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Visceral larva migrans detection using PCR–RFLP in BALB/c mice infected with *Toxocara canis*

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Research Paper

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Abstract

Toxocara canis is an important zoonotic roundworm distributed worldwide. The infective larvae of *T. canis* are one of the causes of visceral larva migrans (VLM), a clinical syndrome in humans. Diagnosing VLM is difficult, and the differential diagnosis of the larval development stage is limited. Therefore, this experimental research aimed to diagnose *T. canis* larvae using a molecular method, not only in liver tissue, which is the most commonly affected tissue, but also in the limb muscles, lungs and brain tissues. For this purpose, 24 BALB/c mice were infected with 1000 embryonated *T. canis* eggs. Necropsies were performed on the second, fourth, seventh and 14th days post-infection. While a part of the samples were digested with pepsin-HCl, the molecular method was used for the remainder of the samples to replicate the mitochondrial DNA adenosine triphosphate (ATP) synthase subunit-6 gene region of *T. canis*. BbsI, a restriction endonuclease, was used to determine the specificity of the amplicons obtained from Polymerase chain reaction (PCR). The detection limit for embryonated eggs was recorded. The PCR results showed that the sensitivity of the PCR analysis was 83.3% in the liver (with 88.8% accuracy), 87.5% in the lungs (with 91.6% accuracy) and 75.0% in the brain, forelimb and hindlimb muscles (with 83.3% accuracy). In all tissues, the test specificity was determined to be 100%. In this study, the molecular method was applied to only experimentally infected BALB/c mice tissues; thus, it is suggested that it can be also employed in different paratenic hosts and materials possibly infected with *T. canis*.

Introduction

Toxocara canis is an important gastrointestinal helminth found in the small intestine of dogs and foxes (Kassai, 1999; Schnieder *et al*., 2011). This helminth has a global distribution, which is investigated with necropsy and faecal examinations in the host. Humans are also paratenic hosts for *T. canis*. In humans, contamination starts with ingesting the embryonated eggs of *T. canis* by accident (which can be found in contaminated playgrounds and parks, in raw vegetables and fruits, in paratenic hosts, such as earthworms and rabbits, etc., and in cat or dog hair); then, the embryonated eggs enter the intestines (L_3 release) and migrate into the organs (Kassai, 1999; Despommier, 2003; Smith *et al*., 2009; Lee *et al*., 2010; Schnieder *et al*., 2011). According to the severity of the infection, toxocariasis is clinically classified as visceral larva migrans (VLM), neurological toxocariasis (NT), ocular larva migrans (OLM) or covert toxocariasis (Strube *et al*., 2013). The diagnosis of VLM is difficult, and the differential diagnosis of the larval development stage is limited. Furthermore, since most cases are rather mild and transient, a case of VLM might be overlooked or misdiagnosed (Roberts & Janovy, 2006).

Today, serological tests (e.g. TES-ELISA and TES-WB) are used in the routine diagnosis of toxocariasis in humans (Smith & Noordin, 2006; Li *et al*., 2007). The results of human toxocariasis seroprevalence studies show the following prevalence ratios: 5.1% in the US (Berrett *et al*., 2017); 50.6% in southern Brazil (Schoenardie *et al*., 2013) and 63.6% in north-eastern Brazil (Silva *et al*., 2017); 38.33% in Argentina (Archelli *et al*., 2014); 15.3% in western Slovakia (Boldis *et al*., 2015); 14.5% in Poland (Jarosz *et al*., 2010); 7.7% in Egypt (Ei-Shazly *et al*., 2013); 44.6% in Swaziland, southern Africa (Liao *et al*., 2010) and 59.9% in Gabon, central Africa (Lötsch *et al*., 2016); 19.27% in Shandong and Jilin Provinces, China (Cong *et al*., 2015); and 2–45.9% in Turkey (Akyol *et al*., 2007; Kustimur *et al*., 2007; Kaplan *et al*., 2008).

However, these serological tests cannot distinguish which species of parasite (e.g. *T. canis* and *T. cati*) have caused this syndrome. In patients with multiparasitism, cross-reactions may be observed. For this reason, it is necessary to use molecular techniques to identify the aetiology and causative agents of human toxocariasis (Gasser *et al*., 2006; Smith & Noordin, 2006; Li *et al*., 2007).

Due to the promising molecular studies that have been carried out in this subject, the following study was devised to examine this topic further. VLM was created with embryonated

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Table 1. Results of pepsin-HCl digestion and PCR reactions during the necropsy days.

		Tissue samples														
		Liver			Lungs			Brain			Forelimbs			Hindlimbs		
Days of necropsy	Sample number	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
2	I-1	370	97	+	70	3	+	170	–	–	400	–	+	630	–	–
2	I-2	520	271	+	50	6	+	140	–	–	280	–	–	560	–	–
2	I-3	690	152	+	120	8	+	140	–	–	550	–	–	660	–	–
2	I-4	460	193	+	60	6	–	160	–	–	500	–	–	730	–	–
2	I-5	440	97	+	80	1	–	130	–	–	250	–	–	500	–	–
2	I-6	470	350	+	40	13	+	180	–	–	550	–	–	740	–	–
2	N-1	410	–	–	20	–	–	100	–	–	600	–	–	120[a]	–	–
2	N-2	360	–	–	50	–	–	120	–	–	240	–	–	620	–	–
2	N-3	350	–	–	30	–	–	60	–	–	580	–	–	700	–	–
4	I-7	460	65	+	250	121	+	220	33	+	450	10	+	780	2	+
4	I-8	590	83	+	360	137	+	150	47	+	530	2	+	750	–	+
4	I-9	350	40	+	230	164	+	110	47	+	540	3	+	660	1	+
4	I-10	470	75	+	150	168	+	150	34	+	520	1	–	650	–	+
4	I-11	230	55	+	280	128	+	120	67	+	470	12	+	600	2	+
4	I-12	510	75	+	240	99	+	130	38	+	600	–	+	1150	–	+
4	N-4	330	–	–	10	–	–	80	–	–	450	–	–	540	–	–
4	N-5	410	–	–	20	–	–	150	–	–	450	–	–	820	–	–
4–	N-6	310	–	–	10	–	–	50	–	–	420	–	–	660	–	–
7	I-13	400	12	–	160	16	+	60	22	+	400	38	+	490	10	+
7	I-14	270	10	+	170	37	+	140	72	+	400	32	+	590	14	+
7	I-15	180	27	+	200	48	+	90	75	+	190	38	+	530	16	+
7	I-16	150	46	+	180	70	+	80	85	+	350	30	+	570	4	+
7	I-17	240	7	+	110	24	+	70	19	+	420	13	+	640	6	+
7	I-18	340	11	+	320	31	+	140	56	+	300	7	+	830	–	+
7	N-7	500	–	–	100	–	–	110	–	–	380	–	–	790	–	–
7	N-8	320	–	–	60	–	–	140	–	–	340	–	–	500	–	–
7	N-9	420	–	–	40	–	–	120	–	–	230	–	–	530	–	–
14	I-19	450	8	+	80	2	+	120	1	+	510	4	+	640	9	+
14	I-20	470	3	–	00	6	–	140	35	+	490	13	+	770	14	+

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14	I-21	390	7	–	140	8	+	110	52	+	430	23	+	450	16	+
14	I-22	590	15	+	160	11	+	100	44	+	420	18	+	650	20	+
14	I-23	580	5	–	150	11	+	140	70	+	640	14	+	680	5	+
14	I-24	510	2	+	50	2	+	120	48	+	490	6	+	910	–	+
14	N-10	450	–	–	80	–	–	90	–	–	440	–	–	670	–	–
14	N-11	430	–	–	40	–	–	60	–	–	420	–	–	610	–	–
14	N-12	300	–	–	100	–	–	60	–	–	260	–	–	690	–	–

A, digested tissue (mg); B, number of larvae; C, PCR results; I, infected BALB/c; N, non-infected BALB/c; +, PCR positive; −, PCR negative.
[a]This tissue was weighted without bone.

T. canis eggs in BALB/c mice to achieve a diagnosis not only for the tissue most commonly affected by larval migration (the liver) but also for the limb muscles, lungs and brain. This study aimed to measure the sensitivity, specificity and accuracy of molecular diagnosis and determine the detection limit of the method. Another aim of the study was to compare molecular diagnosis with a conventional method: pepsin-HCl digestion.

Materials and methods

Sample collections

The ascarid samples were obtained from infected dogs and cats that were referred to parasitological examination at Ankara University, Faculty of Veterinary Medicine. Ascarids were collected after treatment with pyrantel pamoate (Kontil®, 5 mg/kg, peroral) in infected hosts and diagnosed morphologically. The adult *T. canis* females were used for infection of BALB/c and as a positive control for Polymerase chain reaction (PCR), while adult *T. cati* and *T. leonina* were used to measure the sensitivity of the PCR. The mature female *T. canis* were shredded into distilled water with a scalpel, and the eggs were removed. Next, 1% sodium hypochlorite was added to the suspension and stirred for 10 min on a magnetic stirrer. After 10 min of stirring, sodium hypochlorite was removed by repeated centrifugation (2000 rpm × 3 min; three times). Then, 0.5% formalin was added, and the suspension was transferred to Petri dishes and incubated at 26–27°C for 23 days. Egg-larval development was monitored with a microscope (daily control).

VLM syndrome model in BALB/c mice

The syndrome is tolerated, and larvae can survive notably well in the tissues of BALB/c mice. Some researchers have stated that BALB/c mice are the best animal model for toxocariasis studies (Akao, 2006; Strube *et al.*, 2013); therefore, in the present study, BALB/c mice were used as an animal model.

Prior studies have shown that VLM may be induced by a wide range of embryonated *T. canis* eggs (6–5000) in mice (Sprent, 1955, 1958; Oshima, 1961; Sinha, 1966; Burren, 1968, 1971; Bisseru, 1969; Abo-Shehada & Herbert, 1984/1985; Ollero *et al.*, 2008). In light of these studies, BALB/c were firstly infected with 2000 embryonated eggs as a pre-study. However, mass mortality was observed 2–3 days post-infection with this number of embryonated eggs. In the necropsy reports, the infection was identified as the cause of death. Based on this information, the infection dose was decreased to 1000 embryonated eggs.

On the 23rd day of incubation, the suspension was centrifuged three times at 2000 rpm for 2 min with 0.9% isotonic saline solution to remove formalin. Six- to eight-week-old female BALB/c mice were infected with 1000 embryonated *T. canis* eggs in 0.2 ml of 0.9% isotonic saline via intragastric lavage. Non-infected mice were given 0.2 ml of 0.9% isotonic saline solution.

The day upon which embryonated eggs were given to the BALB/c mice was accepted as 'day 0' of the infection. On the second, fourth, seventh and 14th days post-infection, six infected and three non-infected BALB/c mice were sacrificed ($n = 36$). The procedure was conducted by taking blood from the heart of each animal under xylazine–ketamine anaesthesia (xylazine: 5 mg/kg im; ketamine: 45 mg/kg im). The BALB/c mice were necropsied immediately.

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Table 2. Number of larvae detected in different tissues and necropsy days in infected BALB/c groups.

Groups	Tissue	Days of necropsy	Number of larvae (n/g)				
			Mean	Std. dev.	Median	Kruskal–Wallis Test	*P*-value
Infected	Liver	2	398.0	208.7	341.0[a]	18.1	***
		4	157.0	42.8	144.0[ab]		
		7	97.5	113.0	34.5[ab]		
		14	13.3	8.2	13.5[b]		
	Lungs	2	111.3	111.5	83.5[b]	17.1	***
		4	594.6	282.7	470.5[a]		
		7	210.3	107.6	218.0[ab]		
		14	54.0	18.2	58.5[b]		
	Brain	2	0.0	0.0	0.0[b]	14.5	**
		4	327.8	145.6	302.5[a]		
		7	574.6	308.1	457.0[a]		
		14	345.1	187.1	420.0[a]		
	Forelimb muscles	2	0.0	0.0	0.0[b]	18.5	***
		4	10.0	11.1	5.0[ab]		
		7	85.8	63.4	83.0[a]		
		14	27.5	17.5	24.5[a]		
	Hindlimb muscles	2	0.0	0.0	0.0[b]	12.8	**
		4	1.3	1.5	1.0[ab]		
		7	15.0	11.4	14.5[a]		
		14	17.6	13.8	16.0[a]		

**$P < 0.01$, ***$P < 0.001$.
[ab]Different superscripts in the same column for each tissue represents statistically significant difference.

This research was carried out with the approval of the Gazi University Animal Experiments Local Ethics Committee (approval number G.Ü.ET.10.038).

Pepsin-HCl digestion

The liver, lungs, brain, forelimb and hindlimb muscles were removed, and the weight of each organ was recorded. The organs were divided into two parts. One part of each organ was weighed and digested with pepsin-HCl, except for the brain tissue. Instead of using pepsin-HCl, brain samples were squashed and observed with a microscope (10×). The other parts of each organ were stored at −80°C for DNA isolation until they were used.

For digestion, the organs were finely cut, and pepsin-HCl was added. The tissue samples were incubated at 37°C for 2–3 days; next, larval presence/absence was detected, and larvae were counted.

DNA extraction

The 20-mg BALB/c mice tissue samples were homogenized with phosphate-buffered saline (PBS) in an ultrasonic homogenizer (HD2070, Bandelin, Germany). Subsequently, a commercial DNA purification kit was used (K0722, Thermo Scientific, Lithuania) according to the manufacturer's instructions. The amount of DNA was measured with a NanoDrop™ 2000 spectrophotometer (ND2000, Thermo Scientific, USA) and stored at −20°C until use.

The DNA isolation protocol of Sambrook *et al.* (1989) was applied for morphologically diagnosed adult *T. canis*, *T. cati* and *T. leonina*. The amount of DNA was measured and stored at −20°C.

The *T. canis* eggs were incubated in distilled water at 26–27°C for 23 days. During the incubation period, the eggs were checked and ventilated. On the 23rd day of incubation, the eggs were centrifuged with PBS four times for 5 min at 2000 rpm. In this experiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 embryonated eggs were added to each of the ten 1.5-ml tubes. A DNA purification kit (K0722, Thermo Fisher Scientific, Lithuania) was used according to the manufacturer's instructions. The DNA samples were stored at −20°C until use.

PCR–RFLP technique

Forward (*T. canis* ATP-F1: GTTTGTTGTTTTGGGGGCTA) and reverse (*T. canis* ATP-R1: CCAAAGGACGAGAAACCTCA) primers were used to amplify a 217-bp region of the adenosine triphosphate (ATP) synthase subunit-6 (ATPase subunit 6) gene of *T. canis*. The primers were designed for this study. PCR was carried out in a 30-µl total volume mix containing 10× Taq buffer (1.25 ml, including $(NH_4)_2SO_4$), 25 mmol/l of $MgCl_2$, 10 mM of dNTP mix, 5 U/µl Taq DNA Polymerase (Fermentas, Waltham,

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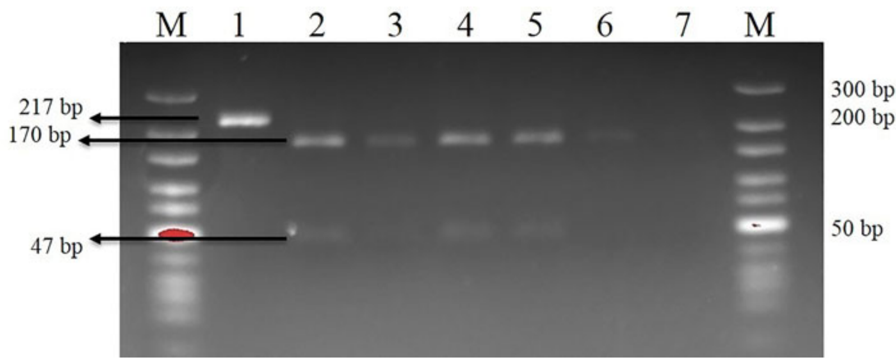

Fig. 1. Bbs-I reaction on the 14th day of necropsy (sample I-22). M, Marker (10–300 bp); 1, *Toxocara canis* positive control (uncut amplicon); 2, *T. canis* positive control (cut amplicon); 3, liver; 4, lungs; 5, brain; 6, forelimb muscles; 7, hindlimb muscles.

MA, USA), molecular biology grade water (18 megohm-cm, Applichem, Darmstadt, Germany) and 10 pmol/µl primers. Ten microliters of template DNA was added to each reaction. Each sample was analysed (T1 thermocyler, Biometra, Germany) under the following conditions: 94°C for 5 min (initial denaturation) followed by 34 cycles at 94°C for 30 s (denaturation), 50°C for 1 min (annealing), 72°C for 1 min (extension) and 72°C for 10 min (final extension). Amplicons were detected on 3% agarose (TAE) gel with ethidium bromide staining. The gel was visualized under ultraviolet light with a gel-imaging system (Kodak Gel Logic 100, USA).

The positive amplicons were digested with BbsI restriction endonuclease (New England BioLabs Inc., UK; 5000 units/ml) at 37°C for 1 h. The fragments were detected on 3% agarose (TAE) gel with ethidium bromide staining and visualized with the gel-imaging system.

Statistical analysis

Descriptive statistics of the pepsin-HCl digestion and PCR results were obtained. The Mann–Whitney *U*-test was used for the PCR results (+/−) of the number of larvae detected in different tissue types. The Kruskal–Wallis test and Bonferroni correction test were performed to determine the differences in larval counts among tissues and the changes in larval counts during independent days of all necropsy. The frequency of occurrence was examined via a chi-square test. *P*-values <0.05 were considered to be statistically significant in the analysis of the results. Specificity, sensitivity and accuracy rates of the PCR results were calculated (SPSS 14.01-9869264).

Results

A total of 36 BALB/c mice were sacrificed on the second, fourth, seventh and 14th days post-infection. The results are shown in table 1.

Results of the VLM syndrome model

The presence/absence of larvae observed in infected BALB/c mice was consistent with the parasite biology. On the second day post-infection, larvae were found in the liver and lung tissues of all infected BALB/c groups; nonetheless, larvae were not found in the brain or the fore- and hindlimbs. However, the average number of these larvae gradually decreased each day due to larval migration (table 2).

Larvae were detected in brain tissue beginning on the fourth day, and the highest average value was recorded on the seventh day post-infection.

The larvae began to appear in the fore- and hindlimb muscles on the fourth day post-infection. Samples I-12 in the forelimb and I-8, I-10, I-12, I-18 and I-24 in the hindlimb were negative on the fourth, seventh and 14th days of necropsy. The highest average larval count for forelimb muscles was detected on the seventh day post-infection (tables 1 and 2). Nonetheless, the average number of larvae in hindlimb tissue established a correlation, which increased daily. A difference was observed between the average larval number in the fore- and hindlimb muscles, with more larvae being observed in forelimb muscle tissue (table 2). No larvae were found in the non-infected BALB/c groups (table 1).

The descriptive statistics of the gram tissue are recorded in table 2. Larvae were encountered in the liver and lung of infected BALB/c mice on all days of necropsy. The highest median values in gram tissue were recorded on the second day of necropsy in the liver (341.0) and on the fourth day of necropsy in the lung (470.5). While larvae were not encountered in the brain or the fore- and hindlimb muscles on the second day of necropsy, the highest median values were recorded on the seventh day of necropsy.

In infected BALB/c mice, the number of larvae in the liver showed a similarity in the first two necropsy days but observed a significant decrease on the following days ($P < 0.001$). In the lungs, a significant increase in the number of larvae was observed between the second and fourth days, while a significant decrease was noted between the fourth and 14th days post-infection. A gradual increase was detected in the number of larvae in the brain tissues depending on the necropsy days. The larvae of the fore- and hindlimb muscles appeared to be observed on the same days, and the number of larvae increased on the seventh and 14th days of necropsy compared to the fourth day (table 2).

Results of PCR–RFLP

The new primers were reacted with cat, dog and human genomes, and no amplicons were observed. Negative results were also obtained for the *T. cati* and *T. leonina* samples.

The sensitivity of the PCR for *T. canis* eggs was determined. The PCR results for the samples taken on the relevant days of necropsy are given in table 1. As expected, the PCR results of all samples from the non-infected BALB/c groups were negative. For the PCR products, BbsI restriction endonuclease was cut from one position. Two DNA fragments were observed at 170 and 47 bp in size (fig. 1).

The difference between the PCR results and the digested amounts of larvae in all tissues was statistically significant (table 3). The

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Table 3. Descriptive statistics of PCR results on digested tissues.

Number of larvae in digested materials (n/g)						
Tissues	PCR results	Mean	Std. dev.	Median	Mann–Whitney *U*-test	*P*-value
Liver	**Negative**	3.9	8.5	0.0	312.5	***
	Positive	196.6	188.2	148.5		
Lungs	**Negative**	11.5	29.0	0.0	302.5	***
	Positive	269.0	270.8	218.0		
Brain	**Negative**	0.0	0.0	0.0	324.0	***
	Positive	415.8	240.5	400.0		
Forelimb	**Negative**	0.1	0.4	0.0	305.0	***
	Positive	41.0	49.3	24.5		
Hindlimb	**Negative**	0.0	0.0	0.0	279.0	***
	Positive	11.3	12.2	7.0		

***$P < 0.001$.

Table 4. Frequency distribution for PCR results.

PCR										
		Negative		Positive						
Tissue	Groups	*n*	%	*n*	%	χ^2 test	*P*-value	Sensitivity	Specificity	Accuracy
Liver	Non-infected	12	100.0	0	0.0	22.5	***	83.3	100.0	88.8
	Infected	4	16.6	20	83.3					
Lungs	Non-infected	12	100.0	0	0.0	25.2	***	87.5	100.0	91.6
	Infected	3	12.5	21	87.5					
Brain	Non-infected	12	100.0	0	0.0	18.0	***	75.0	100.0	83.3
	Infected	6	25.0	18	75.0					
Forelimb	Non-infected	12	100.0	0	0.0	18.0	***	75.0	100.0	83.3
	Infected	6	25.0	18	75.0					
Hindlimb	Non-infected	12	100.0	0	0.0	18.0	***	75.0	100.0	83.3
	Infected	6	25.0	18	75.0					

***$P < 0.001$.

frequency distribution of the PCR results for each tissue was examined. The highest sensitivity and accuracy were found in the lung. The ability to detect negative PCR results in all non-infected tissues in BALB/c mice was 100% (table 4).

Discussion

Non-specific tests (blood count, total serum IgE levels, etc.), direct optical diagnosis and immunodiagnosis are used to detect VLM (Fillaux & Magnaval, 2013). Due to the disadvantages of these tests, molecular techniques have been developed to diagnose VLM (de Moura *et al.*, 2018).

In the current study, VLM was conducted in 36 female BALB/c mice with embryonated *T. canis* eggs, and mice were sacrificed on the second, fourth, seventh and 14th days post-infection. Detection of larvae in the liver, lung, fore- and hind limb muscle tissues was supported by pepsin-HCl digestion, and the brain samples were squashed and observed microscopically to determine the credibility of the study. The study aimed to diagnose larvae in these tissues using a molecular method.

The days of necropsy were determined considering the biology of the parasite. There are studies showing that larvae were observed in liver tissue within the first 24 h (Burren, 1968; Bisseru, 1969); in lung tissues within the first three days and on the sixth day at its highest level (Sinha, 1966; Burren, 1968); in brain tissues after 2–3 days post-infection (Sprent, 1958; Sinha, 1966; Bisseru, 1969; Burren, 1968, 1971; Ollero *et al.*, 2008), and on the seventh day, as well as in brain tissue (Oshima, 1961; Abo-Shehada & Herbert, 1984/1985). Larvae can be observed in muscle tissues on the first two days (Sprent, 1958; Burren, 1968; Abo-Shehada & Herbert, 1984/1985), and some researchers found larvae on the third to seventh days

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post-infection (Oshima, 1961; Abo-Shehada & Herbert, 1984/1985). In our study, in keeping with the biology of the parasite, larvae were found on similar days post-infection as with the aforementioned studies (table 1).

Molecular diagnosis is commonly used. One of the most significant drawbacks of this method is the false negatives that are encountered depending on the DNA or RNA concentration obtained from the sample. Jacobs *et al.* (1997) reported that for Tcan-NC2 primers, the lowest concentration of *T. canis* DNA detectable by PCR was on the order of 0.1–0.5 ng/μl^{-1}. Li *et al.* (2007) reported that in each PCR process diagnosed as *T. canis*, the smallest amount of *T. canis* DNA was 0.14 ng, which was less than a single nematode larva. Furthermore, referring to the related literature, Borecka *et al.* (2008) surmised that 0.8, 0.4, 0.09 and 0.09 larvae could be detected with PCR on the third, fifth, seventh and 14th days post-infection in the liver. However, in the present study, PCR was conducted by morula and/or embryonated eggs into portions of up to ten eggs consecutively, and amplicons were recorded as two or more eggs. The DNA were measured as 5.1–11.2 ng/μl.

Some studies were performed with different amounts of tissues to isolate DNA (Wang *et al.*, 2018). According to our study design, we used 20 mg of tissue and obtained positive PCR results. This finding might be related to the infection dose.

In previous studies, the primers were reacted with other ascaridoid species, and each specific primer had been negative in other parasite samples (Jacobs *et al.*, 1997; Li *et al.*, 2007). In our study, the suitability of the new primers was compared with that of *T. cati* and *T. leonina*, and no amplicons were detected. The negative results suggest that a specific diagnosis of *T. canis* can be achieved with current primer pairs.

When the methods used in the study were compared, the larvae were observed in four samples (I-13, I-20, I-21, I-23) of liver tissue and in three samples (I-4, I-5, I-20) of lungs on the second, seventh and 14th days post-infection with pepsin-HCI digestion, while the PCRs that belong to the samples were negative. Moreover, while the PCR was negative in sample I-10 in the forelimb on the fourth day of necropsy, larvae were observed in same tissue with digestion. These results indicate that the tissues contained no piece of the larva. Some researchers (Borecka *et al.*, 2008; de Moura *et al.*, 2018) have also reported similar data in their studies.

On the second day of necropsy, the PCR result was positive in forelimb sample I-1, and no larvae were observed in the digestion. On the same day, the results of the remaining forelimb muscle samples were negative for both methods (table 1). This finding, which was encountered on the second day of necropsy, shows that there was a piece of larval DNA on the tissue. It is possible to obtain similar findings, as some researchers (Sprent, 1958; Burren, 1968; Abo-Shehada & Herbert, 1984/1985) recorded that larvae were seen on the muscle tissue on the first and second days post-infection. In our study, the PCR results of five hindlimb muscle samples (I-8, I-10, I-12, I-18 and I-24) were positive on the fourth, seventh and 14th days post-infection, but no larvae were observed in their digested samples. The positive PCR results obtained suggest that the utilization of PCR in tissue samples is a suitable method for diagnosing VLM (*T. canis*).

In some VLM patients, the syndrome could be caused by the consumption of raw-uncooked organ/meat from paratenic hosts. Therefore, some studies were performed on this transmission by molecular diagnosis. The organs (liver, heart, lung, kidney, brain and intestine) of paratenic hosts were investigated with the digestion method and PCR technique. The results of both techniques revealed that PCR is more reliable and able to detect species of parasites (Zibaei *et al.*, 2017; Wang *et al.*, 2018). Although larvae could be observed by using the digestion method, this approach took a long time and required extra effort. It was also specified that a piece of larva could not be observed via this method. However, this approach would give positive PCR results, as it has a piece of *Toxocara* DNA (Ishiwata *et al.*, 2004; Wang *et al.*, 2018).

PCR is a convenient procedure for epidemiological studies (detection of eggs to diagnose the source of environmental contamination, etc.) regarding *Toxocara* infection (Smith *et al.*, 2009). Ascaridoid eggs in the soil samples that were distinguished as *T. canis* and *T. cati* can help to measure the prevalence of syndromes that could appear in humans (Borecka, 2004; Fogt-Wyrwas *et al.*, 2007). The results of the present study have shown that *Toxocara* sp. eggs could also be identified as *T. canis* via this protocol. The current primer was also checked to prevent any reactivity that could occur with the cat, dog and human genomes; this step was also taken in similar molecular studies (Jacobs *et al.*, 1997; Li *et al.*, 2007). There were no amplified DNA in the dog, cat or human samples.

In conclusion, the present study shows that the designed primers can be reliable to determine the identity of *Toxocara* sp. eggs as *T. canis* in the faeces, paratenic host, sand and raw vegetables/fruits. Moreover, it is proposed that this method can also be applied in human tissues that were already diagnosed as VLM positive with histopathological examination in order to determine whether *T. canis* was the responsible ethiological factor.

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References

Abo-Shehada MN and Herbert IV (1984/1985) The migration of larval *Toxocara canis* in mice II. Post-intestinal migration in primary infections. *Veterinary Parasitology* **17**, 75–83.

Akao N (2006) Critical assessment of existing and novel model systems of toxocariasis. pp. 74–85 *in* Holland CV and Smith HV (*Eds*) *Toxocara the enigmatic parasite*. Oxfordshire, CABI Publishing.

Akyol A, Bicerol B, Ertug S, Ertabaklar H and Kıylıoglu N (2007) Epilepsy and seropositivity rates of *Toxocara canis* and *Toxoplasma gondii*. *Seizure* **16**, 233–237.

Archelli S, Santillan GI, Fonrouge R, Céspedes G, Burgos L and Radman N (2014) Toxocariasis: seroprevalence in abandoned-institutionalized children and infants. *Revista Argentina de Microbiología* **46**(1), 3–6.

Berrett AN, Erickson LD, Gale SD, Stone A, Brown BL and Hedges DW (2017) *Toxocara* seroprevalence and associated risk factors in the United States. *The American Journal of Tropical Medicine and Hygiene* **97**(6), 1846–1850.

Bisseru B (1969) Studies on the liver, lung, brain and blood of experimental animals infected with *Toxocara canis*. *Journal of Helminthology* **43**(3/4), 267–272.

Boldis V, Ondriska F, Spitalska E and Reiterova K (2015) Immunodiagnostic approaches for the detection of human toxocarosis. *Experimental Parasitology* **159**, 252–258.

Borecka A (2004) Differentiation of *Toxocara* spp. eggs isolated from the soil by the PCR linked RFLP method. *Helminthologia* **41**(4), 185–187.

Borecka A, Gawor J, Niedworok M and Sordyl B (2008) Detection of *Toxocara canis* larvae by PCR in the liver of experimentally infected Mongolian gerbils (*Meriones unguiculatus*). *Helminthologia* **45**(3), 147–149.

Burren CH (1968) Experimental toxocariasis I. Some observations and histopathology of the migration of *Toxocara canis* larvae in mouse. *Zeitschrift für Parasitenkunde* **30**, 152–161.

Burren CH (1971) The distribution of *Toxocara* larvae in the central nervous system of the mouse. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **65**(4), 450–453.

Cong W, Meng QF, You HL, Zhou N, Dong XY, Dong W, Wang XY, Qian AD and Zhu XQ (2015) Seroprevalence and risk factors of *Toxocara* infection among children in Shandong and Jilin provinces, China. *Acta Tropica* **152**, 215–219.

de Moura MQ, de Macedo MRP, da Silva Terto WD, da Costa Avila LF, Leite FPL, Scaini CJ, Natália Berne Pinto N, de Almeida CG, Strothmann AL, MarreiroVillela M and Berne MEA (2018) Detection of *Toxocara canis* DNA in tissues of experimentally infected mice. *Acta Tropica* **187**, 51–56.

Despommier D (2003) Toxocariasis: clinical aspects, epidemiology, medical ecology and molecular aspects. *Clinical Microbiology Reviews* **16**(2), 265–272.

Ei-Shazly AM, Abdel Baset SM, Kamal A, Mohammed KA, Sakr TI and Hammad SM (2013) Seroprevalence of human toxocariasis (Visceral Larva Migrans). *AAMJ* **11**(Supp 3), 16–34.

Fillaux J and Magnaval JF (2013) Laboratory diagnosis of human toxocariasis. *Veterinary Parasitology* **193**(4), 327–336.

Fogt-Wyrwas RF, Jarosz W and Mizgajska-Wiktor H (2007) Utilizing a polymerase chain reaction method for the detection of *Toxocara canis* and *T. cati* eggs in soil. *Journal of Helminthology* **81**, 75–78.

Gasser RB, Zhu XQ, Hu M, Jacobs DE and Chilton NB (2006) Molecular genetic characterization of members of the genus *Toxocara*-taxonomic, population genetic and epidemiological considerations. pp. 18–31 *in* Holland CV and Smith HV (*Eds*) *Toxocara the enigmatic parasite*. Oxfordshire, CABI Publishing.

Ishiwata K, Shinohara A, Yagi K, Horii Y, Tsuchiya K and Nawa Y (2004) Identification of tissue-embedded ascarid larvae by ribosomal DNA sequencing. *Parasitology Research* **92**, 50–52.

Jacobs DE, Zhu X, Gasser RB and Chilton NB (1997) PCR-based methods for identification of potentially zoonotic ascaridoid parasites of the dog, fox and cat. *Acta Tropica* **68**, 191–200.

Jarosz W, Mizgajska-Wiktor H, Kirwan P, Konarski J, Rychlicki W and Wawrzyniak G (2010) Developmental age, physical fitness and *Toxocara* seroprevalence amongst lower-secondary students living in rural areas contaminated with *Toxocara* eggs. *Parasitology* **137**(1), 53–63.

Kaplan M, Kalkan A, Kuk S, Demırdag K, Ozden M and Kılıc SS (2008) *Toxocara* seroprevalence in schizophrenic patients in Turkey. *Yonsei Medical Journal* **49**(2), 224–229.

Kassai T (1999) *Veterinary helminthology*. 1st edn. Oxford, Butterworth-Heinemann.

Kustimur S, Dogruman Al F, Oguzulgen K, Bakır H, Maral I, Turktas H and Tuzun H (2007) *Toxocara* seroprevalence in adults with bronchial asthma. *Transactions of the Royal Society of Tropical Medicine & Hygiene* **101**, 270–274.

Lee ACY, Schantz PM, Kazacos KR, Montgomery SP and Bowman DD (2010) Special issue: Zoonoses of people and pets in the USA. Epidemiologic and zoonotic aspects of ascarid infections in dogs and cats. *Trends in Parasitology* **26**(4), 155–161.

Li MW, Lin RQ, Chen HH, Sani RA, Song HQ and Zhu XQ (2007) PCR tools for the verification of the specific identity of ascaridoid nematodes from dogs and cats. *Molecular and Cellular Probes* **21**, 349–354.

Liao CW, Sukati H, D'lamini P, *et al.* (2010) Seroprevalence of *Toxocara canis* infection among children in Swaziland, southern Africa. *Annals of Tropical Medicine & Parasitology* **104**(1), 73–80.

Lötsch F, Obermüller M, Mischlinger J, *et al.* (2016) Seroprevalence of *Toxocara* spp. in a rural population in Central African Gabon. *Parasitology International* **65**(6), 632–634.

Ollero MD, Fenoy S, Cuellar C, Guillen JL and Aguila C (2008) Experimental toxocariosis in BALB/c mice: effect of the inoculation dose on brain and eye involvement. *Acta Tropica* **105**, 124–130.

Oshima T (1961) Standardization of techniques for infecting mice with *Toxocara canis* and observations on the normal migration routes of the larvae. *Journal of Parasitology* **47**, 652–656.

Roberts LS and Janovy J (2006) *Gerald D. Schmidt & Larry S. Roberts' foundations of parasitology*. International edn. New York, Mc-Graw Hill Press.

Sambrook J, Fritschi EF and Maniatis T (1989) *Molecular cloning: a laboratory manual*. New York, Cold Spring Harbor Laboratory Press.

Schnieder T, Laabs EM and Welz C (2011) Larval development of *Toxocara canis* in dogs. *Veterinary Parasitology* **175**, 193–206.

Schoenardie ER, Scaini CJ, Brod CS, Pepe MS, Villela MM, McBride AJ, Borsuk S and Berne ME (2013) Seroprevalence of *Toxocara* infection in children from southern Brazil. *Journal of Parasitology* **99**(3), 537–540.

Silva MB, Amor AL, Santos LN, *et al.* (2017) Risk factors for *Toxocara* spp. seroprevalence and its association with atopy and asthma phenotypes in school-age children in a small town and semi-rural areas of Northeast Brazil. *Acta Tropica* **174**, 158–164.

Sinha BN (1966) The migratory behaviour of the larvae of *Toxocara canis* (Werner 1782) in white mice. *Indian Veterinary Journal* **43**(12), 1101–1105.

Smith H, Holland C, Taylor M, Magnaval JF, Schantz P and Maizels R (2009) How common is human toxocariasis? Towards standardizing our knowledge. *Trends in Parasitology* **25**(4), 182–188.

Sprent JFA (1955) On the invasion of the central nervous system by nematodes. II. Invasion of the nervous system in ascariasis. *Parasitology* **45**, 41–55.

Sprent JFA (1958) Observations on the development of *Toxocara canis* (Werner, 1782) in the dog. *Parasitology* **48**, 184–209.

Strube C, Heuer L and Janecek E (2013) *Toxocara* spp. infections in paratenic hosts. *Veterinary Parasitology* **193**, 375–389.

Wang Z, Shibata M, Nguyen YTH, Hayata Y, Nonaka N, Maruyama H and Yoshida A (2018) Development of nested multiplex polymerase chain reaction (PCR) assay for the detection of *Toxocara canis*, *Toxocara cati* and *Ascaris suum* contamination in meat and organ meats. *Parasitology International* **67**(5), 622–626.

Zibaei M, Sadjjadi SM and Maraghi S (2017) The occurrence of *Toxocara* species in naturally infected broiler chickens revealed by molecular approaches. *Journal of Helminthology* **91**(5), 633–636.

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