any of the analyzed chronic strains, in the most concentrated infectious doses. So, considering that the responsiveness of the mice in this experiment is the same, the difference lies in the genotype of the chronic strain of *T. gondii*, on the one hand, and in the dose of the inoculum on the other. The characterization of these strains are necessary for future epidemiological studies in toxoplasmosis.

**Keywords:** *Toxoplasma gondii*; Chronic Strains; Virulence; Survival; Histopathology; Costa Rica

### Abbreviations

PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; *T. gondii*: *Toxoplasma gondii*;

FFIP: Formalin-Fixed and Paraffin-Embedded; I.P: Intraperitoneal Inoculation

### Introduction

*Toxoplasma gondii*, an obligate intracellular protozoan parasite belonging to the Apicomplexa phylum, is a parasite that infects warm-blooded vertebrates, including humans [1,2]. *T. gondii* infection not only produces reproductive and economic losses in livestock, but also

has implications for human public health [2,3]. Although most infections are asymptomatic in healthy individuals, this parasite can cause serious complications in immunocompromised individuals, such as AIDS patients or those receiving immunosuppressive therapy. Furthermore, *T. gondii* can cause miscarriage, stillbirth, and birth defects if infection occurs during pregnancy [4].

Substantial variation in disease progression and severity is observed in toxoplasmosis cases, both congenital and immunocompromised, and these differences are presumably due to several variables, including host and parasite genetics [5-8]. However, it is still unclear why most of *T. gondii* infected remain asymptomatic. Recently, attention has focused on genetic diversity among *T. gondii* isolates from apparently healthy and diseased hosts. Serious cases of toxoplasmosis in immunocompetent individuals are considered as result of infection with atypical *T. gondii* isolates [9]. In experimental animals, the virulence in this parasite, depends on the strain characteristics [10]. Therefore, the finding of a correlation between the severity or type of disease and the genotype of the strain, could be important in determining the correct treatment in each human case [11].

Strains of *T. gondii* have been subdivided into three groups or lineages, denominated type I, II, and III by using different characterization methods, such as: (a) isoenzyme analysis (zymodemes), (b) virulence in the Swiss white mouse, and (c) analysis of the Restriction Fragment Length Polymorphism (RFLP) [12]. From the molecular perspective, earliest studies were based on the genetic analysis of the polymorphic locus of the SAG2 surface antigen [8] and related to virulence in different hosts [5,13]. In mice, Type I lineages are uniformly lethal (LD) 100 = 1, contrary to types II and III lineages, which are significantly less virulent (LD 50  $\geq$  1000) [5]. In humans, manifestations of the disease vary widely, from asymptomatic to acute severe toxoplasmosis [14,15]. The Type II lineage causes, predominantly, human toxoplasmosis. However, there are biases between the symptoms of the disease and the genotypes of the parasite. For example, atypical type III isolates are more likely to be involved in severe toxoplasmic chorioretinitis in human patients [16], and causing, severe acute disease in disseminated toxoplasmosis in immunocompetent patients [14]. Differences of clinical presentations, in each host, is probably due to genotype variations that may occur within the different lineages [17]; this fact could be explained by the recombination of genes that are produced during the parasite sexual reproduction in the intestine of felines, natural hosts of *T. gondii* [18,19].

The house mouse (*Mus musculus*), has been used as the primary laboratory animal model for determining the virulence of *T. gondii* strains. Epidemiological evidence also suggests a potential association between virulence in mice and disease severity in human toxoplasmosis. However, many factors can affect virulence measurements, including route of infection, life stage of the parasite, number of passages of the parasite in mice or cell culture, and the mouse host line used [20].

The biological characterization, through virulence, consequence of the initial inflammatory response and the encysting capacity [21], will allow to define some of the characteristics of the strains and the inoculum standardization for carrying out tests with different medications.

### Aim of the Study

The objective of this work is was to characterize the virulence of three chronic *T. gondii* strains, molecularly characterized by PCR-RFLP of the L358, SAG 2 and SAG genes [22], according to survival and histopathology in tissues from Swiss CD-1 mice, orally infected with tissue cysts. In addition, this work to provide genetic and virulence patterns of the isolated chronic strains in Costa Rica.

### Material and Methods

#### Strains of *Toxoplasma gondii*

Three chronic strains and one acute strain were used through. The chronic strains were obtained from the feces of a domestic cat (*Felis catus*, TFC-1;), a "caucel" (popular name) (*Leopardus wiedii*, TLW-1) and from white mice (Strain CD-1, *Mus musculus* Swiss) fed beef (*Bos taurus*, TBT-1). All the chronic strains were isolated from February to October 2007. Molecular characterization of the strains was performed using PCR-RFLP for L358, SAG 2 and SAG genes and reported by Vethencourt, *et al* [22]. For strain A and D, they were char-

acterized as type I but not pure and variant genotypes and strain B as a variant of genotype III [22]. The strain RH (ATCC 50174 D) of *T. gondii* was introduced as an acute control strain. Strains of *T. gondii* were maintained in the laboratory by intraperitoneal inoculation (i.p.) in white mice (strain CD-1, *Mus musculus Swiss*). Strain RH (acute) was passed twice a week with inoculations of 0.4 mL of a peritoneal exudate diluted 1:10 ( $1 \times 10^{-1}$ ), 1:200 ( $2 \times 10^{-2}$ ) and 1:4000 ( $4 \times 10^{-3}$ ), in groups of 2 mice by dilution. The chronic strains TFC-1, TLW-1, TBT-1, denoted in this work as strain A, B, and D, respectively, was processed as follows: brain tissue cysts obtained from the of CD-1 mice, previously infected with these strains, were dilacerated and resuspended in 5 mL of saline; from this suspension, two serial dilutions were made ( $1 \times 10^{-1}$  and  $1 \times 10^{-2}$ ), and 2 mice were inoculated with 0.2 mL of each dilution. The chronic strains were passed every 3 months and the acute strain every 4 days, following the protocols of the Basic Research Laboratory Bioterio of the Medical Sciences University (BLIB-UCIMED), which follows all the regulations established for Costa Rica. The infected mice were maintained with the usual concentrated feed supply and water *ad libitum*, according to the national and international laws for animal care [23-25].

### Virulence

The survival of the mice was recorded up to 30 days post-infection. For chronic strains, 10 mice (five females and five males), four weeks old, were infected orally, with 0.5 mL of the inoculum (tissue cysts) undiluted and with dilutions  $1 \times 10^{-1}$ ,  $1 \times 10^{-2}$  or  $1 \times 10^{-3}$ . For the RH strain, two mice were inoculated with 0.4 mL i.p. of peritoneal exudate, diluted,  $2 \times 10^{-2}$  and  $4 \times 10^{-3}$ .

### Histopathological study

The mice infected with the different dilutions of the chronic strains, which survived or showed clear signs of infection by *T. gondii* (piloerection, atelectasis or paresis of the hind legs), were euthanized and the tissues (brain, lung, heart, spleen, liver, kidney) were fixed in 10% buffered formalin. The fixed tissues were processed by routine methods and stained with Harry's hematoxylin-eosin (H and E). All tissues were observed under a microscope (Labomed; Ce: 7GA9) and calibrated (one optical unit with a 4X objective represented 25 microns ( $\mu\text{m}$ )). Tissue volume was considered after measuring length by width in microns by 5  $\mu\text{m}$  thick and transformed into cubic millimeters ( $\text{mm}^3$ ). The consolidation areas (CA), presence of tachyzoites (Tq) or tissue cysts (Q) were estimated in  $\text{mm}^3$  of analyzed tissue.

### Statistical analysis

The results will be represented in terms of proportion, and the frequency of molecular detection with respect to microscopy will be evaluated with Fisher's Exact Test. The data obtained in the study of tissue findings were represented in terms of mean and standard error of the mean. Any probability less than or equal to 0.05 will be considered significant. Survival analysis was performed using Kaplan-Meier curves and the relationship between strain and virulence was made with Odds Ratio. The analysis was carried out with the help of the SPSS statistical program version 19.

## Results

### Virulence

All chronic strains induced mortality in mice infected with undiluted inoculations. Mortality was observed in 60% (6/10), 70% (7/10) and 100% (10/10) for strains A, B, and D, respectively, at 10 days post-infection. At 15 days there was only mortality in mice infected with strains B and D. For strain B, after 15 days, all mice infected with the undiluted inoculum had died and 100% (10/10) of the mice infected with the  $1 \times 10^{-1}$  diluted inoculum. For strain D, 60% (6/10) of the mice inoculated with  $1 \times 10^{-1}$  dilutions of the inoculum had died. In general terms, between inoculum  $1 \times 10^0$  and  $1 \times 10^{-1}$ , strain A induced mortality in 30% (6/40), strain B in 50% (20/40) and in the strain D in 53,3% (16/30), 15 days post infection. All mice with inoculations of the chronic strains, at  $1 \times 10^2$  and  $1 \times 10^3$  dilutions, survived for more than 30 days (Table 1). All mice infected with the acute strain (RH) of *T. gondii* died 3 days after infection, from a diluted exudate  $2 \times 10^{-2}$  (approximately  $1 \times 10^5$  Tq per inoculum) and at 7 days with a diluted exudate  $4 \times 10^{-3}$  (approximately  $4 \times 10^3$  Tq per inoculum).

On relation to the cumulative mortality rate between the different strains, it can be seen how layer A was significantly less virulent than strain B ( $p < 0.001$ ) and D ( $p < 0.001$ ). Between strain B and D, although the cumulative mortality rate was similar, it was observed that

Strain (inoculum)	Mice survived <sup>α</sup>						
	A (204 Q/mL)		B (578 Q/mL)		D (354 Q/mL)		
Days post-infection	10	15	10	15	10	15	
Dosages inoculum	1 x 10 <sup>0</sup>	4	4	3	0	0	0
	1 x 10 <sup>1</sup>	10	10	10	0	10	4
	1 x 10 <sup>2</sup>	10	10	10	10	10	10
	1 x 10 <sup>3</sup>	10	10	10	10	Np	Np
Cumulative mortality <sup>β</sup>	30 (6/40)		50 (20/40)		53,3 (16/30)		

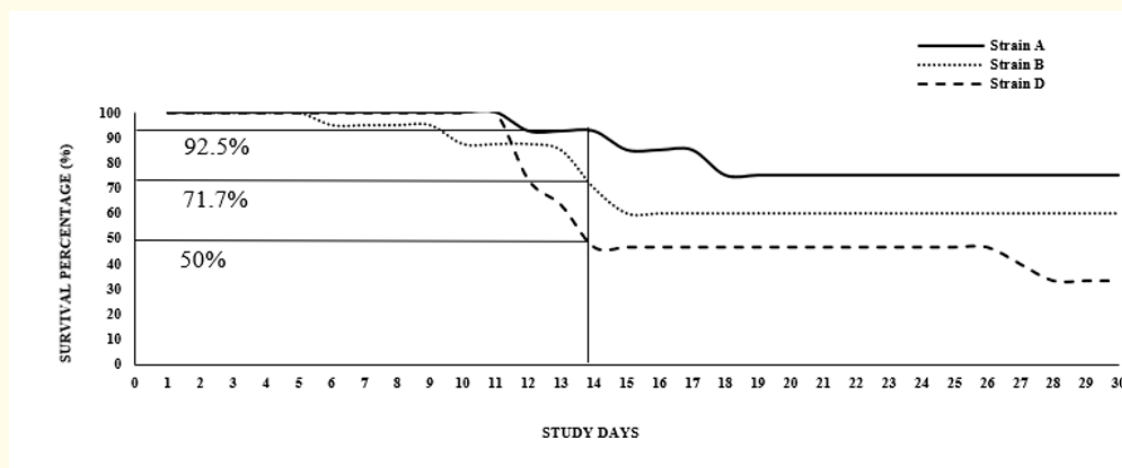
Legend: α: 10 mice/ group. β: The cumulative mortality is mice (survived/mice total\*100). Np: Not Processed.

**Table 1:** Virulence of the chronic strains of *Toxoplasma gondii* estimated based on the mortality rate at different inoculum dilutions and post-infection times.

despite having a relatively minor inoculum (354 Q/mL) with respect to strain B (578 Q/mL), strain D induced the 100% mortality of mice inoculated with the 1 x 10<sup>0</sup> dilution, being significantly different from strain A (p = 0.043), but not when compared to strain B (p = 0.105).

The survival curve Kaplan Meier (Figure 1) shows how only 50% of the mice infected with Strain D survived after 15 days post-inoculation compared to 71.7% of the mice inoculated with Strain B and 92.5% of mice inoculated with strain A, respectively. The OR analysis showed that there is a 38.8 times greater probability that a mouse infected with strain D will not survive (OR: 38.8; 95% CI: 13.86 - 106.3; p < 0.05) compared to strain A, and 2.67 times more likely that it will not survive (OR: 2.67, 95% CI: 1.09 - 6.5; p < 0.05) compared to strain B, after an oral inoculum between 35.4 and 354 Q/mL. No significant difference was found in the OR analysis between Strain A and Strain B (OR: 1.76; 95% CI: 0.34 - 5.08; p ≥ 0.05).

The average days of survival for each strain and inoculum dilution were estimated and differed in terms of the sex of the infected mice. In this regard, it was observed that females infected with strain A and Strain B with the undiluted inoculum (204 Q/mL and 578 Q/mL, respectively) had a significantly lower average days of survival than males (p = 0.009 and p = 0.008, respectively). For strain D, only a difference was observed in terms of days of survival between females and males for the dilution of the inoculum 1 x 10<sup>-1</sup> (35.4 Q/mL) with p = 0.015 (Figure 1 and 2).



**Figure 1:** Survival curve of mice infected with chronic strains. The parallel lines with the X axis represent the survival rate of mice infected with strain A and B, when only 50% of mice infected with strain D have survived post-infection. Inoculum: Strain A: 204 Q/mL, Strain B: 578 Q/mL; strain D: 354 Q/mL.

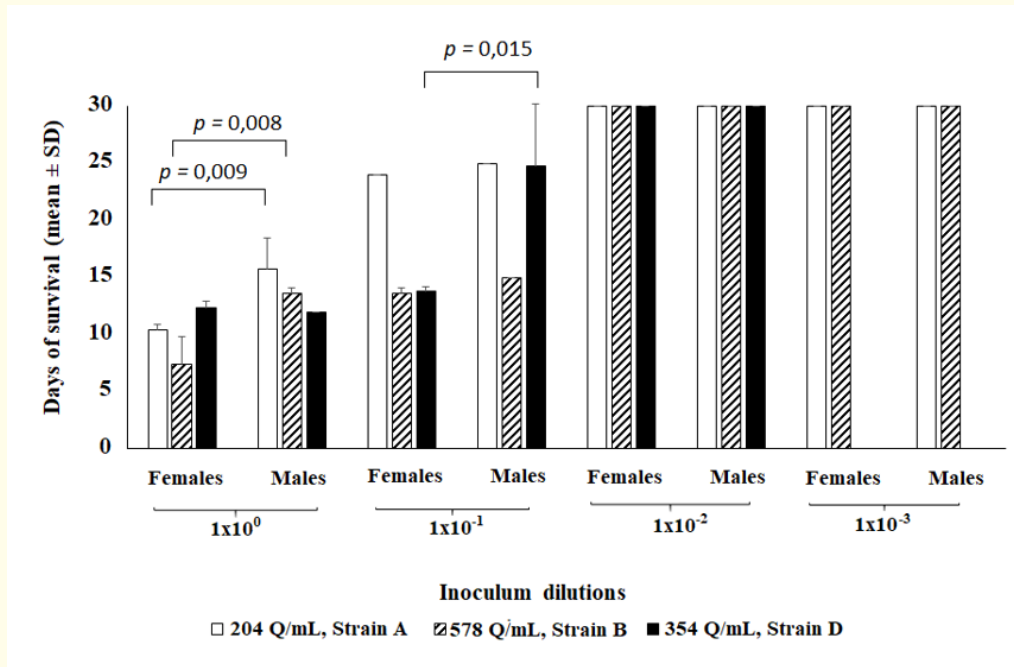


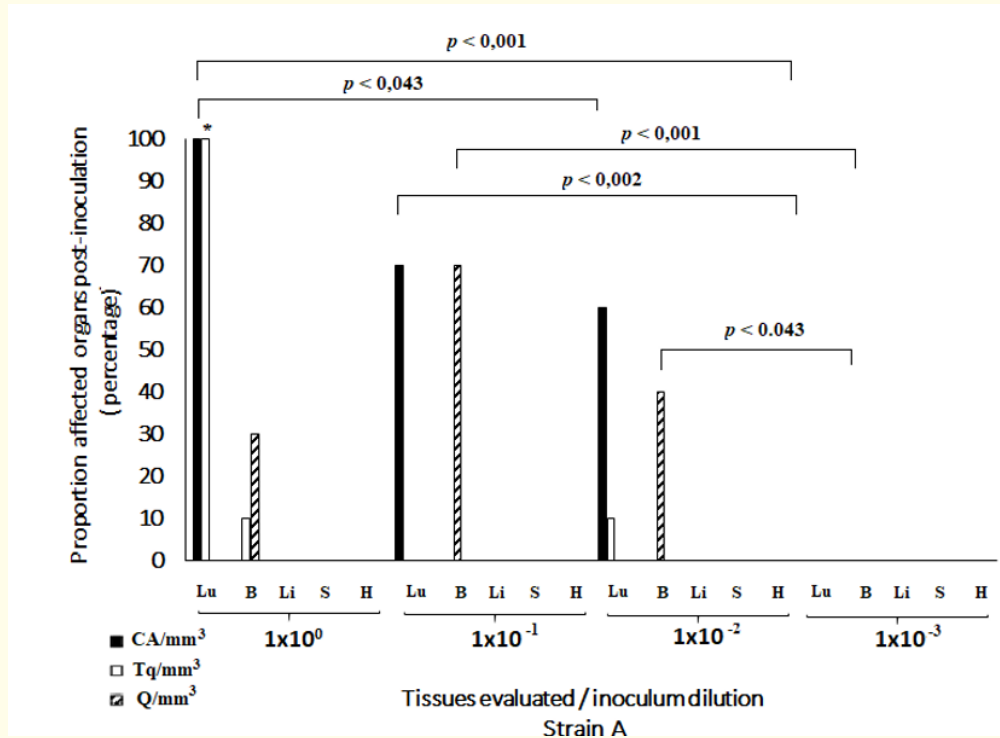
Figure 2: Days of survival of infected mice according to sex, strain and inoculum dilution.

## Histopathological study

### Proportion of affected tissues from each group evaluated with respect to strain and inoculum dilution

In the studio of the organs that were affected after infection with the different chronic strains of *T. gondii* was evaluated, it was observed that for strain A 100% of the lung sections of the mice inoculated with 204 Q/mL had CA, this proportion was significantly higher than that observed in the lungs of the mice inoculated with the dilution of the inoculum  $1 \times 10^{-2}$  (60%; 6/10) or  $1 \times 10^{-3}$  (0%; 0/10) with  $p < 0.043$  and  $p < 0.001$ , respectively. Likewise, the number of mice with CA in lungs was proportionally higher after infection with the diluted  $1 \times 10^{-1}$  inoculum on relation to the diluted  $1 \times 10^{-3}$  inoculum with  $p = 0.002$ . The proportion of the lungs of the mice with tachyzoites was 100%, after being infected with the undiluted inoculum and this was statistically significant ( $p < 0.001$ ; for all dilutions) when compared with the proportion of lungs with tachyzoites at dilutions at  $1 \times 10^{-1}$  (0%), and  $1 \times 10^{-2}$  (0%) and  $1 \times 10^{-3}$  (0%). When we evaluate the brain as an affected organ, it was found that only 10% (1/10) and 30% (3/10) of the brains of mice infected with the undiluted inoculum were affected with tachyzoites and cysts tissue, respectively. The highest proportion of brains with tissue cysts was found after infection with diluted  $1 \times 10^{-1}$  inoculum (70%; 7/10), followed by  $1 \times 10^{-2}$  dilution (40%; 4/10). No brains with cysts were found at the  $1 \times 10^{-3}$  dilution. A statistically significant difference was found when comparing the proportion of brains with tissue cysts between the  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$  dilutions with respect to the  $1 \times 10^{-3}$  dilution of the inoculum, with  $p < 0.001$  and  $p = 0.043$ , respectively (Figure 3).

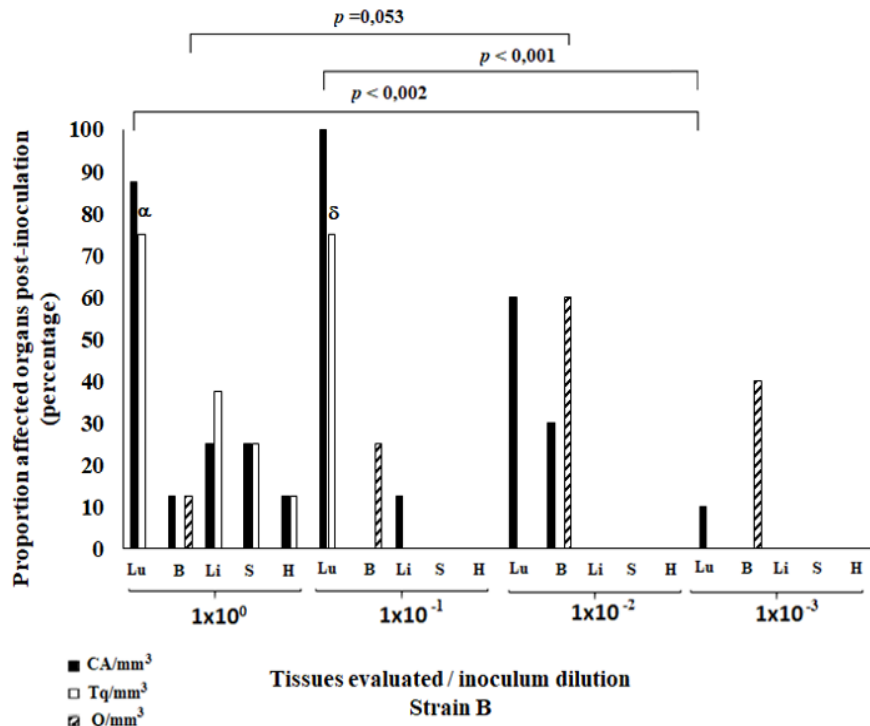
When the proportion of affected tissues of mice inoculated with strain B (578 Q/mL) was evaluated, it was observed that unlike strain A, all the tissues evaluated after the undiluted inoculum, were affected, being predominantly the lung (CA; 87.5% (7/8); Tq; 75% (6/8)), followed by the liver (CA: 25% (2/8)  $p = 0.019$ ; Tq; 37.5% (3/8)), Spleen (CA: 25% (2/8)  $p = 0.019$ ; Tq; 25% (2/8), heart (CA: 12.5% (1/8)  $p = 0.005$ ; Tq; 0% (0/8), brain (CA: 12.5 (1/8)  $p = 0.003$ ; Q; 12.5% (1/8) and heart (CA: 12.5 (1/8); Tq; 12.5% (1/8)  $p = 0.019$ ). The only organ that showed tissue cysts with the undiluted inoculum was the brain. When the proportion of affected organs was compared



**Figure 3:** Proportion of affected organs of mice (n = 10) infected with strain A, with respect to the dose of the inoculum and its dilutions. Strain A inoculum 204 Q/mL). CA/mm<sup>3</sup>: Consolidation areas; Tq/mm<sup>3</sup>: tachyzoites/mm<sup>3</sup>; Q/mm<sup>3</sup>: Cyst/mm<sup>3</sup>. Lu: lung; B: brain; Li: liver; S: spleen; H: heart. \*: statistical difference in the proportion of tissues with Tq/mm<sup>3</sup> (p < 0.001) between 1 x 10<sup>0</sup> and the dilutions of the inoculum 1 x 10<sup>-1</sup> and 1 x 10<sup>-2</sup> and 1 x 10<sup>-3</sup>.

between the different dilutions of the inoculum, the proportion of lungs with affected with CA remained at the 1 x 10<sup>-1</sup> dilution (100% (8/8) but decreased as the dilutions of the inoculum increased. The proportion of affected lungs being significantly lower and between 1 x 10<sup>-0</sup> and 1 x 10<sup>-2</sup> (10% (1/10); p < 0.002) and between 1 x 10<sup>-1</sup> and 1 x 10<sup>-3</sup> (10% (1/10); p < 0.001). Regarding the presence of tachyzoites, the proportion of lungs with this life form was significantly higher in the undiluted inoculum with respect to that observed at the 1 x 10<sup>-2</sup> (0%; 0/10) and 1 x 10<sup>-3</sup> (0%; 0/10) with p < 0.002 and p < 0.002, respectively and between the 1 x 10<sup>-1</sup> dilution (75%; 6/8) and the diluted 1x10<sup>-2</sup> and 1x10<sup>-3</sup> inoculum with p < 0.002 and p < 0.002, respectively. The proportion of tissues with *T. gondii* tissue cysts, were only observed in the brain, increasing progressively at the dilution of 1 x 10<sup>-1</sup> (25%; 2/8) and 1 x 10<sup>-2</sup> (60%; 6/10) and decreasing at the 1 x 10<sup>-3</sup> dilution (40%; 4/10). A significant trend was only found between the proportion of brains affected with tissue cysts between the 1 x 10<sup>-0</sup> and 1 x 10<sup>-2</sup> dilutions with p = 0.053 (Figure 4).

When the proportion of affected tissues of mice inoculated with strain D (352 Q/mL) was evaluated, it was observed that similarly to strain A, after the undiluted inoculum 100% (2/2) of the lungs evaluated had CA and 50% (1/2) presence of tachyzoites. It should be noted that for this strain, only 2 mice from the group of animals could be evaluated with the undiluted inoculum, since 80% (8/10) did not survive prior to euthanasia. Also highlighted is the fact that in this group of mice one (1) of them had *T. gondii* cysts in the heart and not in the brain. The proportion of lungs with CA was significantly lower in mice with dilutions of the inoculum at 1 x 10<sup>-2</sup> (88.9%; 8/9)

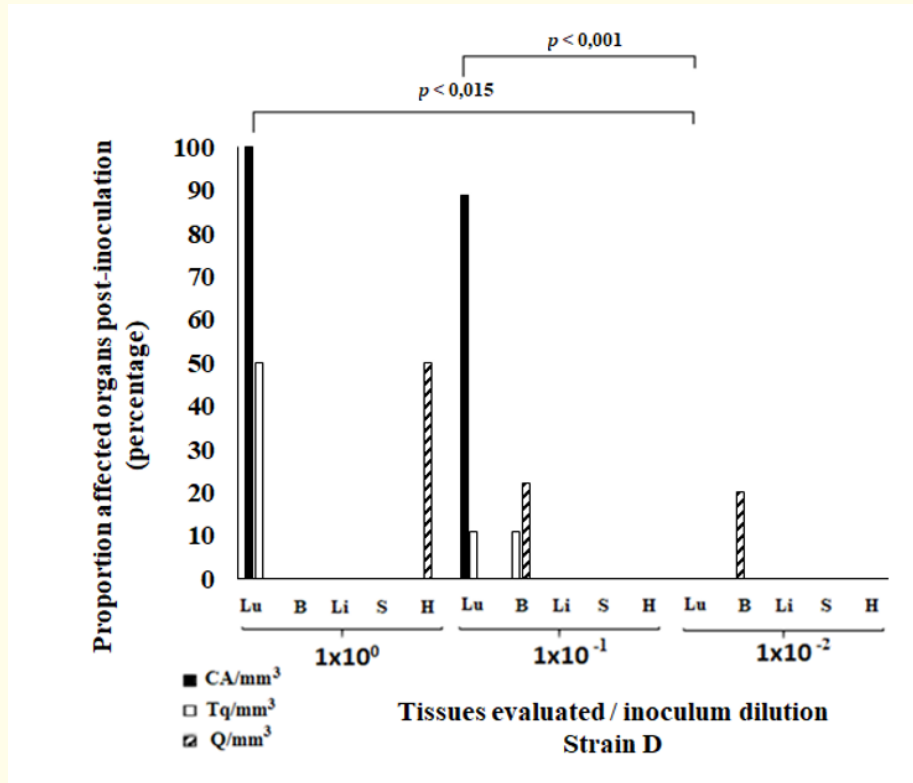


**Figure 4:** Proportion of affected organs of mice ( $n = 10$ ) infected with strain B, with respect to the dose of the inoculum and its dilutions. Strain B inoculum 578 Q/mL). CA/mm<sup>3</sup>: Consolidation areas; Tq/mm<sup>3</sup>: tachyzoites/mm<sup>3</sup>; Q/mm<sup>3</sup>: Cyst/mm<sup>3</sup>. Lu: lung; B: brain; Li: liver; S: spleen; H: heart.  $\alpha$ : Statistical difference in the proportion of tissues with Tq/mm<sup>3</sup> ( $p < 0.002$ ) between  $1 \times 10^0$  and  $1 \times 10^{-3}$  dilutions.  $\delta$ : statistical difference in the proportion of tissues with Tq/mm<sup>3</sup> ( $p < 0.002$ ) and between  $1 \times 10^{-1}$  and  $1 \times 10^{-3}$  dilutions.

compared to  $1 \times 10^0$  with  $p = 0.015$  and proportionally between  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$  (0%; 0/10) with  $p < 0.001$ . The proportion of tissues with *T. gondii* tissue cysts was observed in 22% (2/9) and 20% (2/10) of the brains of infected mice with the dilutions of the inoculum at  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$ , respectively, without statistically significant differences being observed (Figure 5).

#### Quantification of consolidation areas (CA), tachyzoites (Tq) or cysts (Q) in the tissues of mice inoculated with the chronic strains

The intensity of infection of the different strains was studied by analyzing the number of areas of consolidation (CA), Tq or Q per tissue volume (mm<sup>3</sup>). For strain A (inoculum 204 Q/mL), the count was only performed in the affected tissues such as the lung and brain. It was observed that CA/mm<sup>3</sup> in the lung was greater in mice infected with undiluted inoculum ( $1 \times 10^0$ ) with respect to the  $1 \times 10^{-1}$  dilutions ( $p = 0.027$ ),  $1 \times 10^{-2}$  ( $p = 0.027$ ) and  $1 \times 10^{-3}$  ( $p = 0.008$ ) (Table 2). A higher Tq/mm<sup>3</sup> count was found in the lungs of mice infected with the undiluted inoculum that decreased significantly after inoculating mice with inoculum dilutions of  $1 \times 10^{-1}$ ,  $1 \times 10^{-2}$ ,  $1 \times 10^{-3}$  with  $p < 0.001$ , for all comparisons (Table 2). The Q were observed only in the brain both in the undiluted inoculum ( $1 \times 10^0$ ) and to dilutions  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$  of the inoculum. The number of cysts observed with the  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$  inoculum dilutions was significantly greater than the cysts observed in the brain of the mice inoculated with the  $1 \times 10^{-3}$  dilution ( $p = 0.023$  and  $p = 0.040$ , respectively, Table 2).



**Figure 5:** Proportion of affected organs of mice ( $n = 10$ ) infected with strain D, with respect to the dose of the inoculum and its dilutions. Strain D inoculum (9354 Q/mL). CA/mm<sup>3</sup>: Consolidation areas; Tq/mm<sup>3</sup>: tachyzoites/mm<sup>3</sup>; Q/mm<sup>3</sup>: Cyst/mm<sup>3</sup>.

Lu: lung; B: brain; Li: liver; S: spleen; H: heart.

CA/mm<sup>3</sup> and Tq/mm<sup>3</sup> predominated in the lung in the absence of tissue cysts (Q/mm<sup>3</sup>), both at the dose of the undiluted inoculum and at the dilutions  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$ . Regarding Q/mm<sup>3</sup>, they were only observed in the brain, independent of inoculum concentration but predominated over CA/mm<sup>3</sup> at the dilutions  $1 \times 10^{-1}$  and  $1 \times 10^{-2}$ . No CA, Tq or Q were found in lung or brain at  $1 \times 10^{-3}$  (Table 2).

For strain B (inoculum 578 Q/mL), CA/mm<sup>3</sup> was found in all evaluated tissues of mice infected with the undiluted inoculum, and although it was not statistically significant, a higher amount of CA/mm<sup>3</sup> was observed in the lung, followed by spleen and heart and, to a lesser extent in liver and brain (Table 3). The amount of CA/mm<sup>3</sup> observed in the lung tissue sections of infected mice with  $1 \times 10^{-1}$  dilution was significantly higher than that observed in the lung sections of infected mice infected with the undiluted inoculum ( $p = 0.007$ ), and with the inoculum dilutions at  $1 \times 10^{-2}$  ( $p = 0.006$ ) and  $1 \times 10^{-3}$  ( $p < 0.001$ ) (Table 3). Unlike strain A, CA/mm<sup>3</sup> was observed at the  $1 \times 10^{-1}$  dilution in the liver.

On relation to the Tq/mm<sup>3</sup>, the highest amount was observed in the lung of the mice infected with the undiluted inoculum, followed by the  $1 \times 10^{-1}$  dilution. Both results were higher than those observed in mice infected with dilutions of inoculum at  $1 \times 10^{-2}$  and  $1 \times 10^{-3}$  ( $p = 0.022$  and  $p = 0.015$ , respectively, Table 3). Tq/mm<sup>3</sup> were present a lesser extent in liver, heart and spleen tissues and only in mice infected with the undiluted inoculum.



Tissue revised	Inoculum Dilution	No. of mice per group	Strain A						Statistical significance		
			CA/mm <sup>3</sup>		Tq/mm <sup>3</sup>		Q/mm <sup>3</sup>		CA vs Tq	CA vs Q	Tq vs Q
			Average	SEM	Average	SEM	Average	SEM			
Lung	1 x 10 <sup>0</sup>	10	6040 <sup>αβδ</sup>	1560	980 <sup>αβδ</sup>	109	0	0	0.010	0.004	0.001
Brain			0	0	11	11	65	46	0.343	0.198	0.285
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1
Lung	1 x 10 <sup>-1</sup>	10	1839 <sup>ελ</sup>	515	0	0	0	0	0.006	0.006	1
Brain			0	0	0	0	184 <sup>λ</sup>	67	1	0.024	0.024
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1
Lung	1 x 10 <sup>-2</sup>	10	712	263	82	82	0	0	0.043	0.024	0.348
Brain			0	0	0	0	178	75	1	0.041	0.041
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1
Lung	1 x 10 <sup>-3</sup>	10	0	0	0	0	0	0	1	1	1
Brain			0	0	0	0	0	0	1	1	1
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1

**Table 2:** Consolidation areas (CA), tachyzoites (Tq) or cysts (Q) presence in the FFIP tissue of mice infected with the chronic strain A of *T. gondii*.

Legend: The Greek letters indicate the presence of statistical difference ( $p < 0.05$ ) between the comparisons of CA/mm<sup>3</sup>, Tq/mm<sup>3</sup> or Q/mm<sup>3</sup> between the same FFIP tissues between the different dilutions of the inoculum.  $\alpha$ : 1 x 10<sup>0</sup> vs 1 x 10<sup>-1</sup>;  $\beta$ : 1 x 10<sup>0</sup> vs 1 x 10<sup>-2</sup>;  $\delta$ : 1 x 10<sup>0</sup> vs 1 x 10<sup>-3</sup>;  $\epsilon$ : 1 x 10<sup>-1</sup> vs 1 x 10<sup>-2</sup>;  $\lambda$ : 1 x 10<sup>-1</sup> vs 1 x 10<sup>-3</sup>;  $\gamma$ : 1 x 10<sup>-2</sup> vs 1 x 10<sup>-3</sup>. SEM: standard error of the mean. Statistical analysis: Test T.

Tissue cysts were only observed in brain from infected mice. Mice inoculated with the 1 x 10<sup>-2</sup> dilution had significantly more Q/mm<sup>3</sup> than brain from mice inoculated with 1 x 10<sup>0</sup> ( $p = 0.033$ ) and 1 x 10<sup>-1</sup> ( $p = 0.038$ ). In contrast to strain A, brain cysts in strain B were observed in infected mice with a 1 x 10<sup>-3</sup> dilution of the inoculum and their count was higher ( $p = 0.033$ ) than observed after infecting the mice with the undiluted inoculum (Table 3).

Similar to strain A, the CA/mm<sup>3</sup> and Tq/mm<sup>3</sup> in strain B predominated significantly in the lung of infected mice up to the 1 x 10<sup>-2</sup> dilution of the inoculum, Exceeding the CA/mm<sup>3</sup> count over the Tq/mm<sup>3</sup> count. Q/mm<sup>3</sup> were only in the brain and predominated in the 1 x 10<sup>-2</sup> dilution of the inoculum (Table 3).

For strain D (354 Q/mL inoculum), the CA/mm<sup>3</sup> were observed in lung of mice infected with diluted 1x10<sup>-1</sup> inoculum and were greater than of mice infected with the undiluted inoculum. CA/mm<sup>3</sup> were not observed in the lung of mice inoculated with 1 x 10<sup>-2</sup> dilu-

Tissue revised	Inoculum Dilution	No. of mice per group	Strain B						Statistical significance		
			CA/mm <sup>3</sup>		Tq/mm <sup>3</sup>		Q/mm <sup>3</sup>		CA vs Tq	CA vs Q	Tq vs Q
			Average	SEM	Average	SEM	Average	SEM			
Lung	1 x 10 <sup>0</sup>	8	3136 <sup>α</sup>	900	222 <sup>βδ</sup>	76	0 <sup>βδ</sup>	0	0.014	0.010	0.022
Brain			244	244	0	0	13	13	0.352	0.376	0.386
Liver			326	214	37	19	0	0	0.219	0.171	0.098
Spleen			1822	1810	42	36	0	0	0.358	0.348	0.286
Heart			1370	1371	42	41	0	0	0.365	0.351	0.351
Lung	1 x 10 <sup>-1</sup>	8	9960 <sup>ελ</sup>	1796	150 <sup>ελ</sup>	47	0	0	0.001	0.001	0.015
Brain			0	0	0	0	33 <sup>ε</sup>	23	1	0.198	0.198
Liver			28	28	0	0	0	0	0.351	0.351	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1
Lung	1 x 10 <sup>-2</sup>	10	2795 <sup>γ</sup>	1200	0	0	0	0	0.045	0.045	1
Brain			855	439	0	0	529	204	0.083	0.512	0.030
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1
Lung	1 x 10 <sup>-3</sup>	10	54	54	0	0	0	0	0.343	0.352	1
Brain			0	0	0	0	317	161	1	0.080	0.080
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1

**Table 3:** Consolidation areas (CA), tachyzoites (Tq) or cysts (Q) in the FFIP tissue of mice infected with the chronic strain B of *T. gondii*. Legend: the Greek letters indicate the presence of statistical difference ( $p < 0.05$ ) between the comparisons of CA/mm<sup>3</sup>, Tq/mm<sup>3</sup> or Q/mm<sup>3</sup> between the same FFIP tissues between the different dilutions of the inoculum.  $\alpha$ : 1 x 10<sup>0</sup> vs 1 x 10<sup>-1</sup>;  $\beta$ : 1 x 10<sup>0</sup> vs 1 x 10<sup>-2</sup>;  $\delta$ : 1 x 10<sup>0</sup> vs 1 x 10<sup>-3</sup>;  $\epsilon$ : 1 x 10<sup>-1</sup> vs 1 x 10<sup>-2</sup>;  $\lambda$ : 1 x 10<sup>-1</sup> vs 1 x 10<sup>-3</sup>;  $\gamma$ : 1 x 10<sup>-2</sup> vs 1 x 10<sup>-3</sup>. SEM: standard error of the mean. Statistical analysis: Test T.

tions of the inoculum (Table 4). Tq/mm<sup>3</sup> were only observed in the lung, higher in mice infected with the undiluted inoculum than in mice inoculated with 1 x 10<sup>-1</sup> dilution (Table 3). Similar to strain B, the count highest Q/mm<sup>3</sup> were on the brain at the mouse infected with the dilution 1 x 10<sup>-2</sup> relative to the 1 x 10<sup>-1</sup> dilution. Regardless of CA/mm<sup>3</sup>, Tq/mm<sup>3</sup> or Q/mm<sup>3</sup>, the observed differences were not statistically significant (Table 4).

Unlike strain A and B, in strain D no cysts were observed in the brain of mice infected with the undiluted inoculum. Only in this strain, a mouse infected with the undiluted inoculum had cysts in the heart, finding no statistical differences from the same tissue in other dilutions of the inoculum (Table 4). A difference with strain A and comparable to strain B, CA/mm<sup>3</sup> and Tq/mm<sup>3</sup> was predominated in the lung in infected mice up to the 1 x 10<sup>-1</sup> dilution of the inoculum, significantly exceeding the count of CA/mm<sup>3</sup> over Tq/mm<sup>3</sup>. Q/mm<sup>3</sup> were predominant in the brain of mice infected with the 1 x 10<sup>-2</sup> dilution of the inoculum (Table 4).

The three chronic *T. gondii* strains were compared as a function of CA/mm<sup>3</sup> and Tq/mm<sup>3</sup> in lung and Q/mm<sup>3</sup> in brain at different dilutions of the infecting inoculum. Regarding CA/mm<sup>3</sup>, mice were infected with strain A, with the undiluted inoculum had a higher count

Tissue revised	Inoculum Dilution	No. of mice per group	Strain D						Statistical significance (p)		
			CA/mm <sup>3</sup>		Tq/mm <sup>3</sup>		Q/mm <sup>3</sup>		CA vs Tq	CA vs Q	Tq vs Q
			Average	SEM	Average	SEM	Average	SEM			
Lung	1 x 10 <sup>0</sup>	2	1527	420	69	69	0	0	0,171	0,171	0,5
Brain			0	0	0	0	0	0	1	1	1
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	130	130	1	1	1
Lung	1 x 10 <sup>-1</sup>	9	1905	652	23	23	0	0	0,020	0,019	0,347
Brain			0	0	12	12	64	52	0,347	0,255	0,357
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1
Lung	1 x 10 <sup>-2</sup>	10	0	0	0	0	0	0	1	1	1
Brain			0	0	0	0	144	104	1	0,199	0,199
Liver			0	0	0	0	0	0	1	1	1
Spleen			0	0	0	0	0	0	1	1	1
Heart			0	0	0	0	0	0	1	1	1

**Table 4:** Consolidation areas (CA), tachyzoites (Tq) or cysts (Q) in the FFIP tissue of mice infected with the chronic strain B of *T. gondii*. Legend: SEM: Standard Error of the Mean. Statistical analysis: Test T.

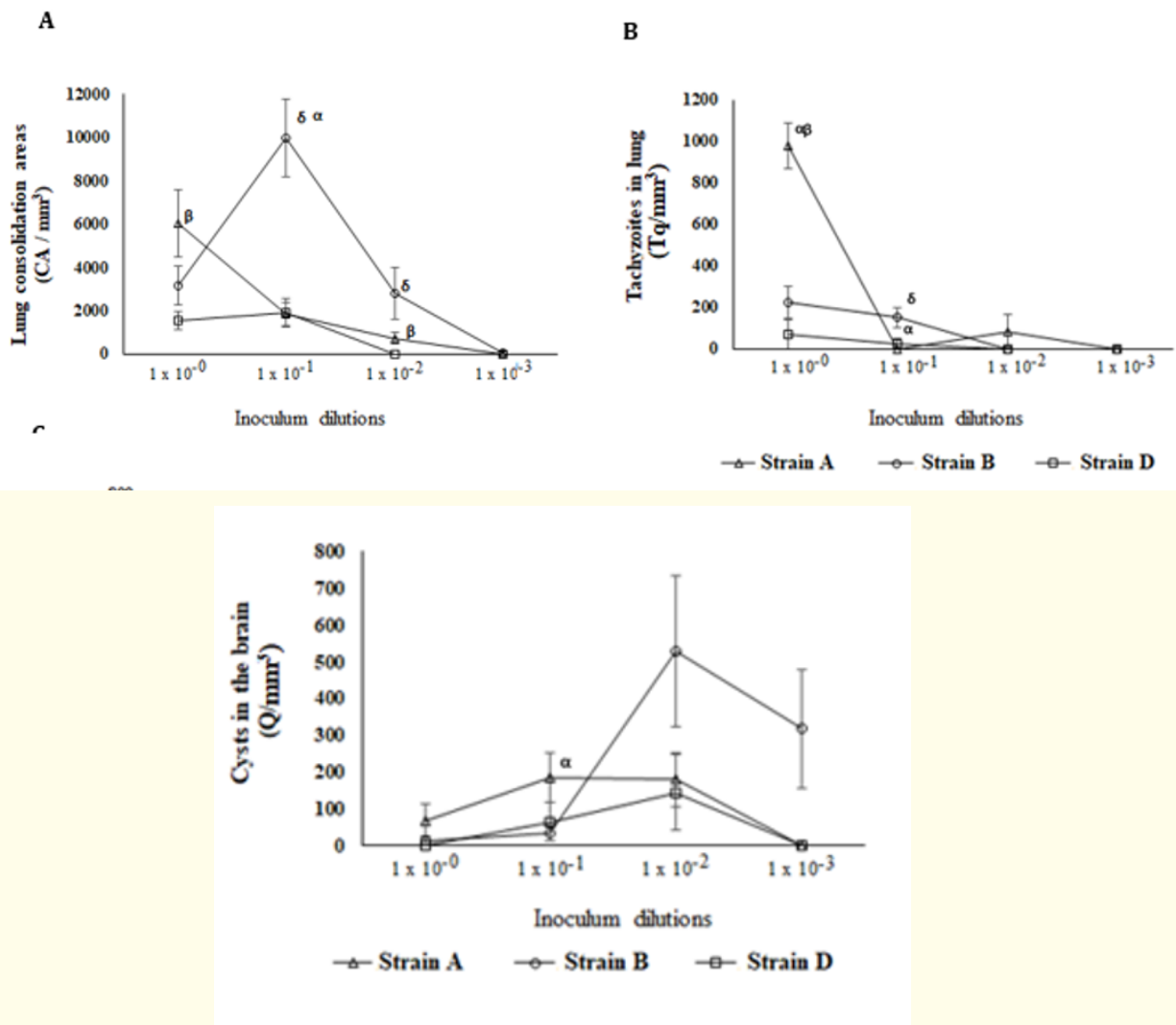
than strain D ( $p = 0.019$ ), but similar to that of strain B ( $p = 0.129$ ). By diluting inoculum  $1 \times 10^{-1}$ , a higher CA/mm<sup>3</sup> count was observed in strain B, relative to strain A ( $p = 0.002$ ) and strain D ( $p = 0.002$ ). When mice were infected with inoculum diluted to  $1 \times 10^{-2}$ , similar CA/mm<sup>3</sup> counts were observed between strain A and B ( $p = 121$ ), but in turn higher than those observed in strain D ( $p = 0.024$  (A vs D) and  $p = 0.045$  (B vs D), (Figure 6A). No statistical difference was found in the CA/mm<sup>3</sup> count at the  $1 \times 10^{-3}$  dilution of the inoculum between strain A and B.

On relation to the Tq/mm<sup>3</sup>, higher amount was observed, at the infection dose of the undiluted inoculum in strain A compared to strain B ( $p < 0.001$ ) and strain D ( $p < 0.001$ ). At the  $1 \times 10^{-1}$  dilution of the inocula, the Tq/mm<sup>3</sup> count was higher in strain B with respect to strain A ( $p = 0.015$ ) and strain D ( $p = 0.036$ ). No statistical differences were found in the Tq/mm<sup>3</sup> count at the  $1 \times 10^{-3}$  dilution between strain A and B (Figure 6B).

When comparing the count of Cysts in the brain, the count was higher in strain A with respect to strains B and D, at the infection dose of the undiluted inoculum or diluted  $1 \times 10^{-1}$ , being only statistically different when relating strain A and B ( $p = 0.028$ ; for a tail). For dilution of the inoculum  $1 \times 10^{-2}$  and  $1 \times 10^{-3}$  and although more cysts count was observed between strain B and strains A and D, this was not statistically different (Figure 6C).

## Discussion

In this study the characterization of the virulence of three chronic strains of *T. gondii* was performed according to survival, histopathology detection studies. The strains used in this study had previously been characterized molecularly using PCR-RFLP [22].



**Figure 6:** Comparison of chronic *T. gondii* strains, based on consolidation areas (CA/mm<sup>3</sup>) and tachyzoites (Tq/mm<sup>3</sup>) counts in lung and brain FFIP tissue cysts in the (Q/mm<sup>3</sup>) at different dilutions of infecting inoculates. A. Consolidation areas in the lung. B. Tachyzoites in the lung. C. Cystis in the brain. The Greek letters indicate the presence of statistical difference ( $p < 0.05$ ) between the comparisons the different strains  $\alpha$ : differences between strain A and strain B;  $\beta$ : differences between strain A and strain D;  $\delta$ : differences between strain B and strain D. Statistical analysis: Test T.

Regarding to virulence of the studied strains, the immunosuppressive effect of *Trypanosoma musculi* infection on *Mus musculus* mice (Swiss strain) in experimental toxoplasmosis had already been investigated. Piccolo-Johanning, *et al.* [26] demonstrated that the chronic *T. gondii* strains whose virulence was evaluated in this study, could induce the mortality of mice in less than 30 days. In addition, in the absence of immunosuppression with *T. musculi*, mice infected with strain A (TFC-1) and D (TBT-1) showed similar amounts of tissue cysts

in the brain, but after immunosuppression in strain D (TBT-1) more brain cysts in the infected mice. This previous study demonstrated different biological behavior, mostly related to the differential genotype reported by Vethencourt., *et al* [22]. The present study confirms the differential behavior of the three chronic strains in terms of mortality, since the probability of surviving was higher in mice infected with by strain A, followed by strain B and Strain D.

Morbidity rates, different degrees of histopathological lesions, the presence of *T. gondii* DNA and tissue cysts are associated with virulence in mouse models [27-30], demonstrated that the index invasion-replication could be inversely correlated with virulence and morbidity in mice. The RH strain and the atypical isolates present lower invasion index values that are associated with greater virulence. In the present study, strain D, characterized by its deficiency in the production of cysts, would have greater virulence, which was demonstrated. On the other hand, it was confirmed that strain A, less virulent was the one that manifested a greater number of cysts in the brain with the least diluted inoculum, despite the manifested very high tissue inflammatory response in the lung, determined by a greater proportion of tissues affected and by the number of CAs found per group of mice evaluated.

One possible explanation for these differences in virulence would be that the mice responded differently immunologically to infection with the chronic *T. gondii* strains tested in this study, which has been confirmed in other studies both *in vivo* and *in vitro* [31-33]. On the other hand, it has been reported for the non-lethal clonal strains, that the multiplication of tachyzoites was controlled by the host's immune system that finish the process in the formation of cysts with bradyzoites inside the CNS. [21,27]. The investigation of the immune response *in vivo* and *in vitro*, in response to infection with the chronic strains, studied in this work, would be the subject of future research.

In this study, higher mortality was observed in female mice compared to males infected with any of the analyzed chronic strains, at the most concentrated infectious doses. This result was similar to those reported more than two decades ago by Roberts., *et al.* [34], who examined a wide range of inbred mice of various genetic backgrounds and complex histocompatibility haplotypes. Their results revealed a dramatic difference in susceptibility between male and female and female mice to *T. gondii* infection. The difference in susceptibility in female mice was attributed to the differential response of cytokines, relative to males, after infection by *T. gondii* with the same chronic strain [35,36]. In this study, neither the immunological response to infection with the chronic strains nor their discrimination between sexes of the mice was made, which would be a subject for future research.

In our study it was demonstrated the predominance of CA and Tq in the lung at dose of the undiluted inoculum, being greater in the strain came from the domestic cat (*Felis catus*, TFC-1; A). In addition, when the inoculum is diluted, the formation of tissue cysts in the brain are presents and this was dose dependent, achieving a maximum peak of cysts at a certain dilution of the inoculum. In this study, the acute response in the lung and the presence of cysts in the brain were similar to those reported in the kinetic experiments [37-41]. Kinetic studies have shown that infection with a virulent strain of *T. gondii* is characterized by early involvement of the lungs, with pneumonia as the leading cause of death, as tachyzoites predominate in the lung early in the infection [37,38,41], and late infection, both tachyzoites and bradyzoites are constantly present in the brain [39,40].

Similar to the results of other authors, and considering that the responsiveness of the mice in this experiment is the same, the difference lies in the genotype of the chronic strain of *T. gondii* and in the dose of the inoculum in the other. Probably for this reason, we detected an acute response at the lung level, lower mortality (200 Q/ml, 100 Q/infectious dose) and a higher generation of cysts (20 Q/ml; 10 Q/infectious dose) in strain A. And the other hand, the D strain despite less amounts of cysts in the brain and the most virulent after infection with approximately 300 Q/mL (150 Q/infective dose), both strains were molecularly characterized as variants different of genotype type I [22]. For strain B, less virulent than D strain, the highest number of cysts in the brain was obtained with a higher dilution of the inoculum (6 Q/mL; 3 Q/infective dose) but was molecularly characterized as a variant of type III genotype [22].

On the other hand, since we have standardized the techniques for the extraction and molecular detection of *T. gondii* DNA [22,41]. A next step in this investigation will be the molecular detection of *T. gondii* in these FFIP tissues from mice infected with the chronic strains of *T. gondii*, and if this methodology can delve into the differences between the chronic strains analyzed histologically in this study.

### **Conclusion**

In this work we can conclude that strain A, less virulent (characterized as a type I variant, different from strain D), was the one that manifested a greater number of cysts in the brain at the lowest dilution of the inoculum, despite the fact of having manifested a very high tissue inflammatory response in the lung, observed both by a higher proportion and by the number of AC present in this tissue. The highest mortality was observed in female mice compared to males infected with any of the analyzed chronic strains, at the most concentrated infectious doses.

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### **Conflict of Interest**

We declare that there is no financial interest or conflict of interest.

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