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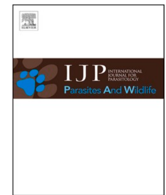

Toxoplasma gondii contamination at an animal agriculture facility: Environmental, agricultural animal, and wildlife contamination indicator evaluation

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ABSTRACT

Toxoplasma gondii is a parasite of significant public health importance. We attempted to detect *T. gondii* contamination and assess advantages and disadvantages of contamination indicators through surveilling soil, wildlife, cats (*Felis catus*), and cows (*Bos taurus*) on a farm in Tennessee, U.S. in 2016 and 2017. Twenty-two soil samples were collected from the farm and subjected to oocyst flotation, DNA extraction, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) targeting 18S ribosomal RNA (18S rRNA) gene to detect and identify *T. gondii*. Three samples (13.6%) were positive for the parasite; however, *T. gondii* DNA was not consistently detected from repeated tests. Blood samples were collected from small mammals, cats, and mesopredators live-trapped on the farm, and serum from 30 of the farm's cows were obtained. Serological testing by the modified agglutination test (MAT; cutoff 1:50) found 2.5% (1/40) of small mammals, 52.9% (9/17) of raccoons (*Procyon lotor*), and 50% (1/2) of domestic cats were seropositive for *T. gondii* antibodies. No antibodies were found in 16 opossums (*Didelphis virginiana*), two skunks (*Mephitis mephitis*), and 30 cows. Small mammal tissue samples were subjected to PCR-RFLP detection. Four out of 29 (13.7%) tissue samples were positive for *T. gondii*; however, *T. gondii* DNA was not consistently detected during repeated PCR-RFLP testing. Our results indicate the ability to detect *T. gondii* varies greatly by contamination indicator. We found detection of soil oocysts to be challenging, and results suggest limited utility of the method performed. The ability to detect *T. gondii* in animals was highly variable among species. Our research emphasizes the importance of a holistic approach when surveilling for *T. gondii* to compensate for shortcomings of each contamination indicator. Future research should be conducted to further investigate the most effective *T. gondii* surveillance methods and species with increased sample sizes at other agricultural facilities.

1. Introduction

Toxoplasma gondii is a coccidian parasite that has a broad spectrum of hosts and is of public health importance (Dubey, 2010; Aguirre et al., 2019). Felids are the definitive hosts of *T. gondii*, being the only hosts where sexual reproduction takes place (Dubey, 2010). Estimating domestic cat (*Felis catus*) population sizes are difficult, but there are approximately 100 million owned cats and estimates of 60 to over 100 million feral and free-ranging cats in the United States (Flockhart and Coe, 2018; Lepczyk and Duffy, 2018). As many as 74% of adult cat populations worldwide are seropositive for *T. gondii*, indicating a current or previous shedding of oocysts (Tenter et al., 2000). Within one to three weeks following ingestion of sporulated oocysts or tissue cysts, felids can shed 3 to 810 million oocysts into the environment (Dubey, 2010). In addition to the risks presented by their abundance, oocysts are environmentally resistant with a high refractory nature to disinfectants (Dubey, 1998; Mirza Alizadeh et al., 2018). Oocysts can be dispersed into environmental matrices including water, soil, and plants with all

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posing risks for human infection (Tenter et al., 2000). The soil contamination rate has been reported to be closely associated with the activities of cats, and the chance of detecting oocysts in randomly selected areas is likely to be low (Afonso et al., 2008; Gotteland et al., 2014; Davis et al., 2018). Therefore, the surveillance of *T. gondii* contamination in the soil is of great importance; however, the extent and prevalence of environmental contamination of oocysts is largely undefined.

The high infectivity of oocysts further perpetuates the transmission of the parasite. Intermediate hosts, including mammals and birds, can contract *T. gondii* by ingestion of tissue cysts through predation, scavenging, or via ingesting oocysts in the environment disseminated in food, water, and soil (Weigel et al., 1995; Lehmann et al., 2003). The parasite chronically infects one-third of the global human population and 6.7% of U.S. born individuals aged 12 to 49 (Jones et al., 2014). Human infection of *T. gondii* occurs through ingestion of sporulated oocysts or tissue cysts, with primary fomites being contaminated meat, plants, or inadvertent oral contact with contaminated soil (Dubey, 2010). One oocyst alone can experimentally infect a pig (*Sus scrofa*; Dubey et al., 1996) or a rat (*Rattus norvegicus*; Dubey, 1996). Due to the high infectivity of *T. gondii* for many species, the seroprevalence of peridomestic animals can help assess the presence of *T. gondii* contamination in addition to soil surveillance.

Collectively, the environmental resistance, high infectivity, and abundance of oocysts contribute to the successful transmission of *T. gondii* and pose a threat to public health and wildlife conservation. Surveillance and control of *T. gondii* contamination is critical to assess infection risks in both humans and non-human animals and alleviate the social and economic burden of toxoplasmosis (Torgerson et al., 2015). The understanding of the eco-epidemiology of *T. gondii* environmental contamination is fundamental for the development of parasite control methods (Tenter et al., 2000). *Toxoplasma gondii* is well studied, with over 15,000 original research articles as of 2000 (Tenter et al., 2000). However, many studies attempt to detect *T. gondii* contamination without a holistic approach or determining the most effective contamination indicator in the study region thereby ignoring the interconnectedness of humans, non-human animals, and the environment in *T. gondii* eco-epidemiology. To this end, when assessing *T. gondii* infection risk it is imperative to investigate a plethora of viable means of detecting *T. gondii* contamination in a region of interest to monitor the parasite and potential transmission routes. The lack of studies using a holistic approach reduces the ability to compare various contaminator indicators in a single area during the same time. Our study aims to elucidate the benefits and challenges of different *T. gondii* detection methods utilizing a variety of indicators (i.e., soil, wildlife, domestic cats, and agricultural animals) while using an agricultural facility as a case study.

2. Materials and methods

2.1. Study site

The Little River Animal and Environmental Unit, located in Walland, Tennessee on two square kilometers of land (Fig. 1), was selected as a representation of a modern small-scale dairy farm. Field work at the study site primarily occurred between September 2016 and April 2017 which comprise fall, winter, and part of spring. Monthly temperatures ranged from 3.8 °C to 22.4 °C and precipitation ranged from 9.9 cm to 13 cm, with snowfall occurring rarely and in limited quantities with the highest monthly average in January and February at 4.1 cm (Young et al., 2018). The farm was owned by the University of Tennessee and was primarily used for research and education, with an emphasis on milk and Holstein cow (*Bos taurus*) production. The farmland was unfenced allowing wildlife and felids to be on the premises. Cows were kept both in open air structures and on pasture. There were reportedly at least six cats on the farm, which were kept for rodent control. The cats were fed by the farmers but had freedom to predate wild animals. To surveil for *T. gondii* contamination at the farm we conducted environmental soil surveillance, peridomestic wildlife surveillance, and food animal surveillance to evaluate the most effective indicators for detecting contamination in the study region.

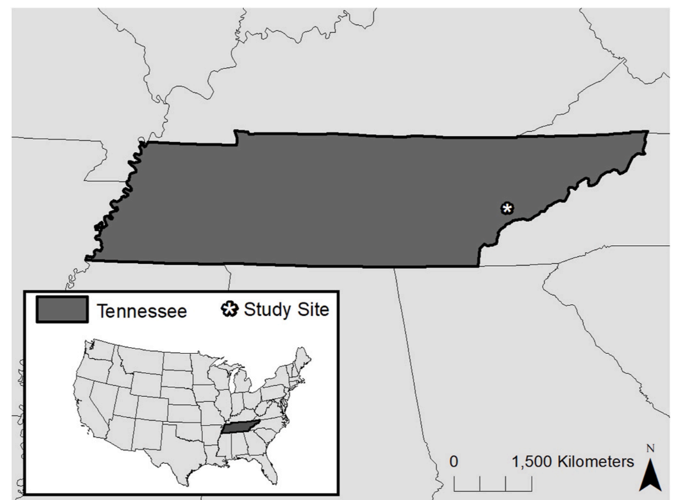

Fig. 1. The geographic location of the study site, Little River Animal and Environmental Unit in Walland, Tennessee, United States.

2.2. Soil surveillance

Twenty-two soil samples were collected from two sampling locations in May 2016 (Fig. 2). The first sampling location was the calf raising area (n = 10 samples) and the second was the maintenance shop and silage storage area (n = 12 samples). Both locations were selected for their high incidence of human and cow contact in conjunction with where cats were fed, observed resting, and seen free-roaming. A targeted random sampling scheme was chosen as the basis for soil contamination detection as focusing on cat habitats provided the best potential of detecting contamination with limited preliminary sampling. In each location, soil sampling sites were chosen to represent different vegetation and soil types. Approximately 20 g of surface soil were collected from each site from a depth of no more than 2 cm in an area 10 cm by 10 cm and stored at 4 °C.

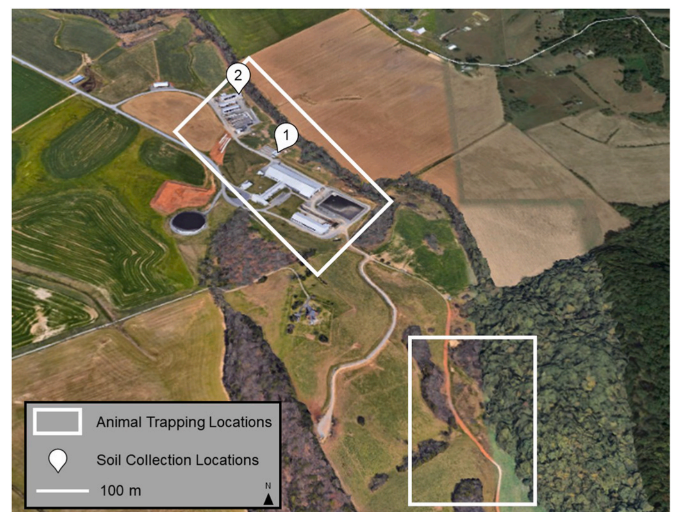

Fig. 2. Soil sampling and animal trapping locations at Little River Animal and Environmental Unit in Walland, Tennessee, United States.

Sheather's sugar solution was used to concentrate the oocysts and prepare them for DNA extraction through soil flotation (Lelu et al., 2011; Zajac and Conboy, 2012). First, soil samples were dried at room temperature and sifted using sieves with mesh size of 1 × 1 mm. For each sieved soil sample, 5 g were placed in a 50 ml tube and was mixed with 5 ml of 2% sulfuric acid. The mixture was then incubated at 4°*C* overnight and diluted with 15 ml ddH_2O the following day. In a new 50 ml tube, 40 ml cold sucrose solution (cane sugar; density: 1.20) was added to the soil and overlaid with distilled water, followed by subsequent centrifugation at 1500×*g* for 20 min as previously described (Gerhold et al., 2015). To attempt collection of as many oocysts as possible, the water-sugar interphase (approximately 10 ml) was obtained for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The content was washed, centrifuged, and eventually resuspended in 100 μl ddH_2O.

The concentrated samples were subjected to three cycles of freeze (−80°*C*) and thaw (20°*C*) with no fewer than 4 h at each temperature. Detection of *T. gondii* directly from lysed oocysts without DNA extraction was attempted according to a publication by Gerhold et al. (2015). Detection with DNA extraction was also attempted on the oocyst lysates using NucleoSpin soil DNA extraction kit (Macherey-Nagel, Duren, Germany; Cat: #740780.50), following the manufacturer's instructions. DNA extraction was performed on two independent subsamples for each of the 22 soil samples.

For each extracted DNA subsample, two independent PCR-RFLP were performed. PCR-RFLP targeting 18S ribosomal RNA (18S rRNA) gene was used for *T. gondii* detection using extracted DNA (Silva et al., 2009). This PCR-RFLP method was used to differentiate *T. gondii* from other closely related apicomplexans (*Hammondia hammondi, Neospora caninum, Sarcocystis* spp.) without sequencing. The external primers Tg18s48F/Tg18s 359R and the internal primers Tg18s 58F/Tg18s 348R were used for nested PCR amplification (Silva et al., 2009). The nested PCR product was approximately 290 base pairs for *Sarcocystis neurona*, *N. caninum*, *T. gondii*, and *H. hammondi*; approximately a 310 base pair product was generated for *Sarcocystis* spp. Restriction enzymes DdeI, Hpy188III, and MspI were used to digest PCR-products to differentiate among *T. gondii*, *H. hammondi*, *N. caninum* and *Sarcocystis* spp. Positive controls included in our study were DNA from *T. gondii* (PTG strain) cell lysates, extracted DNA from *H. hammondi* oocysts, and DNA from *N. caninum* cell lysates. In addition, 25% of negative controls (3:1 sample to negative control ratio) were incorporated in the PCR-RFLP, for which DNA was replaced by water in the PCR-RLFP reaction mix.

For validation, we investigated if doubling the amount of soil would yield different results. Ten grams from each of the 22 samples subjected to the same procedure as mentioned above for oocyst detection. To test the efficacy of the methodology, namely oocyst extraction in conjunction with PCR-RFLP detection, we spiked two *T. gondii*-negative soil samples, one with 10^5 *H. hammondi* oocysts and the other with 10^6. *Hammondia hammondi* was used as a substitute for *T. gondii* due to its inability to infect humans and its close genetic relation to *T. gondii* (Silva et al., 2009). The spiked soil samples were subjected to the same procedure as described above for oocyst detection.

2.3. Animal surveillance

Small mammal trapping occurred from September 2016 to November 2016 and both small mammal and mesopredator trapping occurred from December to April 2017. Animal trapping and handling was conducted in compliance with the procedures approved by the Institutional Animal Care and Use Committee (IACUC; Protocol No. 2438-0716). Trapping was conducted in areas with varying proximities to human activity/farm buildings in differing habitat types (Fig. 2) to investigate contamination across habitat types represented on the property.

Small mammals were trapped using Sherman traps and anesthetized with cotton balls infused with isoflurane. A divider was used in the trap to separate the isoflurane-soaked cotton balls from physically touching the small mammals due to the potentially caustic nature of isoflurane. Trapped small mammals were then euthanized by heart injection of pentobarbital (Fatal-Plus Solution, 390 mg/mL). Small mammals were euthanized due to concerns of serum-based diagnostics potentially having less sensitivity in small mammals and in attempts to genotype the parasite (Dubey et al., 1995, 1997; Dubey 2010) Animals were dissected in the lab under a biosafety hood. Heart and brain tissues, and approximately 100 μl of blood from the chest cavity, were collected from each small mammals. Small mammal trapping was terminated after March 5th, 2017 due to budgetary constraints and low seroprevalence in the animals.

Mesopredators were trapped using Tomahawk traps and sedated using Ketamine (100 mg/ml) and Xylazine (20 mg/ml). Racoons received 16 mg of Ketamine per kg of body weight and 3.2 mg of Xylazine per kg of body weight. Opossums and skunks received 10 mg of Ketamine per kg of body weight and 2 mg of Xylazine per kg of body weight. After sedation, mesopredators had blood samples collected from the cephalic vein apart from the opossum (*Didelphis virginiana*), from which blood was collected from the ventral caudal (tail) vein. Farm cats were not anesthetized but restrained for blood collection. Mesopredators received numbered ear tags for recapture identification. Mesopredators had Atipamezole (5 mg/ml; 0.15 mg per kg of bodyweight) administered intramuscularly to reverse anesthesia and after fully conscious, were released back into their habitat. Recaptured mesopredators previously determined to have a high titer (≥1:50) of *T. gondii* antibodies, would be euthanized and the isolation of parasites from collected tissues would be attempted. Sample sizes for mesopredators were only limited by the number of animals able to be trapped within five months of near daily trapping. Lastly, to monitor incidence of livestock infection and potential human health risk, all available banked serum samples (n = 30) originating from cows at the study site were tested for seroprevalence.

Serum samples were tested via Modified Agglutination Test (MAT) using two-fold dilutions from 1:25 to 1:3200 (Dubey and Desmonts, 1987). A cutoff value of 1:25 is considered seropositive (Dubey et al., 1995; Su and Dubey 2020). However, the MAT antigen used for this study is approximately 1–2 titers higher than the bioMérieux antigen commonly used. Therefore, a cutoff value of 1:50 was also summarized as comparable to 1:25 using the bioMérieux antigen. Dilutions beyond 1:3200 were performed to identify the seropositive titer for samples seropositive at 1:32000. To detect *T. gondii* in tissues of trapped small mammals, PCR-RFLP was utilized. Five grams of tissues (brain and heart combined) were homogenized and DNA was extracted from 100 μl (100 μg) of homogenized tissues using DNeasy Blood & Tissue Kit (Qiagen, Cat. No. 69504) and eluted in 100 μl elution buffer. DNA extraction was performed on two independent subsamples for each animal specimen, and two independent PCR-RFLP were performed for each extracted DNA subsample.

3. Results

3.1. Soil *T. gondii* detection

We were able to detect *H. hammondi* from both soil samples spiked with oocysts, indicating the methodology was effective at that concentration. However, the results of PCR-RFLP detection of *T. gondii* oocyst were not consistently positive (Table 1). In the first test, *T. gondii* appeared in the positive controls (PTG strain) and in two soil samples. In the repeat experiment, an additional soil sample was *T. gondii* positive, and earlier positive samples were not consistently positive (Table 1). When testing if the addition of five more grams of soil from each of the 22 samples increased oocyst detection, no positive samples were detected (Table 2).

Table 1
PCR-RFLP results of 22 soil samples collected.

Sample ID	[a]The first 5 g of soil			
	Test #1		Repeat #1	
	Top	Interphase	Top	Interphase
[c] #1	[b]N	Not done	N	No done
[d] #2	*T. gondii*		N	
#3	N	N	N	N
#4	N	N	N	N
#5	N	N	N	N
#6	N	N	N	N
#7	N	N	N	N
#8	N	N	N	N
#9	N	N	N	N
#10	N	N	N	N
#11	N	*T. gondii*	N	N
#12	N	Band pattern unknown	N	N
#13	N	N	N	N
#14	N	N	N	N
#15	Band pattern unknown	Band pattern unknown	Band pattern unknown	N
#16	N	N	N	N
#17	N	N	N	N
#18	N	N	N	N
#19	N	N	N	N
#20	N	N	N	N
#21	N	N	*T. gondii*	N
#22	N	N	N	N
[e]*T. gondii*	*T. gondii*		*T. gondii*	
[e]*H. hammondi*	*H. hammondi*		*H. hammondi*	
[e]*N. caninum*	*N. caninum*		*N. caninum*	
Negative controls	Total 18 negative controls were included, and all were negative		Total 18 negative controls were included, and all were negative	

H. hammondi oocysts were spiked into 5 g of soil. *Hammondia hammondi* DNA was detected from positive controls.
[a] DNA was extracted from the enriched oocysts from 5 g of soil and used for PCR-RFLP. Positive controls in which 10^5or 10^6.
[b] N denotes negative result.
[c] Only top aqueous layer (T) was collected for this sample.
[d] Top aqueous layer (T) and interphase (I) were combined for this sample.
[e] Positive controls.

Table 2
PCR-RFLP results of repeat testing of 22 soil samples collected.

[a]Sample ID	[b]5g of soil		[c]10g of soil	
	Test #1	Repeat #1	Test #1	Repeat #1
#1	Not done	Not done	N	*T. gondii*
#2	Not done	Not done	N	N
#3	[d]N	N	*H. hammondi*	*H. hammondi*
#4	Not done	Not done	N	N
#5	N	N	N	*H. hammondi*
#6	Unknown band pattern	N	*H. hammondi*	N
#7	N	N	N	N
#8	N	N	*H. hammondi*	*H. hammondi*
#9	N	N	N	N
#10	N	N	N	N
#11	N	N	N	Unknown band pattern
#12	Unknown band pattern	N	Unknown band pattern	Unknown band pattern
#13	N	N	Unknown band pattern	Unknown band pattern
#14	N	N	Unknown band pattern	Unknown band pattern
#15	N	N	Unknown band pattern	Unknown band pattern
#16	N	N	N	N
#17	N	Unknown band pattern	N	*T. gondii*
#18	N	N	Unknown band pattern	Unknown band pattern
#19	N	N	N	N
#20	N	N	N	N
#21	N	N	Unknown band pattern	*T. gondii*
#22	N	N	Unknown band pattern	*T. gondii*
[e]*T. gondii*	*T. gondii*		*T. gondii*	
[e]*H. hammondi*	*H. hammondi*		*H. hammondi*	
[e]*N. caninum*	*N. caninum*		*N. caninum*	
Negative controls	Total 15 negative controls were included, and all were negative		Total 18 negative controls were included; one was false positive with *T. gondii,* and one was false positive with *H. hammondi*	

H. hammondi oocysts were spiked into five or 10 g of soil. *Hammondia hammondi* DNA was detected in positive controls.
[a] Postive samples were included in which 10^6.
[b ,c]DNA was extracted from a new set of 5 g or 10 g of soil.
[c] To test if increasing the quantity of soil would yield better results, 10 g of soil were subjected to sugar floating, DNA extraction, and PCR-RFLP.
[d] N denotes negative result.
[e] Positive controls.

3.2. Animal T. gondii detection

A total of 40 small mammals were trapped and sampled. Serum, blood, and heart tissue samples were obtained from 27 house mice (*Mus musculus*), one Norway rat (*Rattus norvegicus*), five white-footed mice/ deer mice (*Peromyscus* spp.), six cotton rats (*Sigmodon hispidus*), and one eastern mole (*Scalopus aquaticus*) (Table 3). The majority of house mice (96.3%, 26/27) and one Norway rat were captured in developed farm areas, whereas other species of rodents were captured next to the woods.

Serological tests via MAT revealed that with a cutoff at 1:25, 18.5% (95% CI: 7.03–38.75%) (5/27) of the house mice, 40% (95% CI: 7.26–83.0%) (2/5) of white-footed mice/deer mice were positive for *T. gondii* antibodies (Table 3). However, the positivity of rodents significantly decreased with a cutoff set at 1:50. With a cutoff at 1:50, 3.7% (95% CI: 0.19–20.9%) (1/27) of house mice and 0% (95% CI: 0–53.71%) (0/5) of white-footed mice/deer mice were *T. gondii* seropositive (Table 3). The remaining small mammal species were seronegative at all cutoff points. Seroprevalence of small-mammals in more anthropogenically developed habitat was 17.9% (95% CI: 6.77–37.58%) at a cutoff value set at 1:25 and 3.6% (95% CI: 0.19–20.24%) using a 1:50 cutoff. Comparatively, seroprevalence in the less developed trapping area was 16. 7% (95% CI: 2.94–49.12%) at a cutoff value set at 1:25 and 0% (95% CI: 0.00–30.13%) using 1:50.

Brain and heart tissues from 29 small mammals, including seventeen house mice, five white-footed mice/deer mice, five cotton rats, one Norway rat, and one eastern mole, were subjected to PCR-RFLP. Four samples were positive for *T. gondii* during the first test (Table 4). However, these four samples were not consistently positive in the subsequent repeat tests (including DNA extracted from separate tissue homogenates) (Table 4). Two samples were consistently positive with one being *H. hammondi* and the other being *Sarcocystis* spp. (Table 4). No sample was found consistently positive for *T. gondii* (Table 4). The four tissue samples positive in PCR-RFLP detection were all MAT negative for antibodies. Bioassays in lab mice were unable to be conducted due to concerns of introducing wild rodent viruses into the animal testing facility.

Seventeen raccoons (*Procyon lotor*), 16 opossums (*Didelphis virginiana*), two skunks (*Mephitis mephitis*), and two adult farm cats were trapped (Table 3). With the cutoff value set at 1:25, 70.6% (95% CI: 44.05–88.62%) (12/17) of raccoons and 50% (95% CI: 9.45–90.55%) (1/2) of farm cats were serologically positive for *T. gondii* (Table 3). With the cutoff value set at 1:50, 52.9% (95% CI: 28.53–76.14%) (9/17) of raccoons and 50% (95% CI: 9.45–90.55%) (1/2) of farm cats positive for

Table 3
Seroprevalence of *Toxoplasma gondii* in captured animals in Tennessee from 2016 to 2017.

Species	No. of sera tested	Titer										% positive sera (cutoff at 1:25)	% positive sera (cutoff at 1:50)
		<25	25	50	100	200	400	800	1600	10,000	20,000		
House mouse	27	22	4	1	0	0	0	0	0	0	0	18.5% (5/27) (CI 7.03–38.75%)	3.7% (1/27) (CI 0.19–20.9%)
Norway rat	1	1	0	0	0	0	0	0	0	0	0	0% (0/1) (CI 0–94.54%)	0% (0/1) (CI 0–94.54%)
White-footed mice/ deer mouse	5	3	2	0	0	0	0	0	0	0	0	40% (2/5) (CI 7.26–83.0%)	0% (0/5) (CI 0–53.71%)
Cotton rat	6	6	0	0	0	0	0	0	0	0	0	0% (0/6) (CI 0–48.32%)	0% (0/6) (CI 0–48.32%)
Eastern mole	1	1	0	0	0	0	0	0	0	0	0	0% (0/1) (CI 0–94.54%)	0% (0/1) (CI 0–94.54%)
Raccoon	17	5	3	0	2	0	2	1	2	1	1	70.6% (12/17) (CI 44.05–88.62%)	52.9% (9/17) (CI 28.53–76.14%)
Opossum	16	16	0	0	0	0	0	0	0	0	0	0% (0/16) (CI 0–24.07%)	0% (0/16) (CI 0–24.07%)
Domestic cat	2	1	0	0	0	0	0	0	1	0	0	50% (1/2) (CI 9.45–90.55%)	50% (1/2) (CI 9.45–90.55%)
Skunk	2	2	0	0	0	0	0	0	0	0	0	0% (0/2) (CI 0–80.21%)	0% (0/2) (CI 0–80.21%)

Table 4
PCR-RFLP results of 29 tissue samples collected from small animals.

[a]Sample ID	[c]The first DNA extraction			[c]The second DNA extraction	
	Test #1	Repeat #1	Repeat #2	Test #1	Repeat #1
925HM2	*T. gondii*	Unknown band pattern	Unknown band pattern	[b]N	*T. gondii*
1023HM1	*H. hammondi*	*H. hammondi*	*H. hammondi*	*H. hammondi*	*H. hammondi*
1029HM1	*T. gondii*	*T. gondii*	*T. gondii*	N	N
1029CR1	*T. gondii*	N	*T. gondii*	N	N
1113HM1	*T. gondii*	N	N	N	*N*
1113HM2	*Sarcocystis* spp.	*Sarcocystis* spp.	*Sarcocystis* spp.	*Sarcocystis* spp.	*Sarcocystis* spp.
1113CR1	Unknown band pattern	N	N	N	*N*
Negative controls	Seven negative controls were included, and all were negative	Eight negative controls were included, and one was false positive for *T. gondii*			
[d]*T. gondii*	*T. gondii*			*T. gondii*	
[d]*H. hammondi*	*H. hammondi*			*H. hammondi*	
[d]*N. caninum*	*N. caninum*			*N. caninum*	

[a] Twenty-nine tissue samples were included for *T. gondii* detection using PCR-RFLP. These 29 tissues were from 17 house mice, five cotton rats, five white-footed mice/deer mice, one Norway rat, and one eastern mole. DNA was extracted from tissue homogenates. The seven samples listed in the table were those had the potential to be positive for any of three parasites (*T. gondii, H. hammondi, and N. caninum*) based on the results of first test and thus were included for further testing. Twenty-two samples that were initially tested negative were not included in this table; these twenty-two included 94HM1, 910rat, 925HM3, 925HM4, 917HM1, 924DM1, 917HM2, 924DM2, 924HM1, 924HM2, 924DM3, 924HM3, 925HM1, 1023HM2, 1023HM3, 925DM1, 1022HM1, 1022DM1, 1029CR2, 1029 mol, 1113CR2, 1114CR1.
[b] N: negative result.
[c] DNA was extracted from another 100 μl of tissue homogenates.
[d] Positive controls.

T. gondii (Table 3). No skunks or opossums were seropositive. Attempts to isolate parasites and bioassays from mesopredators were not conducted because no animal was recaptured with a known high titer (≥1:50) of *T. gondii* antibodies. Lastly, all 30 cow serum samples tested with MAT were negative for *T. gondii* antibodies.

4. Discussion

While our study was limited to a single area for a limited time period with smaller sample sizes, our results elucidated the difficulties in consistently identifying *T. gondii* contamination even when using a variety of indicators. From the 22 soil samples collected from two cat habitats, no sample was consistently detected positive for oocysts. The lack of positive samples signifies the absence of oocysts (due to cats potentially not defecating in the area or oocysts no longer being viable after the cessation of cat shedding), limited sensitivity of detection method used, or the low concentration of oocyst in the soil which falls below the detection limit of our tests. Our results coincide with a previous publication in which no positive samples were detected from 120 soil samples collected from the University of Hawaii at Mānoa (Davis et al., 2018). However, other studies have successfully identified *T. gondii* oocysts detected at cat defecation sites (Afonso et al., 2008; Gotteland et al., 2014) and prevalences as high as 66.3% in soil at dairy farms with cats in France (Simon et al., 2017) indicating variability in soil contamination levels and/or detection.

The sugar floating technique has been widely used to isolate oocysts from soil samples to eliminate PCR-RLFP inhibitors such as humic acid (Lelu et al., 2011). The method we used in our study was based on methods by Lelu et al. (2011). In this study, the conventional floating method was modified by underlying a sugar solution with the soil suspension, generating a 10-fold higher yield (Lelu et al., 2011). An experimental seeding of soil had shown that 100–1000 oocyst/g was detectable in 50% of assays (Afonso et al., 2008). In our study, we were able to detect soil spiked with *H. hammondi* oocysts, indicating the efficacy of the detection method. However, further testing of soil spiked with lower numbers of *H. hammondi* oocysts is necessary to determine the detection limit of the method.

Three cycles of freeze-thaw were shown to be effective to lyse oocysts for subsequent DNA extraction (Manore et al., 2019). NucleoSpin soil DNA extraction kit (Macherey-Nagel, Cat. No. 740780) outcompeted four other DNA extraction kits to detect 50 spiked *T. gondii* oocysts per 1 g of fecal sample (Herrmann et al., 2011). However, the DNA extraction

process also lost DNA as evidenced by a 100-time detection limit difference between groups with and without DNA extraction using lysed oocysts (Gerhold et al., 2015). In our study we were not able to detect oocysts directly from lysates without DNA extraction probably due to PCR-RLFP inhibition (inhibitors in the soil). Therefore, DNA extraction was conducted. Despite not detecting soil contamination, environmental surveillance remains important. Previous research indicates that soil contamination poses a risk for human and animal *T. gondii* infection with increased risk for children due to frequent soil contact (Dattoli et al., 2011; Stagno et al., 1980). The increased demand for meat products from organically raised animals also increases the odds of human infection both for farm workers and consumers (Weigel et al., 1999). Contacting contaminated soil rather than handling swine has been suggested as the primary route of *T. gondii* infection in farm workers (Weigel et al., 1999). Organically (non-confinement) raised pigs have been observed to have higher rates of *T. gondii* infection likely due to the increased risk of contacting the parasite in soil with more contact with a larger environment (Dubey et al., 2012; Guo et al., 2016). Therefore, soil contamination surveillance is an important aspect to understanding *T. gondii* transmission in an area.

Ease of collection and availability presents strong advantages for soil as a contamination indicator. However, low detection of oocysts in soil requires large sample sizes (much greater than the sample size used in our investigation) to provide the best chance of parasite detection. Furthermore, care must be taken when designing an effective soil sampling protocol to target areas most likely to have contamination (i.e. defecation areas of cats) which necessitates knowledge of animal use in the area. Even if defecation sites are known, it's estimated that only 1% of cats are shedding oocysts at a time (Dubey 2010), which often necessitates the detection of older oocysts that have persisted in the soil. The detection sensitivity of oocysts in soil varies depending on an array of factors including oocyst sporulation status, oocyst age, as well as soil features. Sporulated oocysts, young oocysts, and soil with less sand reveal higher recovery rates (Lelu et al., 2011). Temporal variation in oocyst detection has also been observed indicating the need to sample a study area across multiple years for thorough data collection (de Wit et al., 2020), presenting obvious limitations. The small soil sample size is a limitation of our study and emphasizes the drawbacks of doing small scale preliminary contamination surveillance using this method. Soil contamination surveillance is warranted, but challenges in using the indicator are present.

In addition to surveilling soil, the infection rate in animals was investigated. A geographical partition of small animals was evidenced by increased capture of rodents associated with human dwellings near human activity in less forested landscape. Twenty-seven house mice and one Norway rat were captured in the developed farm area; whereas other species of rodents, including five white-footed mice/deer mice and seven cotton rats, were captured at the forest edge. Seroprevalence in small-mammals was similar between the areas with varying anthropogenic influence despite differences in the species captured. Given that small rodents are the prey of cats, partitioning of rodent species may lead to partitioning of *T. gondii* genotypes through different transmission cycles (Jiang et al., 2018). Apparent spatial segregation of small-mammal species in the area could be the focus of future study to investigate the potential for *T. gondii* genotype partitioning. Home ranges of meso-mammals likely extended the entire property; therefore, differences in seroprevalence by trapping location were not examined for meso-mammals.

MAT is widely used to detect *T. gondii* infection in animals and shows high sensitivity and specificity (Shaapan et al., 2008; Afonso et al., 2006; Gamble et al., 1999). Attempts to detect *T. gondii* in small mammals using PCR did not yield consistent positive results. For the first set of PCR-RFLP negative controls, there were zero out of seven false positives, meaning the contamination rate was lower than 14.3%. For the second set of PCR-RFLP negative controls, one out of eight samples was a false positive, giving a contamination rate of approximately 12.5%. Some samples conducted during the second set yielded inconsistent positive results. Inconsistent results could be due to having low DNA concentration or contamination, given the background contamination level. Therefore, only samples that had consistent results were considered reliable and used, resulting in no PCR positive small animals. Bioassay in mice or cats and a robust sample size were required to truly elucidate rodent infection rates but were unable to be conducted in our study due to concerns of viral transfer and a lack of success recapturing seropositive mesopredators.

Among the trapped mesopredators, one farm cat (50%) was infected, suggesting the potential for previous shedding of oocysts onto the farm. Raccoons had the highest infection rate (52.9%), likely because they have a broad spectrum of food sources. In addition, raccoons are susceptible to *T. gondii* infection. A recent study has shown that 59.2% (32/54) of raccoons and 71.4% of skunks (5/7) captured in the state of Wisconsin were seropositive for *T. gondii* using MAT (Dubey et al., 2007). Viable *T. gondii* was isolated from five of the 30 seropositive raccoon samples, and one of the five skunk samples (Dubey et al., 2007). In Canada, *T. gondii* was isolated from tissues of two raccoons, two feral cats, and one skunk by bioassay in mice (Dubey et al., 2008). In our study, both trapped skunks were seronegative for *T. gondii*.

It is perplexing that the sixteen opossums in our study were all *T. gondii* negative. The seroprevalence in opossums is unknown, and very few isolates were obtained from opossums (Jiang et al., 2018). Dubey et al. (1995) reported a 22.7% (29/128) seroprevalence (MAT, cutoff at 1:25) in opossums trapped on 47 swine farms in Illinois; however, the bioassay was not deployed to isolate the parasite. Similarly, Gerhold et al. (2017) had reported a 50% (6/12) seroprevalence in opossums from the southeastern U.S. (Gerhold et al., 2017). It is unknown why all sixteen trapped opossums were negative in our study, but these results further emphasize the need to obtain region-specific data prior to using a single indicator as means for contamination identification. Further study to obtain a larger sample size is imperative to elucidate the seroprevalence in opossums.

Studying *T. gondii* contamination by trapping animals can be costly, time intensive, require personnel training, and has ethical considerations. Sample sizes need to be large to have a robust understanding of prevalence, but sample sizes are limited by trapping success and animal abundance. Detecting *T. gondii* contamination in our study widely depended on the species trapped. This can present potential challenges for researchers when selecting target species, especially when considering the seroprevalence we found in opossums was quite different than that found by similar studies in the region. Wildlife and domestic cat surveillance can be beneficial in that infected food sources make *T. gondii* contamination potentially more easily detected than environmental detection alone. The seroprevalence of cats is also effective in determining potential sources of *T. gondii*, but cats and other seropositive animals may not be actively shedding the parasite. Conversely, seronegative animals can harbor parasites (Dubey et al., 2002), and it is difficult to determine the best MAT cutoff value for different species without specific testing. Diagnosing *T. gondii* infection prevalence in cattle also presents challenges. Studies have indicated that cattle can eliminate viable *T. gondii* from their tissues becoming seronegative over time (Opsteegh et al., 2011; Dubey, 2010). Correlations between commonly used diagnostic techniques are sometimes poor. Additionally, unknown true prevalence rates in natural infections make validation problematic and raise disagreements of standardized MAT cutoff values when analyzing bovine sera (Opsteegh et al., 2011; Dubey et al., 2020). Despite these challenges, the efficacy of MAT is the most understood and is regarded as the most sensitive test for bovines (Dubey et al., 1985, 2020). A wide range of reported prevalences of *T. gondii* antibodies in cattle have been reported, and infections in cattle have been regarded as often low (Dubey et al., 2005). However, seropositive cattle have been identified with herd prevalences as high as 71.3% (Sanati et al., 2012), 73.6% (Sroka, 2001), and 76.3% (Klun et al., 2006) using MAT (Dubey et al., 2020). No positive sample was identified in the thirty cows in our

study which may be resultant of decreased risk factors of cattle being fed primarily controlled diets with more restricted access to outside water sources and housing (Gilot-Fromont et al., 2009). A benefit of agricultural animal surveillance is that it can more directly present a picture of food-safety risk and health risks to agricultural workers. Additionally, opportunistic surveillance can be accomplished using banked serum from routine veterinary care as was done in our study, thereby eliminating unnecessary stress and handling of animals.

5. Conclusions

In summary, we detected *T. gondii* contamination at the farm with varying degrees of success depending on the contamination indicator. The study of *T. gondii* infection in animals is a critical parameter in detecting contamination, especially in areas where low contamination has been indicated by other means of assessment such as soil surveillance. We found mesopredators, especially raccoons and cats, to be possible suitable indicator species to monitor *T. gondii* presence in our study area. However, our study showed the challenges of obtaining adequate sample sizes and other disadvantages of using non-human animals for surveillance. Additionally, seropositivity varied widely among species.

Our study encountered multiple challenges of different *T. gondii* contamination indicators. The method of detecting *T. gondii* should depend on specific study aims; however, if general surveillance for presence and risk of *T. gondii* infection is the goal then targeting only one potential source of contamination is ill-advised. A non-holistic approach fails to address the complex epidemiology of the parasite and varying risk factors for differing cultures. Rather, in the absence of study area-specific data on effective indicators of contamination an integrative surveillance approach to attempt detection of *T. gondii* is recommended. Our study emphasizes the importance of a holistic approach when surveilling for *T. gondii* to compensate for shortcomings of each contamination indicator.

Authors' contributions

Soil sample collection was done by TJ and CS. Animal trapping was mainly conducted by KK and RWG; TJ and CS also contributed to animal trapping. TJ conducted soil oocyst detection as well as *T. gondii* detection from blood and animal tissues. KK and TJ wrote the manuscript draft, which received edits from CS, RWG, and LM.

Declarations of interest

None.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

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