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Nematode *larva migrans* caused by *Toxocara cati* in the North Island brown kiwi (*Apteryx mantelli*)

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ABSTRACT

Sporadic cases of visceral and neural nematode *larva migrans* have been diagnosed at necropsy in the endangered New Zealand kiwi (*Apteryx* spp.), but the causative organisms have not yet been definitively identified. From an initial group of five affected kiwi, PCR was performed on DNA extracted from archival formalin-fixed paraffin-embedded tissue sections in which larval nematodes had been histologically identified. Sequencing of positive results from four out of the five kiwi aligned with sequences from *Toxocara cati*, a nematode parasite whose definitive host is the domestic cat. PCR was then performed on a second group of 12 kiwi that had histologic inflammatory lesions consistent with *larva migrans*, but variable larval presence. Repeatable positive PCR results were only achieved in one tissue, in which larval organisms were histologically confirmed. This study supports the use of PCR as an alternative or adjunct to the morphological identification of nematode larvae in formalin-fixed histopathological samples, as well as showing that in investigation of *larva migrans*, PCR has greatest chance of success from sections where nematode larvae are evident histologically. The identification of *Toxocara cati* from lesions of *larva migrans* in kiwi reflects an indirect, parasite-mediated effect of an invasive mammalian species on a native species.

1. Introduction

The mammalian invasion of New Zealand has caused major declines in native fauna, primarily through direct predation. Although often overlooked, alterations in parasite ecology that accompany the establishment of introduced species may also have a significant impact on native ecosystems (Chalkowski et al., 2018). The spillover of parasites from introduced hosts into New Zealand avifauna has been studied for avian malaria (Schoener et al., 2014), but there has been little, if any, work performed on the effects of introduced nematodes.

Cases of visceral and neural nematode *larva migrans* (LM), which may be defined as the prolonged migration of a larval parasite in the internal organs of an abnormal host (Beaver, 1956), have been diagnosed by histopathology in the endangered New Zealand kiwi (family *Apterygidae*, genus *Apteryx*) with increasing recognition over the past 15 years (Reid and Williams, 1975; Anonymous, 1978; Boardman, 1995; Alley and Gartrell, 2003, 2006; Alley et al., 2004; van Zyl, 2014). Kiwi are nocturnal, flightless palaeognaths, and are unique to New Zealand. Five genetically and geographically distinct species are currently recognised, all of which have suffered population decline of varying severity since the occupation of New Zealand by humans and predatory mammals (Holzapfel et al., 2008). The consequent establishment of intensive conservation programmes has been associated with outbreaks of parasitic disease such as coccidiosis (Morgan et al., 2012) and avian malaria (Banda et al., 2013); however, reported cases of LM have remained sporadic, involving both wild and intensively managed kiwi.

Based on histomorphology of the larval nematodes, *Toxocara* spp. have been proposed as the most likely cause, but a recent study was unable to confirm this by molecular analysis (van Zyl, 2014). *Toxocara canis* is the major cause for non-cutaneous LM in humans worldwide, and while *Toxocara cati* has only infrequently been associated with similar clinical syndromes, it is now considered likely to represent a greater zoonotic risk than has been previously recognised (Fisher, 2003; Fillaux and Magnaval, 2013). The adult nematodes inhabit the intestinal tract of their definitive hosts, the domestic dog (*Canis familiaris*) and cat (*Felis catus*) respectively, both of which are common throughout New Zealand. Infection occurs through ingestion of the infective larvae either within the egg (e.g. ingestion of faecal-contaminated soil) or within a mammalian, avian, or invertebrate paratenic host. Following ingestion of infective eggs by the definitive host, larvae hatch and migrate from the intestine to the liver and lungs, then via the trachea returning to the intestine to mature. In older animals especially, somatic migration may also occur, where larvae are instead disseminated from the lungs through the blood stream into tissues (e.g. skeletal

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muscle) where they remain dormant but infective and may be re-activated e.g. if the animal is pregnant and/or lactating (Wang et al., 2018). In abnormal hosts such as humans, the infective larvae undertake prolonged, aberrant somatic migration (Fillaux and Magnaval, 2013).

Diagnosis of toxocariasis in humans is not straightforward, and may be based on a combination of history, clinical presentation, haematology, fluid analysis, imaging, and serological testing. Definitive diagnosis of infection requires the confirmation of larval presence via biopsy, but this procedure is not routinely performed because of its invasive nature and the low probability of obtaining a diagnostic sample (Fillaux and Magnaval, 2013). In contrast, diagnosis of LM in animals, particularly wildlife, is most commonly achieved via histology following necropsy, and available samples may be restricted to formalin-fixed, paraffin-embedded (FFPE) tissue blocks. In either case, identification of causative organisms has historically been based on morphology, a technique with acknowledged limitations due to the small size and relative lack of distinguishing features among larval nematodes (Nichols, 1956). A further diagnostic dilemma occurs when inflammatory lesions consistent with LM are identified histologically, but no larvae are evident in the sections; in such cases, a pathologist may be suspicious of LM but unable to confirm the diagnosis (Kaplan et al., 2001).

A number of different studies have now demonstrated the utility of PCR in the identification of *Toxocara* DNA present in cerebrospinal fluid (Caldera et al., 2013), bronchoalveolar lavage fluid (Pinelli et al., 2013), and fresh tissue (e.g. Zibaei et al., 2017; Wang et al., 2018) as well as in the assessment of environmental contamination by *Toxocara* eggs (e.g. Borecka and Gawor, 2008). Although formalin fixation has a negative effect on the quality of DNA, PCR can be successfully performed using FFPE samples (Libório et al., 2005; Sengüven et al., 2014), and has been recently applied to confirm identification of *Baylisascaris procyonis* causing neural LM in FFPE sections of brain from a dog (Hazlett et al., 2018), thus providing an adjunct or alternative to histomorphology for the identification of larvae in fixed tissue. The use of PCR on FFPE sections has the additional benefit of allowing assessment of the association between the presence of the organism and histological evidence of disease.

The purpose of this study was to investigate further the cause of visceral and neural nematode LM in archival FFPE necropsy tissue from kiwi, and to validate the use of molecular techniques to isolate nematode DNA from tissue granulomas with and without larval sections present.

2. Methods

2.1. Case selection

A retrospective search of the School of Veterinary Science Pathology database (Massey University, Palmerston North, New Zealand) was performed to identify cases in which histological examination of tissues from kiwi submitted for necropsy resulted in a diagnosis of confirmed (larvae present in section) or suspected (typical inflammatory lesions present but larvae not identified) nematode LM. Cases with lesions present only in gastrointestinal tissue or skin were excluded. The original Haematoxylin and Eosin (H&E)-stained sections were examined to confirm the diagnosis, or new sections cut and examined if the original slides were unavailable.

The archived FFPE tissue blocks of suitable cases were located and "sandwich" sections cut, comprised of a 4 μm section mounted on a slide and stained with H&E, followed by two or three 10 μm tissue scrolls taken for molecular analysis, then a further 4 μm section mounted and stained with H&E. The H&E-stained sandwich sections were examined to assess the probability of larval tissue being present within the tissue scrolls. An initial group of cases (group I) were chosen for molecular analysis using the following criteria: a) nematode larvae histologically confirmed to be present in at least one affected tissue in sandwich sections taken both before and after the tissue scrolls used for DNA extraction; and b) cases diagnosed within the past six years. The primary aim of the analysis of group I samples was the specific identification of the larval organisms associated with lesions of LM.

Following the results from group I, a further group of cases (group II) were chosen, removing the previous criteria, in order to further evaluate the utility of performing PCR on lesions without histologically identifiable larvae. This second group of tissues had confirmed characteristic inflammatory lesions present in histological sandwich sections but variable larval presence.

2.2. DNA extraction

DNA extraction was performed on the FFPE tissue scrolls using a commercial kit (Roche High Pure FFPET DNA isolation kit, Roche, Switzerland or NucleoSpin DNA FFPE XS kit, Macherey-Nagel, Germany), per the manufacturer's instructions with minor modifications, most notably variable extension of the lysis step in a 56 °C water bath from two or three hours to overnight. DNA was also extracted from fresh specimens of adult *T. canis* and *T. cati* for use as positive controls, using a commercial kit (NucleoSpin Tissue kit, Macherey-Nagel, Germany) per the manufacturer's instructions. The adult *Toxocara* were sourced from the gastrointestinal tract of a domestic dog and cat respectively at routine necropsy and identified morphologically by the School of Veterinary Science parasitology laboratory (Massey University, Palmerston North, New Zealand), based primarily on the size of the organisms and conformation of the cervical alae.

2.3. Molecular analysis

Primer sets were sourced from literature, including one set designed for the gender identification of kiwi tissues (Huynen et al., 2003) and multiple sets targeting either the internal transcribed spacer (ITS)-2 region or 18S gene of nuclear ribosomal DNA of ascaridoid nematodes

Table 1
Primers used in this study, including a range of primers designed to amplify parts of the ITS-2 region or the 18S gene of nuclear ribosomal DNA of ascaridoid nematodes, and one set of primers designed to differentiate the gender of kiwi.

Primer name	Primer sequence (5′-3′)	Target	Approximate amplicon size (bp)	Reference
W5	AATCACCCTTTAAACAAGCTGTTAAAGCAA	Uncertain – Kiwi W-linked and Z-linked	350 (males and females) ± 200	Huynen et al. (2003)
W7	CCTTTCTCAAATCTCTCTTTTGTTCTAGACAC	or autosomal	(females only)	
NC2	TTAGTTTCTTTTCCTCCGCT	Nematode ITS-2 region	N/A (reverse primer)	Gasser et al. (1993)
NC13	ATCGATGAAGAACGCAGC	Ascaridoid ITS-2 region	520 (with NC2)	Jacobs et al. (1997)
XZ1	ATTGCGCCATCGGGTTCATTCC	Ascaridoid ITS-2 region	450 (with NC2)	Li et al. (2006)
T cat1	GGAGAAGTAAGATCGTGGCACGCGT	*Toxocara cati* ITS-2 region	400 (with NC2)	Jacobs et al. (1997)
YY1	CGGTGAGCTATGCTGGTGTG	*Toxocara canis* ITS-2 region	330 (with NC2)	Li et al. (2007)
18SF	CCATGCATGTCTAAGTTCAA	Ascaridoid 18S gene	325	Dangoudoubiyam et al. (2009)
18SR	TTATTCTCCGTTACCCGTTA			
Nemo 18S F	GGCTAAGCCATGCATGTC	Ascaridoid 18S gene	265	Pinelli et al. (2013)
Nemo 18S R	ACTTGATAGACACGTCGCC			

(Gasser et al., 1993; Jacobs et al., 1997; Li et al., 2006, 2007; Dangoudoubiyam et al., 2009; Pinelli et al., 2013), with target sequences of variable base pair (bp) length (Table 1). Each PCR contained 1X of 5X HOT FIREPol Blend Master Mix (10 mM $MgCl_2$, Solis Biodyne, Estonia), 300 nM each of forward and reverse primers (IDT, IA, USA), and 1 μl of template DNA (or nuclease free water), made to a total of 20 μl with nuclease free water.

PCR was performed on either a Labcycler (SensoQuest, Germany) or Mastercycler Nexus GX2 (Eppendorf, Germany). The protocol used for the kiwi gender-specific primer set (W5–W7) was as follows: initial activation of 15 min at 95 °C, 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final elongation at 72 °C for 7 min. A touchdown PCR protocol was used for all nematode primer sets under the following conditions: initial activation of 15 min at 95 °C, 12 cycles of 95 °C for 30 s, annealing for 30 s (starting at 60 °C, reducing by 0.5 °C per cycle), and 72 °C for 30 s, followed by a further 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and final elongation at 72 °C for 7 min. Positive controls, consisting of DNA extracted from *T. canis* and/or *T. cati*, and a negative (blank) control containing no DNA were run simultaneously. The PCR product was separated by electrophoresis on a 1% w/v agarose gel (Bioline, UK) using RedSafe (iNtRON Biotechnology, South Korea) and visualised with a gel image system (MultiDoc-It Imaging System, UVP, CA, USA). Size of PCR products was estimated in comparison to a HyperLadder™ 100 bp molecular ladder (Bioline, UK).

2.4. Sequencing and BLAST analysis

Amplicons of the appropriate size were cut from the gel, eluted overnight in elution buffer (10 mM Tris-HCl, pH 8.0), and the eluate submitted to the Massey Genome Service (Massey University, Palmerston North, New Zealand) for bi-directional Sanger sequencing. The resultant forward and reverse sequences were aligned using Geneious 10.2.3, manually trimmed, and subjected to BLAST (Basic Local Alignment Search Tool) analysis through the NCBI (National Center for Biotechnology Information) database (GenBank®). Nucleotide sequences obtained in this study have been deposited in GenBank under the accession numbers MN585764 to MN585772.

3. Results

3.1. Cases

Group I samples consisted of 16 tissues in total from five kiwi, necropsied between 2011 and 2017. Of these, six tissues (one from each of four kiwi, and two from one kiwi) had nematode larvae histologically confirmed in sandwich sections taken both before and after the tissue scrolls cut for molecular analysis (group Ia). Other tissues from the same birds in which lesions were present but larvae absent or only identified in one of the two histological sandwich sections were also subsequently analysed (group Ib). Group II samples consisted of 20 tissues from 12 kiwi, necropsied between 2004 and 2017, in which typical inflammatory lesions with or without larvae were confirmed present in both histological sandwich sections.

All selected kiwi were North Island brown (*Apteryx mantelli*), which comprise the vast majority of the kiwi species submitted to the necropsy service. They originated from various regions within the North Island of New Zealand, and the majority (15/17; 88.2%) were categorised as wild, from areas that practice no to variably extensive (but incomplete) predator control (Table 2). A majority (14/17; 82.4%) were females, and age cohorts (as reported in the database) included an even mix of juvenile and adult birds along with a single subadult; the age of kiwi is impossible to estimate beyond these broad categorisations unless the bird has been tracked since hatch.

3.2. Histology

The characteristic histological lesion was a discrete granuloma of variable size with central accumulation of brightly eosinophilic, necrotic cellular and pyknotic nuclear debris and a peripheral rim of epithelioid macrophages and multinucleated giant cells (Fig. 1A). Granulomata were most commonly identified in liver, lung, and/or brain, where they were focal, multifocal, or regionally clustered to confluent and appeared randomly located within the parenchyma. In a few cases, focal to regionally extensive acute inflammation was present in addition to these lesions within liver and lung sections, consisting of granulocytic infiltrates with or without acute necrosis. Less specifically, it was also common to see lymphoid aggregates adjacent to portal areas or airways, and brain sections sometimes included mild perivascular lymphoid cuffing and/or small foci of malacia and gliosis.

Larvae, where present, were most commonly found within the necrotic centre of a granuloma, more rarely within foci of acute inflammation. Cross-sections or near cross-sections of larvae were identified and measured in four of the five group I kiwi, ranging in diameter from 9.3 to 17.6 μm and exhibiting single, small, bilaterally symmetrical alae (Fig. 1B). In the fifth kiwi (#4), where only longitudinal sections were identified, the larvae were of similar size but presence of alae could not be confirmed.

3.3. Molecular analysis

3.3.1. Group I

All 16 tissues from the five group I kiwi showed appropriate amplification using the kiwi gender-specific primers (W5–W7), confirming amplifiable kiwi DNA of up to 350bp at least.

3.4. Group Ia

Touchdown PCR using the range of selected nematode primer sets was performed only on those tissues from each bird in group I in which larvae had been confirmed to be present in both histological sandwich sections (Table 3). The positive controls, *T. cati* and *T. canis*, amplified appropriately with all primer sets. One tissue (kiwi #1, lung) was PCR negative for all nematode primer sets. All other kiwi tissues tested produced amplicons of the appropriate size with two or more of the nematode primer sets. Successful amplification decreased with increasing target product size and all tissues were negative for the primer set with the largest target product (NC13-NC2, ~520bp). Additionally, all tissues were negative for the *T. canis*-specific primer set (YY1-NC2).

From the positive results, representative amplicons were selected for sequencing and the resultant sequences were subjected to BLAST analysis. At least one of the nematode primer sets for each tissue yielded a sequence that aligned with the sequence from the *T. cati* positive control, as well as with sequences from *T. cati* present in GenBank (Table 4).

3.3.1.2. Group Ib

The four group Ia kiwi with positive nematode PCR results also had additional tissues in which characteristic inflammatory lesions were present but larvae were absent or identifiable in only one of the two histological sandwich sections. PCR was performed on these tissues using only the primer set with the smallest product (Nemo 18S F-Nemo 18S R, ~265bp); based on group Ia results this was deemed the most likely to produce a positive result if nematode DNA was present (Table 5). Three out of four tissues in which larvae were identified in histological sections taken either before or after (but not both) the tissue scrolls collected for molecular analysis were positive, while one was negative. Sequencing and BLAST analysis of the positive amplicons in all three cases aligned with *T. cati* (EF180059, bit-scores 468.332–483.096), as for the group Ia tissues from the same birds. All

Table 2
Signalment and origin of kiwi in group I (#1 to 5) and group II (#6 to 17).

Kiwi #	Year of necropsy	Species	Age	Gender	Regional location	Origin	Tissues affected
1	2017	*A. mantelli*	Juvenile (30D)	Female	Hawkes Bay	Crèche[a]	Lung
2	2016	*A. mantelli*	Juvenile	Female	Northland	Wild	Lung, liver, brain
3	2016	*A. mantelli*	Adult (2Y)	Female	Waikato	Zoo	Lung, liver, brain, muscle, spinal cord
4	2015	*A. mantelli*	Adult	Female	Bay of Plenty	Wild	Liver, brain
5	2011	*A. mantelli*	Juvenile (6M)	Male	Northland	Wild	Lung, liver, brain
6	2017	*A. mantelli*	Subadult	Male	Coromandel	Wild	Brain
7	2016	*A. mantelli*	Adult	Female	Bay of Plenty	Wild	Liver
8	2013	*A. mantelli*	Juvenile	Female	Northland	Wild	Lung, brain
9	2012	*A. mantelli*	Adult	Female	Northland	Wild	Lung
10	2012	*A. mantelli*	Juvenile (5M)	Female	Hawkes Bay	Wild	Lung, brain
11	2011	*A. mantelli*	Juvenile	Male	Auckland	Wild	Liver, heart
12	2011	*A. mantelli*	Adult	Female	Northland	Wild	Lung
13	2009	*A. mantelli*	Adult	Female	Wanganui	Wild	Liver, heart
14	2006	*A. mantelli*	Adult	Female	Northland	Wild	Lung, liver, brain
15	2005	*A. mantelli*	Adult (2Y)	Female	Northland	Wild	Liver
16	2005	*A. mantelli*	Juvenile (6M)	Female	Northland	Wild	Lung, brain
17	2004	*A. mantelli*	Juvenile (3M)	Female	Northland	Wild	Lung, liver, brain

a = a predator-free area used for raising juveniles.

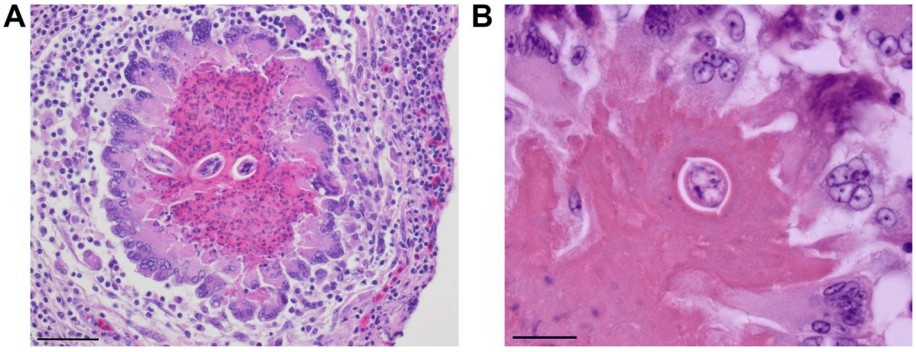

Fig. 1. Histology. (A) Typical inflammatory granuloma in the lung of a kiwi, containing several oblique nematode larval sections (H&E, bar = 50 μm). (B) Cross-section of a nematode larva within an inflammatory granuloma in the brain of a kiwi, showing bilateral alae (H&E, bar = 20 μm).

five tissues in which larvae could not be identified histologically were negative, despite confirmation of the presence of characteristic inflammatory lesions in the histological sandwich sections.

3.3.3. Group II

In nine out of the 12 group II kiwi, larvae were identified in at least one tissue, in at least one of either the original diagnostic or sandwich histology sections, supporting the diagnosis of LM as the cause of the inflammatory lesions in these birds. Where cross-sections were present, the larvae resembled those previously described in group I. PCR was performed on all tissues using the kiwi gender-specific primers (W5–W7) and the nematode primer set with the smallest product (Nemo 18S F-Nemo 18S R, ~265bp), and results compared with the presence or absence of larvae in the sandwich sections (Table 5).

The nematode PCR was performed three times on all samples to test repeatability of results and any with positive results were repeated a further three times. A total of nine tissues originating from seven different kiwi produced a positive amplicon of appropriate size in at least one of the initial three PCR runs, but only one was positive in all three, and subsequently all six total PCR runs (kiwi #10, lung). Larvae in this

Table 3
PCR results from group Ia kiwi, tissues in which larval organisms were identifiable in histological sections taken before and after the sample used for molecular analysis, and for positive control organisms *T. cati* and *T. canis*. Includes a range of primers designed for amplification of nematode DNA, and one set of primers (forward primer W5) designed for differentiating the gender of kiwi. F = PCR positive female, M = PCR positive male, - = negative result, + = amplicon of appropriate size, +/− = faint and/or non-repeatable amplicon.

Kiwi		Forward primer (approximate size of target)						
#	Tissue	NC13 (520bp)	XZ1 (450bp)	Tcat1 (400bp)	W5 (350bp (M&F) & 200bp (F))	YY1 (330bp)	18SF (325bp)	Nemo 18S F (265bp)
1	Lung	–	–	–	F	–	–	–
2	Lung	–	+/−	+	F	–	+	+
3	Brain	–	+/−	+	F	–	+	+
4	Brain2	–	–	–	F	–	+	+
5	Lung	–	+/−	+	M	–	+	+
5	Brain	–	–	+/−	M	–	+	+
	T. cati	+	+	+		–	+	+
	T. canis	+	+	–		+	+	+

Table 4
Results of BLAST analysis for group Ia kiwi, tissues in which larval organisms were identifiable in histological sections taken before and after the sample used for molecular analysis.

Kiwi # (Tissue)	Forward Primer	Sequence length	Aligned to GenBank#	Organism (Target)	Cover	Pairwise Identity	Bit score	E value
2 (Lung)	Tcat1	320	MH043958	*T. cati* (ITS-2)	100%	100%	592.048	4^{e-165}
2 (Lung)	18S	300	EF180059	*T. cati* (18S)	100%	100%	555.115	5^{e-154}
2 (Lung)	Nemo 18S F	215	EF180059	*T. cati* (18S)	100%	100%	398.150	6^{e-107}
3 (Brain)	Tcat1	305	MH043958	*T. cati* (ITS-2)	100%	100%	564.348	8^{e-147}
3 (Brain)	Nemo 18S F	212	EF180059	*T. cati* (18S)	100%	100%	392.610	3^{e-105}
4 (Brain2)	Nemo 18S F	260	EF180059	*T. cati* (18S)	100%	100%	481.249	7^{e-132}
5 (Lung)	Tcat1	322	MH043958	*T. cati* (ITS-2)	100%	100%	595.741	3^{e-166}
5 (Lung)	18SF	231	EF180059	*T. cati* (18S)	97%	100%	427.696	8^{e-116}
5 (Brain)	Nemo 18S F	261	EF180059	*T. cati* (18S)	100%	100%	483.096	2^{e-132}

tissue were present in both histological sandwich sections, and the sequence of the product aligned with *T. cati* (EF180059, bit-score 448.010). Just one other kiwi (#14) had larvae confirmed in both histological sandwich sections, in all three tissues tested; however, this was also one of four birds in group II that failed to amplify using the kiwi gender-specific primers, suggesting overall poor quality DNA in the FFPE samples. Only one of these three tissues was positive with the nematode primers, in only two out of six total PCR runs (once in the initial three runs, and once in the additional three runs). Sequencing results were of poor quality.

The remaining seven positives only amplified in one out of six total PCR runs: three in the first run, and two each in the second and third

Table 5
PCR results in relation to the presence or absence of larvae identified in histological sections taken before and after the sample used for molecular analysis, for all groups. Y = larvae identified histologically, F = PCR positive female, M = PCR positive male, + = amplicon of appropriate size in at least one PCR run (primer set Nemo 18S F-Nemo 18S R), - = negative result.

Group	Kiwi #	Tissue	Presence of larvae		PCR results		Sequencing result
			Before	After	Kiwi DNA	Nematode DNA	
Ia	1	Lung	Y	Y	F	–	
	2	Lung	Y	Y	F	+	*T. cati*
	3	Brain	Y	Y	F	+	*T. cati*
	4	Brain2	Y	Y	F	+	*T. cati*
	5	Lung	Y	Y	M	+	*T. cati*
	5	Brain	Y	Y	M	+	*T. cati*
Ib	2	Liver	Y	–	F	+	*T. cati*[d]
	2	Brain1	–	–	F	–	
	2	Brain2	–	Y	F	+	*T. cati*[d]
	3	Muscle1	–	–	F	–	
	3	Muscle2	–	–	F	–	
	3	Lung	Y	–	F	+	*T. cati*[d]
	3	Liver	–	–	F	–	
	4	Liver	–	–	F	–	
	4	Brain1	–	–	F	–	
	5	Liver	–	Y	M	–	
2	6	Brain	–	–	M	–	
	7	Liver	–	–	F	+[a]	*T. cati*[d]
	8	Lung	–	Y	F	+[a]	*T. cati*[d]
	8	Brain	–	–	F	–	
	9	Lung	–	Y	F	–	
	10	Lung	Y	Y	F	+[c]	*T. cati*[d]
	10	Brain	–	–	F	+[a]	Unsuccessful
	11	Liver	Y	–	–	–	
	12	Lung	–	–	–	–	
	13	Heart	–	–	F	–	
	13	Liver	–	–	F	+[a]	*T. cati*[d]
	14	Brain	Y	Y	–	–	
	14	Liver	Y	Y	–	–	
	14	Lung	Y	Y	–	+[b]	Poor quality
	15	Liver	–	–	F	–	
	16	Brain	–	–	–	+[a]	*T. cati*[d]
	16	Lung	–	Y	–	+[a]	Poor quality
	17	Liver	–	–	F	+[a]	No attempt
	17	Lung	–	–	F	–	
	17	Brain	–	–	F	–	

a = positive in 1/6 PCR runs; b = positive in 2/6 PCR runs; c = positive in 6/6 PCR runs; d = aligned with *T. cati* (EF180059, bit-scores ranging from 204.252 to 483.096).

runs. Two were from tissues in which larvae were identified in a single sandwich section while the other five had no larvae identified in either sandwich section. Amplicons as visualised in the gel were very faint in several cases (e.g. kiwi #10, brain and kiwi #17, liver), and sequencing was either unsuccessful or not attempted. The 11 tissues that were negative in all three initial runs included seven tissues in which larvae were not identified in either sandwich section and two tissues with larvae identified in a single sandwich section; one of each also failed to amplify using the kiwi gender-specific primers.

4. Discussion

This study provides strong evidence that the cause of visceral and neural LM in North Island brown kiwi is the nematode parasite *Toxocara cati* whose definitive host, the domestic cat, is an introduced and invasive species in New Zealand. In a previous study, molecular analysis failed to amplify *Toxocara* DNA in 29 tissues from 18 kiwi diagnosed histologically with LM (van Zyl, 2014). PCR was limited to the use of two species-specific primer sets for *T. canis* and *T. cati* with approximate target sizes of 390bp and 400bp respectively, and did not specifically evaluate the sections tested for the presence or absence of larvae, only for the presence of characteristic inflammatory lesions. Thus for this current study, it was decided to initially test only cases with a high probability of nematode DNA being present in the sections taken for molecular analysis, and to trial a range of primer sets of varying target size, including species-specific and more generic primers.

The predominant histological lesion associated with LM in kiwi resembles the “eosinophilic granuloma” described in human visceral LM (Kaplan et al., 2001). Although not pathognomonic for LM, 14 out of the 17 kiwi also had nematode larvae confirmed histologically in at least one tissue supporting the diagnosis of LM as cause for the lesions. Histomorphology of the nematode larvae in sections from affected kiwi, where cross-sections were available for evaluation, was consistent with that described for *T. canis* and *T. cati* based predominantly on the cross-sectional diameter and presence of single, small, bilaterally symmetrical alae (Nichols, 1956; Bowman, 1987). Differentiating these two *Toxocara* species in histologic section relies on a minor size difference, with one study finding that cross sections through the mid-body of *T. cati* larvae never exceeded 18 μm in diameter, while *T. canis* larvae were often greater than 18 μm but ranged in diameter from 14 μm to 20 μm (Nichols, 1956). The nematodes in kiwi tissues were therefore more consistent with *T. cati*, but not definitively identifiable given the overlap in size ranges and the fact that the level of the larvae measured in diagnostic (as opposed to experimental) sections can't be readily distinguished.

Based on the histomorphology, however, primer sets were chosen that had been designed for identification of ascaridoid nematodes, including one set each designed for the specific identification of *T. canis* and *T. cati*. All of the nematode primer sets evaluated amplified the control organisms *T. canis* and *T. cati* appropriately. It is worth noting that two further primer sets that were initially tested and which reliably amplified the controls were subsequently found to be unsuitable as they also amplified similarly sized target sequences from kiwi tissue (data not shown). This highlights the importance of sequencing to confirm validity of PCR results, especially when using primer sets on a previously untested species.

Successful amplification from FFPE tissue decreased with increasing product size, until no samples were positive for the product of ~520bp length. Formalin fixation causes cross-linkage between DNA and protein (Sengüven et al., 2014); the quality of DNA in formalin-fixed samples degrades over time spent in formalin, and degradation may continue at a low level even after the fixed tissue is processed and embedded within paraffin (Libório et al., 2005). A number of commercial kits designed specifically for the extraction of DNA from FFPE samples are now available, but the quality of DNA extracted also relies heavily on pre-extraction factors including the condition of the fixative, fixation time, and post-fixation storage (Sengüven et al., 2014). Despite this, successful DNA extraction, particularly of small molecular weight fragments, has been proven feasible even from tissue blocks that have been stored for up to 40 years (Libório et al., 2005). In this small sample of cases, the tissue blocks had been stored for up to 14 years. Specific information regarding pre-extraction handling was not readily available although, as is common with wildlife submissions, the time from death to necropsy in the kiwi examined did vary widely which may also result in degradation of DNA quality due to decomposition prior to fixation. However, extraction of kiwi DNA fragments to a size of at least 350bp was successful from all cases archived for five years and less, but only four out of the eight earlier cases, using commercially-available kits.

The only group Ia tissue that was negative for all nematode primer sets was one of the more recent cases (archived less than a year at the time of extraction) and PCR successfully amplified kiwi DNA up to ~350bp in size. Despite the histological confirmation of larvae in both sandwich sections, it is possible that nematode DNA was not included within the extraction sample, or that the quality of nematode DNA present was not equivalent to that of the kiwi tissue. A further consideration is that the nematode present was not *T. cati, T. canis*, or other ascaridoid nematode likely to be amplified by the chosen primers, although the morphology of the larvae in section was compatible with *Toxocara* spp. as previously described.

Results from group I otherwise suggested that PCR is only likely to be successful where there are histologically identifiable nematode larvae in sections directly adjacent to the tissue scrolls taken for molecular analysis, with no amplification from any of the six tissues in which no larvae were evident. Studies evaluating the long-term survival of migrating *T. canis* larvae indicate well-developed strategies for host immune evasion, including the production of a mucin-rich coating over the surface of the cuticle that can be shed in response to adherence by host antibody and inflammatory cells, leaving the organism free to continue migration (Fillaux and Magnaval, 2013; Maizels, 2013). It is likely that such mechanisms reduce the chance that recoverable nematode DNA will be present in tissue migration tracts once the larvae have transited. However, inevitably, a proportion of the larvae will become ‘trapped’ and destroyed by the host's immune response (Parsons and Grieve, 1990; Fillaux and Magnaval, 2013).

Results from the second group of kiwi did not entirely support the hypothesis that PCR is of no diagnostic value if no larvae are identified in histological sections. The one repeatable positive case was the only tissue in which larvae were present in both histological sandwich sections and that also produced successful amplification of kiwi DNA. There were, however, five tissues that were positive despite a lack of histologically identifiable larvae. Each tissue was positive in only one out of three initial duplicate PCR runs. Because of this inconsistency, PCR was repeated on all positives a further three times and was negative in each of these five cases. Considerations for this finding would have to include the possibility of sample contamination causing the original positive results or, in the cases where sequencing was unsuccessful, potential non-specific amplification. Alternatively, it may be that very small amounts of nematode DNA of an amplifiable size were present in the extractions but not consistently included in aliquots taken for PCR. This was suspected to be the case for two tissues with larvae identified in a single sandwich section and one tissue with larvae confirmed in both sandwich sections but with evidence of poor quality DNA, which showed similarly inconsistent positive results.

Two of the positive tissues with no identifiable larvae contained large numbers of granulomata ± extensive acute inflammation histologically, and it is possible that larval sections were obscured or simply missed at histological evaluation, especially if not intact. Additionally, the small size of *Toxocara* larvae (less than 20 μm diameter) could allow for the possibility of larvae or larval fragments present in samples taken for molecular analysis but not in histological sandwich sections. Nonetheless, while it may be worth attempting PCR in cases lacking histologically identifiable larvae, the chances of a reliable positive

result appear to be low, and replication of PCR results is recommended.

In all cases subjected to BLAST analysis, the nematode sequences from kiwi tissues with lesions of LM aligned with sequences from *T. cati* available in the GenBank database. In three of the group Ia kiwi, this included sequences from both the ITS-2 region and 18S gene of nuclear ribosomal DNA, lending weight to the identification. The ITS-2 primer set Tcat1-NC2 was specifically designed for the differentiation of *T. cati* from other ascaridoid nematodes (Jacobs et al., 1997); in contrast there are only two bases different between the sequences of *T. cati* and *T. canis* in the smaller product amplified by the generic 18S primers Nemo 18S F-Nemo 18S R (Pinelli et al., 2013). There is, as yet, no single target sequence that is universally agreed to allow species-level identification, although most investigations in the field of molecular nematology have centred on the "high-copy" sequences present in nuclear ribosomal and mitochondrial DNA (Seesao et al., 2017). Use of BLAST for organism identification relies heavily on the accuracy of the data submitted to the database, including both the integrity of the sequences and the reliability of the accompanying identification (Harris, 2003). The scope of available data is also somewhat limited as, in general, databases of nematode genomics remain relatively sparsely populated. The possibility of a nematode closely related to *T. cati* but unrepresented in GenBank could not be entirely ruled out. A number of other, lesser known *Toxocara* spp. have been described from wildlife but lack molecular studies and remain of uncertain zoonotic potential; for example, *Toxocara pteropodis* in fruit bats (*Pteropus* spp.) (Clark and McKenzie, 1982; Prociv, 1989), and *Toxocara mackerrasae* in the Australian bush rat (*Rattus fuscipes*) (Warren, 1970) (neither of which have been documented in New Zealand).

The identification of *T. cati* as a cause for LM in kiwi is both biologically and ecologically feasible, given the extensive range of the definitive host, the domestic cat, throughout New Zealand (Aguilar et al., 2015). One study into the potential for spread of parasites by feral cats found that 67% of feline faecal samples collected within an area of New Zealand farmland contained *T. cati* eggs (Langham and Charleston, 1990). Another known parasite of cats, *Toxoplasma gondii*, has also been shown to spill over into New Zealand fauna, including kiwi as well as other birds, marine mammals, and shellfish (Roe et al., 2013; Howe et al., 2014; Coupe et al., 2018). The indirect effect of invasive species on native species and ecosystems through co-invading parasites is gaining increasing attention, and the variety of influences that such parasites may have is still being explored (Chalkowski et al., 2018). While LM due to *T. cati* is most likely a sporadic cause of mortality and illness in kiwi and is unlikely to be driving population level changes, its significance may increase where kiwi populations are reduced and managed intensively for conservation.

Infective larvae of *Toxocara* spp. develop within the egg after it is passed in faeces, and remain viable under the right environmental conditions for 6–12 months, potentially longer (Macpherson, 2013). Kiwi feed by probing the surface litter and deeper soil with their long bills and would have a high potential for exposure in contaminated areas. Additionally, the kiwi diet in the wild is composed predominantly of soil and surface-dwelling invertebrates, particularly earthworms (Sales, 2005) which have been shown to act as either transport or paratenic hosts for *T. canis* (Pahari and Sasmal, 1991). Those cases arising in captivity or where kiwi inhabited predator-free sanctuaries at the time of death would have to be investigated on a case-by-case basis for the potential of exposure to *Toxocara* eggs: for example, the length of time the kiwi had been present at the controlled site; the potential for contamination by cat faeces around the perimeter of the controlled site; or any transfer of surface litter or insects from uncontrolled sites.

To the authors' knowledge, this is the first report of *T. cati* causing LM in an avian species other than the chicken (*Gallus gallus domesticus*), where published reports predominantly describe experimental infection (Azizi et al., 2007; Oryan et al., 2010; Taira et al., 2011), largely driven by the zoonotic potential for humans ingesting undercooked or raw muscle or liver from infected animals. The significance of LM to any host depends on the localisation and number of migrating larvae. Early studies on *T. canis* indicated that infections in various laboratory animal species were often well tolerated, up to 10 larvae per gram of body weight in tissue other than the brain (Beaver, 1956), and in humans, subclinical infections are considered common (Fillaux and Magnaval, 2013). Species differences in larval distribution have also been also described (Beaver, 1956). In one six-month study of *T. cati* larval migration in chickens, larvae were most commonly identified in liver, lung, and muscle but seldom in the brain (Taira et al., 2011); no clinical signs or changes in behaviour were observed in this or another long-term study (Oryan et al., 2010), however it has been suggested that the severity of disease may be dose dependant and more significant in natural infections (Azizi et al., 2007).

In this small retrospective study of necropsy cases, brain lesions were histologically confirmed in 10 of the 17 kiwi, however the tissues examined were not uniform in each case and based on the original necropsy reports it appears that only 12 of the kiwi may have had brain histology performed. Other potential predilection sites are not routinely collected at necropsy, and only a single kiwi in this study (#3), which had presented with ataxia prior to death, had sections of spinal cord and skeletal muscle taken for histological evaluation. While subclinical visceral infections may also be common in kiwi, neural infections are likely to have the most impact with the potential to contribute to mortality either directly or through neurological deficits that could interfere with the ability to forage or predispose to death by misadventure. Experimental studies with *T. canis* have also demonstrated lower levels of risk and predator aversion in infected mice than control animals (Holland and Cox, 2001); this is thought to be a pathological side effect rather than true host-manipulation, but is still an effect that could have increased consequence for an endangered species such as the kiwi, already significantly at risk from predation.

There is a single report of a kiwi presenting as a possible definitive host for *T. cati* (Clark and McKenzie, 1982). Nematodes consistent with *T. cati*, as identified by detailed morphological examination, were found at necropsy within the small intestine of a North Island brown kiwi. Both larval and adult forms were present, apparently the first published finding of adult *Toxocara* in birds. The authors speculate that the reported lower body temperature of kiwi relative to similarly sized avian species might have contributed to the ability of kiwi to behave as a definitive host for an organism that is more typically adapted to a mammalian host. None of the pathology reports in the cases from the study presented here describe adult *Toxocara* in the intestinal tract, although a complete parasitological examination is rarely performed for a routine necropsy. The nature of most cases of LM is that migrating larvae are unable to complete their life cycle in an abnormal host and do not mature to adult nematodes. While somatic migration can occur as part of the normal life cycle of *T. cati* in the definitive host, it is worth noting that none of the larvae in the kiwi tissues presented here showed any indication of development beyond the size of the infective larval stage. Further research is required to characterise the nematodes inhabiting the kiwi gastrointestinal tract, to more precisely define the role of kiwi as a host for *T. cati*.

5. Conclusion

The results of this study provide strong evidence for *T. cati* as a cause for visceral and neural LM in the North Island brown kiwi (*Apteryx mantelli)*, and additionally support the utility of PCR using archival FFPE tissue blocks in the investigation of this disease. PCR is most likely to yield a meaningful result when larvae can be identified histologically in sections directly adjacent to those used for molecular analysis, and when primer sets with a small target product (e.g. less than 400bp) are used. The identification of *T. cati* as a cause of LM in native New Zealand kiwi represents an indirect parasite-mediated effect of an invasive mammalian species, the domestic cat.

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Declaration of competing interest

None.

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