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Avanços da pesquisa e inovação e do empreendedorismo em medicina veterinária

Alécio Matos Pereira
Davy Frazão Lima
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Atena Editora
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Telefone: +55 (42) 3323-5493
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APRESENTAÇÃO

A produção vegetal e animal supre uma necessidade básica para a sobrevivência da espécie humana, a alimentação. A busca por uma produção sustentável, que vise o bem-estar animal e alta produtividade animal e vegetal requer um desenvolvimento técnico-científico especializado nas áreas zootécnicas, veterinárias e agronômicas.

Essas pesquisas complementam o conhecimento do corpo acadêmico, profissionais e estudantes das ciências agrárias, dando suporte para a tomada de decisões no manejo alimentar, no tratamento e prevenção de doenças e no controle de qualidade desses alimentos.

Este livro demonstra profundamente os diversos assuntos pertinentes a produção animal, bem como doenças que podem afetar seu bem-estar e/ou potencial produtivo da espécie. Os parâmetros produtivos, reprodutivos e comportamentais dos animais são abordados por especialistas renomados nas mais diversas áreas da ciência animal de forma clara e objetiva.

O livro possui 9 capítulos sendo estes baseados de diversos trabalhos científicos, levando sempre em consideração os aspectos pedagógicos, técnicos e científicos com o objetivo de oportunizar uma melhor compreensão dos profissionais das ciências agrarias.

Esse livro vem com intuito é agregar e atualizar os conhecimentos dos estudantes e profissionais dos cursos de Medicina Veterinária e Zootecnia para auxiliar na tomada de decisões na clínica animal e produção animal. Boa leitura!

Alécio Matos Pereira
Davy Frazão Lima

SUMÁRIO

CAPÍTULO 2

DETECTION OF *Toxocara canis* DNA IN TISSUES OF EXPERIMENTALLY INFECTED MICE

Data de aceite: 01/02/2022

Micaele Quintana de Moura
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Marcia Raquel Pegoraro de Macedo
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Wesley Douglas da Silva Terto
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Luciana Farias da Costa Avila
Faculdade de Medicina (FAMED) da Universidade Federal do Rio Grande (FURG)
Rio Grande, Brazil

Fabio Pereira Leivas Leite
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Carlos Jaime Scaini
Faculdade de Medicina (FAMED) da Universidade Federal do Rio Grande (FURG)
Rio Grande, Brazil

Natália Berne Pinto
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Gabriela de Almeida Capella
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Adriane Leites Strothmann
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Marcos Marreiro Villela
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

Maria Elisabeth Aires Berne
Instituto de Biologia, Universidade Federal de Pelotas (UFPEL)
Pelotas, Brazil

ABSTRACT: The main etiological agent of toxocariasis is the helminth *Toxocara canis*. Several difficulties are found in the diagnosis of this disease, because of nonspecific clinical signs and possible cross-reactions that may occur in the available test, the indirect ELISA. Therefore, molecular diagnosis has been indicated as an alternative to conventional diagnosis. The purpose of this study was to evaluate the polymerase chain reaction (PCR) technique for the identification of *T. canis* in tissues of experimentally infected mice. To this end, nine mice were inoculated with 1500 embryonated eggs and were divided into two groups, the first euthanized 48 hours (G1) and the other 30 days post inoculation (G2). Lungs, brain, liver and blood were collected from all the animals for DNA Extraction and tissue digestion,

also was collected blood samples for DNA extraction and ELISA test (serum). *Toxocara canis* DNA was identified in all the inoculated animals using the ITS-2 target gene. The PCR test successfully identified the parasite in the brain, lung and liver of the animals euthanized 48 h PI and 30 days PI. This technique yielded good results in the identification of the parasite in the brain, being more sensitive than the method for the recovery of larvae, in the group with acute infection (48 h PI). The infection was confirmed by PCR within 48 h after infection, while the ELISA indicated serological conversion occurred only 14 days after inoculation. This study demonstrates the ability of PCR to identify *T. canis* in the liver, lungs and brain during acute and chronic infection.
KEYWORDS: Toxocariasis, Brain, PCR, Diagnosis.

RESUMO: O principal agente etiológico da toxocaríase é o helminto Toxocara canis. Diversas dificuldades são encontradas no diagnóstico desta doença, devido a sinais clínicos inespecíficos e possíveis reações cruzadas que podem ocorrer no teste disponível, o ELISA indireto. Portanto, o diagnóstico molecular tem sido indicado como uma alternativa ao diagnóstico convencional. O objetivo deste estudo foi avaliar a técnica de reação em cadeia da polimerase (PCR) para a identificação de T. canis em tecidos de camundongos experimentalmente infectados. Para tanto, nove camundongos foram inoculados com 1500 ovos embrionados e divididos em dois grupos, o primeiro eutanasiado 48 horas (G1) e o outro 30 dias pós-inoculação (G2). Pulmões, cérebro, fígado e sangue foram coletados de todos os animais para extração de DNA e digestão de tecidos, também foram coletadas amostras de sangue para extração de DNA e teste ELISA (soro). O DNA de Toxocara canis foi identificado em todos os animais inoculados usando o gene alvo ITS-2. O teste de PCR identificou com sucesso o parasita no cérebro, pulmão e fígado dos animais eutanasiados 48 h PI e 30 dias PI. Essa técnica rendeu bons resultados na identificação do parasita no cérebro, sendo mais sensível que o método de recuperação de larvas, no grupo com infecção aguda (48 h PI). A infecção foi confirmada por PCR em até 48 h após a infecção, enquanto o ELISA indicou que a conversão sorológica ocorreu apenas 14 dias após a inoculação. Este estudo demonstra a capacidade da PCR para identificar T. canis no fígado, pulmões e cérebro durante a infecção aguda e crônica.
PALAVRAS-CHAVE: Toxocaríase, Cérebro, PCR, Diagnóstico.

- The PCR technique was evaluated to identify *Toxocara canis* DNA in tissues of experimentally infected mice.
- All inoculated animals had the infection confirmed by PCR during acute and chronic infection.
- The identification of *T. canis* in the brain was promising.

1 | INTRODUCTION

Toxocariasis is a zoonotic disease (Abdi & Sayehmiri, 2012) whose main etiological agent is *Toxocara canis* (Fu et al., 2014; Lee et al., 2010), but it can also be caused by *Toxocara cati* (Hossack et al., 2008). The definitive hosts of *T. canis* and *T. cati* nematodes

are dogs and cats, respectively, in which the adult forms are located in the small intestine (Despommier, 2003).

In non-preferential hosts, such as humans, the larval form of this parasite migrates through the tissues, causing various clinical conditions. Depending on the organ in which the larvae are found, the disease can be characterized as visceral, neurological or ocular toxocariasis, and covert or asymptomatic toxocariasis may occur (Rubinsky-Elefant et al., 2017; Deshayes et al., 2016; Kuenzliet al., 2016; Ranasuriya et al., 2014; Raffray et al., 2013).

Toxocariasis is difficult to diagnose because it lacks specific symptoms and produces a variety of clinical manifestations (Moreira et al., 2014; Fillaux & Magnaval, 2013). Today, serological methods are the techniques normally used for the laboratory diagnosis of human toxocariasis, while the test with the greatest specificity is indirect ELISA, which uses excretory antigens and L3 larvae secretion (TES), confirmed by Western blotting (Deshayes et al., 2016; Moreira et al., 2014; Schoenardie et al., 2013). However, immunological cross reactions may occur between *Toxocara* spp. and other helminths, such as *Ascaris* spp. and *Fasciola hepatica* (Jinet al., 2013; Hotez & Wilkins, 2009; Romasanta et al., 2003). Studies with recombinant antigens are promising, but the diagnostic protocols still indicate the use of the TES as standard (Varghese et al., 2017; Zahabium et al., 2015).

In addition to barriers related to cross-reactions, antibodies may not be detected in the patient's serum (Watthanakulpanich, 2010), as in ocular toxocariasis, in which antibody titers are typically low (Morais et al., 2012; Sharkey & McKay, 1993). Moreover, available ELISA tests using crude TES cannot differentiate between the two species that are agents of toxocariasis (Zibaei et al., 2016; Rubinsky-Elefantet al., 2010).

Several studies have demonstrated the diagnostic capacity of molecular techniques, based on DNA amplification of parasites from different types of biological samples, such as *Ancylostoma caninum*, *Ancylostoma braziliense* and *Baylisascaris transfuga* isolated from feces (Gawor et al., 2017; Oliveira-Arbex et al., 2016;), *Trichinella* spp. in samples of muscle (Marucci et al., 2013) and *Angiostrongylus vasorum, Dirofilaria immitis* and *Dirofilaria repens* from blood samples (Albonico et al., 2013; Jefferies et al., 2009).

The identification of *Toxocara* spp. via molecular techniques, and its differentiation from other *Ascaris* species whose larvae can also migrate through the body's tissues, have contributed to studies on the diagnosis and control of these parasites (Gasser et al., 2013; Zibaei et al., 2013; Chen et al., 2012; Pinelli et al., 2013; Borecka et al., 2008).

In this context, this study focused on identify *T. canis* DNA in different tissues of experimentally infected mice, in order to diagnose toxocariasis.

2 | MATERIAL AND METHODS

A total of 11 female Swiss albino mice (*Mus musculus*), 4 to 8 weeks old, were used

in this study. The animals were kept under controlled temperature in a 12-12 hour light-dark cycle, with free access to food and water.

From natural *T. canis* infected dog (six to eight weeks of age), Pirantel Pamoate (15 mg/kg) was administered orally, for recovery of adult forms. The females were subjected to hysterectomy, and their eggs were incubated for 30 days in 2% formalin, under 80% relative humidity and daily aeration. Nine mice were inoculated with 1500 embryonated eggs by means of gastric gavage. The mice were divided into 2 groups: G1, comprising 5 animals, were euthanized 48 h post inoculation (PI), and G2, comprising 4 mice, were euthanized 30 days PI. A control group of two animals that were not inoculated (negative control) was also maintained.

Whole blood samples with EDTA were collected from G1 immediately before euthanasia, while from G2 these samples were collected 0, 7, 14, 21 and 28 days after inoculation with *T. canis* embryonated eggs. The blood samples from G1 were used solely for molecular analysis, while those from G2 were collected in two tubes, one for molecular analysis (EDTA whole blood) and the other for serological (serum) analysis.

The two infected groups (G1 and G2) were necropsied and their brains, lungs and livers were removed. Each organ was macerated separately and weighed in 5 fractions (quintuples) of 10mg to 20mg, for subsequent extraction of genomic DNA. The remainder of each organ was subjected to tissue digestion to count the number of *T. canis* larvae (Wang & Luo, 1998). The study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Pelotas (CEEA – 7921).

2.1 DNA extraction

All the quintuple samples of brain, lungs, and liver, as well as the blood samples, were frozen to -20ºC for at least 24 h prior to initiating the DNA extraction procedure.

DNA extraction was performed using a commercial Wizard Genomic DNA Purification Kit (Promega®), with modifications. The samples were incubated at 65ºC for 3 h under shaking at 70 rpm/m (except for blood samples, which were held for 1 h). The protein precipitation step was extended to 1 h, and the samples were stored overnight in isopropanol at -20ºC. The other procedures were performed as recommended by the manufacturer.

2.2 PCR procedure

Primers (F: AGTATGATGGGCGCGCCAAT and R: TTAGTTTCTTTTCCTCCGCT) designed by Jacobs et al. (1997) were used for DNA amplification of the ITS-2 gene. Each PCR reaction was performed with 25 µL of Master Mix 2X (Promega®), 1.5 of each primer (20 µM), 300 ng of genomic DNA, and DNase/RNase free water (Promega®) to complete one volume of 50 µL. The temperatures employed were as follows: initial denaturation at 95 °C (5 min), followed by 35 cycles of denaturation at 95 ºC (30 s), annealing at 55 ºC (30 s), extension at 72 °C (30 s), and final extension at 72 °C (7 min), and cooling to 4 °C. Liver

samples were diluted to a concentration of 1: 5 and lungs to 1: 2, while brain samples were not diluted. DNA extracted from 500 larvae of *T. canis* (L3) was used as positive control for PCR, while DNA extracted from the tissues (brain, lungs, liver and blood) of uninfected mice served as negative control. Electrophoresis was performed with a final volume of 6µL on 2% agarose gel and was observed in an UV transilluminator. An organ was considered positive for *T. canis* infection when an amplicon was observed in at least one of the quintuple samples.

2.3 Sequencing

Positive samples (one from each tissue, in the two different groups), were subjected to a new PCR reaction to increase the amount of DNA of interest. They were then purified using a commercial GFX® PCR DNA and Gel Band Purification kit, and sent to Macrogen®, a company specializing in genetic sequencing. The sequences thus obtained were analyzed using ContigExpress® and Mega7® sequence editing software.

2.4 Serological testing

The serological study involved only group G2 and the negative controls. To investigate class G immunoglobulins (anti-*Toxocara*), indirect ELISA was performed using secreted and excreted antigen (TES), at a concentration of 1 µg/ mL and a serum dilution of (1:50), as described by Avila et al. (2011). The serum samples were examined in duplicate and reading was carried out at a wavelength of 492 nm. Data analysis was performed by Student's *t*-test, considering p value of <0.05.

3 | RESULTS

Toxocara canis infection was successfully confirmed in all the tested animals by PCR (Table 1). Amplified *T. canis* DNA was detected in the three analyzed tissues, i.e., brain, lungs and liver in G1 (table 1), as well as in the positive controls. Amplification was absent in the negative controls (tissues of uninoculated controls), indicating that only amplification of the DNA of interest occurred. However, no amplification occurred in any of the blood samples of the two tested groups.

As can be seen in Table 1, *T. canis* DNA was detected in the brain of three mice in G1; in one of these three brains it was not possible to identify larvae by the technique of tissue digestion, and the other two contained only one larva each. The two animals in whose brains no amplification was visualized (PCR negative) also contained no larvae. In G2, infection of the brain was confirmed by PCR in all the animals (Table 1).

The presence of the parasite was identified through PCR in the liver of all animals of groups G1 and G2. However, in one animal of G2 group showed no DNA amplification in the lungs, despite the presence of two larvae in this organ (Table 1).

In the experimentally infected animals, *T. canis* infection was confirmed by PCR 48

h post infection (Table 1). In contrast, in the ELISA using TES, a statistical difference was found in the kinetics of immunoglobulin production among infected animals and controls only 14 days after infection (Figure 1).

	PCR result / Number of recovered larvae								
	G1 (48 h Post inoculation)					G2 (30 days Post inoculation)			
Identification of mice	A1	A2	A3	A4	A5	B1	B2	B3	B4
Brain	+ / 1	+ / 0	- / 0	- / 0	+ / 1	+ / 107	+ / 157	+ / 86	+ / 20
Lungs	+ / 38	+ / 25	+ / 8	+ / 16	+ / 24	+ / 5	+ / 2	+ / 3	- / 2
Liver	+ / 135	+ / 72	+ / 77	+ / 75	+ / 45	+ / 3	+ / 2	+ / 26	+ / 1

Table 1: PCR results for the determination of *T. canis* infection and number of larvae retrieved from experimentally infected mice.

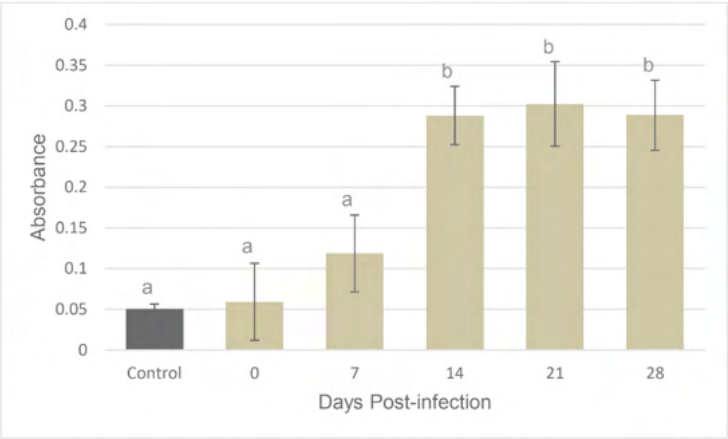

Figure 1: Kinetics of immunoglobulin (IgG) production against *Toxocara canis* in mice experimentally inoculated with 1500 *T. canis* eggs on days 0, 7, 14, 21 and 28 post infection. Different letters indicate a significant difference (<0.05%).

The alignment of the sequences obtained from the PCR/ITS-2 with the *T. canis* sequence obtained from GenBank (Y09489.1) demonstrates the correct amplification and DNA of *T. canis* in the tissues of the animals tested (Table 2).

Toxocara canis	A	T	T	C	G	G	T	G	A	G	C	T	A	T	G	C	T	G	G	T	G	T	G	G	[24]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[24]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[24]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[24]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	[24]
Toxocara canis	T	A	A	T	G	G	A	T	A	T	T	G	T	G	C	A	A	T	T	G	T	A	C	A	[48]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[48]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[48]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[48]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[48]
Toxocara canis	G	C	G	T	A	C	C	T	T	G	C	C	A	A	G	G	A	A	A	T	A	T	T	C	[72]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[72]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[72]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[72]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[72]
Toxocara canis	G	C	A	C	A	A	G	A	A	A	T	G	G	C	T	G	T	C	G	T	T	T	G	C	[96]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[96]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[96]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[96]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[96]
Toxocara canis	T	C	G	T	A	A	A	G	A	G	G	C	A	A	A	A	T	T	G	G	C	C	A	T	[120]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[120]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[120]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[120]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[120]
Toxocara canis	G	A	G	T	G	T	A	T	G	T	T	G	C	G	T	T	G	C	T	T	C	A	C	G	[144]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[144]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[144]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[144]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[144]
Toxocara canis	A	T	A	C	G	G	C	C	T	C	C	A	G	C	A	A	A	C	G	T	T	G	T	T	[168]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[168]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[168]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[168]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[168]
Toxocara canis	T	A	T	T	G	T	T	T	G	G	T	T	G	T	G	G	C	A	G	C	A	T	C	C	[192]

T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[192]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[192]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[192]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[192]
Toxocara canis	A	G	G	T	T	G	G	A	G	G	T	G	G	C	G	T	T	A	T	C	G	G	T	C	[216]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[216]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[216]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[216]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[216]
Toxocara canis	G	C	T	T	G	A	A	T	G	A	G	G	A	A	T	G	C	A	T	G	G	C	G	A	[240]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[240]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[240]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[240]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	[240]
Toxocara canis	A	T	G	G	T	T	G	A	A	A	T	G	A	G	A	T	T	T	T	-	-	-	-	-	[264]
T. canis (Larvae)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	[264]
Brain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	[264]
Lungs	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	[264]
Liver	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	T	T	[264]
Toxocara canis	-	-	-																						[267]
T. canis (Larvae)	T	T	T																						[267]
Brain	T	T	T																						[267]
Lungs	T	T	T																						[267]
Liver	T	T	T																						[267]

Dots (.) represent corresponding nucleotides, divergences are represented by letters, and dashes (-) signify gaps.

Table 2: DNA alignment of *Toxocara canis* (GenBank– Y09489.1) sequences with sequences derived from PCR products (ITS-2) isolated 500 *T. canis* larvae and from brain, lung and liver tissue of mice inoculated with 1500 eggs, confirming the experimental infection by *T. canis*.

4 I DISCUSSION

Polymerase chain reaction (PCR) procedures have been useful in the identification and differentiation of *Toxocara* spp. (Durant et al., 2012). In this study, using the ITS-2 of the rDNA, we identified *T. canis* in tissues of mice infected with this nematode, and the DNA of interest was detected in three of the four tested tissues, during both the acute and chronic infection. In this study was possible to identify by PCR the presence of the larvae of *T. canis* even in very low amounts (Table 1). Noteworthy, that identification, by molecular biology, of parasite DNA in the brain during the acute infection, where the presence of larvae is typically

low, as well as in the viscera during chronic infection has been reported (Strube et al., 2013; Kolbeková et al., 2011; Hamilton et al., 2006).

The performance of the PCR technique in the evaluation of the brain was very promising; in fact, the identification of the parasite was more sensitive than the tissue digestion technique, in the conditions tested. In this study, infection in the brain of one of the animals was detectable only by PCR, given that the tissue digestion technique yielded negative results.

The identification of *T. canis* larvae in the brain is corroborated by Zibaei et al., (2017) that have identified *Toxocara* spp. larvae in brain of naturally infected chickens, however, in the study conducted by these authors the larvae were previously isolated from the tissue for subsequent molecular identification. In the present study the DNA search of the parasite occurred together to the encephalic tissue of the animals, dispensing the previous stage to DNA extraction. Previous studies with other parasites have also been able to identify parasitic DNA in the brain of birds (*Streptopelia semitorquata*, *S. senegalensis*, *Tockus leucomelas*), raccoon dogs (*Nyctereutes procyonoides*), and murids (Montazeri et al., 2016; Zhou et al., 2017; Lukásová et al., 2018)., demonstrating that this type of technique may be useful for the identification of parasites in this tissue.

Molecular evaluation has also been used to confirm *Schistosoma haematobium* infection in the brain of a human patient with inconclusive serological results, from biopsy of the parasitic cyst (Imai et al., 2011).

Despite promising results in this study, been able to identify the parasite DNA from different tissues, *T. canis* DNA could not be identified in the whole blood of the animals, contrary to what has been described previously for other parasites such as *Angiostrongylus vasorum,* Dirofilaria immitis and Dirofilaria repens in blood samples from dogs (Albonico et al., 2013; Jefferies et al., 2009) and *Brugia malayi*, *Dirofilaria immitis*, *Dirofilaria repens* and *Acanthocheilonema reconditum* in blood samples from cats (Wongkamchai et al., 2014). We believe that the difference in results is due to the life cycle of these parasites, given that *T. canis* has no free-living phase in the blood, and reaches the circulation only when migration through the tissues occurs (Despommier, 2003).

We were able to identify the parasite's DNA from the liver of all the animals tested, however, only in one animal it was not possible to identify *T. canis* DNA by PCR in the lungs (table 1). Borecka et al. (2008) reported similar results in the amplification of *T. canis* DNA in gerbil liver (*Meriones unguiculatus*). Moreover, in Borecka et al.'s study (2008), the first analysis was performed three days and the last one 14 days post infection. Conversely, our study demonstrated that molecular identification was possible in just 48 h, and that it remained positive during the chronic phase of the disease, 30 days post inoculation.

Amplification of *T. canis* DNA in lung tissue may also be diagnostically advantageous, since this organ is usually affected by this disease (Lee et al., 2015; Mazur-Melewska et al.,

2015; Walsh & Haseeb, 2014). The results presented here are similar to those of Pinelli et al., (2013), that recovered *T. canis* from three out of six lungs whose molecular diagnosis was negative. In our study, only one animal with confirmed larvae in the lungs was not positive by PCR, an explanation for this is that probably none of the of the quintuplicate's obtained from this organ presented larva or fragments of larva.

In a study by Ishiwata et al. (2004) to identify the nematode responsible for visceral larva migrans (VLM) in a human patient, the possible source of infection (domestic turkeys) was isolated and a molecular study of the nematode was carried out on the livers of these animals, which revealed that the agent was *T. canis*. It should be noted that the serological evaluation had erroneously identified the agent as *Ascaris suum*. The identification of *T. canis* DNA in the liver of different host species confirms the feasibility of using PCR for the diagnosis of this nematode.

Laboratory analyses for the diagnosis of toxocariasis are still the focus of study in the search for a completely effective method (Varghese et al., 2017; Deshayes et al., 2016; Moreira et al., 2014; Watthanakulpanich, 2010). Application of recombinant TES is useful (Zahabium et al., 2015), but the method most commonly employed is TES ELISA, which presents cross-reactions because it uses crude antigen from the parasite (Jin et al., 2013; Hotez & Wilkins, 2009; Romasanta et al., 2003). Therefore, molecular techniques have been increasingly used as an alternative for the diagnosis of *T. canis* in different hosts (Pinelli et al., 2013; Marucci et al., 2013; Zibaei et al., 2013; Borecka et al., 2008; Ishiwata et al., 2004).

In this study, the infection of all the animals was confirmed by PCR technique within 48 h after inoculation, whereas the ELISA required 14 days for the serological conversion. This finding is corroborated by Zibaei et al. (2013), who obtained better results in the PCR diagnosis of ocular larva migrans (OLM) in experimentally infected gerbils than by histopathology and serology. Assis et al. (2010) also found that PCR was more sensitive than ELISA in the diagnosis of visceral leishmaniasis in dogs.

To date, toxocariasis is diagnosed by immunodiagnostic methods (specific IgG screening) associated with clinical signs, eosinophil counts and IgE levels, and epidemiological data (Watthanakulpanich, 2010). ELISA is the most widely used test. A positive serological test may be confirmatory, but a negative serological test cannot be used to rule out toxocariasis due to the low titration of antibodies typical of some forms of the disease, as in the case of OLM (Rubinsky-Elefant et al., 2017; Morais et al., 2012; Sharkey & McKay, 1993).

Another advantage of molecular techniques is the possibility of identifying the *Toxocara* species involved (Fogt-Wyrwas et al., 2007; Borecka et al., 2004), given that the ELISA cannot distinguish *T. canis* from *T. cati* because of the TES antigens two species share (Rubinsky-Elefantet al., 2010). Although most pathology records on humans refer to the action of *T. canis* (Fu et al., 2014; Lee et al., 2010), there are also cases involving *T.*

cati (Hossack et al., 2008), whose importance in the occurrence of visceral and ocular larva *migrans* may be underestimated because of the difficulty of differentiating between *T. cati* and *T. canis* larvae (Rubinsky-Elefant et al., 2010; Fisher, 2003).

Using conventional PCR, Marucci et al. (2013) diagnosed *T. canis* DNA isolated from the muscle of domestic pigs collected during a routine meat inspection in a slaughterhouse, as well as *T. cati* DNA from the muscle of wild birds. Added to this, Van De et al., (2013) performed confirmation of ocular toxocariasis in a patient who had the parasitic cyst removed surgically. Based on their findings, the authors stated that it is feasible to use molecular techniques for the specific diagnosis of *Toxocara* infection.

The identification of larvae by PCR in all the animals of this study corroborate with previous studies that indicate to the promising potential of the use of molecular biology for identification from larval structures such as those found, for example, in biopsies or fragments of organs from surgery or necropsies (Van De et al., 2013; Imai et al., 2011), but this approach is not currently used in practice (Fillaux & Magnaval, 2013).

5 I CONCLUSION

This study demonstrated the ability of the PCR technique to identify *T. canis* (DNA) in the liver, lung and brain of experimentally infected mice, in the acute and chronic infection. The two most promising tissues for this type of evaluation were liver and brain, in the liver all animals were positive in the two evaluated periods (48 h PI and 30 DPI), and in the brain in the animals that were negative by PCR also not observed presence of larvae in the tissue. It was possible to identify larvae by PCR in all animals 48 h after infection, whereas by ELISA the diagnosis was only possible 14 days after infection. Thus, the results obtained in this study suggest that this type of test can be used to epidemiological studies of the environment and paratenic hosts.

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DISCLOSURES

The authors declare no conflict of interest.

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ÍNDICE REMISSIVO

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