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White-tailed deer (*Odocoileus virginianus*) are a reservoir of a diversity of *Toxoplasma gondii* strains in the USA and pose a risk to consumers of undercooked venison

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Research Article

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Abstract

To assess the role of white-tailed deer (*Odocoileus virginianus*, WTD) in the epidemiology of toxoplasmosis, we conducted a national survey of WTD across the USA for *Toxoplasma gondii* infection. To do this, we combined serology with parasite isolation to evaluate the prevalence and genetic diversity of *T. gondii* in this game species. From October 2012 to March 2019, serum and tissues were collected from 914 WTD across the USA. Serum samples were screened for antibodies to *T. gondii*, and then the tissues of seropositive WTD were bioassayed in mice. Antibodies were detected in 329 (36%) of 914 WTD tested by the modified agglutination test (positive reaction at 1:25 or higher). Viable *T. gondii* was isolated from the heart of 36 WTD from 11 states. Three of the 36 isolates were pathogenic but not highly virulent to outbred Swiss Webster mice and all 36 isolates could be propagated further in cell culture and were genotyped. For genotyping, DNA extracted from cell culture-derived tachyzoites was characterized by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico. Genotyping revealed seven ToxoDB PCR-RFLP genotypes, including 24 isolates for genotype #5 (haplogroup 12), four isolates for #2 (type III, haplogroup 3), three isolates for genotypes #1 (type II, haplogroup 2), two isolates for genotypes #3 (type II, haplogroup 2) and one isolate each for #39, #221 and #224. Genotype #5 was the most frequently isolated, accounting for 66.6% (24 of 36) of the isolates. Combining the 36 isolates from this study with previously reported 69 isolates from WTD, 15 genotypes have been identified. Among these, 50.4% (53/105) isolates belong to genotype #5. Our results indicate moderate genetic diversity of *T. gondii* in WTD. The results also indicate that undercooked venison should not be consumed by humans or fed to cats.

Introduction

The protozoan *Toxoplasma gondii* infects virtually all warm-blooded animals, including birds, humans, livestock and marine mammals (Dubey, 2010). Humans become infected postnatally by ingesting tissue cysts from undercooked meat of infected animals, or by consuming food or drink contaminated with *T. gondii* oocysts. In the USA, white-tailed deer (*Odocoileus virginianus*, WTD) are harvested by hunters and infected meat that is consumed by humans or other hosts can result in *T. gondii* infection. Cases of clinical toxoplasmosis including ocular manifestations have been documented in humans who consumed undercooked venison (Sacks *et al.*, 1983; Ross *et al.*, 2001; Schumacher *et al.*, 2018; Gaulin *et al.*, 2020).

Deer are popular game animals in several countries (Aubert *et al.*, 2010). In the USA, the WTD population is estimated at approximately 30 million, with nearly 6 million deer harvested annually (Adams and Ross, 2013). Viscera are removed from most harvested deer in the field to preserve the meat, and the viscera is left on site or buried in a shallow grave. Tissues of these deer may be scavenged by mesocarnivores, which can then become infected.

Few states have strict rules with respect to disposal of the carcases of deer hit by vehicles. Bobcats (*Lynx rufus*) and cougars (*Puma concolor*) scavenge on deer, and if they eat infected tissues they can excrete *T. gondii* oocysts in the environment. Since deer–vehicle accidents are common in the USA (Adams and Ross, 2013), there are many opportunities for felids to become infected.

Since deer are herbivores, ingestion of food or water contaminated with oocysts is likely the main mode of post-natal transmission. Thus, WTD are considered an excellent indicator of contamination of the environment by *T. gondii* oocysts among other hosts, including feral chickens, wild swine and many other species wild ungulates. The objective of the present investigation was to estimate seroprevalence, isolate and characterize *T. gondii* from WTD across the USA.

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Table 1. Prevalence of *T. gondii* in WTD (*O. virginianus*) collected across the USA from 2012 to 2019

Year[a]	Samples received	Male	Female	Juvenile	Sub-adult	Adult	MAT positive [%; (95% CI)]	Samples bioassayed	*T. gondii* isolates
2012	241	121	119	97	0	143	21.2 (16.5–26.8)	40	12
2013	602	217	382	131	0	468	43.2 (39.3–47.2)	110	21
2015	12	4	8	1	0	11	50.0 (25.4–74.6)	6	0
2016	2	2	0	0	0	2	0 (0–65.8)	0	0
2017	48	14	34	7	3	38	22.9 (13.3–36.5)	11	3
2018	4	2	2	1	0	3	25.0 (4.6–69.9)	0	0
2019	5	2	3	2	0	3	0 (0.0–43.5)	0	0
Total (% positive)	914	362 (29.8)	548 (40.0)	239 (19.2)	3 (0.0)	668[b] (42.1)	36.0 (33.0–39.2)	167	36

[a]No samples received in 2014.
[b]Age and sex not recorded for four WTD.

Materials and methods

Animals and sampled areas

The United States Department of Agriculture's Wildlife Services (WS) removes deer from select areas where populations are not naturally regulated due to the lack of predators and hunting restrictions. Samples are opportunistically collected from these deer by WS' National Wildlife Disease Program (NWDP). For this study, sera and hearts were collected from 914 WTD from October 2012 through March 2019 in 19 states (Table 1) following NWDP protocols (Cervid Health Procedures Manual, June 2015). Sex, age group (juvenile or adult), date of killing and location information were recorded for most WTD; not all data were available for all WTD (Table 1). Samples were submitted for *T. gondii* testing to the USDA's Animal Parasitic Diseases Laboratory (APDL) in Beltsville, Maryland.

Serology

Sera were tested for antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Sera were screened at 1:25, 1:50, 1:100 and 1:200 dilutions or higher. Deer with positive reaction at 1:25 dilution were considered infected with *T. gondii*.

Isolation by bioassay in mice

The heart was selected as the organ for isolation of *T. gondii* because of convenience and because in food animals it is most commonly infected with *T. gondii* (Dubey, 2010). Myocardium samples (50 g) were homogenized in saline, digested in acidic pepsin, centrifuged and aliquots of homogenates were inoculated subcutaneously into 3–5 outbred albino Swiss Webster (SW) mice, and/or one or two interferon gamma gene knock out (KO) mice, which are especially susceptible to toxoplasmosis (Dubey, 2010). Inoculated mice that became ill were euthanized and tissue imprints of lungs and brains were examined for *T. gondii* tachyzoites or tissue cysts, respectively (Dubey, 2010). Survivors were bled at the earliest on 45 days post-inoculation (p.i.) and a 1:25 dilution of serum was tested for *T. gondii* antibodies by MAT. Mice were euthanized 46 days p.i. or later and brains of all mice were examined for tissue cysts as described previously (Dubey, 2010). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were detected in their tissues.

Ethical considerations

All experimental procedures were approved by the Institutional Animal Care and Use Committee (Protocol no. 15-017), United States Department of Agriculture, Beltsville, Maryland. Outbred SW and KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) in compliance with the Institutional Animal Ethics Committee guidelines. The WTD were euthanized in the field, often in remote locations and tissues were shipped overnight by the collector with ice packs. Consequently, by the time tissues were received at the USDA laboratory, they often were contaminated with bacteria and not suited for cell culture to isolate *T. gondii*. The number of *T. gondii* in tissues of naturally infected large animal tissues is low (estimated one tissue cyst per 50–100 g) and the probability of isolation of *T. gondii* is very low unless large numbers of mice (10 or more) are used (Dubey *et al.*, 1995; Dubey, 2010). To balance the possibility of isolating parasites and using the minimum number of mice, we decided to use 3–5 mice for the bioassay of each deer sample in the current study.

All mice used in the current study were treated humanely and examined twice daily for any signs of illness. A veterinarian was assigned exclusively to the toxoplasmosis project. Any sick mice were euthanized because our objective was isolation of *T. gondii* and not testing for mortality. We wanted to collect mouse tissues aseptically for cultivation in cell culture or subpassage to other mice.

In vitro *cultivation*

Lungs or brain from infected mice were seeded on to CV1 cell culture flasks and tachyzoites were harvested from the medium as previously described (Dubey, 2010).

Genotyping of DNA samples

In our experience, for successful genotyping of *T. gondii* strains from asymptomatic naturally infected animals, it is necessary to obtain good quality parasite DNA with minimal contamination of host tissue. Therefore, parasites isolated from mouse tissues were expanded in cell culture. Genotyping of DNA samples by multi-locus polymerase chain reaction (PCR)-restriction fragment polymorphism (RFLP) markers were carried out following the previously reported protocol (Su *et al.*, 2010; Su and Dubey, 2020). Samples with missing data for one to three of the 10 PCR-RFLP markers, which otherwise matched with previously reported genotype were designated as 'likely' of that genotype.

Table 2. Collection information for *T. gondii* isolates from WTD (*O. virginianus*) collected across the USA from 2012 to 2019

WTD					Bioassays in mice		
Isolate number	State[a]	County	Collection date	MAT	SW (no. of infected/no. of inoculated)	KO (no. infected/no. inoculated)	Genotype
(1) TgWTDAL1	AL	Clay	4/8/2013	800	3/3	ND	#5
(2) TgWTDAL2	AL	Coosa	4/2/2013	800	3/3	ND	#5
(3) TgWTDAL3	AL	Tallapoosa	5/23/2013	400	2/3	2/2	#5
(4) TgWTDGA1	GA	Greene	11/5/2012	>3200	4/4	1/1	#5
(5) TgWTDGA2	GA	Greene	2/13/2013	200	1/3	ND	#5
(6) TgWTDGA3	GA	Gwinnett	11/27/2012	200	3/4	1/1	#224
(7) TgWTDIN1	IN	Porter	3/20/2013	>3200	2/2	ND	#2
(8) TgWTDIN2	IN	Porter	3/13/2013	400	2/3	ND	#2
(9) TgWTDKS1	KS	Jewell	10/8/2017	>3200	4/4	1/1	#5
(10) TgWTDKS2	KS	Wabaunsee	10/3/2017	1600	0/4	1/1	#5
(11) TgWTDKY1	KY	Scott	1/20/2013	>3200	1/4	1/1	#39
(12) TgWTDLA1	LA	Bossier	12/12/2012	800	3/4	1/1	#5
(13) TgWTDLA2	LA	East Feliciana	11/10/2012	1600	1/4	0/1	#5
(14) TgWTDLA3	LA	East Feliciana	11/10/2012	>3200	3/4	1/1	#5
(15) TgWTDLA4	LA	East Feliciana	11/10/2012	400	4/4	1/1	#5
(16) TgWTDLA5	LA	East Feliciana	11/10/2012	800	1/4	0/1	#5
(17) TgWTDLA6	LA	East Feliciana	4/24/2013	800	3/3	1/1	#5
(18) TgWTDLA7	LA	East Feliciana	4/24/2013	200	3/3	1/2	#5
(19) TgWTDLA8	LA	Iberville	11/24/2012	1600	4/4	1/1	#5
(20) TgWTDLA9	LA	Union	10/27/2012	1600	4/4	1/1	#5
(21) TgWTDLA10	LA	Vernon	10/27/2012	800	4/4	1/1	#5
(22) TgWTDLA11	LA	Vernon	10/27/2012	1600	4/4	1/1	#5
(23) TgWTDLA12	LA	Vernon	10/27/2012	400	2/4	0/1	#5
(24) TgWTDMI1	MI	Alpena	4/2/2013	200	2/3	1/1	#3
(25) TgWTDMI2	MI	Alpena	4/6/2013	1600	3/3	ND	#5
(26) TgWTDMI3	MI	Alpena	6/10/2013	1600	2/3	1/1	#5
(27) TgWTDMI4	MI	Alpena	7/8/2013	200	1/3	1/1	#5
(28) TgWTDMI5	MI	Alpena	7/9/2013	800	1/3	1/1	#5
(29) TgWTDMI6	MI	Alpena	7/23/2013	1600	3/3	1/1	#5 likely
(30) TgWTDMN1	MN	Hennepin	2/15/2013	1600	3/3	1/1	#1
(31) TgWTDNJ1	NJ	Essex	1/22/2013	400	0/3	1/1	#2
(32) TgWTDNJ2	NJ	Essex	1/29/2013	1600	0/3	1/1	#1
(33) TgWTDNJ3	NJ	Essex	1/29/2013	200	0/3	1/1	#221
(34) TgWTDNY1	NY	Dutchess	1/13/2017	>1600	1/4	0/1	#2
(35) TgWTDOH1	OH	Cuyahoga	1/15/2013	1600	0/4	1/1	#1
(36) TgWTDOH2	OH	Cuyahoga	1/15/2013	1600	1/4	1/1	#3

AL, Alabama; GA, Georgia; IN, Indiana; KS, Kansas; KY, Kentucky; LA, Louisiana; MI, Michigan; MN, Minnesota; NJ, New Jersey; NY, New York; OH, Ohio.
SW, Swiss Webster albino mice; KO, interferon-γ knockout mice; ND, not done.
[a]No isolates from FL, Florida; IL, Illinois; PA, Pennsylvania; ME, Maine; NC, North Carolina, WA, Washington; WI, Wisconsin and WY, Wyoming.

Results

Antibodies to *T. gondii* were detected in 36% (329 of 914) of WTD (Table 1). Among 362 male and 548 female WTD tested (sex was unidentified for four), seroprevalence was higher in females (40.0%) than in males (29.8%). Seroprevalence were higher in adults than juveniles (Table 1).

Of the 329 seropositive deer, hearts from 167 were bioassayed (Table 1; Supplementary Table 1). The selection was based on antibody titres and the number of samples received and the availability of mice. Viable *T. gondii* was isolated from 36 WTD collected in 11 states (Table 2, Fig. 1) (Supplementary Table 1). The rate of isolating viable parasites was positively associated with MAT titres, with the isolation rates of 10.7, 17.9, 33.3, 59 and 50% for MAT titres of 1:200, 1:400, 1:800, 1:1600 and $\geqslant$1:3200, respectively. Tissue cysts were found in brains of all seropositive mice.

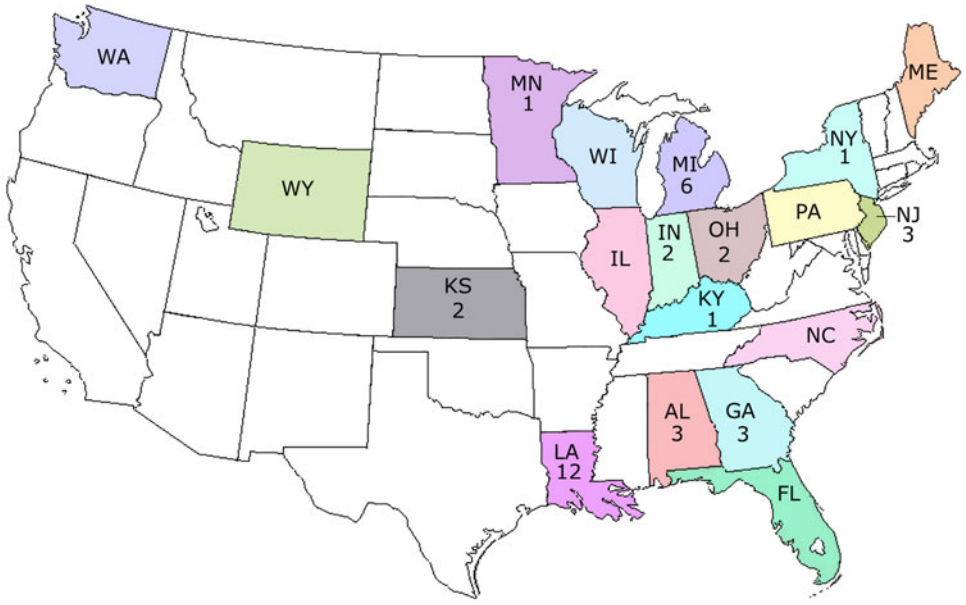

Fig. 1. Map of USA showing the *T. gondii* isolates from WTD.

Table 3. Isolates of pathogenic *T. gondii* identified in WTD collected across the USA from 2012 to 2019

								Bioassayed in SW mice[a]		
Isolate number	WTD ID	Collection date	State	County	Age class	Sex	MAT	No. of mice infected with *T. gondii*	No. of dead mice (day of death)	Genotype
15 TgWTDLA4	TOX00684	11/10/2012	LA	East Feliciana	Adult	Female	400	4	1 (46)	#5
20 TgWTDLA9	TOX00667	10/27/2012	LA	Union	Adult	Female	1600	4	1 (13)	#5
21 TgWTDLA10	TOX00660	10/27/2012	LA	Vernon	Adult	Female	800	4	1 (41)	#5

The SW mice that were inoculated with digest of hearts from three of the 36 infected WTD had clinical signs of *T. gondii* infection, and three mice died or were euthanized at 13, 41 and 46 days p.i. (Table 3). All three isolates were from Louisiana but from different counties and hunted on different dates (Table 3).

Thirty-six isolates were genotyped (Table 4); typing results for individual isolates are shown in Supplementary Table 2. The results revealed seven ToxoDB genotypes, including 24 isolates for genotype #5 (haplogroup 12), four isolates for genotype #2 (type III, haplogroup 3), three isolates for genotype #1 (type II, haplogroup 2), two isolates for genotypes #3 (type II, haplogroup 2), one isolate each for #39, #221 and #224. Genotype #5 was the most frequently isolated, accounting for 66.6% (24 of 36) of the isolates.

Table 4. *T. gondii* isolates from WTD (*O. virginianus*) bioassayed samples and PCR-RFLP genotype per state collected from 2012 to 2019

State[a]	Samples bioassayed	No. of isolates	ToxoDB-PCR-RFLP genotype
AL	7	3	#5
FL	5	0	–
GA	9	3	#5, #224
IL	10	0	–
IN	9	2	#2
KS	4	2	#5
KY	1	1	#39
LA	24	12	#5
MI	32	6	#3, #5
MN	10	1	#1
NC	1	0	–
NJ	34	3	#1, #2, #221
NY	3	1	#2
OH	5	2	#1, #3
PA	12	0	–
WA	1	0	–
Total	167	36	#1 ($n = 3$), #2 ($n = 4$), #3 ($n = 2$), #5 ($n = 24$), #39 ($n = 1$), #221 ($n = 1$), #224 ($n = 1$)

[a]No samples were bioassayed from ME, WI and WY.

Discussion

Nearly one-third of humans have been exposed to *T. gondii*, but not all develop clinical symptoms of disease. It is unknown how the severity of toxoplasmosis in immunocompetent hosts is linked to the parasite strain, host variability or to other factors. Attention has been focused on the genetic variability among *T. gondii* isolates from apparently healthy and sick hosts (Grigg and Sundar, 2009). Historically, *T. gondii* was considered clonal with low genetic diversity and grouped into three types, namely I, II and III (Howe and Sibley, 1995; Sibley and Ajioka, 2008). However, recent studies have revealed a greater genetic diversity of *T. gondii*, particularly isolates from Brazil (Shwab *et al.*, 2018). A recent update indicated 279 genetic variants (Su and Dubey, 2020).

Table 5. Summary of prevalence of *T. gondii* in WTD from previous reports in the USA[a]

State	No. tested	No. of seropositive (MAT, ⩾1:25)	% pos. samples	No. of *T. gondii* isolates	ToxoDB PCR-RFLP genotypes	Reference
Alabama	16	7	44.0	ND	ND	Lindsay *et al.* (1991)
Alabama	50	17	34.0	4	ND	Lindsay *et al.* (1997)
Illinois	443	247	55.9	ND	ND	Hollis-Etter *et al.* (2019)
Iowa	62	20	32.2	ND	ND	Dubey *et al.* (2009)
Iowa	Foetuses from 61 deer	43	70.5	6	#1 (type II) – 4 isolates #4–2 isolates	Dubey *et al.* (2008)
Minnesota	Foetuses from 27 deer	15	55.5	9	#4–5 isolates #2 (type III) – 2 isolates #54–1 isolate #74–1 isolate	Dubey *et al.* (2008)
Minnesota	1367	410	30.0	ND	ND	Vanek *et al.* (1996)
Minnesota	170	91	53.5	ND	ND	Dubey *et al.* (2009)
Minnesota	487	110	22.5	1	#146	Dubey *et al.* (2014*a*)
Mississippi	73	21	46.0	19	#2 – 1 isolate #5 – 18 isolates	Dubey *et al.* (2004, 2014*a*)
New Jersey	264	76	28.7	9	#2 – 2 isolates #3 – 1 isolate #4 – 1 isolate #216 – 1 isolate #220 – 1 isolate #221 – 3 isolates	Dubey *et al.* (2013*a*)
Ohio	749	557	74.4	4	#1 – 2 isolates #2 – 1 isolate #3 – 1 isolate	Dubey *et al.* (2014*a*)
Ohio	444	261	58.8	ND	ND	Ballash *et al.* (2015)
Pennsylvania	79	49	62.0	7	#3 – 2 isolates #4 – 3 isolates #216 – 2 isolates	Dubey *et al.* (2014*b*)
Pennsylvania	593	357	60.0	ND	ND	Humphreys *et al.* (1995)
Southeast	241	99	41.0	13	#5 – 10 isolates #154 – 1 isolate #167 – 1 isolate #216 – 1 isolate	Gerhold *et al.* (2017)
Nationwide	**914**	**329**	**35.9**	**36**	**7 ToxoDB genotypes**	**Current study**

[a]In addition to these reports, Yu *et al.* (2013) reported ToxoDB PCR-RFLP genotype #5 for the isolate TgWtdAL from the heart and tongue of a WTD from Alabama.

Severe cases of toxoplasmosis have been reported in immunocompetent patients in association with atypical *T. gondii* genotypes (Ajzenberg *et al.*, 2004; Demar *et al.*, 2007; Elbez-Rubinstein *et al.*, 2009; Lorenzi *et al.*, 2016). However, little is known about the association of genotype and clinical disease in animals and humans in the USA (Dubey, 2010).

In the current study, 36% of WTD were seropositive, and these data are in accord with several regional surveys with seroprevalences of 28.7–74.4% (Table 5). These results are comparable because in all surveys listed in Table 5, the same MAT was used to detect *T. gondii* antibodies. WTD are the most common wild cervids in the USA and they are spreading from rural to urban areas. WTD have been imported to Europe (Jokelainen *et al.*, 2010). Nothing has been published concerning the genotypes of *T. gondii* in WTD in Europe, but it would be most interesting to learn whether they may have played any role in introducing or propagating parasite strains otherwise considered endemic to North America.

In the current study, there was relatively a low to moderate diversity of the isolates identified. Genotypes #1, #2, #3, #4, #39 and #221 have previously been identified in animals in the USA, with the first three being most common in animals on farms (Jiang *et al.*, 2018). Genotype #224 was previously reported in dog from Grenada (Dubey *et al.*, 2013*b*).

From previously published reports, 69 isolates from WTD in the USA have been genotyped (Table 5). Among these isolates, 13 ToxoDB PCR-RFLP genotypes were identified, including #1, #2, #3, #4, #5, #54, #74, #146, #154, #167, #216, #220 and #221. From these genotypes, #5 (haplotype 12) accounted for 42% (29 of 69), was the most frequently isolated. Genotype #4 (also known as haplotype 12) accounted for 16% (11 of 69). Genotypes #1 and #3 together known as type II and haplogroup 2, accounted for 15% (10 of 69). Combining the 36 isolates from our current study with previously reported 69 isolates from WTD, 15 genotypes are identified, including #1, #2, #3, #4, #5, #39, #54, #74, #146, #154, #167, #216, #220, #221 and #224. Among these, 50.4% (53/105) isolates were characterized as genotype #5. Genotypes #1 and #3 (together as type II, haplogroup 2) accounted for 14.3% (15/105) of the isolates. Genotype #4 (haplotype 12) accounted for 10.5% (11/105) and genotype #2 (type II, haplogroup 3) accounted for 9.5% (10/105). The other 10 genotypes were less frequently identified. A recent national survey of

feral swine in the USA indicated a similar pattern, in which genotype #5 was dominant and accounted for 57% of 76 isolates genotyped (Dubey *et al.*, 2020). Based on these two studies, dominance of genotype #5 suggests sylvatic transmission of *T. gondii* in wildlife in the USA.

Although bobcats are the most likely hosts involved in the sylvatic cycle of *T. gondii* in the USA (Dubey, 2010; VanWormer *et al.*, 2014; Verma *et al.*, 2017), other wild cats may participate in both feral and domestic cycles introducing atypical genotypes to domestic cats, thereby facilitating transmission of potentially more pathogenic genotypes to humans, domestic animals and wildlife (VanWormer *et al.*, 2014). The partition of *T. gondii* genotypes such as #1, #2 and #3 among domestic animals and #5 in wildlife is mainly due to distinct sylvatic and domestic transmission cycles, though both cycles overlap to a certain degree (Jiang *et al.*, 2018; Shwab *et al.*, 2018). Recent evidence indicates that the strains of *T. gondii* prevalent in wildlife can also cause clinical disease in humans (Jokelainen *et al.*, 2018; Pomares *et al.*, 2018) and domesticated animals (Dubey and Prowell, 2013; Crouch *et al.*, 2019), indicating a plausible route for introduction of virulent strains of *T. gondii* from the sylvatic cycle.

Our results revealed low to moderate genetic diversity of *T. gondii* in WTD in the USA, with genotype #5 (haplogroup 12) identified as the most dominant type in the USA. The contribution of WTD to the epidemiology of *T. gondii* deserves additional scrutiny. Considering the high rate of exposure of WTD to *T. gondii* in the USA, results affirm that the environment is highly contaminated with oocysts.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020000451.

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Conflict of interest. None.

Ethical standards. All experimental procedures were approved by the Institutional Animal Care and Use Committee (Protocol no. 15-017), United States Department of Agriculture, Beltsville, Maryland.

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