Toxoplasma gondii screening in cats and mice using PCR as part of the Cape to City Initiative

Executive Summary

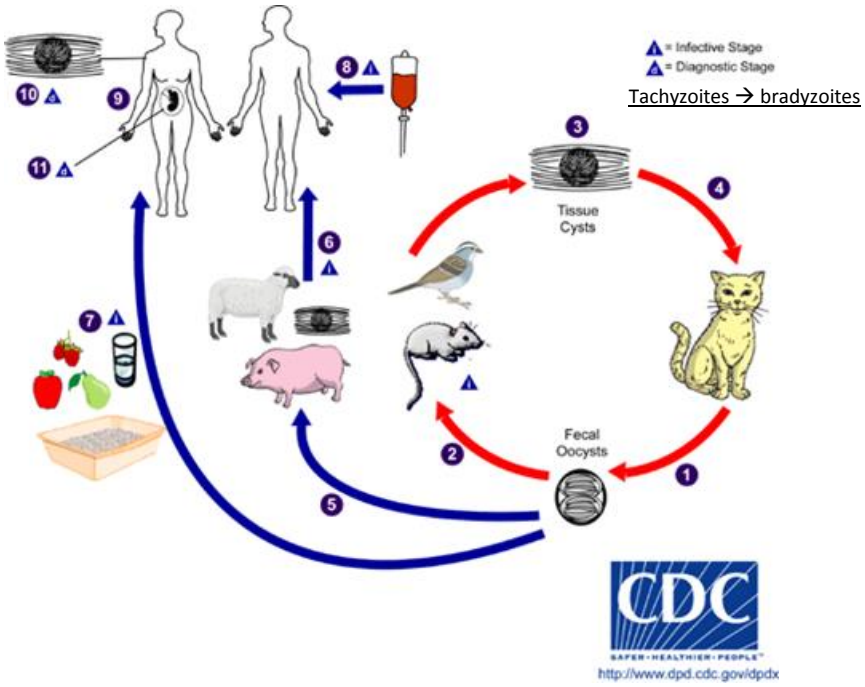
- Cape to City is a collaborative landscape scale project between partners HBRC, DOC, Cape Sanctuary, Landcare Research and various landowners and businesses, covering 26,000ha of land with an aim to 1. Leverage the native species and research success of privately owned Cape Sanctuary to deliver significant conservation outcomes within the project area; 2. Trial large scale, low cost (<$3/ha) predator control (stoats, ferrets and feral cats) techniques for biodiversity enhancement within an agricultural landscape in Hawke's Bay; 3. Drive a long term positive step change in regional biodiversity profile, funding, community engagement and conservation outcomes.
- In this study we aim to determine the impact of feral cat control on the prevalence of *Toxoplasmosis gondii* in ewes, and associated abortion rates, by reducing the abundance of this definitive host, thereby reducing opportunity for infection.
- We optimised a PCR assay for *T. gondii* using a vaccine as a positive control, and developed an effective *T. gondii* DNA extraction protocol from the brain tissue of cats and mice (an intermediary host).
- Ewes were tested for *T. gondii* using a serological assay in 6 farms – 3 case farms within the C2C footprint that included trapping, and 3 outside the footprint that had no trapping. Cats and mice were trapped and tested for *T. gondii* using PCR in the case farms only.
- Across all 6 farms *T. gondii* prevalence ranged from 22% to 80% in ewes. In the 3 case farms prevalence ranged from 26% to 83% in feral cats. The PCR was not successful for mice.
- These high prevalence rates provide ample opportunity to measure reductions in infection of ewes as a result of feral cat control.
- Future work includes, over the next several years, maintaining trapping in the case farms, confirming presence of *T. gondii* in cats and recording prevalence of *T. gondii* in ewes to determine if a trapping program is reducing *T. gondii* in ewes and *T. gondii*-associated abortions. An assessment will also be made on the value of further developing the PCR assay for mice.

Background

Toxoplasmosis gondii is a protozoan parasite with a complex life cycle. Felids (domestic cats and their relatives) are the definitive hosts, which are the only place where the parasites can undergo sexual replication to generate oocysts (Figure 1). A large amount of unsporulated oocysts are shed in the cat's faeces; however, oocyst shedding occurs for only 1-2 weeks. Within 1-5 days, oocysts in the environment sporulate and become infective. All warm-blooded vertebrates can serve as intermediate hosts, which acquire infection through ingesting soil, water or plant contaminated with oocysts – this includes pigs, sheep and humans. Parasites in an intermediate host undergo asexual

replications. First, oocysts transform into tachyzoites shortly after ingestion. Then, tachyzoites develop into tissue cyst bradyzoites, which often localize in neural and muscle tissue. While cats may be infected through ingestion of sporulated oocysts, the infection to cats is much more effective through consuming intermediate hosts containing tissue cysts. Disease manifests more easily in cats with compromised immune systems, which struggle to contain the spread of the tachyzoite form. Humans can be infected by ingesting undercooked meat containing tissue cysts or from food/water contaminated by sporulated oocysts. Humans can also be infected through blood transfusion or organ transplantation. In addition, infection can be transmitted from mother to foetus. In the human host, tissue cysts may remain throughout the life of the host and are most commonly found in skeletal muscle, myocardium, brain, and eyes. *T. gondii* can infect a wide range of intermediary hosts, infecting tissue in cysts, but felines are the primary host that pass the infective oocytes on in their faeces. It can cause a high rate of abortion in sheep on first infection.

Figure 1 The life cycle of T. gondii (figure from the Centers for Disease Control and Prevention)



Objectives

In this study we aim to reduce the prevalence of *T. gondii* and the opportunity for infection of ewes by eradicating its definitive host, thereby reducing abortion rates in ewes.

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1. To develop a molecular PCR assay for the detection of *T. gondii* from host tissue
2. To detect *T. gondii* and determine prevalences in various life history stages (namely feral cats as definitive hosts and mice as intermediary hosts) via PCR, and in 1 year old ewes as additional infections via serology.

Future years

1. To repeat detection of *T. gondii* in cats, mice and sheep to determine if the cat trapping program has been successful at reducing prevalence on farms
2. To assess abortion rates in ewes

Methods

Faecal matter that enters the soil can result in oocysts being distributed by precipitation or can be taken up by earthworms which can carry the infective *T. gondii* [1]. Screening of the feral cat population for the infection will give an indication of whether feral cat control may reduce the impact of the disease in naïve ewes. Many studies have used qPCR or PCR to screen brain tissue for the presence of *T. gondii* DNA.

T. gondii detection in 1 year old ewes by serology

Blood from 60 ewes was sampled from each of three case farms within the C2C footprint (with active feral cat trapping), and three control farms outside the footprint. Samples were collected by local vets and sent to Gribbles, Palmerston North, for serological testing.

Review of brain processing methods for DNA extraction

A challenge with extracting *T. gondii* DNA from an infected brain is that there is a relatively small proportion of target DNA compared to host. To select the appropriate approach, five methods used previously were reviewed. Krijger et al (2014) [1] homogenised brain tissue of moles with an ultra turrax. Although the volume of sample is unclear, the Qiagen DNeasy blood and tissue protocol was used and incubated with lysis buffer for 2.5h at 56°C, agitated with glass beads present, as per booklet. Garcia et al [2] took 15g pig brain sample and homogenised it in 10ml TE, then homogenised with glass beads for 3 min. 500ul homogenate + 500ul extraction buffer were incubated at 56°C for 1h and then a phenol-chloroform extraction done in triplicate. Homan et al 2000 [3] extracted brain, heart and muscle tissue from mice in PBS, on a metal sieve. The homogenate was concentrated by centrifugation and extracted using guanidine thiocyanate. Montoya et al [4,5] digested 30-50g of cat brain in trypsin (0.25%) EDTA (0.025%) in PBS for 1h at 37°C. Samples were then filtered through gauze and centrifuged for 10min at 2000xg before digestion with proteinase K and DNA extracted. Esteves et al [6] also used a trypsin digest (0.2% w/v) in PBS for 3h. The sample was then centrifuged at 3000g for 10min, washed 3 times and DNA extracted using guanidinium thiocyanate using the silica spin column method. Yan et al [7] took 50mg of rat brain tissue and extracted DNA using a commercial kit including a proteinase K digest at 55°C for 4h. Bradyzoites can also be isolated from brain tissue [8] using a 20% dextran (mwt162000) solution in HBSS, which may increase the sensitivity of the PCR assay by concentrating the toxoplasma out of the brain tissue. This can be checked by spiking some brain tissue with infective material followed by a DNA extraction.

Review of PCR methods for DNA amplification and detection

Burg et al 1989 [10] used primers from the B1 region of the sequence (oligo 1 (F) 5'-GGAACTGCATCCGTTCATGAG; oligo 2 (F) 5'-TGCATAGGTTGCAGTCACTG and oligo 4 (R) 5'-TCTTTAAAGCGTTCGTGGTC). These were also used by Montoya et al [5] and Esteves et al 2014 [6]. Reischl et al 2003 [9] compared the detection of the B1 region of the gene to the 529 bp fragment identified by Homan et al [3]. The 529 bp fragment appeared to be more sensitive at detecting the

presence of toxoplasma in the brain tissue. The Tox-8 and Tox-5 primers were used to amplify DNA from a variety of strains and gave a 450 bp product. Quantitative PCR (qPCR), a highly sensitive version of PCR, was completed using Tox-9 and Tox-11 primers, which gave a 162bp region. Garcia et al 2006 [2] used the Tox-4 and Tox-5 primers from Homan et al AFI46527 and an annealing temperature of 55°C which generated a product of size 529 bp. Krijger et al [1] used Tox -9 and Tox-11 as qPCR primers and an annealing temperature of 60°C for 1 min, based on Reischl [9]. Primers are summarised in Table 1.

Table 1 Summary of historical *T. gondii* PCR primers

Primer label	Primer seq (5'-3')	Nt position	Product size	Reference
Oligo 1 (F)	GGAACTGCATCCGTTCATGAG	694-714	193	Burg et al [10]
Oligo 2 (F)	TGCATAGGTTGCAGTCACTG			
Oligo 4 (R)	TCTTTAAAGCGTTCGTGGTC	887-868		
Oligo5 (F) nest	TGCATAGGTTGCAGTCACTG	757-776	96	Montoya et al [5]
Oligo6 (R) nest	GGCGACCAATCTGCGAATACACC	853-831		
Tox-4 (F)	CGCTGCAGGGAGGAAGACGAAAGTTG		533	Homan et al [3]
Tox-5 (R)	CGCTGCAGACACAGTGCATCTGGATT			
Tox-8 (F)	CCCAGCTGCGTCTGTCGGGAT		450	Reischl et al [9]
Tox-5a (R)	GACGTCTGTGTCACGTAGACCTAAG			
Tox-9 (F)	AGG AGA GAT ATC AGG ACT GTA G	143-164	162	Reischl et al [9]
Tox-11 (R)	GCG TCG TCT CGT CTA GAT CG	304-285		

DNA extraction method development

Multiple methods were trialled to find a successful method to extract the brain cysts. Initially the method outlined by Montoya et al [5] was tested with brain tissue that was spiked with the toxovax vaccine. The trypsin digest did not appear to make any difference, and cysts were found in both the supernatant and the pellet. A percol gradient was also trialled. The toxovax tachyzoites were stained with Giemsa's stain before spiking brain tissue and centrifugation. No obvious band of tachyzoites was produced using the gradient. The successful method involved the extraction of 1g of brain tissue using a scaled up CTAB extraction method:

1. Make up fresh CTAB extraction buffer containing proteinase K (Table 2).
2. Add 4.2mL buffer to 1g homogenised brain tissue (homogenise frozen tissue using a scalpel) and vortex well.

3. Incubate at 65°C for 3h, vortexing intermittently.
4. Centrifuge at 13000xg for 3min at 4°C.
5. Transfer supernatant to a clean tube and add 1/3 volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge 5min at 13000xg.
6. Remove top lay and put in new tube with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), vortex and centrifuge at 13000xg for 10min at 4°C. Remove top layer and put in clean tube.
7. Add 1 volume of chloroform:isoamyl alcohol (24:1), vortex and centrifuge and 13000xg for 10min at 4°C.
8. Remove top layer and put in clean tube, then add 2/3 x volume of isopropanol. Place at -20°C for 1/2h.
9. Spin at 13000xg for 5min at 4°C. Discard supernatant.
10. Add 1mL 70% ethanol to the pellet, vortex and centrifuge at 13000xg for 3min. Discard supernatant .
11. Dry pellet and reconstitute in 200µL of ultrapure water.
12. Approximately 600µg (3000ng/µL) DNA was extracted per 1g of brain tissue from the cat samples and 300µg (1500ng/µL) DNA from the mouse brains.

Table 2 Reagent combination for CTAB extraction buffer containing proteinase K

Reagent	1x volume (µL)
0.5M EDTA	155
1M Tris Base	388
10% CTAB	775
5M NaCl	1160
Proteinase K 600µg/mL	450
1M DTT	180
Water	1390
TOTAL VOLUME	4498

PCR primer selection

DNA was extracted from the toxovax vaccine, using the extraction method outlined above, and used to optimise the PCR. We trialled six different primer sets (Table 3), with the aim of adopting the most sensitive set for this study. The PCR product was amplified using the Biomix reagent (Bioline #BIO-25012). PCR steps and conditions were conducted as outlined in the Biomix product insert (Table 4). Primer set 3 appeared the most sensitive and gave the least number of multiple bands (Figure 2). Final annealing temperature was determined using a temperature gradient, with 65°C giving the highest sensitivity and least number of multiple bands for the primer set.

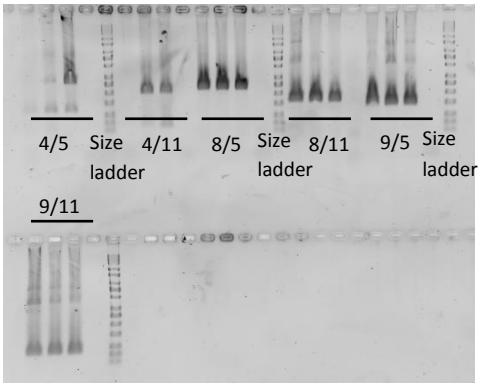
Table 3 Primer sets used in this study. PCR product size was determined by aligning the primer sequences to the partial *T. gondii* sequence from Homan et al [3] using Sequencher 5.4.1 (Gene Codes Corporation © 1991-2015)

Primer set	Primer	direction	Product size
1	Tox4	F	533
	Tox5	R	
2	Tox4	F	407
	Tox11	R	
3	Tox8	F	451
	Tox5	R	
4	Tox8	F	325
	Tox11	R	
5	Tox9	F	288
	Tox5	R	
6	Tox9	F	162
	Tox11	R	

Table 4 PCR conditions

Temperature	Time	Cycles
95	5 min	1
95	30 s	40
65	30 s	
72	1 min	
72	5 min	1
4	∞	1

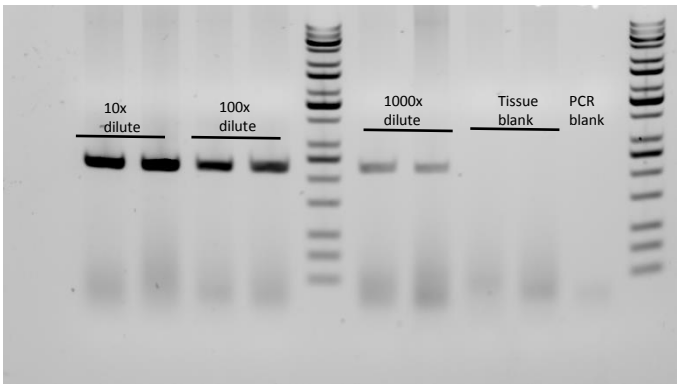
Figure 2 PCR fragments produced by the six primer sets. Each number combination represents a primer combination. Bands in the lanes of the gel represent PCR fragments, separated by size ladders to enable size estimation of bands.



Assessment of PCR detection limits

To assess the detection limit of our assay, we created several test samples with known concentrations of parasite DNA by spiking cat brain tissue with diluted toxovax vaccine. Three 1g brain tissue samples were spiked with 10µL of vaccine diluted either 10, 100 or 1000 fold in ultrapure water. Our assay produced clear and distinct PCR products of the correct size at even the lowest test dilution (Figure 3).

Figure 4 Assessment of PCR assay detection limits using serial dilution. Bands in the lanes of the gel represent PCR fragments, separated by size ladders to enable size estimation of bands.



Results

T. gondii detection in wild caught animals

Cat and mouse samples were collected and analysed from the three case farms (Table 5). Three mouse samples were also collected and analysed from a control farm (Robinson).

Table 5 Summary of samples, sites and PCR results for wildlife *T. gondii* detection on case farms

Farm	Species	Number	Number infected	Confirmed sex
Gordon	Cat	12	4	2 male: 2 female
	Mouse	3	na	na
Radley	Cat	6	5	1 female
	Mouse	4	na	na
Palmer	Cat	23	6	6 male: 12 female
	Mouse	1	na	na

Cat samples were easily identified as positive or negative, however results for mouse samples were ambiguous. Sequencing showed the amplified PCR products from the cat brain to be 99% identical to

the *T. gondii* microsatellite sequence, however amplified PCR products from the mouse brain were 100% identical to the *Mus musculus* chromosome 9.

Summary of *T. gondii* detection

Across all 6 farms *T. gondii* prevalence ranged from 22% to 80% in ewes. Prevalence in cats ranged from 26% to 83% (Table 6). Sample sizes and percentages of cats and ewes that returned positive from the three case farms are outlined in Table 6. No mouse samples could be identified as positive for toxoplasmosis.

Table 6 *T. gondii* prevalence in ewes in 3 control farms, and in ewes and feral cats in 3 case farms. A case farm is defined by an active feral cat trapping program

	Control Farms			**Case Farms**		
Farm Names	Robinson	Steve Tait Jamieson	Mt. Erin	Radley	Gordon	Palmer
Date sent	1/10/2015	16/10/2015	19/11/2015	27/08/2015	18/09/2015	9/10/2015
Toxo prevalence in ewes (%)	14/60	13/60	48/60	19/60 (31)	18/60 (30)	30/60 (50)
Toxo prevalence in cats (%)				5/6 (83)	8/12 (33)	6/23 (26)

Summary

Overall, the year 0 work has revealed a substantial level of toxoplasmosis in 1 year old ewes and feral cats. Levels are high enough in our opinion to enable a change in prevalence in future years to be measured. The required infrastructure is in place to investigate the impacts of feral cat trapping program on the *T. gondii* prevalence rate in ewes susceptible to infection. Abortion rates in the case farms can then be monitored and compared to national averages.

References

1. Krijger I, Cornelissen J, Wisselink H, Meerburg B (2014) Prevalence of Toxoplasma gondii in common moles (Talpa europaea). Acta Veterinaria Scandinavica 56: 1-4.

2. Garcia JL, Gennari SM, Machado RZ, Navarro IT (2006) Toxoplasma gondii: Detection by mouse bioassay, histopathology, and polymerase chain reaction in tissues from experimentally infected pigs. Experimental Parasitology 113: 267-271.

3. Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR1. International Journal for Parasitology 30: 69-75.

4. Montoya A, Miró G, Blanco MA, Fuentes I (2010) Comparison of nested PCR and real-time PCR for the detection of Toxoplasma gondii in biological samples from naturally infected cats. Research in Veterinary Science 89: 212-213.

5. Montoya A, Miró G, Mateo M, Ramírez C, Fuentes I (2009) Detection of Toxoplasma gondii in cats by comparing bioassay in mice and polymerase chain reaction (PCR). Veterinary Parasitology 160: 159-162.

6. Esteves F, Aguiar D, Rosado J, Costa ML, de Sousa B, et al. (2014) Toxoplasma gondii prevalence in cats from Lisbon and in pigs from centre and south of Portugal. Veterinary Parasitology 200: 8-12.

7. Yan C, Liang L-J, Zhang B-B, Lou Z-L, Zhang H-F, et al. (2014) Prevalence and genotyping of Toxoplasma gondii in naturally-infected synanthropic rats (Rattus norvegicus) and mice (Mus musculus) in eastern China. Parasites & Vectors 7: 591.

8. Freyre A (1995) Separation of Toxoplasma Cysts from Brain Tissue and Liberation of Viable Bradyzoites. The Journal of Parasitology 81: 1008-1010.

9. Reischl U, Bretagne S, Kruger D, Ernault P, Costa J-M (2003) Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infectious Diseases 3: 7.

10. Burg JL, Grover CM, Pouletty P, Boothroyd JC (1989) Direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction. Journal of Clinical Microbiology 27: 1787–1792.