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Molecular Detection of *Toxoplasma gondii* Cyst Formation in Fresh or Paraffin-Embedded Tissues of Three Chronic Strains

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Abstract

The purpose of this study was to compare molecular detection of *T. gondii* cyst formation in fresh or paraffin-embedded tissues of three chronic strains. Molecular detection was carried out using a nested PCR that detects the *B1* gene of *T. gondii*. Parasite DNA was extracted from fresh tissue and formalin-fixed and paraffin-embedded (FFEP) tissues and from CD1 strain mice (Swiss) infected with three chronic strains. Ten (10) mice (5 female and 5 male) were orally infected with serial dilutions of brain tissue cysts from previously infected mice. The chronic strains of *T. gondii* came from a domestic cat (*Felis catus,* TFC-1; A), from *Leopardus wiedii* (TLW-1; B) and from white mice fed with *Bos taurus* beef (TBT-1; D). The FFEP tissues of 10 mice that survived after oral infection with different dilutions of tissue cysts, were used respectively. The presence of tachyzoites (Tq) or tissue cysts (Q) was estimated per mm^3 of tissue analyzed. Three 10 um sections of the FFEP tissues were used for DNA extraction with two manual protocols (salt and ammonium acetate) and with a commercial one, using silica columns. DNA was detected with the nested PCR that amplified the *T. gondii B1* gene, after its standardization. In this work, it was found that standardized nested PCR could amplify at least 0.1 fg/µL of DNA. Only with the commercial methodology was it possible to detect the parasite DNA, after applying a nested PCR for the *T. gondii B1* gene, although the quality of the DNA after extraction was similar with the three extraction protocols. In unfixed tissues, the highest molecular detection of the parasite occurred in the brain and skeletal muscle of mice infected with chronic strains A and B. The molecular detection in FFEP tissues follows, like the kinetic tests, a maximum detection curve that depends on the inoculum. A higher molecular detection was observed in the tissues at lower doses of the inoculum, which was related to a higher reading of cysts per mm^3 of tissue and with a lower virulence, previously studied. It is concluded that the molecular detection of the *T. gondii B1* gene, with nested PCR of FFEP tissues, was 10 times more sensitive than the histological detection of cysts by microscopy. In addition, the molecular and histological detection of cysts of a chronic strain of *T. gondii* were inversely proportional to the virulence of the previously characterized strain.

Keywords: *Toxoplasma gondii; Chronic Strains; Molecular Detection; FFEP; Cyst Formation; Costa Rica*

Abbreviations

PCR: Polymerase Chain Reaction; *T. gondii: Toxoplasma gondii*; FFEP: Formalin-Fixed and Paraffin-Embedded; i.p.: Intraperitoneal Inoculation

Introduction

Toxoplasmosis, caused by the organism, *Toxoplasma gondii,* Apicomplexa [1] is an infection for human and animals that produces abortion and other symptoms; this generates important economics losses and public health problems [2,3]. In human beings, this disease is usually present as a subclinic infection, but in special low immune conditions, ocular lesions, encephalitis besides abortion, can be observed [1,4]. In addition, 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].

T. gondii strains 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) [9]. In experimental animals, the virulence in this parasitosis depends on the strain characteristics [10]; Type I lineages are uniformly lethal (LD) 100 = 1, contrary to types II and III lineages, which are significantly less virulent (LD 50 ≥ 1000) [5].

Certain low virulence strains of Type II and III of *T. gondii* show a predisposition to encystment *in vitro* under cell culture conditions [11]. These strains has been used in the study of the differentiation process from tachyzoites to bradyzoites and in the process of cell encystment or cystogenesis. Considering the importance of these processes in the pathogenesis of the disease, different methods have been developed to induce the differentiation of tachyzoites to bradyzoites and tissue cysts in infected cell cultures [12]. *In vitro* studies have shown that immunological factors are not always necessary for the induction of tissue cyst formation [13,14]. So far, stress factors that induce the development of bradyzoites in infected cells have been studied, such as alkaline pH, IFN-gamma and other pro-inflammatory cytokines, high temperature, drugs, and nutrient depletion [15]. Certainly, the conversion of tachyzoites into bradyzoites plays a fundamental role in the maintenance of *T. gondii* in nature, especially with the transmission of the parasite through carnivorism between intermediate hosts [16].

In vivo encystment of *T. gondii* in mice has been evaluated after infection with different strains of *T. gondii* isolated from animals and man [17] and in the absence of molecular characterization, the strains can be divided into three groups according to their virulence and encysting capacity in brain tissue. A first group is made up of strains that present high virulence, similar to the RH strain (AS28, BV and N strains), with high mortality and the absence of brain cyst production. A second group of strains that, despite being virulent for mice, killing 100% of the animals; brain cysts are observed in mice that died after infection, but the time to death is greater than that observed for mice inoculated with the strains of the first group. The EGS, RAR, and SAF strains isolated from cases of human congenital toxoplasmosis formed the second group of strains observed in this study. And a third group of strains an avirulent behavior (ME49 strain), whose tachyzoites inoculated in mice led to the development of brain cysts without killing the mice, even with the inoculum of 10^3 tachyzoites [17]. Some of these strains located in the virulence and encysting capacity groups have been molecularly characterized as genotype I (Rh strain), with genotype I/III (EGS strain) [18] and genotype II (ME49 strain) [19] or genotype III (strain NED) [20,21], for groups 1, 2 and 3, respectively.

Regarding to the obtention of DNA from formalin-fixed and paraffin-embedded (FFEP) tissue samples, a large number of studies have been carried out evaluating the methodology for DNA extraction, and the ultimate goal of which is to achieve good quality DNA (pure and less degraded), that allows to apply Molecular Biology techniques, and detect genes of interest [22,23] or non-coding DNA sequences [24-26]. The molecular detection of genes in FFEP tissues has been a useful tool for the diagnosis of genes related to carcinoma presence [22,23]. For pathogenic protozoa, molecular detection in FFEP tissues has been carried out to *Trypanosoma cruzi* [24], *Leishmania* sp. [26], *Entamoeba histolytica* [27], *Naegleria fowleri* [28] and in *T. gondii*, the molecular detection of this parasite has been carried out in FFEP tissues of the placenta of women with recurrent abortions [29]. In our Basic Research Laboratory of the UCIMED of Costa Rica (LIB-UCIMED), we obtained *T. gondii* DNA from FFEP tissue samples of CD-1 Swiss White mice infected with a chronic strain of *T. gondii*, which served to its molecular characterization, by PCR-RFLP [30]. Among the methods used to extract DNA from FFEP tissues, are manual

methods where extraction with high concentrations of salts such as NaCl [31] or ammonium acetate are used [32] to promote protein precipitation. The use of phenol-chloroform as a deproteinizer [33] or the use of commercial kits in which detergents, chaotropic salts and silica columns are used to favor the purification of the extracted DNA, previous to the deparaffinization process which can use different reagents such as Xylol [34,35] or mineral oil [36]. Regardless of the methodology used to extract DNA from FFEP, tissue fixation, paraffin embedding, and storage contribute to the fragmentation, cross-linking, and chemical modification of tissue-derived nucleic acids, which can interfere with classical molecular testing requiring high quality of extracted nucleic acids [35]. Therefore, the standardization of the extraction and the measurement of the yield and quality of the extracted DNA [37], is a necessary process in any research work with FFEP tissue samples that uses molecular biology techniques to detect or characterize the DNA present in the tissue [34,35,37].

In our laboratory, three chronic strains of *T. gondii* have been molecularly characterized, two for genotype I and one for genotype III [30] and their virulence has also been characterized in white mice (strain CD-1) by evaluating mortality and histological studies [38].

Purpose of the Study

The purpose of this study was to compare the encysting capacity chronic strains observed in tissues through the molecular detection of the *T. gondii B1* gene, using as a source of DNA FFEP tissue from CD-1 mice infected with the chronic strains, as part of the molecular and biological evaluation of the same strains previously studied. Molecular detection of *T. gondii* in FFEP tissues involved the standardization of DNA extraction from FFEP tissues and the standardization of nested PCR that amplifies the *T. gondii B1* gene described by Burg., *et al* [39].

Material and Methods

Strains of *Toxoplasma gondii*: Three chronic strains and one acute strain were used through all the experiment. 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); the chronic strains were isolated from February to October 2007. 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 (1×10^{-1}), 1:200 (2×10^{-2}) and 1:4000 (4×10^{-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 (1×10^{-1} and 1×10^{-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 [40-42].

Histopathological study: The same FFEP tissues from 10 mice, which survived after being orally infected with the chronic strains with the inoculum (tissue cysts) undiluted and with dilutions 1×10^{-1}, 1×10^{-2} or 1×10^{-3} and that have been histologically analyzed by Vethencourt., *et al* [38]. The presence of tachyzoites (Tq) or tissue cysts (Q) was estimated in mm^3 of tissue analyzed according to Vethencourt., *et al* [38]. The unstained FFEP tissues were saved for DNA extraction.

DNA extraction with commercial kits: The DNA from all samples was extracted with the commercial NucleoSpin® Tissue Kits (Machery-Nagel, USA). For the RH strain, the DNA was extracted from the tachyzoites of 1 ml of peritoneal exudate. The DNA was extracted according to the specifications of the commercial house, with the methodology used to obtain DNA from culture cells, after washing with 1 mL of phosphate buffered saline (PBS, Gibco), by centrifugation (11,000 rpm for 5 minutes; Eppendorf® Microcentrifuge 5415, USA) and remov-

ing the supernatant. For the fresh tissues of the strains chronic strains TFC-1 (A) and TLW-1 (B), the tissue was placed in cold mortar and dilacerated in 2 mL of cold PBS, transferred to a 1.5 mL tube and centrifuged to remove the supernatant. The DNA was extracted according to the specifications of the commercial house, described for obtaining DNA from tissues. For all strains, a piece of tissue of the FFEP organs, approximately 5 µm, was placed in a 1.5 mL eppendorf tube. According to the instructions of the commercial house, it was deparaffinized with xylol for 30 minutes. After washing the tissue with absolute ethanol and dried at 37°C for 15 minutes. DNA was extracted using the commercial protocol for obtaining DNA from tissues already described.

Non-commercial DNA extraction from fixed and paraffin-embedded tissues (FFEP): Enzymatic digestion for extraction protocol 1 and 2: The process of paraffin removal and enzymatic digestion was carried out according to the protocol of Rivero., *et al*. [43] and modified by Baena Del Valle., *et al*. [32], as follows: three sections of approximately 5 µm, of all mouse tissues included in a paraffin block, were placed in a 1.5 mL eppendorf tube and 1 mL of preheated Xilol (approximately 50°C) was added, incubated at 56°C for 10 minutes and centrifuged at 10,000 rpm, at room temperature (RT) for 5 minutes, discarding the supernatant. This procedure was performed two or three times until the paraffin was completely removed. The tissue was washed with ethanol in descending concentrations (absolute ethanol, 95% and 70% ethanol). Each change was preceded by vortex homogenization and centrifugation at 10,000 rpm, RT for 5 min. Subsequently, 400 µL of digestion buffer (1M NaCl, 1M Tris-HCl, 0.5M EDTA, pH 8; 10% sodium dodecyl sulfate) was added with 2 µL of proteinase K (20 mg/mL). Digestion was carried out at 60°C/24 hours. At the end of the incubation period, proteinase K was inactivated at 95°C/10 min. After the process of deparaffinization and enzymatic digestion, DNA extraction was performed with two non-commercial protocols.

Protocol 1: DNA extraction by salting-out: After enzymatic digestion was complete, 500 µL of 5M NaCl was added, vortexed and incubated on ice for 5 minutes. After centrifugation at 13,000 rpm/RT/5 min., the supernatant was transferred to a sterile tube containing 600 µL of isopropanol. The mixture was incubated on ice for 10 minutes and centrifuged at 13,000 rpm/RT/5 min. The supernatant was discarded, and the DNA button was washed with 70% ethanol and centrifuged at 13,000 rpm/RT/5 min. The supernatant was discarded and dried at 37°C for 45 min and then dissolved in TE buffer (10mM Tris-HCl, pH 1.4 and 1mM EDTA, pH 8). DNA was stored at -20°C until used.

Protocol 2: Extraction with ammonium acetate: Once the enzymatic digestion was completed, 250 µL of 0.5 M ammonium acetate was added and incubated at -20°C for 5 minutes and centrifuged at 13,000 rpm/3 min. The supernatant was transferred to another eppendorf tube with 300 µL of 100% isopropanol, centrifuged at 13,000 rpm/5 min and the supernatant discarded; then, 300 µL of 70% ethanol were added and centrifuged at 13,000 rpm /2 min. The discarded and dried supernatant, the DNA was dissolved and stored similarly to protocol 1. For comparison of the three DNA extraction methodologies from FFEP, this was done with the chronic strain D only. Three sections of the 5 µm tissue blocks from 10 mice infected with the undiluted inoculum or diluted $1x10^{-1}$ or $1x10^{-2}$ were placed in triplicate in eppendorf-type tubes. Each triplicate was extracted with a different methodology.

Quality of extracted DNA and electrophoresis: DNA was quantified by fluorometry (Qubit, ThermoFisher, USA) following the manufacturer's instructions. DNA integrity (10 µl) was observed after electrophoresis in agarose gel (SeaKem LE, Cambrex, USA) at 1%, dissolved in Tris-Acetate-EDTA (TAE) buffer and stained with GelRed (Biotium, USA). Electrophoresis was executed at 100 volts for 30 minutes (Power Source FB1000, Fisher Scientific). For PCR amplifications, electrophoresis was performed on 2% agarose gels dissolved in TAE and the size of the amplified size was compared with 75 bp marker (Thermofisher cat. No: #SM1331, USA) or 100 pb (Thermofisher cat. No: #SM0323, USA). DNA and PCR amplifications were observed with a UV transilluminator (Slimline Series; Spectroline). The images were captured with an image digitizer (Enduro TM GDS, Labnet International, Inc.) and analyzed with TotalLab 1D software, version 14.0.

Molecular detection of *T. gondii* in fresh tissue and paraffin: Molecular detection in the fresh tissue was performed with a PCR endpoint for the B1 gene of *T. gondii*. The endpoint PCR (external PCR) was standardized in the LIB-UCIMED [44] to be as sensitive as the

nested PCR described by Burg., *et al* [39]. The molecular detection of *T. gondii* by FFEP was carried out with the nested PCR described by Burg., *et al* [39] using 1 µL of the PCR product performed with the ToxoN1 and ToxoC1 primers. The nested PCR used ToxoN2 (5´-TGCATAGGTTGCCAGTCACTG-3´) and ToxoC2 (5´-GGCGACCAATCTGCGAATACACC-3´) as primers, which gave rise to an amplification of 96 bp, after 14 amplification cycles: 94ºC/30 sec, 53 C/30 sec and 72ºC/30 sec, with a previous denaturation step at 94ºC/5 min and a final polymerization step at 72ºC/10 min. The reaction was performed at a final volume of 25 µL, adjusted with nuclease-free water (Life Technologies, USA), with the same final concentrations of the reagents, described for external PCR by Vethencourt., *et al* [44]. For both reactions The Apllied Biosystems (2720 Thermal Cycler, USA) equipment was used as a thermal cycler. For nested PCR, sensitivity tests were performed for minimal DNA detection, making serial dilutions in factor 10 of a DNA extracted from tachyzoites from the acute strain of *T. gondii* (strain RH). The intensity of the infection in the fresh tissue of mice infected with the chronic strains was carried out through PCR to detect the *T. gondii* B1 gene (primers N1 and C1) from 10 ng of DNA. The amount of amplified observed, after electrophoresis of 10 µL of the amplified, was quantified by densitometry when quantifying the area under the histogram curve.

Statistical analysis

The results will be represented in terms of proportion, and the frequency of molecular detection 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. The analysis was carried out with the help of the SPSS statistical program version 19.

Results

Quality of extracted DNA

Quality of DNA extracted from FFEP tissues according to the extraction method: Mouse tissues infected with strain D were used to compare the quality of the extracted DNA. The concentrations obtained from the three extraction protocols were similar regardless of inoculum dilution (Figure 1A). Protocol # 1 (NaCl) resulted in a mean concentration of 0.034 ± 0.038 ng/µL; range: 0 - 0.095 ng/µL, similar to that obtained after DNA extraction with Protocol # 2 (ammonium acetate) 0.045 ± 0.040 ng/µL; range: 0 - 0.092 ng/µL (p = 0.553) or with the commercial kit (0.052 ± 0.042 ng/µL; range: 0 - 0.136 ng/µL (p = 0.343) (Figure 1B).

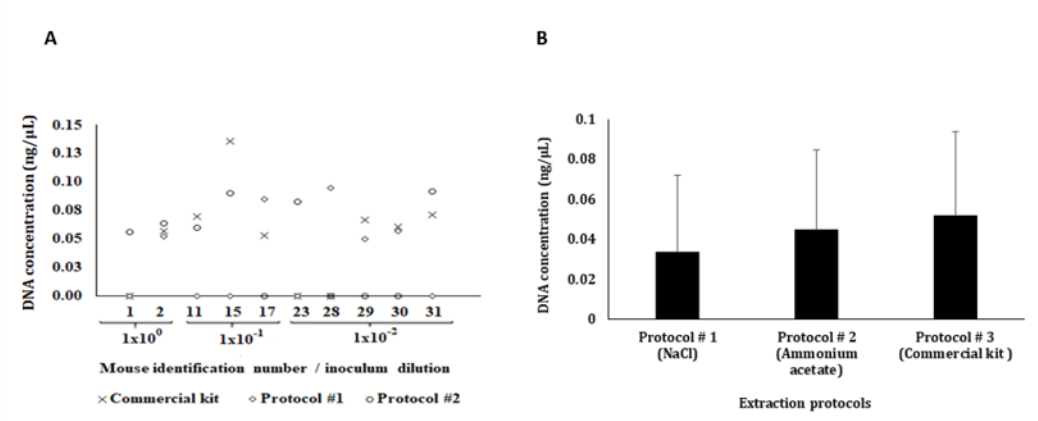

Figure 1: *Concentration of DNA extracted from FFEP, according to extraction method. A. Concentration of the extracted DNA according to the concentration of the inoculum. B. DNA concentration (ng/µL obtained according to the extraction method regardless of the dilution of the inoculum.*

Quality of DNA extracted from fresh tissue of mice infected with chronic *T. gondii* strains: DNA quality was assessed for strains A and B only, since strain D was lost due to its high virulence [38]. For both strains, DNA extracted from the lung, heart, brain and kidney was the least degraded. The most degraded DNAs were generally obtained from the liver and spleen (Figure 2A and 2B). In terms of DNA concentration and regardless to the tissue extracted, an average of 12.9 ± 11.8 ng/µL (range: 4.37 - 35.5 ng/µL) was obtained, for strain B and 20.3 ± 18, 9 ng/µL (range: 0.797 - 46.8 ng/µL), which was statistically similar (p = 0. 404).

Quality of DNA extracted from fresh or non-FFEP tissue compared to that obtained from FFEP tissue, obtained with the commercial kit: For strain A and strain B, DNA concentration from fresh or non-FFEP mouse tissue was significantly higher than that found in FFEP tissues. For strain A, the DNA concentration obtained from FFEP tissues was significantly lower (0.120 ± 0.185 ng/µL; range: 0 - 0.508 ng/µL) compared to the concentration of DNA extracted from non-FFEP (p = 0.016), figure 2A and 2C respectively. For strain B, the DNA concentration obtained from FFEP tissue was significantly lower (0.097 ± 0.028 ng/µL; range: 0 - 0.140 ng/µL) than the concentration of DNA extracted from non-FFEP tissue (p = 0.041), (Figure 2B and 2D, respectively). The integrity of the DNA extracted from FFEP tissues was similar for both strains (Figure 2C and 2D).

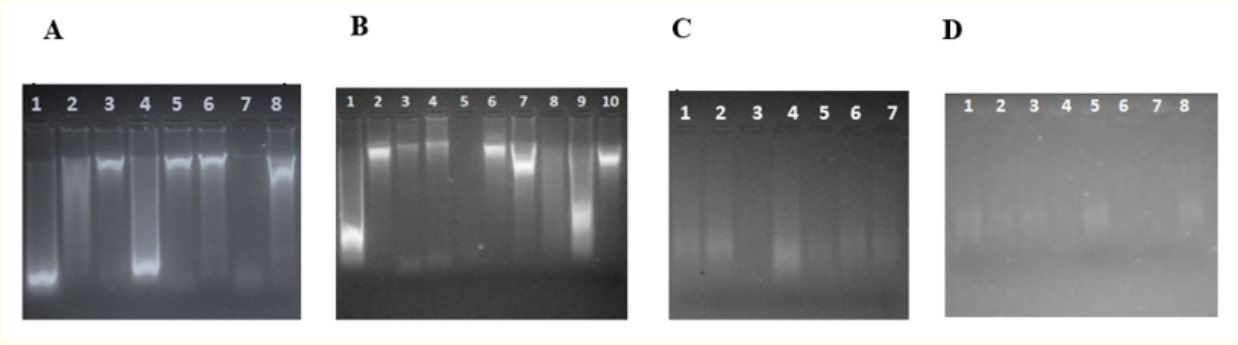

Figure 2: *DNA integrity. A: Extracted from non-FFEP tissues mouse infected with chronic T. gondii strain A. Lines: 1: Liver; 2: Muscle; 3: Lung; 4: Spleen; 5: Heart; 6: eye; 7: brain; 8: Kidney. B. Extracted from non-FFEP tissues mouse infected with chronic T. gondii strain B. Lines 1: Spleen; 2: Heart; 3: Brain1; 4: Brain2; 5: Liver1; 6: Muscle; 7: Lung; 8: Kidney; 9: Liver2; 10: Muscle. C. Extracted from FFEP tissues mouse infected with chronic T. gondii strain A. Lines: 1 to 7 DNAs of mice 33, 31, 32, 37, 35 (1 x 10-3), 41, 43 (healthy controls), respectively. D: Extracted from FFEP tissues mouse infected with chronic T. gondii strain B. Lines: 1 to 8 DNAs from mice 42, 41 (healthy control), 39, 38 (1 x 10-3), 25, 23 (1 x 10-2), 8 and 2, (1 x 10-0), respectively.*

Sensitivity of nested PCR detecting the *T. gondii B1* gene

The detection limit of *T. gondii* DNA was evaluated after performing the nested PCR that detects the *T. gondii B1* gene. This was performed with 7 dilutions in factor 10, from a DNA extracted from tachyzoites and from a *T. gondii* RH strain. DNA titrated by fluorometry at 1 ng/µL was brought up to 0.1 fg/µL. Nested PCR resulted in the amplification of a 196 bp fragment and then performed external PCR. Weak amplification was observed when 10 fg/µL (10 pg/rx) or 1 fg/µL (10 fg/rx) was amplified, (Figure 3A), but the amplification performance increased after performing internal PCR (Figure 3B). Thus, standardized PCR was able to detect up to 0.1 fg/µL of *T. gondii* DNA or 1 fg/PCR reaction (rx).

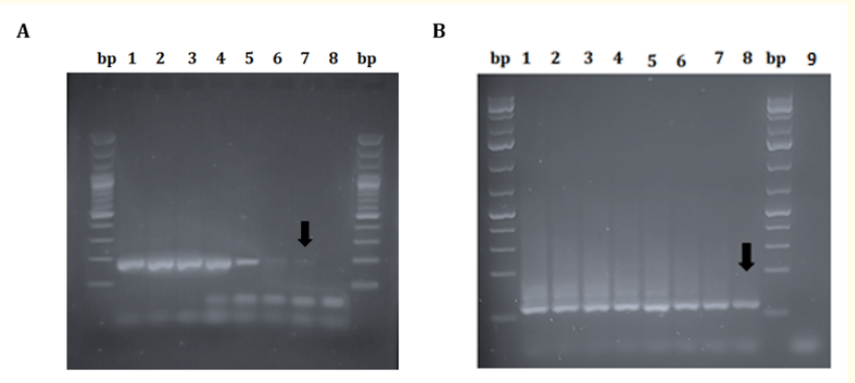

Figure 3: *Sensitivity of nested PCR that detects the T. gondii B1 gene. A. PCR performed with external primers. (196 bp) B. PCR performed with the internal primers (96 bp). Lines: 1-7: serial dilutions factor 10 of a DNA of 1 ng/uL concentration or 10 ng/per reaction by PCR reaction (rx). 1: 10 ng/rx; 2: 1 ng/rx; 3: 0.1 ng/rx; 4: 10 pg rx 5: 1 pg/rx; 6: 0.1 pg/rx; 7: 10 fg/rx; 8: 1 fg/rx; 9: Mix control. Arrows indicate limit of detection. Base pair marker A: 100 bp (# SM0323) B: 75 bp (# SM1333).*

Molecular detection of *T. gondii* DNA in FFEP tissue according to the DNA extraction methodology used

The figure 4A, shows that regardless of the extraction method used, *T. gondii* DNA was not detected after amplifying it with the N1 and C1 primers, which amplify a 196 bp fragment of the *B1* gene from *T gondii*. The figure 4B presents the molecular detection of DNA with nested PCR that detects the *T. gondii B1* gene and amplify a 96 bp fragment. In addition, this figure shows that the detection of *T. gondii* DNA using the commercial extraction protocol (60%; 6/10) was higher than that observed by both manual extraction protocols.

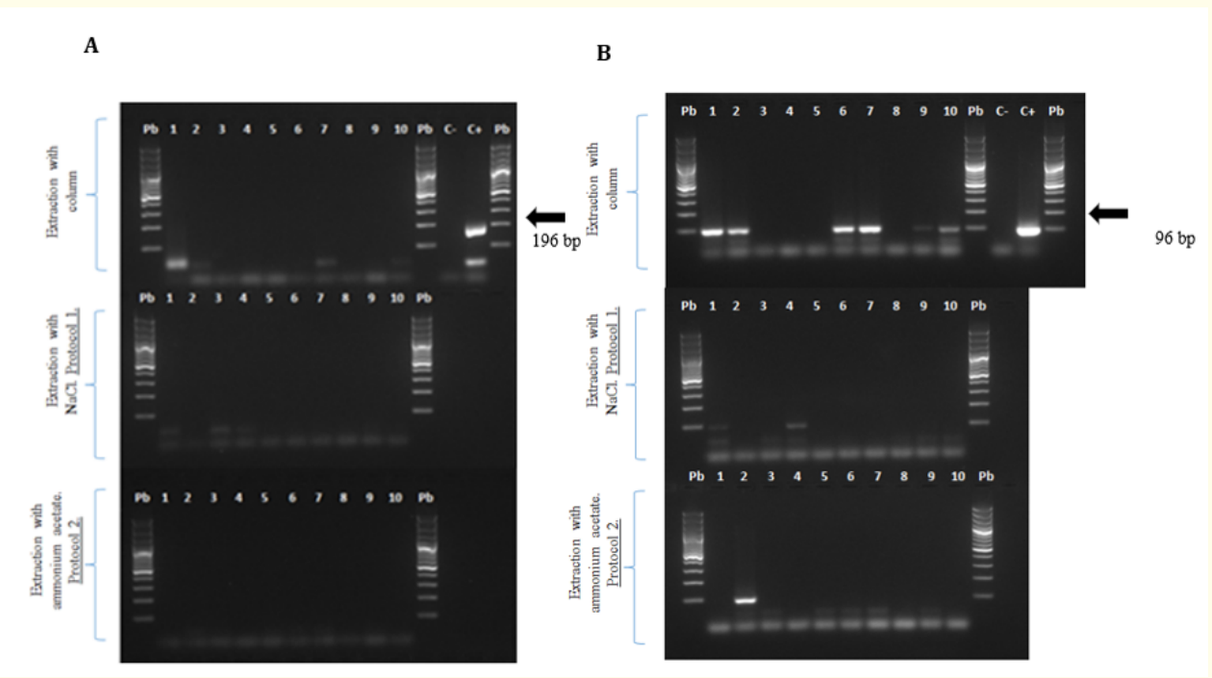

Figure 4: *Molecular detection of T. gondii Strain D DNA in FFEP tissue with respect to the DNA extraction methodology used. A. External PCR (primers N1 and C1) that amplify the B1 gene. B. Nested PCR for B1 gene. The arrow indicates the size of the expected amplified. Lines: 1 and 2: mice inoculated with the 1 x 100 inoculum (mouse 1 and 2); lanes 3-5: mice inoculated with the inoculum diluted 1 x 10-1 (mice 11, 15 and 17); lanes 6-10: mice inoculated with the inoculum diluted 1 x 10-2 (mice 23, 28, 29, 30). C-: mix control; C +: Tq DNA from the T. gondii strain RH. Pb: 100 bp marker (Thermofisher cat. No: #SM0323, USA).*

Molecular detection of *T. gondii* DNA in fresh or non-FFEP tissue from mice inoculated with chronic *T. gondii* strains: An amplification of 196 bp was obtained in the brain, muscle, lung and kidney of strain B and in the heart, brain, muscle, lung and kidney of strain A. The semi-quantitative evaluation (see methodology) showed that for both strains, the organs with the highest amount of DNA were the brain and muscle followed by the kidney and the lung (Figure 5A and 5B). In liver or spleen, parasite DNA was not detected in none of the strains. Strain B DNA was detected in 57.1% (4/7) of the organs and tissues evaluated and in 71.3% (5/7) for strain A, and although the semi-quantification of the density of the amplified was higher in strain A, it was not statistically significant ($p = 0.367$).

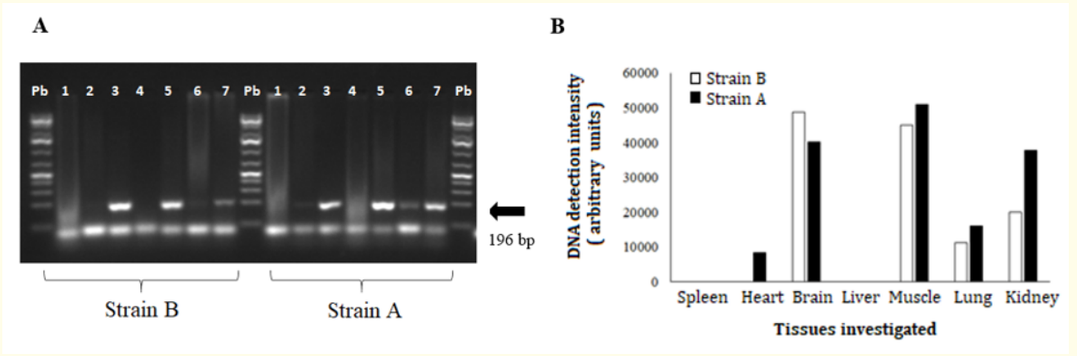

Figure 5: *Molecular detection of T. gondii DNA in fresh tissue from mice inoculated with chronic T. gondii strains. A. External PCR (primers N1 and C1) that amplify the B1 gene. B. Nested PCR for the B1 gene. The arrow indicates the size of the expected amplified. B. Lines: 1: spleen; 2: heart; 3: brain; 4: Liver; 5: muscle; 6: lung; 7: kidney. Tissues collected from mice infected with strain A and B from mice infected after 3 months, see methodology. Pb: 75 bp marker (Thermofisher cat. No: SM133, USA).*

Molecular detection of *T. gondii* DNA in FFEP from mice inoculated with chronic *T. gondii* strains with nested PCR that detected *B1* gene

Amplifications obtained by performing nested PCR to detect the *T. gondii B1* gene, from FFEP tissue DNA obtained from A, B and D strains can be seen in figure 6A-6C, respectively. The detection rate of *T. gondii* DNA is summarized in figure 6D. For strain A, the highest detection rate was found in the FFEP tissues of the mice that were infected with a 1 x 10^{-2} dilution of the inoculum (204 Q/mL), which corresponded, approximately to an inoculum of 2 Q/mL. For strain B, detection was higher in FFEP of mice infected with near to one cyst (0.5 Q/mL) and corresponding to the 1 x 10^{-3} dilution of the inoculum (578 Q/mL). For strain D, *T. gondii* DNA could be detected in mice infected with the undiluted inoculum (354 Q/mL). When mice were infected with diluted 1 x 10^{-2} inoculum, corresponding to approximately 2 Q/mL, 6 Q/mL or 4 Q/mL, for strains A, B and D respectively, the DNA detection *T. gondii* was 60%. No significant difference was found in detection between strain D and strain A or B, when mice were infected with the undiluted inoculum ($p = 0.142$, for both comparisons) and neither between strain A and B, when mice were infected with the diluted inoculum 1 x 10^{-3} ($p = 0.238$) (Figure 5D).

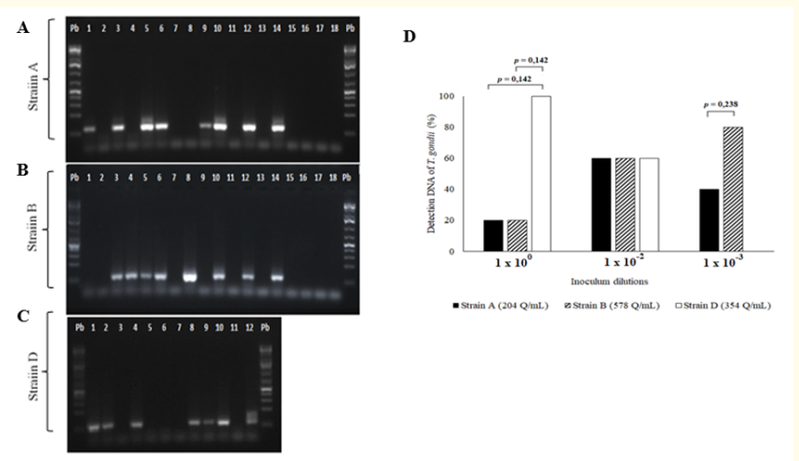

Figure 6: *Molecular detection of T. gondii DNA in FFEP of mice inoculated with chronic strains of T. gondii with nested PCR that detected the B1 gene. A. Strain A: Lines: 2: healthy control; 1 and 3: PCR positive control; 4-8: inoculated with the 1 x 10-3 dilution; 9-13: inoculated with the 1 x 10-2 dilution; 14-18: inoculated with the 1 x 100 dilution. B. Strain B: Lines: 1, 2, 9: healthy controls; 3, 4, 10-12: inoculated with the 1 x 10-3 dilution; 5, 6, 13-15: inoculated with the 1 x 10^{-2} dilution; 7.8, 16-18: inoculated with the 1 x 100 dilution. C. Strain D: Lines: 1, 2: inoculated with the 1 x 100 dilution; 3-5: inoculated with the 1 x 10^{-1} dilution; 6-10: inoculated with the 1 x 10^{-2} dilution; 11: PCR negative control; 12: PCR positive control. D. Percentage of DNA detection in FFEP tissues of mice infected with chronic T. gondii strains. Pb: 75 bp marker (Thermofisher cat. No: #SM133, USA).*

Regardless of the dilution of the inoculum, T. gondii DNA was detected in 33.3% (5/15) of the FFEP tissues analyzed for strain A, which was lower than the proportion observed for strain B (53%; 8/15) and strain D (71.4%; 5/7), but these differences were not statistically significant ($p = 0.161$ and $p = 0.097$, respectively).

Comparison between the molecular detection of *T. gondii* DNA and the presence of tachyzoites (Tq) in lung or cysts (Q) in brain, of mice infected with the chronic *T. gondii* strains.

On relation to the Tq/mm^3, in lung higher amount was observed in the infection dose of the undiluted inoculum in strain A (980 ± 109 Tq/mm^3) compared to strain B (222 ± 76 Tq/mm^3; $p < 0.001$) and strain D (69 ± 69 Tq/mm^3; $p < 0.001$). At the 1 x 10^{-1} dilution of the inoculum, the Tq/mm^3 count was higher in strain B (150 ± 47 Tq/mm^3) with respect to strain A (0 Tq/mm^3; $p = 0.015$) and strain D (23 ± 23 Tq/mm^3; $p = 0.036$). At the 1 x 10^{-3}, no lung Tq were observed for strains A and B (Figure 7A).

Regarding to the presence of cysts in the brain, the count was higher in strain A (65 ± 43 Q/mm^3) with respect to strains B (13 ± 13 Q/mm^3) and D (0 Q/mm^3), at the infection dose of the undiluted inoculum or diluted 1 x 10^{-1}, but this was not statistically different (Figure 7B). For inoculum dilution of the 1 x 10^{-2} and 1 x 10^{-3}, although more cysts were observed between strain B (529 ± 204 Q/mm^3 and 317 ±161 Q/mm^3) and strains A (184 ± 67 Q/mm^{3}and 178 ± 75 Q/mm^3), respect to strain D (144 ± 104 Q/mm^3, only 1 x 10^{-2} dilution), but this was not statistically different (Figure 7B).

The sensitivity of the molecular detection of *T. gondii* DNA was compared with the detection of Tq and Q in FFEP tissues. For strain A, the largest number of Q/mm^3 was found after infecting the mice with the 1 x 10^{-1} dilution of the inoculum (Figure 7B), but the molecular detection for the tissues FFEP rate was higher in the 1 x 10^{-2} dilution of the inoculum (Figure 6D). In Strain B, after histological analysis, the highest count cysts were observed in the 1 x 10^{-2} dilution (Figure 7B), while molecular detection was highest at the 1 x 10^{-3} dilution (Figure 6D).

For strain D, similarity was found with the lung tachyzoite count (Figure 7A). The highest Tq/mm^3 was found in the FFEP lung sections of the mice infected with the undiluted inoculum, decreased in the diluted 1 x 10^{-1} inoculum and was not appreciable at the 1 x 10^{-2} dilution of the inoculum (Figure 6D).

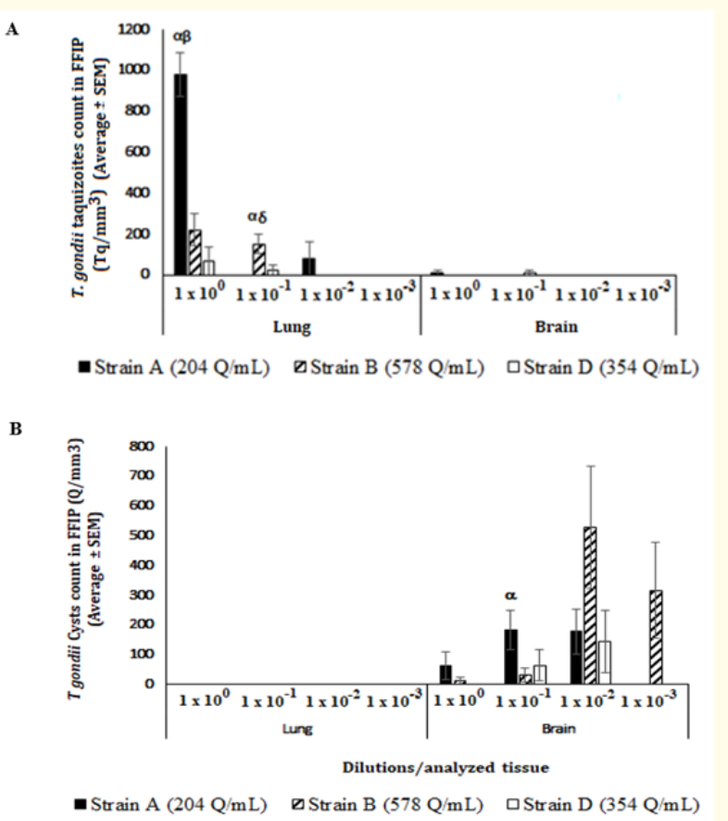

Figure 7: *Tissue count of T. gondii life forms in PPIF tissue sections from mice infected with three chronic T. gondii strains. A. T. gondii tachyzoites (Tq/mm3) B. T. gondii cyst (Q/mm3). α: statistical difference between strain A and Strain B. β: statistical difference between strain A and Strain D. δ: statistical difference between strain B and strain D. SEM: standard error of the mean. Statistical analysis: T Test.*

Discussion

The purpose of this study was to compare molecular detection of *T. gondii* cyst formation in fresh or paraffin-embedded tissues of three chronic strains. As part of the standardization of the technique, it was demonstrated that the best methodology for the extraction of DNA from FFPE tissues was the commercial extraction method. And, although the DNA concentration obtained with the three extraction protocols was similar, only with the commercial methodology utilizing silica columns was possible detect parasite´s DNA, after applying a nested PCR for the *B1* gene of *T gondii*.

The highest purity and best quality obtained from FFPE, have been evaluated by other researchers [34,35,45] Methods for extracting DNA from FFPE using phenol chloroform as an extraction method or commercial kits based on silica columns have been evaluated for some time, the latter being recommended for extraction of FFEP [34]. Routine procedures, which are labor-intensive, time-consuming, and toxic solvents use such as phenol and chloroform, can result in a poor-quality DNA sample [37]. The incorporation of other paraffinic solvents such as mineral oil [36], prior to digestion with Proteinase K, has also yielded good results, when DNA is extracted with a commercial kit containing a silica column compared to extraction-based methodologies with ammonium acetate, with high concentrations of NaCl (salinization) or with phenol-chloroform [31]. According to the results obtained, and in comparison, with other authors [34,35], in the present study the degree of purity of the DNA obtained with the commercial methodology was the best, since DNA can be obtained after deparaffinizing with xylol, washing with alcohol and later purifying it with silica columns.

Nested PCR detecting the *T. gondii B1* gene was initially described by Burg., *et al* (1989). The PCR described by these investigators had a sensitivity of 1 parasite or 10 in the presence of 10^5 white blood cells. In our laboratory and with the aim of standardizing the PCR described by Burg., *et al* [39] one parasite alone and between 10^4 and 10^6 in the presence of meat was obtained by amplifying the external PCR [44]. In this study, the sensitivity of the nested PCR was determined, resulting in 0.1 fg/µL or 1 fg/rx when standardizing the nested PCR, described by Burg., *et al* [39], compared to external PCR that amplifies the 196 bp fragment [44]. Therefore, nested PCR is 10 times more sensitive, thus favoring the detection of DNA that is in very low concentration and degraded, as is the case of DNA obtained from FFEP tissues, which has been described by other researchers [34]. In addition, the detection of specific genes, whose amplifications are less than 300 bp are suitable strategies to be able to detect specific DNAs, after being extracted from FFEP tissues [46-48].

The use of DNA obtained from FFEP, for the detection and molecular typing of parasites such as *Trypanosoma cruzi* [24], *Leishmania* spp. [26], *Entamoeba histolytica* [27] and Free-living amoeba [28], have been reported, in addition to the investigation of cancer biomarkers [23,49]. In the case of *T. gondii*, the molecular detection and molecular characterization of this parasite was reported from fetoplacental FFEP tissues of women with recurrent abortions [29], which puts on the table the applicability of obtaining of FFEP DNA in these retrospective studies. On the other hand, the possibility of PCR amplification incorporates sequencing studies, that not only can genes be detected, but they can also be sequenced, even through new generation sequencing [50].

The application of nested PCR that detects the *B1* gene of *T. gondii*, standardized and used in this work, may be appropriate for the molecular detection of this parasite from FFEP tissues, so that the use of this molecular technique could be used in retrospective studies in the diagnosis of toxoplasmosis in humans and animals. In this regard, 3 decades ago an attempt has been made to use *T. gondii* DNA extracted from FFEP [51]. The amplification of the B1 gene and its hybridization with a radioactive phosphorus-labeled probe (^{32}P), was reported almost 1000 times less sensitive than DNA determination in fresh tissue [51]. Studies that are more recent have been carried out for the detection of *T. gondii* with other molecular markers, which according to the literature, could be the same or more sensitive than the detection of the *B1* gene [52].

In this sense, a 529 bp non-coding DNA fragment has been used, which is repeated in the *T. gondii* genome between 200 and 300 times [52], in comparison with the *B1* gene, which is repeated approximately 35 times [39] and that encodes the enzyme glyceraldehyde 3 phosphate dehydrogenase [53]. Although both markers, with competitive detection limits [54], have been used for the molecular detection of

T. gondii from FFEP tissues, it is preferable to use the *B1* gene as a molecular marker, due to the size of amplification obtained after the application of the nested PCR of 196 bp/96 bp [39]. This ensures the capture of small segments of partially degraded DNA, as consequence of its extraction from FFEP tissues [47,51].

In this regard, the *B1* gene has been used as a molecular marker for the detection of *T. gondii* in FFEP samples in breast cancer [55] or in preserved samples from recurrent abortive women [56]. Therefore, after validating the test, it could be put at the service of Costa Rica, both for the diagnosis of conditions produced by this parasite, such as reactivation by immunosuppression or pregnant women [57], as for the investigation of this parasite in FFEP tissue biopsies [55,56].

In the tissue of a mouse infected with *T. gondii*, the presence of the parasite or the detection of its DNA varies according to the virulence of the strain, being related to the presentation of the infection (chronic or acute) and the genotype or lineage of the strain [30,38,58,59]. In our study, a higher molecular detection of the parasite was demonstrated in the brain and skeletal muscle of mice infected with the chronic strains A and B of *T. gondii*, after 3 months p.i. (dosed with sulfadiazine, to ensure the chronicity of the infection), and in none of the strains *T. gondii* DNA was detected in liver or spleen. Similar study conducted by Djurković-Djaković., *et al* [58], demonstrated that the detection of *T. gondii* DNA quantified by real-time PCR targeting the 529 bp gene fragment is detected depending on the strain and dose with oral infection with 10 tissue cysts of a chronic strain of *T. gondii* (Me49, type II strain). These authors showed high and constant parasite loads in the brain until the end of the experiment (day 42) and the *T. gondii* DNA had been completely removed from the liver. In contrast to our study, the chronic strains A (*Felis catus*, TFC-1) and B (*Leopardus wiedii*, TLW-1), characterized by PCR-RFLP as type I (variant) and type III (variant) lineage strains, respectively [30], were virulent, depending on the dose of infection [38]. The strain B (genotype III variant) was more virulent than strain A (genotype I variant) [38]; therefore, it was expected that it could be found in different tissues of the infected mouse, similar to what occurs in an infection with the acute strain RH (Type I) of *T. gondii* [58]. After inoculation of the mouse with high (10^6 parasites) or low (10^2 parasites) doses, *T. gondii* DNA can be detected in all tissues, including the brain, of the infected mouse [58].

Similar studies carried out by Nishi., *et al*. [59] demonstrated the presence of the parasite, both histologically and molecularly, mainly in brain and muscle, after 50 days post oral infection with 25 cysts of a chronic strain of *T. gondii* (ME-49 strain, type II) and in the absence of treatment with pyrimethamine and sulfadiazine or with rosuvastatin. In addition, these researchers showed necrosis processes at the brain level, which were attenuated after treating the mice for 21 days with rosuvastatin. This supports the idea of the presence of a chronic inflammatory response after infection with chronic strains and that was observed by us when studying the virulence of chronic strains of *T. gondii*, previously [38].

In this work, the molecular detection of *T. gondii* DNA from chronic strains A and B in FFPE tissues shows a curve with a maximum peak at a given dilution. It is similar to kinetic studies in which the highest number of cysts in the tissues [60,61] or the highest molecular detection of *T. gondii* [62,63] was observed at a certain time, after infection. Therefore, molecular detection in mouse tissues infected with chronic strains of *T. gondii* follows, as in kinetic assays, a maximum detection curve that depends on the inoculum.

When the strains are acute, the detection in the tissues does not follow the curve of the chronic strains, as observed in the behavior of the molecular detection of strain D, with respect to strains A and B. In strain D, the greater detection is found in FFEP tissues when mice were infected with the doses of the inoculum undiluted. The curve was similar to that observed for an acute strain of *T. gondii* (strain RH), reported by Costa., *et al* [64].

On the other hand, the encysting capacity and virulence [38] shown by the chronic strains, analyzed in this study, reveal that only if the strains are genotypically pure (Type I, II and III) can they be classified according to the groups described by Sibley and Boothroyd [5] or Ferreira., *et al* [17]. Otherwise, as is the case with the chronic strains studied in this work, the genetic variation of the strain will give rise to virulence and encystment processes that are not comparable with the groups previously described, since the genetic characteristics of the isolates, will influence their biological behavior.

Regarding the molecular detection in the tissues regardless of the dilution of the inoculum, *T. gondii* DNA was detected in a lower percentage for strain A, followed by strain B and strain D. This result is related to the virulence studied for these strains [38], which leads us to think that depending on the virulence of the strain, the control of the immune response during the acute phase of the infection could be different. During the initial phase of the infection, the immune control of the parasite would favor the reduction of the acute forms (tachyzoites) and the establishment of the immune response against the infection would promote the formation of tissue cysts, which has been reported by Costa., *et al* [64]. In this way, the control of the acute infection and the rapid encystment with a greater cyst concentration per tissue, could explain the lower virulence of the strain, when the mice are infected with comparable inoculums. Therefore, the encysting capacity in the tissues of the chronic strains, studied in this work, could indirectly favor the survival of the mice previously studied by Vethencourt., *et al* [38]. The modulation of the immune response during the infection of chronic strains studied in this work and its comparison with the acute strain RH of *T. gondii* would be a future subject of study. Interestingly, when quantifying the amount of tachyzoites or cysts per tissue area in FFEP tissues of infected mice, it was found that despite the fact that strain A showed a higher concentration of tachyzoites in lung followed by strain B and D, and in the brain, the number of tissue cysts obtained with strain D was the lowest. This reinforces the idea that the encysting capacity of a chronic strain of *T. gondii* is inversely proportional to the virulence of the strain.

With respect to the sensitivity of the molecular detection of *T. gondii* and in comparison with microscopy, it should be noted that the molecular detection of the *B1* gene of *T. gondii* with nested PCR from FFEP tissues raised the sensitivity to a logarithm microscopy detection.

The detection and molecular characterization of *T. gondii*, based on FFEP in tissues, has no antecedents in Costa Rica, therefore, after the validation of the technique, it could be put at the service for retrospective studies in humans and animals.

Conclusion

In this work it can be concluded that: 1. the commercial kit that uses silica columns for DNA purification should be used, since the DNA thus obtained is of sufficient quality to perform the molecular detection of the *T. gondii B1* gene by means of nested PCR. 2. The molecular detection in non-preserved mouse tissues infected with chronic strains of *T. gondii* follows, like kinetic assays, a maximum detection curve that is dependent on the inoculum. 3. The molecular detection of *T. gondii B1* gene with nested PCR from FFEP tissues is 10 times more sensitive than detection of cysts by microscopy. 4. The encysting capacity of a chronic strain of *T. gondii* is inversely proportional to the virulence of the strain.

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Conflict of Interest

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

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