Seroprevalence, isolation and co-infection of multiple *Toxoplasma gondii* strains in individual bobcats (*Lynx rufus*) from Mississippi, USA

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Abstract

Toxoplasma gondii causes lifelong chronic infection in both feline definitive hosts and intermediate hosts. Multiple exposures to the parasite are likely to occur in nature due to high environmental contamination. Here, we present data of high seroprevalence and multiple *T. gondii* strain co-infections in individual bobcats (*Lynx rufus*). Unfrozen samples (blood, heart, tongue and feces) were collected from 35 free ranging wild bobcats from Mississippi, USA. *Toxoplasma gondii* antibodies were detected in serum by the modified agglutination test (1:≥200) in all 35 bobcats. Hearts from all bobcats were bioassayed in mice and viable *T. gondii* was isolated from 21; these strains were further propagated in cell culture. Additionally, DNA was extracted from digests of tongues and hearts of all 35 bobcats; *T. gondii* DNA was detected in tissues of all 35 bobcats. Genetic characterization of DNA from cell culture-derived isolates was performed by multiplex PCR using 10 PCR-RFLP markers. Results showed that ToxoDB genotype #5 predominated (in 18 isolates) with a few other types (#24 in two isolates, and #2 in one isolate). PCR-DNA sequencing at two polymorphic markers, GRA6 and GRA7, detected multiple recombinant strains co-infecting the tissues of bobcats; most possessing Type II alleles at GRA7 versus Type X (HG-12) alleles at GRA6. Our results suggest that individual bobcats have been exposed to more than one parasite strain during their life time.

Keywords: Toxoplasmosis; Felids; Bioassay; Isolation; Strain; Genotype

1. Introduction

The protozoan parasite *Toxoplasma gondii* infects virtually all warm-blooded animals including birds, humans, livestock and marine mammals, and causes lifelong chronic infection in both intermediate and feline definitive hosts (Dubey, 2010). Multiple exposures to the parasite are expected to be common in high transmission areas where prevalence among infected intermediate hosts is high and multiple routes of transmission are common, including asexual transmission of bradyzoites by carnivory and exogenous exposure to oocysts defecated by predatory cats. In these highly endemic regions, exposure to multiple independent parasite infections during the lifetime of the host is expected. The degree to which asexual transmission of *T. gondii* strains by carnivorism accounts for co-infection exposure risk versus endemic expansion of infectious clones by self fertilization during the sexual cycle in definitive hosts remains unclear (Su et al., 2003; Grigg and Sundar, 2009; Wendte et al., 2010).

Acquired immunity due to primary infection does not always fully protect against secondary infection. Super infection, particularly by strains with different genotypes, has also been documented (Dubey, 1995; Araujo et al., 1997; Dao et al., 2001; Sibley and Ajioka, 2008; Franco et al., 2015; Jensen et al., 2015). In regions such as Brazil where the genetic diversity of *T. gondii* is greater, the incidence of congenital disease and reactivation of ocular toxoplasmosis is common (Gilbert et al., 2008; Shwab et al., 2014). Little is known about whether reactivation of latent infection or re-infection with a new strain contributes to the severe consequences seen during either congenital toxoplasmosis or patent disease in a seropositive immune compromised host (Elbez-Rubinstein et al., 2009). Occasionally, a few studies have documented natural multiple strain co-infections in individual chickens (Dubey et al., 2003, 2006a; Lindström et al., 2008), sea otters (Sundar et al., 2008), sheep (Ajzenberg et al., 2002; Dubey et al., 2008), cats (Su et al., 2006; Dubey et al., 2007, 2009; Herrmann et al., 2012; VanWormer et al., 2014; Brennan et al., 2016), foxes (VanWormer et al., 2014), Australian marsupials (Pan et al., 2012), and humans (Aspinall et al., 2003). PCR-DNA sequencing of single copy genes has been used previously to detect these multiple strain co-infections within an individual host (Pan et al., 2012), but validation of these results through isolation of more than one strain from a multiply infected host has not yet been done.

To better understand the degree to which multiple strain co-infections and super infections occur in nature within individual hosts, we collected multiple tissues from bobcats from rural Mississippi, USA, to estimate the number of concurrent infections by both PCR-DNA sequencing and isolation. Genetic characterization of direct tissues of naturally infected animals and humans without in vivo and in vitro expansions has rarely been attempted (Aspinall et al., 2003; Pan et al., 2012; VanWormer et al., 2014; Cunha et al., 2016). This study was pursued to understand the dynamics of mixed strain co-infections in a natural setting. Felids are important in the epidemiology of toxoplasmosis because they are the only hosts that can excrete the environmentally resistant oocysts. Here, we present data of *T. gondii* strains within bobcat (*Lynx rufus*) definitive hosts collected from rural areas in Mississippi. Antibodies against *T. gondii* were detected by the modified agglutination test (MAT) and viable parasites were isolated by mouse bioassay. The genetic characterization of *T. gondii* isolates was performed by multiplex PCR using the 10 PCR-RFLP genetic markers, SAG1, SAG2 (5'-3' SAG2, alt.SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico. Additionally, sequence-based typing of isolates and direct tissues (heart and tongue) were compared using two polymorphic markers, GRA6 and GRA7.

2. Materials and methods

2.1. Sample collection

Bobcats ($n=35$) were obtained through legal trapping in February, 2014 by licensed trappers from Mississippi. Samples were collected from remote areas in three Mississippi State counties (Table 1). The bobcats (22 males, 13 females) were older than 18 months and were trapped for fur and food. After necropsy, blood was collected from the chest cavity of individual bobcats and stored at 4 °C. Heart, tongue and feces from individual bobcats were placed separately in plastic bags and refrigerated at 4 °C until sent to the Animal Parasitic Diseases Laboratory (APDL), U.S. Department of Agriculture, Beltsville, Maryland for further experimentation. Samples were protected from freezing to minimize repeated freeze-thaw that can kill *T. gondii* tissue stages. Long exposure of samples under open conditions deteriorates the quality of tissues for bioassay; however, 5-19 days elapsed between sampling and laboratory examination (Supplementary Table S1). Before pepsin digestion, tissues were washed

individually with water to remove blood and fluids, and to minimize the chance of cross contamination. Blood, hearts, tongues and feces from individual bobcats were tested for *T. gondii* infections.

2.2. Serology

Sera were diluted two-fold serially from 1:25 to 1:200 and tested for antibodies to *T. gondii* by MAT as described previously (Dubey and Desmonts, 1987). *Toxoplasma gondii* MAT antigen was mouse-derived as described previously (Dubey et al., 2011a). Seropositivity in 1:25 or higher dilution was considered as *T. gondii* positive (Dubey, 2010).

2.3. Bioassay

Outbred Swiss Webster (SW), and IFN-gamma gene knock out (KO) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) in compliance with the Institutional Animal Ethics Committee guidelines. All procedures were approved by the Beltsville Area Animal Care and Use Committee, United States Department of Agriculture. After serological screening, the myocardium (30 g) of MAT-positive bobcats (all 35 bobcats) was homogenized in saline (0.85% NaCl), and digested in acidic pepsin, washed and aliquots of homogenates were inoculated s.c. into three SW mice (Supplementary Table S1) as described by Dubey (2010). The recipient mice were observed daily for signs of illness. Mice were bled on day 45 p.i. and a 1:25 dilution of serum was tested for *T. gondii* antibodies by MAT. Mice were euthanized on day 46 p.i., and their brain tissue smears were examined microscopically for tissue cysts. Brains homogenates of mice were subpassaged into one SW and one KO mouse. Imprints of lungs of mice that died or were euthanized when moribund were examined for *T. gondii* tachyzoites. The inoculated mice were considered infected with *T. gondii* when tachyzoites and/or tissue cysts were found in their tissues and/or antibodies to *T. gondii* were demonstrable in their sera.

Additionally, 30 g of tongue from individual bobcats were homogenized and digested in acidic pepsin solution separately as described previously (Dubey, 2010). The digested samples (tongue and remaining heart digest after mouse inoculation) were stored at -80 °C until required for molecular assays. We took all possible precautions in order to avoid any chance of cross-

contamination. Before digestion, tissues were washed individually with water to remove blood and fluids. We digested individual tissues in different glass bottles which were washed with detergents, rinsed three to four times with water and autoclaved before reuse. DNA extractions and PCR-RFLP genotyping were done separately in two laboratories.

2.4. Fecal examination and bioassay

Fecal samples from all 35 bobcats were individually subjected to sucrose floatation for examination of *T. gondii* oocysts. After microscopic examination, the floats were mixed with sulfuric acid, aerated and stored at 4 °C (Dubey, 2010). All fecal floats were bioassayed in mice, irrespective of the microscopic examination results, as described previously (Yang et al., 2015).

2.5. In vitro cultivation

African green monkey kidney fibroblast cells (CV-1 cell line) were utilized for in vitro cultivation of *T. gondii*. Lung or brain tissues of bioassayed mice that were found positive for *T. gondii* were homogenized in culture medium and seeded into CV-1 cell culture flasks. Flasks were incubated at 37 °C and 5% CO₂ and observed twice per week under an inverted microscope. Tachyzoites from successfully grown cultures were harvested from the medium for DNA isolation and infected host cells were cryopreserved in liquid nitrogen for future studies as described previously (Dubey, 2010).

2.6. Genetic characterization of *T. gondii*

DNA was extracted separately from pepsin digested heart and tongue of bobcats and cell culture derived tachyzoites using a DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. PCR-RFLP genotyping was done by following the procedures using the 10 genetic markers; SAG1, SAG2 (5'-3'SAG2, and alt.SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico as described previously (Su et al., 2010). Appropriate positive and negative controls were included in all analyses.

2.7. Sequences analysis

We conducted PCR-DNA sequencing of two polymorphic genes, GRA6 and GRA7, to provide sequence-based genotyping. Nested PCR was performed to amplify the GRA6 gene using primer pairs and under conditions described previously (Fazaeli et al., 2000; Miller et al., 2004; Sundar et al., 2008). Primers for amplification were: external forward primer GRA6-S1 5'-CAG GAA AAC AGC TTC GTG GT-3'; internal forward primer GRA6-AS1 5'-CTC TCC CGG TCG TTG CAT G-3'; external reverse primer GRA6-S2 5'-GTA GCG TGC TTG TTG GCG AC-3'; internal reverse primer GRA6-AS2 5'-TAC AAG ACA TAG AGT GCC CC-3'.

GRA7 gene amplification was performed using the specific primer pairs of the GRA7 gene (Bottos et al., 2009): external forward primer GRA7: CCA GCA TGG ATA AGG CAT CT, internal forward primer GRA7: GAC GCT GAA GTG ACT GAC GA, external reverse primer GRA7: GAC ACT GTC CTC GAG CTC CT, internal reverse primer GRA7 TTA GCC CCC ATA TCC TAC TGG.

The PCR amplicons of GRA6 and GRA7 were excised from the gel and purified using QIAquick Gel Extraction (Qiagen) according to the manufacturer's recommendation. The purified PCR products were sent to Macrogen Corporation (Rockville, MD, USA) for direct sequencing using the same primer pair used in amplification to obtain both reads. The resulting sequences were imported, read, edited manually if necessary, and analyzed using the software Geneious version 9.0.4 (Biomatters Ltd. Auckland, NZ).

3. Results

Antibodies against *T. gondii* were detected in all 35 bobcats and all had high titers (≥ 200). After inoculation of mice with digest of hearts, 21 of 35 bobcats were found to contain viable *T. gondii* as indicated by seropositive mice that were euthanized on day 46 p.i. (Table 1, Supplementary Table S1). Neither tissue cysts nor tachyzoites were found in mice inoculated with tissues of one bobcat (#14), but the mice became seropositive. In some groups only one of three mice inoculated was infected, indicating that there were only a few viable *T. gondii* in the inocula (Supplementary Table S1). None of the SW outbred mice inoculated with bobcat tissues died. Brain homogenates of SW mice inoculated with tissues of bobcats were subinoculated into one KO and one SW mouse; all inoculated KO mice died or were euthanized when moribund, the result of acute toxoplasmosis. Enhanced mouse virulence was observed in eight of 21 isolates

in the first subpassage in mice; all SW mice of these groups died during acute toxoplasmosis within 20 days p.i. (Supplementary Table S1). Lung homogenates from KO mice were seeded onto cell cultures and tachyzoites were grown successfully. *Toxoplasma gondii* oocysts were not found in any bobcat feces by microscopy or by bioassay in mice.

Multilocus PCR-RFLP genotyping of cell culture-derived tachyzoites revealed three different genotypes among 21 isolates, including ToxoDB PCR-RFLP genotype #5 (18 isolates), #24 (two isolates), and #2 (one isolate). This result suggests genotype #5 is the dominant genotype (Table 2).

PCR-DNA sequencing-based genotyping using two polymorphic genes, GRA6 and GRA7, revealed significant diversity, with the majority possessing either a Type II allele or one sharing phylogenetic ancestry with Type II (designated IIa or IIb) at GRA7 (see Supplementary Table S2, Bankit ID is 1976823), or one of three different alleles that share phylogenetic ancestry with Type X (designated X1 ($n=15$), X2 ($n=2$), or X3 ($n=27$)) after alignment at GRA6 (see Supplementary Table S3, Bankit ID is 1976817). The detection of three alleles sharing ancestry with Type X at the GRA6 locus identified a higher sequence diversity than previously identified among Type X *T. gondii*. The GRA6 sequence obtained from DNA amplified from bobcat heart (#33) possessed double peaks at known polymorphic sites, indicating that a mixed strain co-infection was present in this tissue. Infection with multiple *T. gondii* strains in the same bobcats was detected in 11 bobcats by the presence of different GRA6 ($n=10$) and GRA7 ($n=2$) alleles between the various tissues examined or between the isolates and tissues from the same bobcats (Table 2). Sequence based typing identified 17 of 21 isolates belonging to atypical strains (Type X, and II). The *MseI* digestion failed to readily differentiate a Type II allele from a Type X allele, which explains why the sequence data is distinct from the RFLP data at the GRA6 locus. In summary, we observed 100% seroprevalence, and frequent multiple *T. gondii* strain co-infections in bobcats from rural Mississippi.

4. Discussion

Bobcats are the most widely distributed wild cat in North America and are important epidemiologically because they can excrete *T. gondii* oocysts (Miller et al., 1972; Marchiondo et al., 1976). The oocysts are shed in the feces of infected felids during the acute phase of infection.

Shedding can persist for up to 2 weeks, during which millions of oocysts can be produced from a single infected animal (Dubey, 2010). Carnivorous, herbivorous and omnivorous animals and humans may become infected by oocysts of *T. gondii*.

Toxoplasma gondii infection is quite frequent in bobcats and high seroprevalence of *T. gondii* in bobcats from North America, Central America and South America has been reported from different surveys with regional variations (Franti et al., 1975, 1976; Riemann et al., 1978; Dubey et al., 1987; Smith et al., 1995; Kikuchi et al., 2004; Mucker et al., 2006). All 35 bobcats from Mississippi were seropositive. The high prevalence of antibodies to *T. gondii* in bobcats is probably a reflection of the abundant source of infected intermediate hosts. In the previous studies, 34 (46.5%) of 73 White-tailed deer (WTD) from six sites spanning a broad geographical area of Mississippi (six counties: Granada, Calhoun, Wilkinson, Stone, Lamar and Tunica) were infected with *T. gondii*. Viable *T. gondii* was isolated from 21 (61.7%) of 34 seropositive deer (Dubey et al., 2004a). *Toxoplasma gondii* was isolated from three of six skunks, one of four Canada geese, and two of two feral cats from Mississippi. Two of three skunk isolates were mouse virulent (Dubey et al., 2004b). One skunk and one goose isolate were Type III, and one of two cats had genotype #5 (also referred to as Type X or HG-12) (Dubey et al., 2011b). Five (83%) of six bobcats had *T. gondii* antibodies and *T. gondii* was isolated from all five seropositive bobcats from Albany County, Georgia, USA (border state of Mississippi) (Dubey et al., 2004a). The high antibody titers in bobcats has been reported previously (Dubey et al., 2004a). Recently, a case of clinical feline toxoplasmosis was reported in a 6 month old domestic male pet cat associated with infection by an HG-12 strain. The cat was hospitalized due to lethargy, anorexia, fever and diarrhea. *Toxoplasma gondii* oocysts were found in feces of the cat and it also had a high serum antibody titer (1:800) against *T. gondii* (Dubey and Prowell, 2013).

No viable *T. gondii* was isolated from the first six (Bobcats#1-6) and seven other samples (Bobcat#8, 11, 13, 22, 26, 30, 34) although they were MAT positive; however PCR-positive amplification was detected in the tissue samples. There are three possible explanations for why viable *T. gondii* was not isolated from these MAT and PCR-positive samples. First, as many as 19 days elapsed between sample collection and laboratory testing, and none of these samples had viable *T. gondii*. Second, in some groups only one of three mice inoculated was infected, indicating that there were only a few viable *T. gondii* in the inocula. Third, the parasite burden

and the ratio of tachyzoites to encysted bradyzoites may vary between strains and thus their survival during transport and susceptibility to acidic pepsin digestion may differ (Dubey, 1998; Fux et al., 2007).

Enhanced mouse virulence was observed in eight of 21 isolates in the first subpassage in mice and all SW mice from these groups died of acute toxoplasmosis within 20 days p.i. (Supplementary Table S1). Whether this observation is related to the number of *T. gondii* in the inocula was not investigated, however, it was not the result of a single strain or genotype. Four distinct genotypes based on typing at the GRA6 and GRA7 loci were identified, seven of the eight strains possessed Type X alleles at GRA6 whereas only one strain possessed Type I alleles at both markers. Mouse virulent strains have been isolated previously from deer (four of 20) and bobcats (one of five) from the same Mississippi region (Dubey et al., 2004a), but only one study has reported the genotypes of *T. gondii* associated with this region (Dubey et al., 2011b). Eighteen of 20 Mississippi deer, and four of five Georgia bobcat isolates were genotype #5 (Type X; HG-12) (Dubey et al., 2011b), which supports the results of this study, that some strains bearing Type X alleles at GRA6 are mouse-virulent. In addition, all five isolates from five seropositive bobcats from Georgia and two isolates from bobcats of Alabama, USA were also genotype #5 (Dubey et al., 2004a, 2011b). Because Alabama and Georgia are geographically close, it is not surprising that the isolates from the two states belong to the same genotype. Recently, we isolated *T. gondii* from two of two seropositive bobcats from Pennsylvania, USA. The first isolate was genotype #5 (Type X; HG-12) whereas the second was clonal Type II (Dubey et al., 2015). Collectively, our results suggest high infection rates of *T. gondii* in bobcats and this enriches the *T. gondii* genotype database from wildlife collected within the USA.

Recent population genetic studies performed on wildlife isolates from North America have suggested the predominance of a fourth clonal lineage, designated HG-12, which includes PCR-RFLP genotypes #4 and #5, and the Type X strain designation based on sequences from GRA6. It has been reported that HG-12 is possibly derived from a single recombination event between a Type II strain and a unique parental lineage (Dubey et al., 2011b; Khan et al., 2011). In the present study, ToxoDB genotype #5 was mainly isolated from bobcats, supporting the previous studies. However, our results detected three separate GRA6 alleles that were not resolved by the PCR-RFLP typing methodology. These alleles in the Mississippi bobcat isolates

share ancestry with the Type X allele originally reported by Miller et al. (2004). We also identified three separate GRA7 alleles that share ancestry with the Type II allele. At both typing loci, the alleles within each ancestry were separated by only a private single nucleotide polymorphism (SNP), an insertion or deletion (INDEL), or both. Given the limited genetic drift that was identified among the Type II alleles at GRA7 and the HG-12 (Type X) alleles at GRA6, our results support a model whereby the single recombination event proposed by Khan et al. (2011) that produced the HG-12 clonal lineage was ancient, and that sufficient time has elapsed to accumulate SNPs and INDELs at the typing loci used in this study. Accordingly, in this model asexual transmission among a clade of genetically related strains via carnivorousism or expansion by self-mating in the definitive felid host has resulted in the endemic transmission of these HG-12 genotypes (Su et al., 2003; Wendte et al., 2010).

The limited polymorphism does not, however, refute a model whereby natural recombination during mating within the intestine of feline definitive hosts has occurred to produce hybrid strains that are circulating in rural Mississippi. The ability to generate experimentally derived genetic hybrids after feeding co-infected prey is well established (Grigg et al., 2001; Su et al., 2002, 2003; Volkman and Hartl, 2003), and recombinants have likewise been isolated from naturally infected cat feces (Herrmann et al., 2012). In fact, strains bearing all pair-wise or admixture combinations between the GRA6 X1, X2 and X3 alleles with the GRA7 Type II, IIa and IIb alleles were detected, which argues in support of transmission by admixed progeny present in oocysts. Such a scenario would support a high incidence of mixed strain co-infection among intermediate hosts, and currently studies are underway to investigate prey species of Mississippi bobcats to see if this is indeed the case.

In an extensive survey of *T. gondii* infections in feral domestic and wild felids in California, USA, *T. gondii* DNA was found in 49 of 166 feral cats (*Felis catus*), 10 of 73 mountain lions (*Puma concolor*), and 11 of 27 bobcats (VanWormer et al., 2014). PCR-DNA sequencing at six loci from DNA extracted directly from tissues of eight infected bobcats identified two bobcats infected with Type II strains and another six infected with Type X *T. gondii*. Of note, the bobcats infected with Type II *T. gondii* were isolated from a semi-urban environment, whereas those infected with Type X were from a rural region where land has not been developed. The researchers concluded that wild cats that come in contact with feral and

domestic cycles may introduce atypical genotypes to domestic cats and thereby facilitate the transmission of potentially more pathogenic genotypes to humans, domestic animals and wildlife (VanWormer et al., 2014). Direct amplification of *T. gondii* DNA from bobcats at other typing loci was not successful in the present study. This is probably because the density of *T. gondii* in tissue of asymptomatic wild and domestic animals was too low and may not be detectable by PCR amplification at less sensitive loci, and even by mouse bioassay.

Bobcats are generalist carnivores and their diet is entirely made up of a wide variety of animals. The majority of individuals have likely been exposed multiple times during their life to *T. gondii* infection, the result of successive predation on infected intermediate hosts that harbor different *T. gondii* strains. Previous work provided indirect evidence, using molecular PCR, that multiple *T. gondii* strain co-infections occur in other naturally infected hosts (Dubey et al., 2003, 2006; Lindström et al., 2008; Sundar et al., 2008; Herrmann et al., 2012; Pan et al., 2012; VanWormer et al., 2014; Brennan et al., 2016) including humans (Aspinall et al., 2003). In our study, the isolation of parasites by mouse bioassay, multilocus PCR-RFLP genotyping and sequencing of polymorphic markers (GRA6 and GRA7) of isolates was compared against PCR-DNA sequencing directly from infected tissues to provide the first known direct evidence for high frequency multiple strain co-infection in a natural host, with different genotypes partitioning to different organs. Our results suggest that co-infection may be more frequent than previously assumed. Primary infection generates strong immunity in intermediate hosts and this is known to reduce the risk of superinfection with the same strain (Sibley and Ajioka, 2008). However, previous infection only confers partial protection against re-infection with different strains of *Toxoplasma* in experimentally infected mice (Araujo et al., 1997; Dao et al., 2001). Additionally, susceptibility of vertical transmission has been demonstrated by Brazilian strains in a rodent (*Calomys callosus*) chronically infected with clonal Type II (Franco et al., 2015). In the present study, the exact mechanism facilitating co-infection of strains in bobcats is unclear. However, the majority (8/11; 73%) of co-infected bobcats harboured superinfections between a Type X or HG-12 strain and either a Type III ($n=5$), Type I ($n=2$) or an altogether new genotype bearing a novel allele at GRA6 ($n=1$). These data support the prevailing model whereby a defect in the induction of protective immunity against the variant superinfecting genotype can explain how the second (or third) genotype escapes pre-existing immunity to establish chronic infection.

Alternatively, the feeding behavior of bobcats, preying simultaneously on more than two intermediate hosts, or a single superinfected prey harboring different strains, could conceivably facilitate such co-infection. Another possibility is the ingestion of oocysts containing admixture progeny from a sexual outcross.

While it has been postulated that the genetic basis determining *T. gondii* superinfection in mice is highly dependent on the exact allelic combination of the ROP5/ROP18 alleles expressed by co-infecting strains (Jensen et al., 2015), this is unlikely to be a significant factor determining multiple strain co-infection in humans for example, since the immunity-related GTPases (IRG) genes targeted by ROP5/ROP18 parasite effector proteins are not encoded within the human genome. The 100% seroprevalence, the sheer number of bobcats infected with admixed strains bearing distinct combinations of GRA6 and GRA7 alleles, and the high incidence of superinfected wild bobcats (11/35; 31%) supports a model whereby sexual recombination or outcrossing may be favored in these sylvatic cycles, so it is of significant interest to determine the genetic diversity and extent to which mixed strain superinfection is occurring among prey of bobcats in this highly endemic niche.

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Table 1

Table 1. Summary of sample collection, serology and isolation of *Toxoplasma gondii* from bobcats from Mississippi, USA. *Toxoplasma gondii* was not isolated from underlined bobcat samples.

Variables		Bobcat#	Total		
			Sampled	MAT positive	<i>T. gondii</i> isolation
County	Claiborne	<u>1, 2, 3, 6</u> , 10, <u>11, 13</u> , 14, 15, 16, 17, 18, 21, <u>22</u> , 23, 24, 25, <u>26</u> , 28, 29, <u>30</u> , 31, 33, <u>34</u> , 35	25	25	14
	Warren	<u>4, 5</u> , 7, 12, 20, 32	6	6	4
	Jefferson	<u>8</u> , 9, 19, 27	4	4	3
Sex	Male	1-3, 5, 8-11, 13, 14, 16, 17, 18, 21-24, 27, 29-31, 33	22	22	12
	Female	4, 6, 7, 12, 15, 19, 20, 25, 26, 28, 32, 34, 35	13	13	9

MAT, modified agglutination test.

Table 2

Table 2. Genetic characterization of *Toxoplasma gondii* DNA from cell culture-derived isolates and tissues of bobcats from Mississippi, USA

Bobcat ID#	Tissue/ Culture	Isolates designation	ToxoDB PCR-RFLP genotype #	GRA6	GRA7	Bobcat ID#	Tissue/ Culture	Isolates designation	ToxoDB PCR-RFLP genotype #	GRA6	GRA7
Bobcat#1	Tongue			-	-	Bobcat#20	Tongue			X1	II
	Heart			X2	-		Heart			X1	II
Bobcat#2 ^a	Tongue			-	-		Culture	TgBobcatMS10	#5	X1	II
	Heart			III	-	Bobcat#21	Tongue			III	III
Bobcat#4	Tongue			III	-		Heart			II	-
	Heart			X1	II		Culture	TgBobcatMS11	#5	X3	IIa
Bobcat#5	Tongue			-	-	Bobcat#22	Tongue			X3	-
	Heart			X3	II		Heart			-	-
Bobcat#6	Tongue			-	-	Bobcat#23	Tongue			-	-
	Heart			X1	II		Heart			X3	IIa
Bobcat#7	Tongue			X1	-		Culture	TgBobcatMS12	#5	X3	IIa
	Heart			X2	IIa	Bobcat#24	Tongue			-	-
	Culture	TgBobcatMS1	#5	X3	IIa		Heart			X1	II
Bobcat#8	Tongue			-	-		Culture	TgBobcatMS13	#5	X1	II
	Heart			X	-	Bobcat#25	Tongue			X3	-
Bobcat#9	Tongue			X3	IIa		Heart			-	IIa
	Heart			X3	IIa		Culture	TgBobcatMS14	#5	X1	IIa
	Culture	TgBobcatMS2	#5	X3	IIa	Bobcat#26	Tongue			-	-
Bobcat#10	Tongue			-	-		Heart			X3	IIb
	Heart			-	II	Bobcat#27	Tongue			-	-
	Culture	TgBobcatMS3	#5	X3	-		Heart			III	IIIa
Bobcat#11	Tongue			-	-		Culture	TgBobcatMS15	#2	III	IIIa
	Heart			III	-	Bobcat#28	Tongue			-	-
Bobcat#12	Tongue			X	-		Heart			X1	II
	Heart			X1	-		Culture	TgBobcatMS16	#5	X1	II
	Culture	TgBobcatMS4	#5	X1	II	Bobcat#29	Tongue			-	-
Bobcat#13	Tongue			-	-		Heart			X3	IIa
	Heart			III	II		Culture	TgBobcatMS17	#5	X3	IIa
Bobcat#14	Tongue			-	-	Bobcat#30	Tongue			X	-
	Heart			X1	II		Heart			-	IIa
Bobcat#15	Tongue			III	-	Bobcat#31	Tongue			III	-
	Heart			X3	-		Heart			X3	-
	Culture	TgBobcatMS5	#5	X3	IIa		Culture	TgBobcatMS18	#5	X3	IIa
Bobcat#16	Tongue			I	-	Bobcat#32	Tongue			-	-
	Heart			X	I		Heart			X3	II
	Culture	TgBobcatMS6	#24	I	I		Culture	TgBobcatMS19	#5	X3	IIa
Bobcat#17	Tongue			-	-	Bobcat#33	Tongue			X3	-
	Heart			X3	II		Heart			IIIxX3	-
	Culture	TgBobcatMS7	#5	X3	-		Culture	TgBobcatMS20	#5	X1	IIa
Bobcat#18	Tongue			X3	-	Bobcat#34	Tongue			III	-
	Heart			-	-		Heart			-	-
	Culture	TgBobcatMS8	#5	U	-	Bobcat#35	Tongue			-	-
Bobcat#19	Tongue			-	-		Heart			X3	-
	Heart			X	-		Culture	TgBobcatMS21	#5	X3	IIa
	Culture	TgBobcatMS9	#24	I	-						

^aData not available for bobcat #3

-, No data