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Research Article

Detection and Molecular Characterization of Three Chronic Strains of *Toxoplasma gondii*, Isolated In Costa Rica

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Abstract

The molecular diagnosis of *T. gondii* should include the molecular detection of the parasite and its characterization. The purpose of this study was to detect and characterize molecularly three chronic strains of *Toxoplasma gondii* isolated in Costa Rica. DNA was isolated from tissues (fresh or fixed and embedded in paraffin (FFIP)) from white mice (Strain CD-1, *Mus musculus* Swiss) infected chronic strains. The chronic strains came from the faeces of a domestic cat (A: *Felis catus*, TFC-1), the stool of a caucel (B: *Leopardis wiedii*, TLW-1), of CD-1 mice fed beef (D: *Bos Taurus*, TBT-1). A strain of *T. gondii* (strain RH) was used as a control strain. Detection and molecular characterization were performed by PCR (gene B1) and PCR-RFLP with genes SAG2, SAG3 and L358, respectively. A good performance was observed when amplifying the B1 gene by PCR from tachyzoite DNA (acute strain) and for strains A and B (fresh tissue), but the yield was lower for strain D, whose DNA was obtained from FFIP. Strains A and D were characterized as type I lineages and strain B as type III lineage. None of the three strains showed pure lineages. The molecular characterization of the *T. gondii* isolates will contribute to the scientific community of Costa Rica, since it will have the molecularly characterized strains necessary to define different epidemiological and diagnoses in both humans and animals.

Keywords: *Toxoplasma gondii; chronic strains; molecular detection; molecular characterization; Costa Rica*

Abbreviations

PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; *T. gondii: Toxoplasma gondii* ; FFIP: Fixed In Formalin And Embedded In; Pb: Molecular Marker In Base Pairs; I.P: Intraperitoneal.

Introduction

Toxoplasma gondii is a wide geographical distribution parasite; present a very high prevalence, especially in countries of tropical climates as in Tropical and Subtropical America [1,2]. Therefore, in Costa Rica, the prevalence of toxoplasmosis is also very high [3,4].

The conventional diagnosis of *T. gondii,* generally employ serological tests and bioassays in cats or mice, or in a combination of both [5]. The diagnosis of toxoplasmosis by detecting the parasite´s DNA, using molecular methods based on the polymerase chain reaction (PCR), has gained popularity in the last two decades. The reason is the major sensitive and profitable than conventional methods [6,7]. The molecular diagnosis of *T. gondii*, should not only include the molecular detection of the parasite, but also its characterization. The identification of a correlation between the severity of the disease and the genotype of the strain can be crucial important for

the determination of appropriate treatment [8]. On the other hand, the molecular characterization of *T gondii* lineages, can provide information on the geographical distribution of different strains and genotypes.

The strains of *T. gondii* have been subdivided into three groups or lineages, namely type I, II and III, by, using different characterization methods, such as: a) isoenzyme analysis (zymodemas), b) virulence in the Swiss white mouse and c) analysis of the Restriction Fragment Length Polymorphism (RFLP) [9]. In recent years, there has been a greater number of molecular studies using PCR-RFLP for different *T. gondii* genes [7,10-12], by allowing to show the variability of the lineages, which define the genotypes and vary depending on the geographical area [13].

At present, approximately 11 genes of *T. gondii* (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3) have been studied to define the lineage of the parasite, using PCR-RFLP [1]; the SAG2 gene is the most frequently studied and correlated with the disease in humans [7,9].

The tissues fixed in formalin and embedded in paraffin (FFIP) can be used for molecular findings in the clinical and scientific fields. The molecular detection of *T. gondii* in FFIP can allow for the correlation between molecular aspects and the pathogenesis, treatment, and even the possibility of carrying out retrospective studies [15-17]. In addition, it has been shown that the DNA extracted from FFIP is suitable for PCR amplification [18]. These background studies lead us to think that we may obtain a quality DNA, allowing for the detection and molecular characterization studies of *T. gondii* in FFIP.

The Basic Research Laboratory of the University of Medical Sciences (LIB-UCIMED) has isolated and maintained three chronic strains of *T. gondii*.

Aim of the study

The purpose of this study was to detect and molecularly characterize three chronic strains of *Toxoplasma gondii* by PCR-RFLP, using the *B1*, *SAG2*, *SAG3* and *L358* genes. The molecular characterization of these *T. gondii* strains will help with defining the lineage.

Materials and Methods

Toxoplasma gondii strains

Three chronic and one acute strain were worked on. The chronic strains were obtained from the feces of a domestic cat (Felis catus, TFC-1), the stool of a caucel (*Leopardis 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 between February and October 2007. The strain RH (ATCC 50174 D) of *T. gondii* was introduced as an acute control. All the strains were maintained in the laboratory by intraperitoneal (i.p.) inoculation in white mice (strain CD-1, *Mus musculus* Swiss). For RH strain (acute) the procedure was inoculation of 0.2 ml of a peritoneal exudate diluted 1/10, 1/200 and 1/4000, in groups of 2 mice by dilution. For chronic strains (TFC-1, TLW-1, TBT-1), tissue cysts obtained from the brains of CD-1 mice, previously infected with these strains, were dilacerated, and tissue was resuspended in 5 ml of saline; from this suspension, two serial dilutions were made (1/10 and 1/100) 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), established for this purpose. The infected mice were maintained with the usual concentrated feed supply and water ad libitum, in the BLIB-UCIMED, according to the national and international laws for animal care [19-21].

DNA source

For the chronic strains TFC-1 (A) and TLW-1 (B), the DNA was extracted from the brains of mice previously i.p. inoculated with tissue cysts. For strain, TBT-1 (D) the DNA was extracted from tissue sections of FFIP organs, from orally infected mice with undiluted tissues or with dilutions 1/10 or 1/100. For the RH strain, we used the tachyzoites from peritoneal exudate.

DNA extraction

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 g for 5 min) and removing the supernatant. For the chronic strains TFC-1 (A) and TLW-1 (B), the DNA was obtained from the brains of mice previously i.p. infected. The tissue was placed in cold mortar and dilacerated in the presence of 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 strain D (TBT-1), a piece of tissue of the FFIP organs, approximately 10 µm, was placed in a 1.5 mL ependorf. According to the instructions of the commercial house, it was deparaffinized with xylol for 30 min. After washing the tissue with absolute ethanol and allowing to dry at 37 °C for 15 min. The DNA was extracted using the protocol of the commercial house already described.

Molecular detection of *T. gondii in tissue*

Molecular detection was performed with a PCR endpoint for the B1 gene of *T. gondii*. The endpoint PCR was standardized in the LIB-UCIMED [22] to be as sensitive as the nested PCR described by Burg., *et al.* [23]. The ToxoN1 primer (5'-GGA ACT GCA TCC GTT CAT GAG-3 ') and ToxoC1 (5'-TCT TTA AAG CGT TCG TGG TC-3') were used, which generate a product of 196 bp, after 40 cycles amplification at: 94°C/1 min, 53 °C/1 min and 72°C/1 min, with a previous denaturation step of at 94 °C/5 min and a final polymerization step at 72°C/10 min. The Apllied Biosystems equipment (2720 Thermal Cycler, USA) was used as a thermal cycler. PCR was performed with the following final concentrations: 1.25 U of Dream Taq Polymerase (Thermo Scientific, USA), 0.5 µM of each of the primers, 0.2 mM of the dNTPs (Sigma, USA), adjusted to a final volume of 50 µl with nuclease-free water (Life Technologies, USA) [22].

Molecular characterization by PCR-RFLP

This methodology was performed with the SAG2, SAG3 and L358 genes, according to the recommendations by Su., *et al.* [11]. Table 1 shows the location of the gene in the genome of *T. gondii*, sequence of primers, size of the product obtained by PCR, restriction enzymes, digestion protocol and polymorphisms obtained for each genotype. PCR amplifications were obtained after 35 amplification cycles of: 94 ° C / 30 sec, 55 ° C / 1 min and 72 ° C / 2 min, with a previous of denaturation step at 94 ° C / 4 min and a final polymerization step to 72 ° C / 7 min. The PCRs were carried out with the following final concentrations: 0.75 U of Dream Taq Polymerase (Thermo Scientific, USA), 0.3 µM of each of the primers, 0.2 mM of the dNTPs (Sigma, USA), adjusted to a final volume of 50 µl with nuclease-free water (Life Technologies, USA)., Sensitivity and specificity were tested for each PCR. According to the amplification conditions it was possible to obtain high yield amplifications with DNAs between 20 pg and 1.6 ng per reaction and all the PCRs were 100% specific (data not shown). The digestions with the restriction enzymes for SAG2 and SAG3 were performed at a final volume of 25 µL, adjusted with nuclease-free water, BE 1X buffer (Buffer CutSmart, BioLabs, New England, USA) and with Hae III buffer for the digestion of amplified L358 (Table 1). Simple or double digestion was performed with 5 units of each of the fast-digesting restriction enzymes (BioLabs, New England, USA) and 5 to 10 µl of the amplification obtained by PCR at 37 ° C or 65 ° C, according to the amplified to digest (Table 1).

Gene	Gene Location in the genome of *T. gondii*α	Sequence of sense primers β	Sequence of anti-sense primers β	Pb	Restriction enzymes and procedure	Size of fragments obtained after digestion, in base pairsγ		
						I	II	III
SAG2	VIII	SAG2-Fa: 5´ACCCATCTGC-GAAGAAAACG 3´	SAG2-Ra: 5´ ATTTCGAC-CAGCGGGAG-CAC 3´	546	*Hinf* I (5 U) , *Taq* I (5U), Buffer BE 1X, 15 min at 37°C, 10 min at 65°C	349, 165	349, 118	404, 165
L358	V	L358-F2: 5´ AG-GAGGCGTAGCG-CAAGT 3´	L358-R2: 5´ CCCTCTGGCTG-CAGTGCT 3´	418	*Hae* III (5U), *Nla* III (5U), Buffer *Hae* III, 1X, 15 min at 37°C	288	212, 113	170, 113
SAG3	XII	P43S1: 5´ CAACTCTCAC-CATTCCACCC 3´	P43AS1: 5´ GCGCGTTGT-TAGACAAGACA 3´	311	*Nci* I (5U), Buffer BE 1X, 15 min at 37°C	139, 98, 64	319	208, 108

Table 1: *Location in the genome of T. gondii, sequence of primers, size of the product obtained by PCR, restriction enzymes, digestion protocol and polymorphisms obtained for each genotype. α: location in the chromosome of the genome. β: Sequence primers, accoding to Su., et al. [11]. γ: Sizes of the fragments expected according to Su., et al. [11]. Image analyzed with TotalLab 1D software, version 14.0. Pb: base pairs. BE: Buffer CutSmart (Biolabs inc. New England, USA).*

Quality of extracted DNA and analysis of PCR-RFLPs

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, stained with GelRed (Biotium, USA). Electrophoresis was executed at 100 volts for 30 minutes (Power Source FB1000, Fisher Scientific). For PCR amplifications and RFLPs, electrophoresis was performed on 2% agarose gels dissolved in TAE and the size of the amplified size was determined by PCR and RFLP when compared with a 50 bp ladder (FastRuler ™ Low Range DNA, Thermo Scientific) or 1kb (GeneRuler, Plus DNA Ladder, USA). DNA, PCR and RFLPs 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.

Results

DNA detection of *T. gondi* by PCR of the B1 gene

T. gondii DNA was detected in the tissue of mice infected with the different chronic strains, through the amplification of the B1 gene, which gave rise to an amplification of 196 bp. Good performance was observed when amplifying from the tachyzoites RH strain (acute strain) and for strains A and B (fresh tissue), but the yield was considerably lower for strain D, whose DNA was obtained from cuts of tissue FFIP. For the strain D, DNA was detected in the tissues of mice infected with the undiluted inoculum, but not in the tissues of mice infected with 1/10 or 1/100 dilutions, neither in the first round of PCR nor in amplifications (Figure 1).

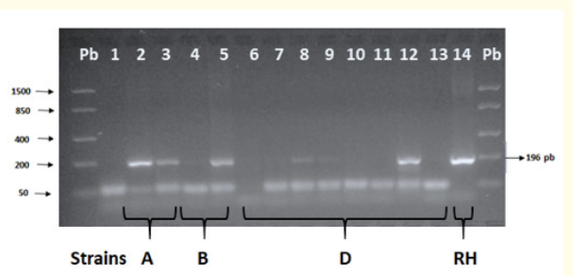

Figure 1: *DNA detection of different T. gondi strains from fresh tissue samples (Strain A and B) or from FFPI tissue (Strain D). Lines: 1. PCR mixing control; 2 and 3: strain A; 4 and 5: strain B; 6-11: strain D; lines 6, 8, 9: undiluted inoculum. 10 and 11: diluted inoculum 1:10; 7. inoculum diluted 1: 100; 12. Re-amplified an undiluted inoculum. 13. Re-amplification of a diluted inoculum 1: 100; 14: strain RH. Pb: 50 bp ladder.*

PCR of the L358, SAG2 and SAG3 genes

The PCR for the different genes gave rise to amplifications with the sizes in expected base pairs (Table 1). For chronic strains A and B and acute (strain RH) good yields were obtained (Figure 2). For strain D it was necessary to carry out re-amplifications of the PCR products in order to observe the amplification with good performance (Figure 3).

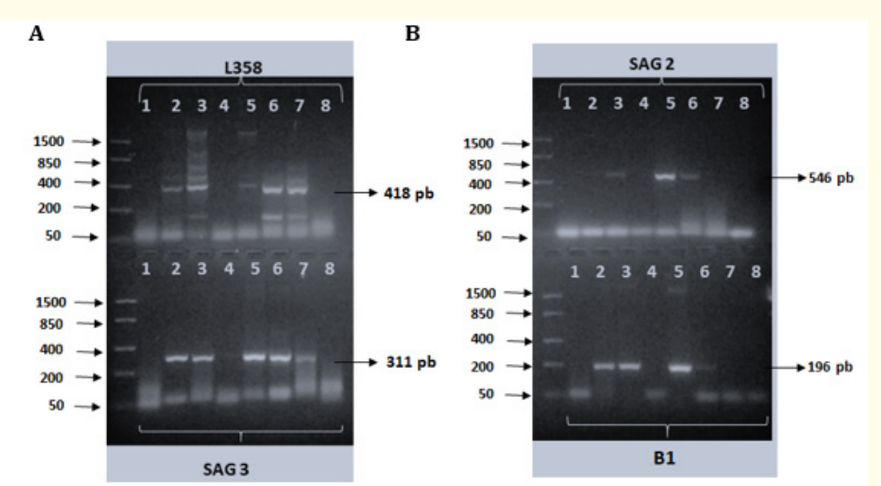

Figure 2: *PCR for the L358, SAG 2, SAG 3 and B1 genes. A. Genes L358 (up) and SAG 3 (down). B. Genes SAG 2 (up) and gene B1 (down). Lines: marker; 1. Control of the mixture; 2. strain A; 3. strain B; 4. strain D; 5. Rh strain; 6. Re-amplified strain A. 7. Re-amplified strain B; 8. Re-amplified strain D.*

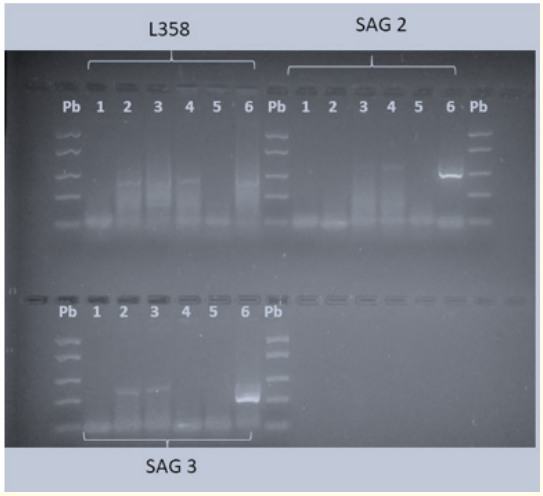

Figure 3: *PCR for the L358, SAG 2, SAG 3 genes from the DNA of T. gondii strain D, obtained from cuts of FFPI tissue. Lines: Pb: 50 bp; 1: control of the PCR mixture; 2 and 3: amplification of DNAs obtained from mice with the inoculum undiluted; 4 and 5: amplification of DNAs obtained from mice with the inoculum diluted 1/10; 6: positive control (DNA of strain RH).*

Obtaining the lineage using RFLP

The polymorphism obtained for genes L358, SAG 2 and SAG 3 confirmed the type I lineage for the RH strain (ATCC) (Figure 4A and Table 2). For strain A, a polymorphism compatible with type I lineage was obtained for the SAG2 and SAG3 genes and a variant polymorphism with the L358 gene (u-1). For strain B, a polymorphism compatible with type III lineage was obtained for the L358 and SAG2 genes and a variant (u-1) when carrying out the PCR RFLP of the SAG3 gene (Figure 4B and Table 2). For strain D an RFLP compatible with type I lineage was obtained, for SAG2 and SAG3 genes, and a variant after digestion L358 gene, but different from that obtained with strain A, so that it was named as u-2 (Figure 5 and Table 2).

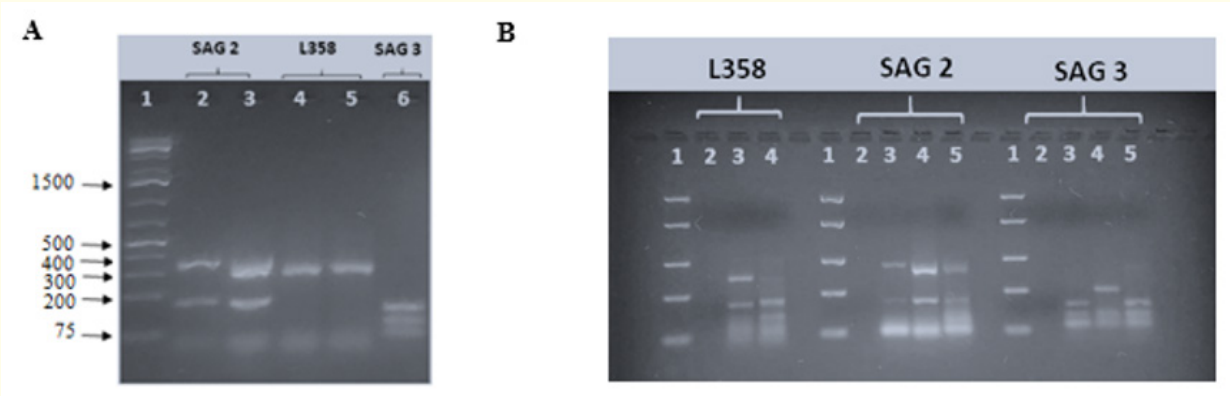

Figure 4: *Genotyping of strain RH, A and B by RFLP. A: RFLP of strain RH. Lines: 1. 1kb marker; 2 and 3. RFLP of the SAG 2 gene; 4 and 5. RFLP of the L358 gene; 6: RFLP of the SAG gene 3. B. RFLP of strain A and B. Lines: 1. 50 bp marker. 2. Control of digestion mixture; 3. strain A; 4. strain B; 5. RH strain.*

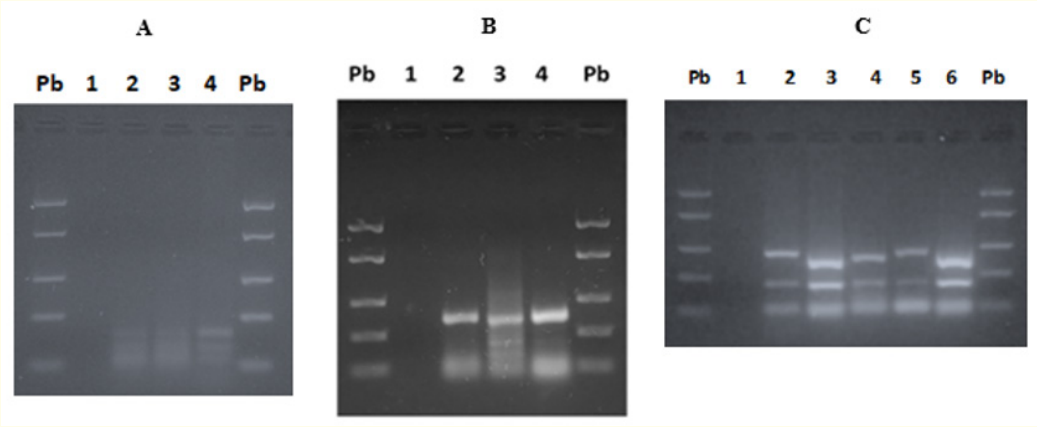

Figure 5: *Gentotyping of strain D by RFLP. A. RFLP of the L358 gene with the enzymes Nae III and Nla III. Lines: Pb: 50 base pair marker; 1: digestion control; 2 and 4: strain RH; 3: strain D; B. RFLP of the SAG3 gene with the Nci I enzyme. Lines: Pb: 50 base pair marker; 1: digestion control; 2: strain D (dilution 1: 1); 3: strain D (dilution 1:10); 4: strain RH; C. RFLP of the SAG2 gene with Hinf I and Taq I. Lines: Pb: 50 base pair marker; 1: digestion control; 2 and 5: strain B; 3: strain A; 4: strain D; 6: strain RH.*

				Genes			
	L358		**SAG2**		**SAG3**		
Identification /Strain	**RFLP (Pb)**	**Lineage**	**RFLP (Pb)**	**Lineage**	**RFLP (Pb)**	**Lineage**	**Lineage definition**
TFC-1/ A	300,157,75	u-1	320, 165	I	133, 94, 56	I	I/u-1
TLW-1/ B	171, 107	III	371, 165	III	198, 89, 56	u-1	III/u-1
TBT-1/ D	247, 160, 75	u-2	320, 165	I	133, 94, 56	I	I/u-2
RH (ATCC)	295	I	320, 165	I	133, 94, 56	I	I

Table 2: *Origin of the strains, identification, polymorphism obtained and genotyping.*

RFLP: Restriction Fragment Length Polymorphism; Pb: base pairs; u: undeterminade variante.

Obtaining the lineage using RFLP

The polymorphism obtained for genes L358, SAG 2 and SAG 3 confirmed the type I lineage for the RH strain (ATCC) (Figure 4A and Table 2). For strain A, a polymorphism compatible with type I lineage was obtained for the SAG2 and SAG3 genes and a variant polymorphism with the L358 gene (u-1). For strain B, a polymorphism compatible with type III lineage was obtained for the L358 and SAG2 genes and a variant (u-1) when carrying out the PCR RFLP of the SAG3 gene (Figure 4B and Table 2). For strain D an RFLP compatible with type I lineage was obtained, for SAG2 and SAG3 genes, and a variant after digestion L358 gene, but different from that obtained with strain A, so that it was named as u-2 (Figure 5 and Table 2).

Discussion

The molecular detection of *T. gondii* through the PCR-nested B1 gene has been frequently used for this purpose [23-25]. The implementation of the final point PCR, standardized in our laboratory [22] reveals the presence of the gene, in both fresh tissues and FFIP, with the inherent limitations to the extracted DNA quality from FFIP, which favors the amplification of DNA segments around 300

bp [17,18]. The detection of *T. gondii* DNA, from FFPI was possible since the B1 gene amplified is below 200 bp (196 bp). In the case of the molecular characterization of *T. gondii* with genetic markers, with larger amplification, the re-amplification strategies used in this study or the use of nested PCRs [7], are necessary to enrich the amplification before digestion with restriction enzymes. The amplification (PCR) and characterization (PCR-RFLP) of *T. gondii* DNA from FFIP will allow retrospective studies, both in animals and in humans. These techniques open a portal for molecular detection and characterization in different biological simples.

The molecular characterization of *T. gondii* linages is essential; since it has been determined that some of the *T. gondii* linages could induce more or less pathological conditions in the infected individuals [26-28]. In regard, the linage type II is the lineage that predominantly causes human toxoplasmosis. However, there are biases between the presentations of the disease and the genotypes of the parasite. For example, atypical type II isolates are more likely to be involved in severe toxoplasmic chorioretinitis in human patients [29], and cause severe disseminated toxoplasmosis in immunocompetent patients [26]. The variation of the clinical presentations or virulence, in the different hosts, is probably due to the different genotypes that may be within the different lineages [27]. it could be explained by the recombination of genes that are produced during the parasite sexual reproduction in the intestine of felines [28].

With the purpose to detect and characterize molecularly the three chronic strains of *T. gondii* by means of PCR-RFLP in mice infected with these, this study determined a similar a linage type I for strains A and D with the SAG2 and SAG3 genes, but with variable polymorphisms for the L358 gene. In the case of strain B, was identified a lineage type III, with SAG2 and L358 genes, and a variability when the polymorphism was performed with SAG3 gene. These results indicate that the characterized chronic strains are not of pure lineages, as has been reported in isolates from other geographical regions [30].

In Costa Rica, Dubey., *et al.* [31] characterized strains of *T. gondii* from chickens (*Gallus domesticus)*. The genotyping of 32 isolates using polymorphisms at the SAG1, SAG2, SAG3, BTUB and GRA6 loci, using PCR-RFLP, revealed five genotypes. Five isolates had type I alleles and one isolate had type III alleles at all loci. The remaining 26 isolates contained the combination of alleles of type I and II or I and III, and were divided into three genotypes and did not find genotype II in any of the five loci investigated. Unlike the studies of Dubey., *et al*. [31]; genotype II was not achieved in the present study, and it was observed that the strains studied are not pure or clonal strains. This confirms that the strains studied like the majority of those studied by Dubey., *et al.* [31], possess genetic variability.

Some studies conducted in South America have shown similar results to those obtained by this work. A study from 18 chicks bred free in the northwestern state of Paraná in Brazil, characterized by PCR-RFLP with 10 genetic markers that included SAG1, SAG2 (5'-3'SAG2, alt SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico. It showed a high genetic diversity [32] when comparing the results obtained with the genotypes included in the Toxo Plasma Database (TOXODB) [33].

Studies where only the SAG2 locus was analyzed, demonstrated the presence of linages type I and type III, from isolates of cats in Brazil [34]. While the genotyping of 13 isolates of *T. gondii* a from chicken, using the SAG2 locus, indicated that seven isolates were of type I and six that were of type III; three of these type III isolates killed all infected mice, suggesting that all virulent strains for mice are not only type I [35]. In Argentina, the genotyping of chicken isolates of *T. gondii* using the SAG2 locus indicated that one was type I, one was type II and seven were type III [36]. The results of these studies are similar to our study. In this work, only Type I and III lineages were found when analyzing the SAG2 gene, but by analyzing two more genes we were able to define that the strains analyzed by us do not have a pure lineage.

In other latitudes, the appearance of *T. gondii* has been observed in pigs and wild boars raised in different production systems in the Czech Republic. The genotyping of SAG2 locus amplified for type II suggests the presence of a clonal genotype circulating in these animals [37]. In Serbia, viable parasites were isolated from two mares; both isolates of parasites were *T. gondii* type III and showed a greater lethality for mice with successive passages [38]. These results demonstrate a great variability between toxoplasma lineages from different geographical areas and hosts.

Studies carried out in some countries of the America and Europe show that molecular characterization by PCR-RFLP allows the identification of the most predominant lineages in animals (type I and III), which may be responsible for the transmission of *T. gondii* to humans, for the consumption of raw or undercooked meat or its derived products. Therefore, the outcome from our study coincides with those studies made in other latitudes.

Conclusion

Three chronic strains of *T. gondii* were detected and characterized, from tissue fresh or FFIP, of the infected mice. The detection of *T. gondii* DNA, from FFPI was possible since the B1 gene amplified is below 200 bp (196 bp), but with genetic markers for molecular characterization, re-amplifications were necessary to obtain a higher performance amplification. Strains A and D were characterized as type I linages and strain B of type III linage, but none of the three strains showed pure lineages with the genetic markers used. The detection and molecular characterization of the chronics strains of *T. gondii* will contribute to the scientific community of Costa Rica, since it will have the molecularly characterized strains, necessary for defining different epidemiological and diagnoses studies in both humans and animals.

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