

**The population genetics, ecology, and transmission of *Toxoplasma gondii* in
North America**

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DEDICATION

I dedicate this dissertation to the people I love and the people who love me.

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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite that can infect a broad range of hosts, including mammals and birds. Felines, including wild and domestic cats, are the sole definitive hosts that can discharge millions of parasites into the environment through their feces in the form of oocysts. Due to the massive number of oocysts one cat can shed, and their highly infectious and resistant nature, the environmental contamination by oocysts poses a serious public health concern. Therefore, a better understanding of *T. gondii* transmission in the environment is essential. To this end, we compiled and analyzed genotypic data from animals in North America across a proximity gradient from human settlements to the wilderness (Chapter 2). We demonstrated that, *T. gondii* genotype distribution was associated with the spatial habitat and host species, and that parasite diversity decreased towards the human environment, suggesting the human impact on parasite transmission. To better understand the environmental contamination by oocysts, we developed a comprehensive protocol to evaluate the contamination by *T. gondii* on a typical farm in Tennessee (Chapter 3). *T. gondii* oocyst contamination in both soil and animals was detected on the farm. The results demonstrated that animals, especially meso-predators (such as raccoons and skunks), were good indicators of *T. gondii* prevalence in the environment. Thirdly, to reduce the environmental contamination, methods were tested to produce a potential cat vaccine in cell culture (Chapter 5). Lower temperature (31°C) was used to generate viable parasites of a vaccine strain (T263) in cell culture. Thus far, laboratory tests have lent evidence of the partial success. Lastly, we investigated 11 cases of fetal toxoplasmosis in captive macropods (kangaroos and wallabies) that occurred between 2014-2018 in the Busch Gardens Zoo, Florida (Chapter 4). Parasites were isolated and characterized using molecular methods. The results revealed over these three years, three independent infection events occurred at the zoo, suggesting frequent contamination by *T. gondii* of the zoo environment.

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CHAPTER 1

INTRODUCTION

Toxoplasma gondii

Toxoplasma gondii is an obligate intracellular parasite that can infect a broad range of hosts. It has been more than 100 years since its discovery in 1908 in rodent in France and in rabbit in Brazil (Dubey, 2010). *T. gondii* belongs to Phylum Apicomplexa and genus *Toxoplasma* and is the only species in the genus (Dubey, 2010). The medical significance of *T. gondii* was not recognized until 1939 when the parasite was found in the tissues of a congenitally-infected infant in New York, USA (Wolf et al., 1939). Likewise, its veterinary significance was not appreciated until it was found to be the culprit of abortion storms in sheep in Australia (Hartley and Marshall, 1957). One-third of the global human population has been chronically infected (Dubey, 2010). Among individuals older than six years in the U.S., 13.2% have been infected in a cross-nation survey in 2009 - 2010 (Jones et al., 2014). Infection in immunocompetent individuals generally induces no or mild symptoms. However, recent studies have reported acute infection in immunocompetent people, which demands intensive treatment. Toxoplasmosis can be life-threatening in immunocompromised individuals, such as HIV patients (Weiss et al., 2013). Likewise, in seronegative women, exposure to *T. gondii* during pregnancy can cause congenital toxoplasmosis, devastating the fetuses and neonates (Remington et al., 2006).

Life Cycle

The *T. gondii* life cycle was not revealed until 60 years after its discovery. In 1970, Frenkel, Dubey, and Miller unraveled its complex life cycle mystery (Frenkel et al., 1970). Felids, including the domestic cats (*Felis domesticus*), are the sole definitive hosts of *T. gondii* and thus play a pivotal role in its transmission (Hunter et al., 2012) (Figure 1.1).

Like many protozoa, *T. gondii* switches between several distinct life stages in response to its environment. Three stages are observed in *T. gondii*. Being the fast-growing stage, tachyzoite propagates and disseminates rapidly in its host, which marks the acute infection. The bradyzoite is the semi-dormant stage in which the parasite replicates slowly in tissue cysts. The sporozoites, existing as oocysts, are the environmental form, which can be widely dispersed and are therefore responsible for the efficient and extensive transmission of the parasite.

Cats can contract infection through eating any of the three stages of parasite: tachyzoites,

bradyzoites, and oocysts. Upon infection, the sexual reproduction occurs in the intestinal epithelial cells of the definitive hosts. Parasites excyst from tissue cysts or oocysts upon digestion by proteolytic enzymes in cat stomach. In the intestinal epithelial cells, tachyzoites sequentially develop into schizonts, merozoites, and gametes. Macrogametes are fertilized by microgametes to form diploid zygots which are enclosed by oocyst walls, released into the intestinal lumen, and ultimately excreted into the environment (Dubey, 2010). In most cases, a parasite can self-mate in cat epithelial cells; however, in rare cases, when a cat eats prey that contains more than one strain of *T. gondii*, a genetic cross can take place. Oocysts disseminate in the environment and contaminate environmental matrices such as water, vegetables, fruits, and soils. Ingesting oocysts accounts for the infection in intermediate hosts such as humans and livestock. Intermediate hosts can also contract infection via transplacental/vertical transmission or by eating meat containing tissue cysts. After a few days to months of acute infection, parasites retire into host tissues and become semi-dormant, which marks the onset of the chronic disease. Bradyzoites in the form of tissue cysts reside in host tissues, especially muscular and neural tissues, typically for the lifetime of the hosts. The life cycle of *T. gondii* is completed when the definitive hosts (felids) eat the parasites residing in the tissues of the intermediate hosts (Weiss and Kim, 2013).

Two geographically separated life cycles are known to exist in *T. gondii* transmission, namely sylvatic and domestic life cycles. The two cycles have wild felids and domestic cats as definitive hosts, respectively (Shwab et al., 2018) (Figure 1.2). Before the rise of agriculture 11,000 years ago, the sylvatic life cycles likely sustained *T. gondii* in the environment with wild felids being the definitive hosts and other wild mammals and birds being the intermediate hosts (Shwab et al., 2018). The prosperity of human society afforded by agriculture engendered the flourish of house mice and domestic cats, both of which formed close commensal relationships with humans. The stored grains from successful agriculture attracted mice, which in turn attracted cats (Rosenthal, 2009). Consequently, the success of human civilization and animal husbandry inadvertently established the domestic cycle of *T. gondii*.

As human society continues to expand and prosper, humans slowly encroach upon the wildness. As a result, the boundary between the wild and domestic spheres became increasingly obscure. The dwindling interspace between the wild and the anthropized environment increased territory overlapping between wild and domestic animals (Taiz, 2013; McDonald et al., 2008). The

modern-day *T. gondii* transmission had featured an unprecedented overlapping of the two cycles (Pongsiri et al., 2009). Two major routes contribute to the transmission overlapping of the two life cycles, namely through the environmentally widespread oocysts or through carnivorism in which wild animals eat domestic animals and vice versa (Figure 1.2). Noteworthy, oocyst contamination of water due to soil runoff has been suggested to be the culprit of sea mammal infection (Sundar et al., 2008). The overlapping of two life cycles is clearly reflected in sea otter (VanWormer et al., 2014) and coyote (Jiang et al., 2018), as the dominant genotypes in domestic and those in wild animals were found to co-dominate in these animals.

Tachyzoites, Bradyzoites, and Oocysts

Tachyzoite and bradyzoite

A tachyzoite, a size of $2 \times 6 \mu\text{m}$, is crescent-shaped with a pointed anterior end and a rounded posterior end (Dubey et al., 2010). A tachyzoite has a centrally located nucleus and many organelles, including rhoptries, micronemes, amylopectins, dense granules, and apicoplasts (Figure 1.3). Although tachyzoites have no visible locomotive structures such as cilia, flagella or pseudopodia, it can slightly move its body, such as gliding, flexing or rotating. Tachyzoites can invade any nucleated mammalian cell. Host cell invasion is a process involving protein secretion and actin-based motility and is divided into seven steps in Figure 1.3. During the invasion, microneme (MI) secrete proteins to engage in host cell attachment and motility, whereas the rhoptry neck (RN) secreted a wide array of proteins (RONs) to form moving junction (MJ), and rhoptry bulb secretes other proteins (ROPs) to modify parasitophorous vacuoles (PVs) and modulate host cell transcription (e.g., the phosphorylation of transcription factors by ROP16) (Figure 1.3).

Once formed moving junction, the parasite invaginates the plasma membrane of host cell and finally enters host cell. Once inside, parasite is enclosed by a parasitophorous vacuole membrane (PVM) to sequester parasites from the host cytosol. In addition to not fusing with lysosomes or becoming acidic, the PVs also recruits host mitochondria and ER which may help parasite obtain nutrients. After a lag period, tachyzoites within PVs start to replicate asexually via endodyogeny, in which two progenies form in a mother cell and gradually consume the mother

cell. A rosette (8 or 16 parasites) is formed in the case of synchronous cell division. The burgeoning tachyzoites soon rupture the host cells and are ready for next round of invasion.

Under the pressure of host immune system, especially the IFN- γ -dependent cell-mediated immune response, tachyzoites convert to bradyzoites six to seven days after infection (Dubey et al., 1998). Bradyzoite, a size of $1.5 \times 7 \mu\text{m}$, are enclosed by tissue cyst walls and become semi-dormant. The cyst wall is formed through the protein modification of PVM, although the exact composition of the cyst wall is unclear. Being non-immunogenic, the cysts reside in host tissues, awaiting a chance for transmission. Without the active and systemic host immune defense, the *in vitro* formation of bradyzoite/cysts was different from that of *in vivo* in many perspectives. In cell culture, tachyzoite and bradyzoite can spontaneously interconvert (Bohne et al., 1993). However, the conversion of tachyzoites to bradyzoites is not a synchronized event *in vitro* as one vacuole can harbor parasites expressing tachyzoite-specific antigens and those expressing bradyzoite-specific antigens (Gross et al., 1996).

T. gondii cysts have a propensity for neural (central nervous system, the eyes) and muscular tissues (skeletal muscles, cardiac muscles), although a broad spectrum of visceral organs can harbor tissue cysts, including lung, liver, kidney, and bone marrow (Skariah et al., 2010). The size of the tissue cysts varies from $5 \mu\text{m}$ to $100 \mu\text{m}$ with varying number of bradyzoites enclosed. A tissue cyst can harbor as few as two bradyzoites in the case of a young tissue cyst and as many as thousands of bradyzoites in the case of an old tissue cyst. However, tissue cysts do occasionally rupture and release bradyzoites which can flourish and cause diseases in the case of immune suppression. The reactivation of bradyzoites is especially noteworthy in AIDS patients and cancer patients who undergo chemo and or radiation therapy (Weiss et al., 2013).

The stage interconversion

The interconversion between tachyzoites and bradyzoites is significant in chronic infection as well as disease reactivation. Understanding the molecular basis of the interconversion can aid in the identification of drug targets to clear bradyzoites in the chronic stage.

Tachyzoites differ from bradyzoites in many perspectives (Dubey et al., 1998). Although both are crescent-shaped, tachyzoites are plump, while bradyzoites are slender. Ultrastructurally, bradyzoites have electron dense rhoptries, higher content of micronemes and amylopectin

granules, posteriorly located nuclei as opposed to centrally located nuclei. Bradyzoites are more resistant to pepsin or trypsin digestion. Furthermore, tachyzoite and bradyzoite express different cell surface proteins.

A wide range of approaches have been employed for tachyzoite and bradyzoite interconversion *in vitro*. It has been known that tachyzoite to bradyzoite conversion is a stress-related phenomenon. The high temperature at 43 °C, high (8.0-8.2) or low (6.6-6.8) pH, chemical stress (i.e., Na arsenite) can result in the upregulation of bradyzoite-specific gene expression and the shutting down of tachyzoite-specific gene expression (Soete et al., 1994). Nitric oxide (NO), an inhibitor of mitochondrial function, was believed to be a potent differentiation inducer. IFN- γ triggered bradyzoite-specific surface protein expression was partially attributed to the downstream induction of NO (Bohne et al., 1994). Sodium nitroprusside, a NO donor, was capable of inducing tachyzoite to bradyzoite differentiation *in vitro* (Bohne et al., 1994). The *in vitro* produced bradyzoites proved to be pepsin resistant (Dubey et al., 1998; Popiel et al., 1996) and can orally infect both mice (Fux et al., 2007) and cats (Hoff et al., 1977). The conversion from bradyzoite to tachyzoite is triggered when the immunological factors (e.g., IFN- γ) or host immune defense, especially T cell response, is lacking (Lyons et al., 2002). Prolonged *in vitro* propagation has favored tachyzoite formation and decreased the efficiency of bradyzoite formation in some avirulent strains of *T. gondii* (Weiss and Kim, 2011).

Tachyzoite and bradyzoite are antigenically distinct (Lyons et al., 2002), which can be utilized for stage identification. Concerning surface proteins, tachyzoite expresses unique proteins including SAG1/p30, SAG2A, and SAG2B, whereas bradyzoite expresses a different repertoire of antigens including SAG4/p18, BSR4/p36, BAG1/hsp30, SAG2C, and SAG2D. BAG1 (bradyzoite antigen 1) is homologous to a small heat shock protein in plants. BAG1 protein expression on the bradyzoite surface is an early event of differentiation and thus is widely used as a differentiation marker. Cells expressing BAG1 were seen after 24 hours of exposure to stress such as pH 8.1 (Weiss and Kim, 2011). Deletion of BAG1 did not prevent cyst formation but only hindered the efficiency of cyst formation (Bohne et al., 1998). Bradyzoites also express lactate dehydrogenase 2 (LDH2), enolase 1 (ENO 1), and matrix antigen 1 (MAG1). In contrast, tachyzoites express LDH1 and ENO2, and have no MAG1 expression. The formation of the cyst wall and cyst matrix are unique to the bradyzoite stage. MAG1 was found in the cyst matrix and

not on bradyzoite. A 116 kDa glycoprotein CST1 containing N-acetylgalactosamine on the cyst wall was capable of binding to *Dolichos biflorus* lectin (DBL). In addition, the cyst wall can also bind to succinylated wheat-germ agglutinin (S-WGA). Consequently, DBL or S-WGA can be used to detect cyst wall formation in mature cysts.

The metabolism of bradyzoites and tachyzoites are also different. While both utilize glycolytic pathways to obtain energy, tachyzoite also utilizes tricarboxylic acid (TCA) cycle and respiratory chain, whereas bradyzoite lacks the TCA cycle and respiratory chain (Denton et al., 1996). As a result, the enzymatic activity in each stage form is different. The activity of lactate dehydrogenase (LDH) and pyruvate kinase is higher in bradyzoite (Denton et al., 1996). Bradyzoite relies on breaking down amylopectin to lactate for energy and therefore resistant to acidic pH. Due to their metabolic differences, antibiotics interfering metabolism can also induce stage differentiation. Oligomycin, an inhibitor of mitochondrial ATP synthase, and antimycin A, an inhibitor of the electron transport chain, were shown to induce bradyzoite formation (Tomavo et al., 1995).

Considering the nature of tachyzoites and bradyzoites, reducing growth rate in some strains can induce tachyzoite to bradyzoite conversion (Bohne et al., 1994). *T. gondii* strain differences were seen in the variations of cyst size, number, and the efficiency of cyst formation. Strains with a slower growth rate and less virulence are predisposed to produce cysts *in vitro*. Slow-growing and less virulent strains such as ME49 tend to have a higher rate of bradyzoite formation than virulent and fast-growing strains such as RH strain (Soete et al., 1994). Drugs (i.e., pyrimethamine) and cytokines (i.e., IFN- γ) that can reduce the growth rate of the parasite can induce tachyzoite to bradyzoite switch (Bohne et al., 1994).

The signaling pathway and molecular mechanism of interconversion are unknown. However, as a stress-induced response, the common stress-related pathways in eukaryotes are implicated. Cyclic nucleotide kinase is believed to partly mediate this interconversion (Eaton et al., 2006). A study has determined that inhibition of cAMP-dependent kinase and cGMP-dependent kinase reduced parasite replication and induced the differentiation (Eaton et al., 2006). Parasite-specific eukaryotic initiation factor-2 (eIF2) kinase was also implicated in the differentiation process (Sullivan et al., 2004). As protein chaperones and transcription regulators, heat shock proteins (HSP) induced in stress response gain research interest in elucidating the

conversion process. After induction at pH 8.1, *T. gondii* hsp70 was induced 3-4 folds more than that of at pH 7.1, consistent with results seen in other Apicomplexans (Weiss et al., 1998; Shiels et al., 1997). Likewise, inhibition of HSP synthesis using quercetin hindered *in vitro* bradyzoite development (Weiss et al., 1998).

Oocyst

Upon being discharged into the environment, an oocyst requires one to five days to sporulate to become mature. One oocyst contains two sporocysts each with four haploid sporozoites. Sporulated oocyst, a size of 11 to 13 μm in diameter, is enclosed by a five-layer oocyst wall (Dubey et al., 1998).

As the environmental form of the parasite, oocysts possess important features which ensures parasite's effective transmission. First, oocysts are large in number. The number of oocysts one cat can shed ranges from 3 to 810 million during a median shedding period of eight days (Dubey, 2010). In California, the annual average oocyst burden in soil ranges from 94 to 4671 oocysts per square meter (Dabritz et al., 2007). Oocysts have been proved to be highly hardy and infectious. Oocysts can survive in the environment for a prolonged period. Outdoors (6°C-36°C), oocysts in cat feces had survived for 46 days when the feces were uncovered, for 334 days when they were covered (Dubey et al., 1998; Frenkel et al., 1975). Oocysts are also resistant to chemical inactivation, including sodium hypochlorite and ozone (Dubey, 2010). However, oocysts can be destroyed by desiccation, or high temperature (55°C for 2 min or 60°C for 1 min) (Dubey, 1998). It has been revealed that oocyst-induced infection is more severe than bradyzoite-induced infection (Dubey et al., 1988; Dubey et al., 1973; Dubey et al., 1997a and 1997b). Oocysts are more infectious than bradyzoite in intermediate hosts. One oocyst can potentially induce infection in a laboratory pig (Dubey et al., 1996) or mouse (Dubey, 2010). Recent studies demonstrated that oocysts caused 78% of congenital infections in North America (Boyer et al., 2011), and 43% in Southern Chile (Munoz-Zanzi et al., 2010).

Infectivity of tachyzoite, bradyzoite, and oocyst

Cats are more susceptible to bradyzoite infection as compared to tachyzoite or oocyst infection; the reverse is true for mice. Although cats can shed oocysts after ingesting any stage of the parasite,

the prepatent period (time from infection to shedding oocysts) varies (Dubey, 2010). The prepatent period is 3 to 10 days with bradyzoite, but ≥ 19 days with tachyzoite, and ≥ 18 days in case of oocyst (Dubey, 2010). Moreover, the dose of tachyzoite required for oocyst shedding in a cat is approximately 100 times higher than that of bradyzoite (Weiss et al., 2011). Oral infection of as few as one bradyzoite can induce oocyst shedding in 16.6% (3/18) of cats, whereas 1,000 bradyzoites can only orally infect 50% (12/24) of mice (Dubey, 2001). One oocyst can cause disease in a mouse, whereas 100 oocysts could not induce oocyst shedding in a cat (Dubey, 2001 and 2006a). *T. gondii* is adapted to be transmitted in cats via bradyzoites, whereas via oocysts in mice (Dubey, 2010).

Population Genetics

The genetic information of *T. gondii* strains from geographically distinct locations and a wide array of host species have been unveiled owing to the advanced molecular techniques. Microsatellite DNA analysis (Ajzenberg et al., 2002 and 2010), polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) (Howe and Sibley, 1995; Su et al., 2006 and 2010), and intron sequencing (Khan et al., 2007 and 2011) have been used to investigate the genetic variation of *T. gondii*. Among these methods, PCR-RFLP is the most commonly employed and has been used on thousands of *T. gondii* strains. Examining the PCR-RFLP data of 1457 samples collected worldwide, Shwab et al. (2014) summarized the population genetic structure of *T. gondii* on a global scale (Figure 1.4). Based on this article, the northern hemisphere is dominated by a few clonal genotypes, whereas the population structure of the southern hemisphere is a mosaic of numerous atypical genotypes without dominant genotypes (Figure 1.4). The details of this pattern are outlined below.

The designation of *T. gondii* genotypes has been streamlined using ToxoDB PCR-RFLP nomenclature in which genotype is numbered based on the order of discovery (Shwab et al., 2014). Genotype #1, #2 and #3 were found worldwide, which are also the dominant genotypes in Europe. In North America, genotype #1, #2, #3, #4 and #5 are prevalent. In Africa, the dominant genotypes are #2 and #3. #9 and #10 are prevalent in East Asia. No genotypes seemingly predominate in Central and South America. In North America, type 12 is found to dominate in wildlife, whereas

type II and type III are believed to dominate in domestic animals. It is unknown as to what factor(s) contribute to this genotype partition.

***T. gondii* Infection in Humans and Animals**

Infection in humans

T. gondii is evolutionarily successful, infecting virtually all warm-blooded animals. In addition to its expansive host range, *T. gondii* is geographically ubiquitous, having been found on every continent, including Antarctica (Dubey, 2010). Seroprevalence of the human population varies from as low as 4% in Korea and as high as 92% in Brazil (Dubey, 2010). In the period 1999-2004, US-born women 15-44 of age had lower seroprevalence (11%) than women of the same age group who are foreign-born (28.1%) (Jones et al., 2007). Infection is higher in Latin America than in North America and East Asia. Infection rates are lower in cold, dry or high latitude regions as compared to warm or low latitude areas (Dubey, 2010). It has been shown that infection prevalence is associated with cooking habit, hygiene, and frequency of soil contact (Stagno et al., 1980; Jones et al., 2008). Incidence at large is declining in the last few decades in many countries (Aspöck et al., 1992; Kortbeek et al., 2004). A serosurvey conducted in the U.S. revealed that the age-adjusted *T. gondii* prevalence of US-born persons 12-49 years old decreased from 14.1% in 1988-1994 to 9% in 1999-2004 and 6.7% in 2009-2010 (Jones et al., 2014).

Infection in immunocompetent people is generally asymptomatic. In some cases, mild symptoms, such as fever, malaise, and lymphadenopathy can develop. In rare cases, the infection causes severe ocular toxoplasmosis (e.g., retinochoroiditis), multi-visceral toxoplasmosis or even fatal outcomes. In the U.S., it has been estimated that 2% of the infections manifested as ocular toxoplasmosis; this number is 17.7% in southern Brazil (Holland, 2003). The underlying reasons for these severe cases in immunocompetent patients are elusive. Variabilities of host immunity, parasite strain, and other factors have been suspected to be relevant. It appears that these severe cases are often associated with highly virulent strains such as those found in South America (Carme et al., 2002; Demar et al., 2007). Over 50 cases of human toxoplasmosis had been reported in French Guiana since 1998; isolates from these patients were found to be atypical and highly virulent in mice; forest-related activities including eating undercooked game meat were suspected

to be the cause (Dardé et al., 1998; Carme et al., 2002; Demar et al., 2007). In Brazilian children, in addition to the higher occurrence, the severity of congenital ocular toxoplasmosis is also higher than that in Europe (Gilber et al., 2008). Two thirds of *T. gondii* isolated from citizens of the United States belong to type I (highly virulent) or atypical genotypes (Grigg et al., 2001).

One of the recent research interests of *T. gondii* is linking *T. gondii* chronic infection with the tendency of patients to take risks (Johnson et al., 2018), tendency for accidents (Flegr et al., 2002), behavior impairments (Sugden et al., 2016), and mental disorder (Brown et al., 2005). In immunocompromised patients, the reactivation of latent infection can be life-threatening. Encephalitis resulting from intracerebral focal lesion and or pneumonia are the hallmarks of toxoplasmosis in AIDS patients (Dubey et al., 1998).

Infection during pregnancy in previously uninfected women can lead to congenital infection in fetus or neonates. The risk of congenital infection is lowest (10-15%) in the first trimester; however, the potential damage is the most severe if infected (Dubey and Jones, 2008). On the contrary, infection risk in the third trimester is the highest (60–90%), and the damage is less severe if infected (Dubey and Jones, 2008). The manifestation of congenital infection varies from spontaneous abortion, stillbirth to congenital disabilities in newborns (Remington et al., 2006). Symptoms of congenital toxoplasmosis in neonates include hydrocephalus, microcephalus, cerebral calcification and retinochoroiditis (Remington et al., 2006). Some children may be healthy at birth but could develop visual or neurological sequels (e.g., learning disability) later in life (McLeod et al., 2006).

Infection in animals associated with human households (cats, rodents, pigs, chickens, cattle, sheep, and goats)

Cats

As the definitive hosts, domestic cats are of utmost importance in the study of epidemiology and transmission of *T. gondii*. Studies showed that *T. gondii* infection is scarce or lacking in areas without cats (Dubey, 1997c; Munday et al., 1972; Wallace et al., 1969). Cats, historically used by humans to protect stored grains from rodents, have long ago formed a commensal relationship with humans dating back approximately 9,000 years (Driscoll et al., 2007). Today, one-third of households in the United States own cats. It has been estimated that there are 78 million domestic

cats and 73 million feral cats in the U.S. (Dubey, 2010).

Cats are also common on farms. A 1999 study demonstrated that a mean of 8.5 cats was present on each pig farm in Illinois, of which six were seropositive for *T. gondii* (Weigel et al., 1999). An average of 30% of cats worldwide has *T. gondii* antibodies, which suggests that they have already shed oocysts and can re-shed oocysts upon reinfection (Dubey, 2010). Cats, regardless of age, are capable of shedding oocysts. Most cats were believed only to shed oocysts for a week once in their lifetime, however; empirical data attested that cats could re-shed oocysts upon reinfection six years after the initial infection (Dubey, 1995). At any given time, 1% of cats were expected to shed oocysts (Dubey, 2010). Taking advantage of the clinical samples accumulated in an animal laboratory in the United States, a study showed that 31% of 12,628 cats had *T. gondii* antibodies, which coincides with the estimated global seroprevalence (30%) in cats (Vollaire et al., 2005). Infection rates in cats can be much higher in other parts of the world. In southern Brazil where a water-associated outbreak of toxoplasmosis had occurred, 84.4% (49/58) of cats were seropositive for *T. gondii* (Dubey et al., 2004).

Unlike many other animals, cats can suffer from clinical toxoplasmosis. Some individuals develop symptoms including lethargy, anorexia, or sudden death. Histological manifestations involve inflammation or necrosis in multiple organs including heart, brain, eyes, liver, and pancreas. At present, *T. gondii* isolates from domestic cats are scarce. Eight isolates were obtained from cats on pig farms in Illinois, among which five were type 12 (Dubey et al., 1995).

Rodents

A large-scale bioassay using tissues from 5,166 rodents across 17 species revealed that only a small proportion (0.9%) of rodents were infected with *T. gondii* (Hejlíček and Literák, 1998). Rodents can harbor *T. gondii* but may have no detectable antibodies in their blood. Viable parasites have been isolated from seronegative rodents (Dubey et al., 1995; Dubey et al., 1997d). Therefore, bioassay is the definitive method for *T. gondii* detection in rodents. Immunological tolerance and short lifespan of rodents are believed to account for the low infection rate in rodents. *T. gondii* susceptibility in rodents varies highly even in closely related species. It has been known that rats (genus *Rattus*) are resistant to *T. gondii* infection, whereas house mice (*Mus musculus*) are highly susceptible (Dubey, 2010).

Pigs

The prevalence of *T. gondii* in pigs varies. Feeder pigs and market pigs are typically raised indoors in well-managed facilities, and thus their seroprevalence is declining (Dubey et al., 1995). However, the seroprevalence of pigs raised in poorly managed or non-confined facilities is as high as 68% (Dubey et al., 2008). Noteworthy risk factors of pig infection include direct contact with oocysts and eating dead mice (Weigel et al., 1995b). Viable *T. gondii* has been frequently isolated from pork for human consumption; isolation rate ranges from 0.3% to 92.7%, depending on the age of pigs and pig farming conditions (Dubey and Jones, 2008).

Chickens

Chicken raised in the backyard are often infected with *T. gondii* with infection rates ranging from 27% to 100% worldwide (Dubey and Jones, 2008; Dubey et al., 2005b; Dubey et al., 2006b). A study conducted in Paraná, Brazil revealed that 12.2% (47/386) of free-range chickens were seropositive for *T. gondii*; PCR-RFLP revealed ten different genotypes from 18 isolates, emblematic of the high genetic diversity in South America (Vieira et al., 2018). However, the infection rate is low in chickens raised in confined and industrialized feeding operations. *T. gondii* was not isolated from chicken breast meat, although *T. gondii* antibodies were found in the meat juice (Dubey et al., 2005a).

Cattle

Cattle are resistant to *T. gondii* infection. Although cattle can be infected with *T. gondii* oocysts, they can gradually clear parasite or reduce the infection to an undetectable level (Dubey and Jones, 2008). The underlying mechanism of parasite resistance in cattle is elusive. In an experimental setting, 10,000 of oocysts were fed to each of the four steers weighing 100-150 kg; the fourth steer became seronegative after 15 months, and no parasite was isolated using the cat bioassay three years post infection (Dubey, 1992). Attempts to detect *T. gondii* antibodies using beef juice has been futile (Dubey et al., 2005a).

Sheep and Goats

Sheep and goats have been susceptible to *T. gondii* infection as evidenced by the frequent reports of abortion incidences and neonatal mortality. Congenitally infected lambs that survived can

remain asymptomatic, which could be a source for human infection (Dubey and Kirkbride, 1989). Besides abortion and neonatal mortality, clinical signs of toxoplasmosis have been observed in adult goats (Dubey and Beattie, 1988), but not in adult sheep.

Infection in wild (white-tailed deer, bobcats) or captive animals

T. gondii infection has been steadily reported in wildlife. White-tailed deer and bobcats are among animals that are frequently sampled (Dubey et al., 2011). The infection in white-tailed deer heralds oocyst contamination in the wilderness because deer are strict herbivores. A recent study surveyed 241 serum samples from white-tailed deer in North America showed that 41.0% of these animals had *T. gondii* antibodies; 13 isolates were obtained with ten being ToxoDB genotype #5 (Gerhold et al., 2017). Infections in bobcats can be either through ingesting oocyst or through carnivorism. In one study, *T. gondii* antibodies were present in 51.7% of 58 bobcats from North America, Central America and South America (Kikuchi et al., 2004). In another study, parasites were isolated from 21 of 35 bobcat samples; the dominant genotype #5 (18/21) was revealed among these isolates (Verma et al., 2017).

The infection in marine mammals indicates land-to-water oocyst contamination. Sea otter, sea lions, dolphins, and seals have been reported to have *T. gondii* associated encephalitis and *T. gondii* antibodies (Dubey et al., 2003). The sea otter was listed as a threatened species in the United States due to its dwindling population. *T. gondii*, capable of causing death in sea otter, had been suspected to be the principal culprit of sea otter's decline. However, *T. gondii* and *Sarcocystis neurona* combined are responsible for the death of a small percentage (39/334=11.3%) of sea otter (Thomas et al., 2007). Seroprevalence in sea otter has been considered high, ranging from 47% to 100% (Dubey and Jones, 2008). Sea otter seroprevalence is higher in California than in Washington. Bottlenose dolphins from both coasts of the United States have high *T. gondii* seropositivity (53%-100%), although not many isolates have been obtained (Dubey et al., 2003).

Marsupials and New World primates are highly susceptible to *T. gondii* infection. Acute toxoplasmosis has been reported in many species including squirrel monkeys (*Saimiri sciureus*) (Oh et al., 2018), ring-tailed lemurs (*Lemur catta*) (Juan-Sallés et al., 2011), marmoset (*Saguinus imperator*) (Epiphanio et al., 2000), woolly monkey (*Lagothrix lagotricha*) (Hessler et al., 1971), and golden-headed lion tamarins (*Leontopithecus chrysomelas*) (Epiphanio et al., 2000).

T. gondii has significant veterinary importance especially in zoos and theme parks. Acute and often fatal infection in captive kangaroos and wallabies was frequently reported worldwide (Basso et al., 2007; Boorman et al., 1977; Miller et al., 1992; Guthrie et al., 2017). As a result, zoos and theme parks had suffered from significant economic loss due to the death of expensive animals. Clinical signs of toxoplasmosis in macropods include blindness, weight loss, respiratory distress, diarrhea, neurological deficits, and sudden death. Necropsy and histological examination reveal hepatitis, inflammation, and necrosis in cardiac, skeletal, and smooth muscles (Portas, 2010). Acutely infected macropods often succumb suddenly, and medicine treatment (e.g., sulfonamides and pyrimethamine, atovaquone) appears to be impotent to save lives (Portas, 2010).

The Arms Race with Its Hosts

T. gondii is an epitome of host-parasite coadaptation. The arms race between host defense and parasite virulence ensues as each imposes intense selective pressures on the other. Mice and cats are *T. gondii*'s natural and ancient hosts, whereas humans and many other intermediate hosts that are not part of natural feline's food chain are considered as accidental hosts. As a result, the mice immune system is under prolonged selective pressures of the parasites, but not the human immune system— the very reason human lacks the immune defense mechanisms found in mice. The toll-like receptor (TLRs) (TLR11, TLR12) and immunity-related GTPase (IRG) proteins are absent or nonfunctional in humans.

Host immunity

Among the host defense mechanism against parasite, Th1 cell-mediated immune response, namely the signaling pathway of IL12 – Th1– IFN- γ – intracellular killing, is the most significant (Figure 1.5). In mice, endosomal TLR 7 and TLR 9 can recognize parasite RNA or DNA, whereas endosomal TLR11 and TLR12 recognize parasite profilin-like protein (PRF) (Gazzinelli et al., 2014). Profilin is an essential protein of the parasite, governing parasite invasion and motility. The detection of profilin by a dendritic cell is a “detection at a distance” scheme. In addition to endosomal TLRs, cell surface TLR 2 and TLR 4 are also involved in the recognition of *T. gondii*. TLR 2 and TLR 4 can both be activated by *T. gondii* glycosylphosphatidylinositol (GPI), or *T. gondii* derived heat-shock protein 70 (HSP70); however, the immune effect initiated by these two

receptors is likely marginal as compared to other toll-like receptors.

Upon sensing the parasite, dendritic cells and monocytes are activated to produce IL-12, which triggers the production of IFN- γ mainly by NK cells, Th1 cells (Figure 1.5). The function of neutrophils in combating parasite includes the secretion of IL-12 and IFN- γ , phagocytosis, and the formation of extracellular DNA net (Bliss et al., 1999; Filisetti and Candolfi, 2004). Although neutrophils can produce reactive oxygen species, the role of ROS in parasite killing remains controversial (Murray and Teitelbaum, 1992). The production of IFN- γ in NK cells and T cells are IL-12 dependent, whereas the secretion of IFN- γ in neutrophils are regulated by TNF- α and IL-1 β . IFN- γ not only induces the production of proinflammatory mediators but also fosters the maturation of Th1 cells, which in turn enhance IFN- γ production. The IRG proteins produced in both myeloid cells and non-myeloid cells induced by IFN- γ are among the most effective parasitocidal effectors. In comparison to the conservative TLR 11 and TLR 12, IRG proteins are relative polymorphic, counteracting the mutative virulent factors of the parasite.

In humans, the TLR11 gene contains three stop codons, which renders the gene unable to encode a functional protein. TLR12 is not present in the human genome. In the case of human immune defense, it is suspected that TLR7, TLR8, and TLR9 play similar roles in parasite recognition and subsequent activation of dendritic cells and monocytes (Gazzinelli et al., 2014). In addition, IRG proteins are missing in humans. It is intriguing and yet perplexing that TLR11, TLR12, and IRG proteins are absent in most mammals that *T. gondii* are capable of parasitizing.

IFN- γ is a major effector in the killing of intracellular parasites through at least three mechanisms (Yarovinsky, 2014). First, IFN- γ induces the production of indoleamine 2, 3-dioxygenase (IDO), which converts tryptophan to N-formylkynurenine, leading to tryptophan deprivation and parasite starvation. Another downstream effect of IFN- γ is the activation of nitric oxide synthase, which can release nitric oxide (NO). NO intermediates are nonpolar and uncharged which allow them to enter PVs to interact with parasites directly. NO modifies metabolic enzymes, rendering them nonfunctional. In addition, during the biosynthesis of NO, arginine is deprived due to the fact that L-arginine is a precursor of NO. *T. gondii* is an arginine auxotroph - the reason why arginine starvation can trigger tachyzoite to bradyzoite switch. Without arginine, the replication of the parasite is stunted. The third mechanism is through the induction of IRG proteins and p65 guanylate-binding proteins (GBPs). Mice seem to highly rely on IRGs for host defense as there

are 22 members of IRG proteins in mice and only one in human (expressed in testes) (Howard et al., 2011). In human, the IRG homolog – IRGM is truncated and is not regulated by IFN- γ (Bekpen et al., 2005). It is unknown whether IRGM is participating in host defense against the parasite. The localization of IRGs onto PVs results in the disruption of PVs (Figure 1.6). Parasites are vulnerable once exposed to the cytosol and soon are disintegrated by lysosome (Figure 1.6). GBPs (seven GBPs in humans; 13 in mice) were believed to indirectly combat parasite by recruiting IRGs onto PVs (Selleck et al., 2013).

In addition to Th1, CD8⁺ T cell is another component of the specific acquired immune response. Upon the activation by IL-2 (secreted by Th1), CD8⁺ T cells exert cytotoxic activity on infected cells or extracellular tachyzoites. The humoral immune response is also involved in the battle with the parasite. IgM appears in serum one week after infection, which is followed by IgG. IgG plays a critical role in maternal protection for the fetus as IgG can pass placenta. Antibodies play a role in killing extracellular tachyzoites through antibody-dependent cytotoxicity or opsonization.

Parasite virulence

T. gondii virulence has apparent strain variation in sensitive hosts (e.g., outbred mice) with type I being highly virulent ($LD_{100}=1$), type II being intermediately virulent ($LD_{50} \geq 10^2 - 10^4$), and type III being avirulent ($LD_{50} \geq 10^5$). The quantitative trait locus (QTL) mapping was used to identify virulence factors using progenies from sexual crosses among Type I, II, and III (Saeij et al., 2006; Taylor et al., 2006; Behnke et al., 2011). *T. gondii* strain virulence in mice seems to be geographically distinct. A recent study pinpoints that 61% of 427 isolates from Central and South America are highly virulent to house mice, whereas only 7% of 193 isolates from North America are virulent (Shwab et al., 2018). There is no apparent association between host species and *T. gondii* virulence.

The polymorphic rhoptry-secreted kinase (ROPs) are first identified using forward genetics approach (Taylor et al., 2006, Saeij et al., 2006) as the significant virulent factors to counteract IRGs and GBPs. ROP18 is functional in Type I and II and not functional in type III due to the insertion in its promoter region (Boyle et al., 2008). ROP18 is capable of phosphorylating specific threonine residues in IRGs and thus hampers the disruption of PVs by IRGs. Using the same

forward genetics approach, ROP5 was identified and was found to be indispensable to parasite resistance as highly virulent parasite completely lost virulence without ROP5 (Reese et al., 2011, Behnke et al., 2011). Despite being a pseudo-kinase, ROP5 forms a complex with ROP18 to facilitate the phosphorylation of IRGs (Figure 1.6) (Etheridge et al., 2014). ROP5 also complexes with ROP17 to target threonine residues of the IRGs (Figure 1.6) (Etheridge et al., 2014). ROP5 is evolutionarily under intense selective pressure and thus being highly polymorphic. The interacting regions of ROP5 and IRGs are also their polymorphic sites, exemplifying the arms race between parasite and its host as each diversifies under selective pressure of the other. Similar to ROP18, the functionality of ROP5 varies in different strains: type I and III have a virulent form of ROP5, whereas ROP5 in type II is avirulent. ROP18 and ROP5 gene alleles combined can be used to predict *T. gondii* strain virulence in mice (Shwab et al., 2016).

Dissertation Objectives

Toxoplasma gondii is an inclusive parasite, inhabiting virtually all warm-blooded animals on every continent. Because of its indiscriminating host range and broad geographical distribution, we can use it as a model organism to study pathogen transmission and ecology in the context of human intervention on the environment. The recent few decades have witnessed an extensive genetic characterization of the parasite and thus enriched our understanding of parasite transmission and distribution on the planet. Studies of population genetics have unveiled an intricate and yet unique *T. gondii* genotype global landscape as a result of the evolutionary adaptation of the parasite to a wide array of distinctive environments. The northern hemisphere is dominated by a few clonal lineages, whereas numerous genotypes exist in South and Central America. Factors that shape the global genetic diversity of *T. gondii* has been addressed in a recent publication (Shwab et al., 2018). According to the paper, the rise of agriculture 11,000 years ago and the concomitant flourishing of cat and mouse engendered the domestic life cycle of *T. gondii*. It was suspected that certain lineages of *T. gondii* were selected in the domestic life cycle and was expanded worldwide through agricultural expansion and human migration. In addition, a superinfection model was proposed to explain the virulence diversity in different continents. Alternatively, it has been speculated that the unique climate and the wide variety of wildlife in South America likely sustain

the high diversity of *T. gondii*. Nonetheless, the cause of the contemporary *T. gondii* genetic structure is multifactorial.

One of the primary implications of understanding transmission is to actively hamper the transmission to control parasite contamination in the environment. As cats are the ancestral and definitive hosts of *T. gondii*, it comes as no surprise that *T. gondii* infection is prevalent in cats, which indicates previous oocyst-shedding events and potential re-shedding upon reinfection. To make matters worse, the oocysts shed by cats are highly infectious and recalcitrant to disinfection. Given the number of domestic and feral cats in the United States and the number of oocysts each cat could shed, the environmental contamination by oocysts poses a threat to public health. The overarching goal of this dissertation is to understand *T. gondii* population genetics, its transmission, and ecology to develop control tactics to reduce its environmental contamination, thus alleviating the social-economic burden of this notorious parasite.

Chapter 2 objectives

To tackle the problem of environmental contamination, a better understanding of *T. gondii* transmission in the environment is fundamental. In recent decades, PCR-RFLP genotyping and microsatellite have considerably advanced the genetic characterization of *T. gondii*. With the accretion of genetically characterized strains of *T. gondii*, the population genetics of *T. gondii* has been steadily revealed, which aids in a better understanding of parasite transmission. Previous studies have indicated that North American *T. gondii* population is clonal and dominated by four archetypal lineages identified as types I, II, III, and type 12. Furthermore, type 12 is mainly found in wildlife; type II and III are mainly found in domestic animals (Dubey et al., 2011b). Factors that shape the genetic structure of *T. gondii* in North America are elusive. To tease them apart, we compiled and analyzed existing genotypic data from animals across a proximity gradient from the anthropized environment to the wilderness in North America. We aimed to investigate the effect of host habitat and host species on *T. gondii* transmission. We asked the following questions:

- What are the genotype compositions of *T. gondii* in animal populations with different proximities to anthropized environment? How are these genotype compositions different?
- How does human activity influence *T. gondii* diversity and genotype distribution?

- Does *T. gondii* genotype distribution have host specificity?

Chapter 3 objectives

To control *T. gondii* environmental contamination, we proposed that sandboxes can be used to reduce the environmental contamination by *T. gondii*. The hypothesis was inspired by the defecation behavior of cats who prefer loose sand to defecate to bury their feces (Turner and Bateson, 2000). The hypothesis was also supported by empirical data in which feral cats frequently used sandboxes (whether experimental set up in parks or those in school playground) to defecate (Lass et al., 2009; Uga et al., 1995 and 1996). To test that hypothesis, a typical dairy farm in East Tennessee was chosen as research site. It was planned that the farm contamination would be evaluated which would be compared to that of two years after sandbox implementation. As the project progressed, the initial idea of using the sandboxes to reduce environmental contamination was abandoned due to the low contamination of the farm by *T. gondii*. However, a systemic evaluation method was developed to assess the environmental contamination by *T. gondii* using animal infection in conjunction with soil oocyst detection. In this study, we sought to answer the following questions:

- Are oocysts detectable in soil on a typical dairy farm harboring cats? Can we use oocyst contamination as an indicator of farm contamination?
- How prevalent is *T. gondii* in domestic and wild animals appearing on the farm? Can we use animal infection rate to assess farm contamination?

Chapter 4 objectives

Captive macropods have suffered from fatal toxoplasmosis worldwide (Basso et al., 2007; Fernández-Aguilar et al., 2013; Guthrie et al., 2017). Recent reports of fatal toxoplasmosis in captive macropods in the United States have accentuated the significance of *T. gondii* infection in these animals and shed light on the urgency of developing control measures to reduce *T. gondii* contamination in zoological settings. Toxoplasmosis in this setting is noteworthy as theme parks/zoos suffer from myriad economic loss due to the death of expensive animals. Furthermore, congregation of humans at oocyst-contaminated themes parks or zoos provide a ready opportunity

for human infection. In this chapter, we attempted to isolate and characterize *T. gondii* from six cases of fatal toxoplasmosis from macropods in a theme park in Tampa, Florida. We sought to investigate the genetic features of the causative agent to understand the infection source of these fatal cases. We attempted to address the following questions:

- Are *T. gondii* antibodies and viable parasite present in macropod sera and tissues, respectively?
- What are the genetic characteristics of these *T. gondii* isolates? Could the genetic characteristics aid in the understanding of infection source?

Chapter 5 objectives

Attempts have been made to use vaccines to reduce *T. gondii* infection. However, these efforts have had limited success thus far. A live cystless strain (S48) of tachyzoite has been used in sheep to reduce neonatal mortality in Europe and New Zealand (Buxton et al., 1993). A vaccine for human use is not yet available as killed strains provide at best marginal protection and attenuated or mutant strains pose the risk of infection especially to fetuses (Dubey, 2010). The most effective vaccination strategy is in favor of the immunization of definitive hosts. A 3-year field trial revealed that vaccination of cats on eight commercial farms using T263 provided promise to reduce the infection in farm animals (finishing pigs and mice) (Mateus-Pinilla et al., 1999). However, the application of this cat vaccine is stalled due to several factors. The key problems are the exacting requirement for storage of tissue cysts produced in mice and the cumbersome procedures for preparation, which render the high price of the vaccine and result in the lack of interest of cat owners. In the conventional vaccine production, a mutant strain, T263, is injected into mice. Brain tissue is harvested, homogenized, digested with pepsin, and preserved in liquid nitrogen. Because parasites cannot survive long at room temperature, immediate administration to cats is required upon thawing of the vaccine for no more than 15 mins (Frenkel et al., 1991).

To simplify the vaccine production and to reduce its price, we sought to develop a methodology to produce live bradyzoites *in vitro*. To accomplish this, developing a way to convert T263 tachyzoite to bradyzoite *in vitro* is essential. In our lab, we tested vacuum conditions or low

temperature to induce the conversion from tachyzoite to bradyzoite. In this chapter, we aimed to answer the following questions:

- Are vacuum conditions capable of inducing tachyzoite to bradyzoite conversion?
- Is low temperature (31°C) capable of inducing tachyzoite to bradyzoite conversion?

To help address the above questions, we used three criteria to distinguish between bradyzoites and tachyzoites and to assess the quality of treated tachyzoites under vacuum conditions or low temperature. We asked the following questions:

- Do treated tachyzoites express BAG1 proteins and form cyst wall?
- Are treated tachyzoites more resistant to pepsin digestion?
- Are treated tachyzoites able to induce infection in mice via oral route?

References

- Ajzenberg, D., Bañuls, A. L., Tibayrenc, M. and Dardé, M. L., 2002. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *Int J Parasitol*, 32: 27–38.
- Ajzenberg, D., Collinet, F., Mercier, A., Vignoles, P. and Dardé, M.L., 2010. Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. *J Clin Microbiol*, 48: 4641–4645.
- Aspöck, H., Pollak, A., 1992. Prevention of prenatal toxoplasmosis by serological screening of pregnant women in Austria. *Scand. J Infect Dis*, S84: 32–38.
- Basso, W., Venturini, M.C., More, G., Quiroga, A., Bacigalupe, D., Unzaga, J.M., Larsen, A., Laplace, R., Venturini, L., 2007. Toxoplasmosis in captive Bennett's wallabies (*Macropus rufogriseus*) in Argentina. *Vet Parasitol*, 144: 157–161.
- Behnke, M.S., Khan, A., Wootton, J.C., Dubey, J.P., Tang, K., Sibley, L.D., 2011. Virulence differences in *Toxoplasma* mediated by amplification of a family of polymorphic pseudokinases. *Proc Natl Acad Sci USA*, 108: 9631–9636.
- Bekpen, C. et al., 2005 The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. *Genome Biol*, 6: 31.
- Bliss, S.K., Zhang, Y., and Denkers, E.Y., 1999. Murine neutrophil stimulation by *Toxoplasma gondii* antigen drives high level production of IFN- γ -independent IL-12. *J. Immunol*, 163: 2081–2088.
- Bohne, W., Heesemann, J., Gross, U., 1993. Induction of bradyzoite-specific *Toxoplasma gondii* antigens in gamma interferon-treated mouse macrophages. *Infect Immun*, 61: 1141–5.
- Bohne, W., Heesemann, J., Gross, U., 1994. Reduced replication of *Toxoplasma gondii* is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. *Infect Immun*, 62: 1761–7.
- Bohne, W., Hunter, C. A., White, M. W., Ferguson, D. J., Gross, U., Roos, D. S., 1998. Targeted disruption of the bradyzoite-specific gene BAG1 does not prevent tissue cyst formation in *Toxoplasma gondii*. *Mol Biochem Parasitol*, 92: 291–301.

- Boorman, G.A., Kollias, G.V., Taylor, R.F., 1977. An outbreak of Toxoplasmosis in wallaroos (*Macropus robustus*) in a California zoo. *J wildl Dis*, 13: 64–68.
- Boyer, K., Hill, D., Mui, E., Wroblewski, K., Karrison, T., Dubey, J.P., Sautter, M., Noble, A.G., Withers, S., Swisher, C., Heydemann, P., Hosten, T., Babiarz, J., Lee, D., Meier, P., McLeod, R., 2011. Unrecognized ingestion of *Toxoplasma gondii* oocysts leads to congenital toxoplasmosis and causes epidemics in North America. *Clin Infect Dis*, 53: 1081–1089.
- Boyle, J. P., Saeij, J. P., Harada, S. Y., Ajioka, J. W., Boothroyd, J. C., 2008. Expression quantitative trait locus mapping of *Toxoplasma* genes reveals multiple mechanisms for strain-specific differences in gene expression. *Eukaryot Cell*, 7: 1403–1414
- Brown, A.S., Schaefer, C.A., Quesenberry Jr., C.P., Liu, L., Babulas, V.P., Susser, E.S., 2005. Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. *Am J Psychiatry*, 162: 767–773.
- Buxton D., Thomson KM., Maley, S., Wright, S., Bos, H.J., 1993. Experimental challenge of sheep 18 months after vaccination with a live (S48) *Toxoplasma gondii* vaccine. *Vet Res*. 133: 310–312.
- Carme, B., Bissuel, F., Ajzenberg, D., Bouyne, R., Aznar, C., Demar, M., Bichat, S., Louvel, D., Bourbigot, A.M., Peneau, C., Neron, P., DardeÅL, M.L., 2002. Severe acquired toxoplasmosis in immunocompetent adult patients in French Guiana. *J Clin Microbiol*, 40: 4037–4044.
- Dabritz, H. A., Miller, M. A., Atwill, E. R., Gardner, I. A., Leutenegger, C. M., Melli, A. C., Conrad, P. A., 2007. Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. *JAVMA*, 231: 1676 – 1684.
- Dardé, M. L., Villena, I., Pinon, J. M., Beguinot, I., 1998. Severe Toxoplasmosis Caused by a *Toxoplasma gondii* Strain with a New Isoenzyme Type Acquired in French Guyana. *J Clin Microbiol*, 36: 324.
- Demar, M., Ajzenberg, D., Maubon, D., Djossou, F., Panchoe, D., Punwasi, W., Valery, N., Peneau, C., Daigre, J.L., Aznar, C., Cottrelle, B., Terzan, L., DardeÅL, M.L., Carme, B., 2007. Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical, and parasitological aspects. *Clin Infect Dis*, 45: e88–e95.

- Denton, H., Roberts, C.W., Alexander, J., Thong, K.W., Coombs, G.H., 1996. Enzymes of energy metabolism in the bradyzoites and tachyzoites of *Toxoplasma gondii*. FEMS Microbiol Lett, 137: 103–8.
- Driscoll, C. A., Menotti-Raymond, M., Roca, A. L., Hupe, K., Johnson, W. E., Geffen, E., Harley, E. H., Delibes, M., Pontier, D., Kitchener, A. C., Yamaguchi, N., O'Brien, S. J., Macdonald, D. W., 2007. The Near Eastern Origin of Cat Domestication. Science, 317: 519–523.
- Dubey, J.P., Frenkel, J.K., 1973. Experimental *Toxoplasma* infection in mice with strains producing oocysts. J Parasitol, 59: 505–512.
- Dubey, J.P., Beattie, C.P., 1988. Toxoplasmosis of animals and man. CRC Press Inc., Boca Raton, FL, USA.
- Dubey, J.P., Kirkbride, C.A., 1989. Economic and public health considerations of congenital toxoplasmosis in lambs. J Am Vet Med Assoc, 195: 1715–1716.
- Dubey, J.P., 1992. Isolation of *Toxoplasma gondii* from a naturally infected beef cow. J Parasitol, 78: 151–153.
- Dubey, J.P., 1995. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. J Parasitol, 81: 410–415.
- Dubey, J.P., Weigel, R.M., Siegel, A.M., Thulliez, P., Kitron, U.D., Mitchell, M.A., Mannelli, A., Mateus-Pinilla, N.E., Shen, S.K., Kwok, O.C.H., Todd, K.S., 1995. Sources and reservoirs of *Toxoplasma gondii* infection on 47 swine farms in Illinois. J Parasitol. 81: 723–729.
- Dubey, J.P., Lunney, J.K., et al., 1996. Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. J Parasitol, 82: 438–443.
- Dubey, J.P., 1997a. Bradyzoite-induced murine toxoplasmosis: stage conversion, pathogenesis, and tissue cyst formation in mice fed bradyzoites of different strains of *Toxoplasma gondii*. J Eukaryot Microbiol, 44: 592–602.
- Dubey, J.P., Speer, C.A., et al., 1997b. Oocyst-induced murine toxoplasmosis: life cycle, pathogenicity, and stage conversion in mice fed *Toxoplasma gondii* oocysts. J Parasitol, 83: 870–882.
- Dubey, J.P., 1997c. Validation of the specificity of the modified agglutination test for toxoplasmosis in pigs. Vet Parasitol, 71: 307–310.

- Dubey, J.P., Shen, S.K., Kwok, O.C.H., Thulliez, P., 1997d. Toxoplasmosis in rats (*Rattus norvegicus*): congenital transmission to first- and second-generation offspring and isolation of *Toxoplasma gondii* from seronegative rats. *Parasitol*, 115: 9–14.
- Dubey, J.P., 1998. *Toxoplasma gondii* oocyst survival under defined temperatures. *J Parasitol*, 84: 862–865.
- Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* Tachyzoites, Bradyzoites, and Sporozoites and Biology and Development of Tissue Cysts. *Clin Microbiol Rev*, 11: 267–299.
- Dubey, J.P., 2001. Oocyst shedding by cats fed isolated bradyzoites and comparison of infectivity of bradyzoites of the VEG strain *Toxoplasma gondii* to cats and mice. *J Parasitol*, 87: 215–9.
- Dubey, J.P., Zarnke, R., Thomas, N.J., Wong, S.K., Van Bonn, W., Briggs, M., Davis, J.W., Ewing, R., Mensea, M., Kwok, O.C.H., Romand, S., Thulliez, P., 2003. *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis*-like infections in marine mammals. *Vet Parasitol*, 116: 275–296.
- Dubey, J.P., Navarro, I.T., Sreekumar, C., Dahl, E., Freire, R.L., Kawabata, H. H., Vianna, M.C.B., Kwok, O. C. H., Shen, S. K., Thulliez, P., Lehmann, T., 2004. *Toxoplasma gondii* infections in cats from Paraná, Brazil: seroprevalence, tissue distribution, and biologic and genetic characterization of isolates. *J Parasitol*, 90: 721–726
- Dubey, J.P., Hill, D.E., Jones, J.L., Hightower, A.W., Kirkland, E., Roberts, J.M., Marcet, P.L., Lehmann, T., Vianna, M.C.B., Miska, K., Sreekumar, C., Kwok, O.C.H., Shen, S.K., Gamble, H.R., 2005a. Prevalence of viable *Toxoplasma gondii* in beef, chicken and pork from retail meat stores in the United States: risk assessment to consumers. *J Parasitol*, 91, 1082–1093.
- Dubey, J.P., Gomez-Marin, J.E., Bedoya, A., Lora, F., Vianna, M.C.B., Hill, D., Kwok, O.C.H., Shen, S.K., Marcet, P.L., Lehmann, T., 2005b. Genetic and biologic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Colombia, South America. *Vet Parasitol*, 134: 67–72.
- Dubey, J.P., 2006a. Comparative infectivity of oocysts and bradyzoites of *Toxoplasma gondii* for intermediate (mice) and definitive (cats) hosts. *Vet Parasitol*, 140: 69–75.

- Dubey, J.P., Patitucci, A.N., Su, C., Sundar, N., Kwok, O.C.H., Shen, S.K., 2006b. Characterization of *Toxoplasma gondii* isolates in free-range chickens from Chile, South America. *Vet Parasitol*, 140: 76–82.
- Dubey, J.P., Hill, D.E., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Pierce, V., Kelly, K., Dulin, M., Thulliez, P., Iwueke, C., Su, C., 2008. Endemic toxoplasmosis in pigs on a farm in Maryland: isolation and genetic characterization of *Toxoplasma gondii*. *J Parasitol*, 94: 36–41.
- Dubey, J.P. and Jones, J.L., 2008. *Toxoplasma gondii* infection in humans and animals in the United States. *Int J Parasitol*, 38: 1257–1278.
- Dubey, J.P., 2010. Toxoplasmosis of animals and humans. 2th edition. CRC Press, Boca Raton, FL, USA.
- Dubey, J.P., Velmurugan, G.V., Rajendran, C., Yabsley, M.J., Thomas, N.J., Beckmen, K.B., Sinnett, D., Ruid, D., Hart, J., Fair, P.A., McFee, W.E., Shearn-Bochsler, V., Kwok, O.C.H., Ferreira, L.R., Choudhary, S., Faria, E.B., Zhou, H., Felix, T.A., Su, C., 2011. Genetic characterization of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. *Int J Parasitol*, 41:1139–1147.
- Eaton, M. S., Weiss, L. M., Kim. K., 2006. Cyclic nucleotide kinases and tachyzoite-bradyzoite transition in *Toxoplasma gondii*. *Int J Parasitol*, 36: 107–114.
- Epiphanio, S., Guimarães, M.A., Fedullo, D.L., Correa, S.H., Catão-Dias, J.L., 2000. Toxoplasmosis in golden-headed lion tamarins (*Leontopithecus chrysomelas*) and emperor marmosets (*Saguinus imperator*) in captivity. *J Zoo Wildl Med*, 31: 231–235.
- Etheridge, R.D., Alagunan, A., Tang, K., Lou, H.J., Turk, B.E., Sibley, L.D., 2014. The *Toxoplasma* pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. *Cell Host Microbe*, 15: 537–550.
- Fernández-Aguilar, X., Ajzenberg, D., Cabezón, O., Martínez-López, A., Darwich, L., Dubey, J.P., Almería, S., 2013. Fatal toxoplasmosis associated with an atypical *Toxoplasma gondii* strain in a Bennett's wallaby (*Macropus rufogriseus*) in Spain. *Vet Parasitol*, 196: 523–527.
- Filisetti, D. and Candolfi, E., 2004. Immune response to *Toxoplasma gondii*. *Ann 1st Super Sanita*, 40: 71-80.

- Flegr, J., Havlíček, J., Kodým, P., Maly, M., Smahel, Z., 2002. Increased risk of traffic accidents in subjects with latent toxoplasmosis: a retrospective case-control study. *BMC Infect Dis*, 2: 11.
- Frenkel, J.K., Dubey, J.P., Miller, N.L., 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. *Science*, 167: 893–896.
- Frenkel, J.K., Ruiz, A., et al., 1975. Soil survival of toxoplasma oocysts in Kansas and Costa Rica. *Am J Trop Med Hyg*, 24: 439–443.
- Frenkel, J. K., Pfefferkorn ER., Smith, D. D., Fishback, J. L., 1991. Prospective vaccine prepared from a new mutant of *Toxoplasma gondii* for use in cats. *Am J Vet Res*, 52: 759–763.
- Fux, B., Nawas, J., Khan, A., Gill, D.B., Su, C., Sibley, L.D., 2007. *Toxoplasma gondii* strains defective in oral transmission are also defective in developmental stage differentiation. *Infect Immun*, 75: 2580–2590.
- Gazzinelli, R.T., Mendonça-Neto, R., Lilue, J., Howard, J., Sher, A., 2014. Innate Resistance against *Toxoplasma gondii*: An Evolutionary Tale of Mice, Cats, and Men. *Cell host and Microbe*, 15: 132–138.
- Gerhold, R.W., Saraf, P., Chapman, A., Zou, X., Hickling, G., Stiver, W.H., Houston, A., Souza, M., Su, C., 2017. *Toxoplasma gondii* seroprevalence and genotype diversity in select wildlife species from the southeastern United States. *Parasit Vectors*, 10: 508.
- Gilbert, R.E., Freeman, K., Lago, E.G., Bahia-Oliveira, L.M., Tan, H.K., Wallon, M., Buffolano, W., Stanford, M.R., Petersen, E., European Multicentre Study on Congenital Toxoplasmosis (EMSCOT), 2008. Ocular sequelae of congenital toxoplasmosis in Brazil compared with Europe. *PLoS Negl Trop Dis*, 2: e277.
- Grigg, M.E., Ganatra, J., Boothroyd, J.C., Margolis., T.P., 2001. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *J Infect Dis*, 184: 633–639.
- Gross, U., Bohne, W., Lüder, C. G. K., Lugert, R., Seeber, F., Dittrich, C., Pohl, F., and Ferguson, D. J. P., 1996. Regulation of developmental differentiation in the protozoan parasite *Toxoplasma gondii*. *J Eukaryot Microbiol*, 43:114–116S.
- Guthrie, A., Rooker, L., Tan, R., Gerhold, R., Trainor, K., Jiang, T., Su, C., 2017. Newly described *Toxoplasma gondii* strain causes high mortality in red-necked wallabies (*Macropus rufogriseus*) in a zoo. *J Zoo Wildl Med*, 48: 694–702.

- Hartley, W.J., Marshall, S.C., 1957. Toxoplasmosis as a cause of ovine perinatal mortality. *N Z Vet J*, 5: 119–124.
- Hejlíček, K., Literák, I., 1998. Long-term study of *Toxoplasma gondii* prevalence in small mammals (Insectivora and Rodentia). *Folia Zool*, 47: 93–101
- Hessler, J.R., Woodward, J.C., Tucek, P.C., 1971. Lethal toxoplasmosis in a woolly monkey. *J Am Vet Med Assoc*, 159: 1588–1594.
- Hoff, R.L., Dubey, J.P., Behbehani, A.M., Frenkel, J.K., 1977. *Toxoplasma gondii* cysts in cell culture: new biologic evidence. *J Parasitol*, 63:1121–4.
- Holland, G.N., 2003. Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease. *Am. J. Ophthalmol.* 136: 973–988.
- Howard, J.C., Hunn, J.P., Steinfeldt, T., 2011. The IRG protein-based resistance mechanism in mice and its relation to virulence in *Toxoplasma gondii*. *Curr. Opin. Microbiol*, 14: 414–421.
- Howe, D. K. and Sibley, L. D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis*, 172: 1561–1566.
- Hunter, C, A., Sibley, L, D., 2012. Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. *Nat Rev Microbiol*, 10: 766–778.
- Jiang, T., Shwab, E.K., Martin, R.M., Gerhold, R.W., Rosenthal, B.M., Dubey, J.P., Su, C., 2018. A partition of *Toxoplasma gondii* genotypes across spatial gradients and among host species, and decreased parasite diversity towards areas of human settlement in North America. *Int J Parasitol*, 48: 611–619.
- Johnson, S.K., Fitza, M.A., Lerner, D.A., Calhoun, D.M., Beldon, M.A., Chan, E.T., Johnson P.T.J., 2018. Risky business: linking *Toxoplasma gondii* infection and entrepreneurship behaviors across individuals and countries. *Proc R Soc Lond, B, Biol Sci*, 285: 20180822.
- Jones, J.L., Kruszon-Moran, D., Sanders-Lewis, K., Wilson, M., 2007. *Toxoplasma gondii* infection in the United States, decline from the prior decade. *Am J Trop Med Hyg*, 77: 405–410.
- Jones, J. L., Kruszon-Moran, D., Won, K., Wilson, M., Schantz, P. M., 2008. *Toxoplasma gondii* and *Toxocara spp.* co-infection. *Am J Trop Med Hyg*, 78: 35–39

- Jones, J. L., Kruszon-Moran, D., et al. 2014. *Toxoplasma gondii* seroprevalence in the United States 2009-2010 and comparison with the past two decades. *Am J Trop Med Hyg*, 90: 1135–1139.
- Jones, E.M., Korcsmaros, T., Carding, S.R., 2017. Mechanisms and pathways of *Toxoplasma gondii* transepithelial migration. *Tissue Barriers*, 5: e1273865-1– e1273865-11.
- Juan-Sallés, C., Mainez, M., Marco, A., Malabia Sanchís, A. M., 2011. Localized toxoplasmosis in a ring-tailed lemur (*Lemur catta*) causing placentitis, stillbirths, and disseminated fetal infection. *J Vet Diagn Invest*, 23: 1041–1045.
- Khan, A., Dubey, J. B., Su, C., Ajioka, J. W., Rosenthal, B. M. and Sibley, L.D., 2011. Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. *Int J Parasitol*, 41: 645–655.
- Khan, A., Fux, B., Su, C., Dubey, J. P., Darde, M. L., Ajioka, J. W., Rosenthal, B. M. and Sibley, L. D., 2007. Recent transcontinental sweep of *Toxoplasma gondii* driven by a single monomorphic chromosome. *Proc Natl Acad Sci*, 104: 14872–14877.
- Kikuchi, Y., Chomel, B.B., Kasten, R.W., Martenson, J.S., Swift, P.K., O'Brien, S.J., 2004. Seroprevalence of *Toxoplasma gondii* in American free-ranging or captive pumas (*Felis concolor*) and bobcats (*Lynx rufus*). *Vet Parasitol*, 120: 1–9.
- Kortbeek, L.M., De Melker, H. E., Veldhuijzen, I.K., and Conyn-van Spaendonck, M.A.E., 2004. Population-based *Toxoplasma* seroprevalence study in The Netherlands. *Epidemiol Infect*, 132: 839–845.
- Mateus-Pinilla N. E., Dubey, J. P., Choromanski, L., Weigel, R. M., 1999. A field trial of the effectiveness of a feline *Toxoplasma gondii* vaccine in reducing *T. gondii* exposure for swine. *J Parasitol*, 85: 855–860.
- Mcdonald, R.I., Kareiva, P., Forman, R.T.T., 2008. The implications of current and future urbanization for global protected areas and biodiversity conservation. *Biol Conserv*, 141: 1695–1703.
- Lass, A., Pietkiewicz, H., Modzelewska, E., Dumètre, A., Szostakowska, B., Myjak, P., 2009. Detection of *Toxoplasma gondii* oocysts in environmental soil samples using molecular methods. *Eur J Clin Microbiol Infect Dis*, 28: 599–605.

- Lyons, R.E., McLeod, R., Roberts, C.W., 2002. *Toxoplasma gondii* tachyzoite–bradyzoite interconversion. Trends Parasitol, 18: 198–201.
- McLeod, R., Boyer, K., Karrison, T., Kasza, K., Swisher, C., Roizen, N., Jalbrzikowski, J., Remington, J., Heydemann, P., Noble, A.G., Mets, M., Holfels, E., Withers, S., Latkany, P., Meier, P. Toxoplasmosis Study Group, 2006. Outcome of treatment for congenital toxoplasmosis, 1981–2004: The National Collaborative Chicago-Based, Congenital Toxoplasmosis Study. Clin Infect Dis, 42: 1383–1394.
- Miller, M.A., Ehlers, K., Dubey, J.P., Steenbergh, K.V., 1992. Outbreak of toxoplasmosis in wallabies on an exotic animal farm. J Vet Diagn Invest, 4: 480–483.
- Munday, B.L., 1972. Serological evidence of *Toxoplasma* infection in isolated groups of sheep. Res Vet Sci, 13: 100–102.
- Munoz-Zanzi, C.A., Fry, P., et al. 2010. *Toxoplasma gondii* oocyst-specific antibodies and source of infection. Emerging Infect Dis, 16: 1591–1593.
- Murray, H. W., Teitelbaum, R. F., 1992. L-arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. J Infect Dis, 165: 513–517.
- Oh, H., Eo, K., Gumber, S., Hong, J. J., Kim, C., Lee, H., Jung, Y., Kim, J., Whang, G., Lee, J., Yeo, Y., Ryu, B., Ryu, J., Lee, S., Kim, U., Kang, S., Park, J., 2018. An outbreak of toxoplasmosis in squirrel monkeys (*Saimiri sciureus*) in South Korea. J Med Primatol, 47: 238–246.
- Popiel, I., Gold, M.C., Booth, K.S., 1996. Quantification of *Toxoplasma gondii* bradyzoites. J Parasitol, 82: 330–2.
- Portas, T., 2010. Toxoplasmosis in macropodids: a review. J. Zoo Wildl. Med, 41: 1–6.
- Pongsiri, M. J., Roman, J., Ezenwa, V. O., Goldberg, T. L., Koren, H. S., Newbold, S. C., Ostfeld, R. S., Pattanayak, S. K., Salkeld, D. J., 2009. Biodiversity Loss Affects Global Disease Ecology. BioScience, 59: 945–954,
- Reese, M.L., Zeiner, G.M., Saeij, J.P., Boothroyd, J.C., Boyle, J.P., 2011. Polymorphic family of injected pseudokinases is paramount in *Toxoplasma* virulence. Proc Natl Acad Sci USA, 108: 9625–9630.

- Remington, J.S., McLeod, R., Thulliez, P., Desmonts, G., 2006. Toxoplasmosis. In: Remington, J.S., Klein, J.O., Wilson, C.B., Baker, C.J. (Eds.), *Infectious Diseases of the Fetus and Newborn Infant*. Elsevier Saunders, Philadelphia, USA. pp. 947–1091.
- Rosenthal, B.M., 2009. How has agriculture influenced the geography and genetics of animal parasites? *Trends Parasitol*, 25: 67–70.
- Saeij, J.P., Boyle, J.P., Collier, S., Taylor, S., Sibley, L.D., Brooke-Powell, E.T., Ajioka, J.W., Boothroyd, J.C., 2006. Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science*, 314: 1780–1783.
- Selleck, E. M. et al., 2013. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against *Toxoplasma gondii*. *PLoS Pathog*, 9: 25.
- Shiels, B., Aslam, N., McKellar, S., Smyth, A., Kinnaird, J., 1997. Modulation of protein synthesis relative to DNA synthesis alters the timing of differentiation in the protozoan parasite *Theileria annulata*. *J Cell Sci*, 110: 1441–51.
- Shwab, E. K, Zhu, X., Majumdar, D., Pena, H., Gennari, S., Dubey, J., and Su, C., 2014. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. *Parasitology*, 141: 453–461.
- Shwab, E.K., Jiang, T., Pena, H.F., Gennari, S.M., Dubey, J.P., Su, C., 2016. The ROP18 and ROP5 gene allele types are highly predictive of virulence in mice across globally distributed strains of *Toxoplasma gondii*, *Int J Parasitol*, 46:141–146.
- Shwab, E. K., Saraf, P., Zhu, X., Zhou, D., McFerrin, B. M., Ajzenberg, D., Schares, G., Hammond-Aryee, K., van Helden, P., Higgins, S. A., Gerhold, R.W., Rosenthal, B. M., Zhao, X., Dubey, J. P., Su, C., 2018. Human impact on the diversity and virulence of the ubiquitous zoonotic parasite *Toxoplasma gondii*. *Proc Natl Acad Sci*, 115: E6956–E6963.
- Skariah, S., McIntyre, M., Mordue, D., 2010. *Toxoplasma gondii*: determinants of tachyzoite to bradyzoite conversion. *Parasitol Res*, 107: 253–260.
- Soete, M., Camus, D., Dubremetz, J.F., 1994. Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of *Toxoplasma gondii* in vitro. *Exp Parasitol*, 78: 361–70.

- Stagno, S., Dykes, A. C., Amos, C. S., Head, R. A., Juranek, D. D., Walls, K., 1980. An outbreak of toxoplasmosis linked to cats. *Pediatrics*, 65: 706–712.
- Su, C., Zhang, X. and Dubey, J. P., 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int J Parasitol*, 36: 841–848.
- Su, C., Shwab, E. K., Zhou, P., Zhu, X. Q. and Dubey, J. P., 2010. Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasitology*, 137: 1–11.
- Sugden, K., Moffitt, T. E., Pinto, L., Poulton, R., Williams, B. S., Caspi, A., 2016. Is *Toxoplasma gondii* Infection Related to Brain and Behavior Impairments in Humans? Evidence from a Population-Representative Birth Cohort. *PLoS ONE*, 11: e0148435.
- Sundar, N., Cole, R.A., Thomas, N.J., Majumdar, D., Dubey, J.P., Su, C., 2008. Genetic diversity among sea otter isolates of *Toxoplasma gondii*. *Vet Parasitol*, 151: 125–132.
- Sullivan, W. J., Narasimhan, J. J., Bhatti, M. M., Wek, R. C., 2004. Parasite-specific eIF2 (eukaryotic initiation factor-2) kinase required for stress-induced translation control. *Biochem J*, 380: 523–531.
- Taiz, Lincoln., 2013. Agriculture, plant physiology, and human population growth: past, present, and future. *Theor Exp Plant Phys*, 25: 167–181.
- Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S.J., Tang, K., Beatty, W.L., Hajj, H.E., Jerome, M., Behnke, M.S., White, M., Wootton, J.C., Sibley, L.D., 2006. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science*, 314: 1776–1780.
- Tomavo, S., Boothroyd, J.C., 1995. Interconnection between organellar functions, development and drug resistance in the protozoan parasite, *Toxoplasma gondii*. *Int J Parasitol*, 25: 1293–9.
- Thomas, N.J., Dubey, J.P., Lindsay, D.S., Cole, R.A., Meteyar, C.U., 2007. Protozoal meningoencephalitis in sea otters (*Enhydra lutris*): a histopathological and immunohistochemical study of naturally occurring cases. *J Comp Pathol*, 137: 102–121.
- Turner, D.C., Bateson, P.B., 2000. The domestic cat: the biology of its behavior. Cambridge University Press, 2nd edition. Cambridge, UK.

- Uga, S., Kataoka, N., 1995. Measures to control *Toxocara* egg contamination in sandpits of public parks. *Am J Trop Med Hyg*, 52: 21-24.
- Uga, S., Minami, T., Nagata K., 1996. Defecation habits of cats and dogs and contamination by *Toxocara* eggs in public park sandpits. *Am J Trop Med Hyg*, 54: 122-126.
- VanWormer, E., Miller, M.A., Conrad, P.A., Grigg, M.E., Rejmanek, D., Carpenter, T.E., Mazet, J.A.K., 2014. Using molecular epidemiology to track *Toxoplasma gondii* from terrestrial carnivores to marine hosts: implications for public health and conservation. *PLoS Negl Trop Dis*, 8: e2852.
- Verma, S.K., Sweeny, A.R., Lovallo, M.J., Calero-Bernal, R., Kwok, O.C., Jiang, T., Su, C., Grigg, M.E., Dubey, J.P., 2017. Seroprevalence, isolation and co-infection of multiple *Toxoplasma gondii* strains in individual bobcats (*Lynx rufus*) from Mississippi, USA. *Int J Parasitol*. 47, 297–303.
- Vieira, F.E.G., Sasse, J.P., Minutti, A.F., Miura, A. C., Barros, L.D., Cardim, S.T., Martins, T.A., Seixas, M., Yamamura, M.I., Su, C., Garcia, J.L., 2018. *Toxoplasma gondii*: prevalence and characterization of new genotypes in free-range chickens from south Brazil. *Parasitol Res*, 117: 681–688.
- Vollaire, M.R., Radecki, S.V., Lappin, M.R., 2005. Seroprevalence of *Toxoplasma gondii* antibodies in clinically ill cats in the United States. *Am. J. Vet. Res.* 66, 874–877.
- Wallace, G.D. 1969. Serologic and epidemiologic observations on toxoplasmosis on three Pacific Atolls. *Am J Epidemiol*, 90: 103–111.
- Weigel, R.M., Dubey, J.P., Siegel, A.M., Kitron, U.D., Mannelli, A., Mitchell, M.A., Mateus-Pinilla, N.E., Thulliez, P., Shen, S.K., Kwok, O.C.H., Todd, K.S., 1995b. Risk factors for transmission of *Toxoplasma gondii* on swine farms in Illinois. *J Parasitol*. 81: 736–741.
- Weiss, L.M., Ma, Y.F., Takvorian, P.M., Tanowitz, H.B., Wittner, M., 1998. Bradyzoite development in *Toxoplasma gondii* and the hsp70 stress response. *Infect Immun*, 66: 3295–302.
- Weigel, R.M., Dubey, J.P., Dyer, D., Siegel, A.M., 1999. Risk factors for infection with *Toxoplasma gondii* for residents and workers on swine farms in Illinois, USA. *Am J Trop Med Hyg*, 60, 793–798.

- Weiss, L.M. and Kim, K., 2011. The development and biology of bradyzoites of *Toxoplasma gondii*. *Front Biosci*, 5: D391–D405.
- Weiss, L.M., Kim, K., 2013. *Toxoplasma gondii*: The model apicomplexan -perspectives and Methods. 2nd edition. Amsterdam, Boston, Heidelberg, London.
- Wolf, A., Cowen, D., Paige, B., 1939. Human toxoplasmosis: occurrence in infants as an encephalomyelitis verification by transmission to animals. *Science*, 89: 226–227.
- Yarovinsky, F., 2014. Innate immunity to *Toxoplasma gondii* infection. *Nat Rev Immunol*, 14: 109–121.

Chapter 1 Appendix

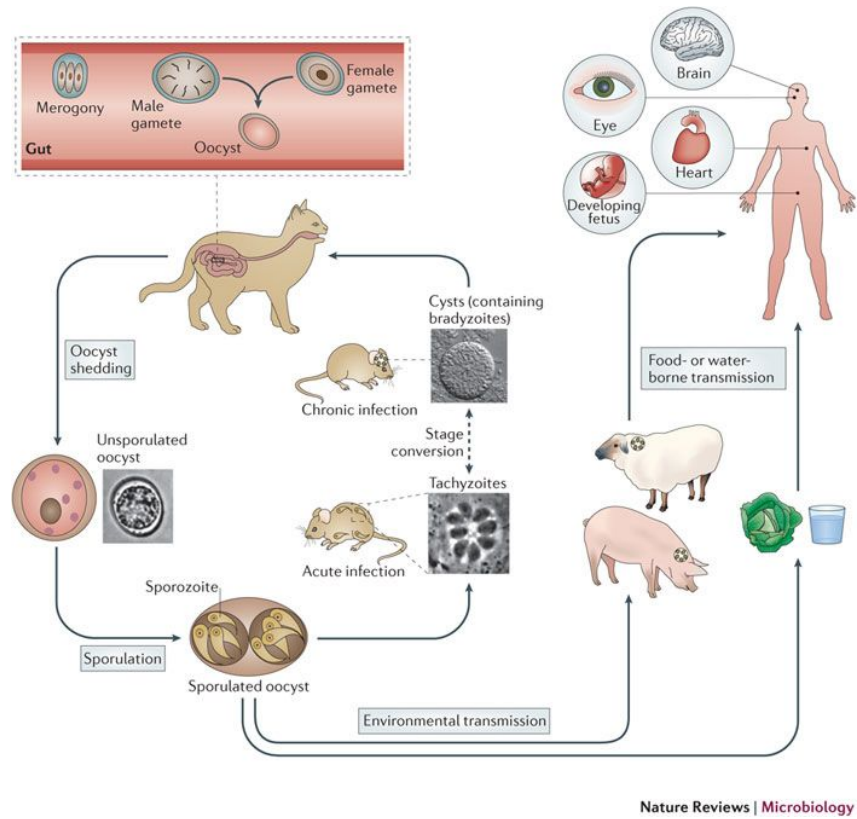


Figure 1. 1 The life cycle of *T. gondii* (Hunter et al., 2012).

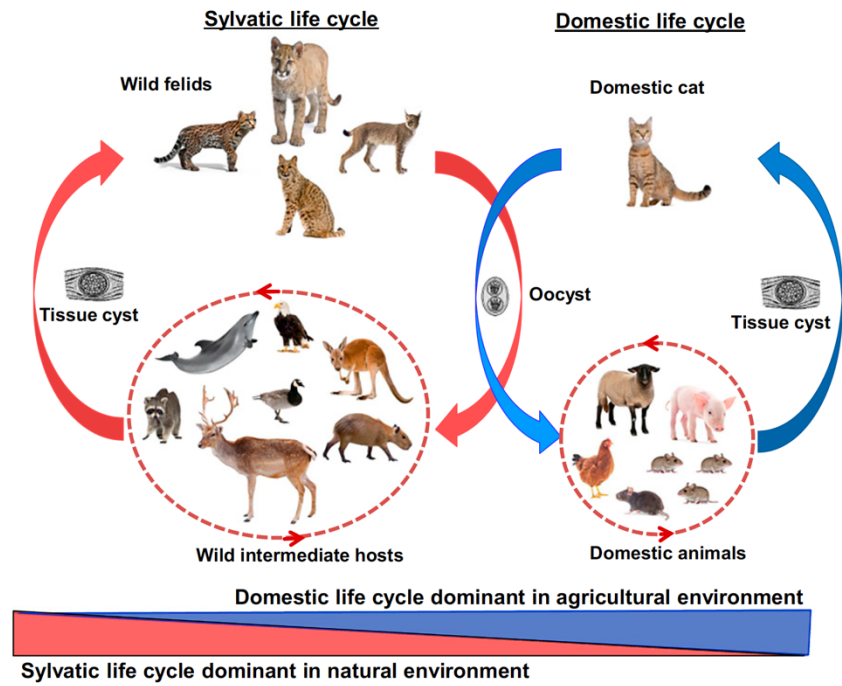


Figure 1. 2 The domestic and sylvatic life cycles of *T. gondii* (Shwab et al., 2018).

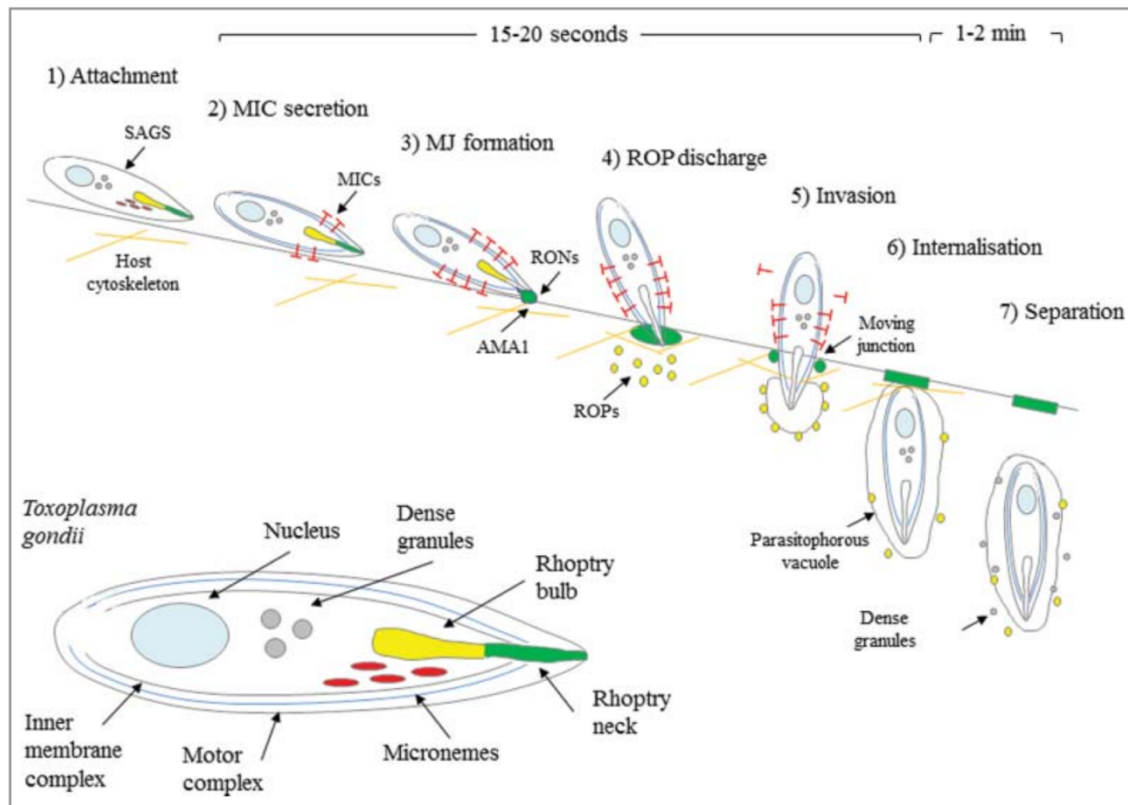


Figure 1. 3 *T. gondii* host cell invasion process (Jones et al., 2017).

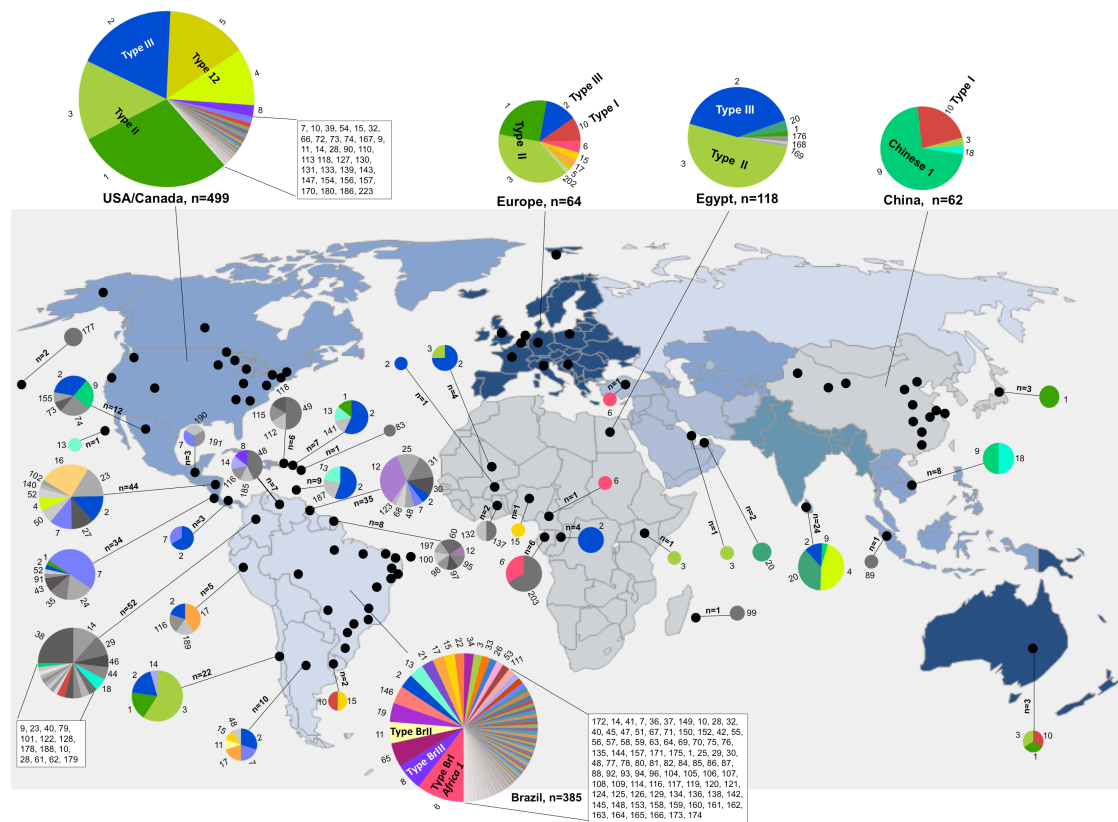


Figure 1. 4 The population structure of *Toxoplasma gondii* (Shwab et al., 2014).

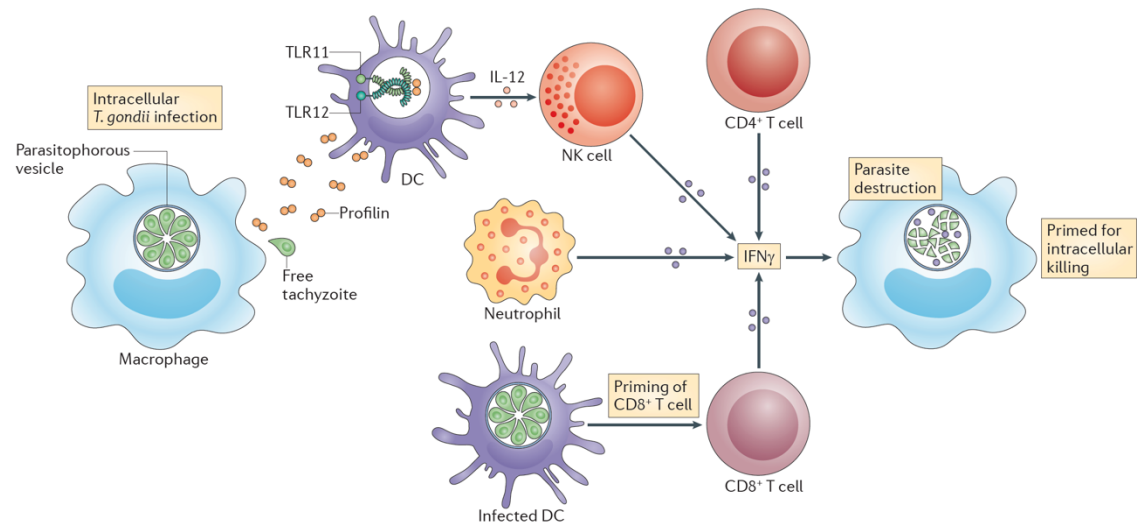


Figure 1. 5 The Th1 mediated immune response (Yarovinsky et al., 2014).

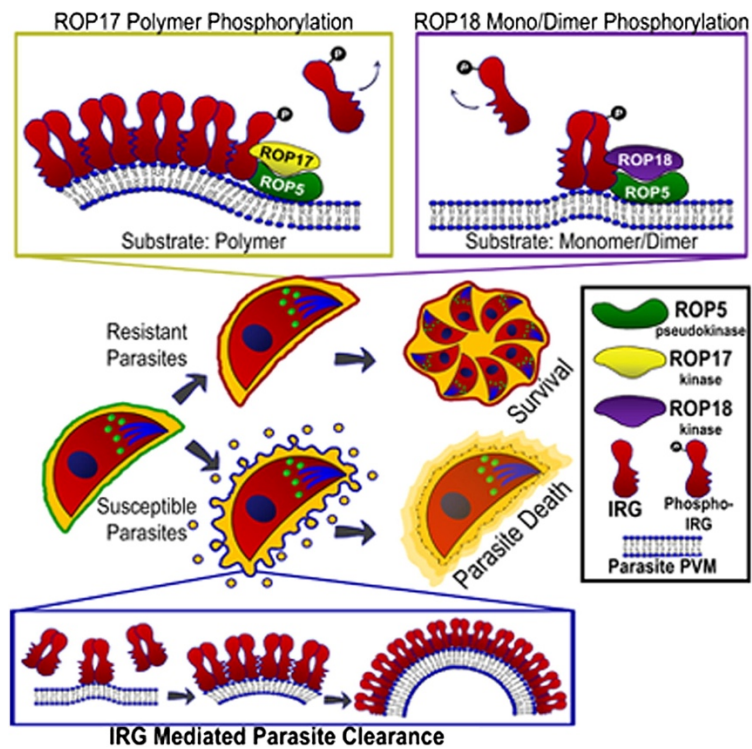


Figure 1. 6 The interaction between IRGs and ROPs (Etheridge et al., 2014).

CHAPTER 2

**A PARTITION OF *TOXOPLASMA GONDII* GENOTYPES ACROSS
SPATIAL GRADIENTS AND AMONG HOST SPECIES, AND
DECREASED PARASITE DIVERSITY TOWARDS AREAS OF HUMAN
SETTLEMENT IN NORTH AMERICA**

Publication Note

This chapter is a peer-reviewed publication in *International Journal for Parasitology* in 2018, authored by Tiantian Jiang, E. Keats Shwab, Robbie M. Martin, Richard W. Gerhold, Benjamin M. Rosenthal, Jitender P. Dubey and Chunlei Su.

My contribution to this work is compiling data, analyzing data, and writing the manuscript. The rest of co-authors helped with the revision of the manuscript. Dr. Chunlei Su is the corresponding author.

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Abstract

Toxoplasma gondii counts among the most consequential food-borne parasites, and although the parasite occurs in a wide range of wild and domesticated animals, farms may constitute a specific and important locus of transmission. If so, parasites in animals that inhabit agricultural habitats might be suspected of harboring genetically distinct parasite types. To better understand habitat effects pertinent to this parasite's transmission, we compiled and analyzed existing genotypic data of 623 samples from animals across a proximity gradient from areas of human settlement to the wilderness in North America. To facilitate such analysis, *T. gondii* isolates were divided into three groups: (i) from farm-bound animals (with the most limited home ranges on farms); (ii) from free-roaming animals (with wider home ranges on or near farms); and (iii) from wildlife. In addition, parasite genotype distribution in different animal species was analyzed. We observed no absolute limitation of any of five major genotypes to any one habitat; however, the frequency of four genotypes decreased across the gradient from the farm-bound group, to the free-roaming group, then the wildlife, whereas a fifth genotype increased along that gradient. Genetic diversity was greater in free-roaming than in farm-bound animals. The genotypic composition of parasites in wildlife differed from those in farm-bound and free-roaming animals. Furthermore, parasite genotypes differed among host species. We conclude that *T. gondii* genotype distributions are influenced by the spatial habitat and host species composition, and parasite diversity decreases towards areas of human settlement, elucidating facts which may influence transmission dynamics and zoonotic potential in this ubiquitous but regionally variable parasite.

Introduction

The ubiquity of certain pathogens can reveal important differences in their local transmission dynamics. Habitat effects may be important in structuring the dynamics of transmission, and these differences may be epidemiologically consequential. Global analyses, and certain regional studies, have suggested that farming has disseminated a peculiar and especially consequential transmission dynamic, overlapping with (but qualitatively distinct from) conditions that prevail among wildlife hosts (Crosby, 2004; Rosenthal, 2009; Cincotta and Gorenflo, 2011). *Toxoplasma gondii*, the agent of human toxoplasmosis, has an extraordinarily broad intermediate host range, and is found in every habitat inhabited by its definitive feline hosts; but differences among habitats (and the composition of potential hosts) may significantly alter which parasites predominate (Mercier et al., 2011). Here, we sought to apply decades of molecular characterization in order to discern the extent and quality of differences among regions and hosts of this widespread parasite.

Toxoplasma gondii is a successful apicomplexan parasite that infects a broad spectrum of hosts including mammals and birds. It is estimated that, on average, one-third of the global human population is infected (Dubey, 2010; Weiss and Kim, 2013). In 12–49-year-old US born individuals, 6.7% have been exposed to *T. gondii* (Jones et al., 2014). Infection can be life-threatening in immunocompromised individuals such as HIV patients and those undergoing chemotherapy or radiation. Similarly, in pregnant women who have not been previously exposed to the parasite, infection during gestation can lead to congenital toxoplasmosis, which occurs in approximately 30–40% of babies born of infected mothers (Dubey, 2010). Manifestations of congenital toxoplasmosis vary from spontaneous abortion and stillbirth to ocular and cerebral damage to the infants. Infection in immunocompetent people generally causes mild symptoms such as fatigue and malaise, followed by chronic infection in which the parasite resides in host tissues and becomes semi-dormant. However, in rare cases, severe diseases may occur which require hospitalization or intensive health care (Demar et al., 2007; Elbez-Rubinstein et al., 2009). The cause of these severe cases is unclear, but they are most likely due to infection by highly virulent strains identified in South America (Ajzenberg et al., 2004; Demar et al., 2007; Elbez-Rubinstein et al., 2009; Grigg and Sundar, 2009; de-la-Torre et al., 2013).

Beside humans, a wide variety of animals are infected by *T. gondii*, ranging from terrestrial and marine mammals to birds. As its sole definitive host, felids (domestic and wild cats) can shed millions of oocysts in their faeces and thereby contaminate the environment. Intermediate hosts, including humans and animals, become infected via accidentally ingesting oocysts disseminated in environmental matrices such as soil, water, fruit and vegetables. Upon infection, parasites reside in the form of tissue cysts in the muscular or neural tissues of the intermediate hosts. *Toxoplasma gondii* can be transmitted horizontally or vertically. In the case of horizontal transmission, bradyzoites in tissues or oocysts in the environment are ingested by intermediate hosts or definitive hosts. Vertically, parasites in pregnant hosts can pass through the placenta and can thus be transmitted to the foetus (Dubey, 2010).

Toxoplasma gondii is genetically diverse among animal populations globally (Shwab et al., 2014). Genetic diversity of this parasite is low in Europe and high in South America, with the diversity in North America being intermediate. There are a few lineages of *T. gondii* dominant in North America (Dubey et al., 2011b; Khan et al., 2011; Schwab et al., 2014). ToxoDB genotypes #4 and #5 (collectively known as type 12) are the dominant genotypes in wildlife in North America (Dubey et al., 2011b), while genotypes #1 and #3 (collectively known as type II) are often identified in domestic animals (Dubey et al., 2008b; Velmurugan et al., 2009). However, a comparison of genetic profiles of *T. gondii* in animal populations with differing degrees of human association has not, to our knowledge, been conducted. Here we have compiled and analyzed genetic data of *T. gondii* isolated from domestic and wild animals in North America. We have categorized *T. gondii* populations into three groups based on spatial distribution of animal hosts, and by animal species. We show a stratified distribution of genotypes along the spatial gradient from areas of human settlement to the wild. In addition, there is an association of *T. gondii* genotypes with animal host species, and parasite diversity decreases towards an area of human settlement. This is of importance in understanding the transmission of *T. gondii* in animals and humans.

Materials and Methods

Data collection

Genotyping data of 623 *T. gondii* samples from animals in the USA and Canada were collected from published reports (Table 2.1). All samples were originally characterized using 10 multilocus PCR-RFLP markers (Su et al., 2010; Shwab et al., 2014). The 623 *T. gondii* samples were classified as having derived from farm-bound animals (n = 195), free-roaming animals (n = 135), or wildlife (n = 293).

The farm-bound group included pigs (*Sus domesticus*), chickens (*Gallus gallus domesticus*), geese (*Anser anser domesticus*), house mice (*Mus musculus*) and domestic cats (*Felis catus*). These samples were collected from six US states including Illinois, Maryland, Iowa, Pennsylvania, Massachusetts and New Jersey (Table 2.1). Among the 195 samples, 168 pig isolates were collected from more than eight farms across six states: 85 were from Iowa (Dubey et al., 1995a; Mondragon et al., 1998; Velmurugan et al., 2009), 29 from Pennsylvania (Velmurugan et al., 2009), 53 from Massachusetts (Dubey et al., 2002, 2005; Velmurugan et al., 2009), and one from New Jersey (Dubey et al., 2005; Velmurugan et al., 2009). The remaining 27 samples were obtained from domestic animals including 11 chickens (Dubey et al., 2007b), nine domestic cats (Dubey et al., 1995b; Dubey and Prowell, 2012), six house mice (Dubey et al., 1995b) and one goose (Dubey et al., 2007b). These animals were either raised on pig farms or raised as pets (Dubey et al., 1995b, 2007b). For data analysis, the farm-bound animal group captures all animals that have limited home ranges on farms; they are not necessarily physically penned.

The free-roaming animal group included goats (*Capra aegagrus hircus*), sheep (*Ovis aries*), alpacas (*Vicugna pacos*), feral cats (*Felis catus*), organic pigs (*Sus domesticus*) and free-range chickens (Table 2.1). Given feral cats have larger home ranges and are likely to be away from farms, we assigned them to this group for data analysis. From the 135 samples, two were collected from Canada and the rest were collected from nine states in USA including Maryland, Virginia, West Virginia, Pennsylvania, Ohio, Michigan, Massachusetts and Texas. Fifty-five of these samples originated from sheep in a slaughterhouse in Baltimore, Maryland. These sheep were between 6 and 12 months old and were raised in small flocks in Maryland, Virginia and West Virginia (Dubey et al., 2008c). Three sheep isolates were obtained from Virginia (Dubey et al.,

2014a). One *T. gondii* strain was isolated from a congenitally infected lamb during a lambing season in Texas, USA (Edwards and Dubey, 2013). Twenty-nine *T. gondii* strains were collected from goat meat sold in retail stores in Maryland. These goats were raised in small flocks in Maryland, Virginia or Pennsylvania (Dubey et al., 2011a). Additionally, 22 chicken isolates were from free-range chickens from Ohio and Massachusetts (Ying et al., 2017). Nineteen isolates were obtained from organic pigs which were free roaming on the farms. Among these isolates, 15 were from Michigan from two organic pig farms where pigs were raised according to organic standards (Dubey et al., 2012); four were from Maryland, USA from a poorly managed farm in which pigs with poor nutritional diets scavenged on the premises (Dubey et al., 2008a). Four *T. gondii* isolates were obtained from feral cats with two from Canada (Dubey et al., 2008b), and two from Pennsylvania (Dubey et al., 2015). Additionally, two *T. gondii* isolates were collected from alpacas grazing on a pasture in Virginia (Dubey et al., 2014a).

Of the 293 *T. gondii* isolates from wildlife, 288 were collected from 23 states in the USA and five samples from Canada (Table 2.1). The host animals from which parasites were successfully isolated and genotyped included 54 white-tailed deer (*Odocoileus virginianus*), 46 sea otters (*Enhydra lutris*), 30 grey wolves (*Canis lupus*), 27 bobcats (*Lynx rufus*), 21 coyotes (*Canis latrans*), 13 black bears (*Ursus americanus*), 13 red foxes (*Vulpes vulpes*), 12 raccoons (*Procyon lotor*), 11 Canadian geese (*Branta canadensis*), and 24 additional wildlife species from which 66 strains were obtained (Table 2.1).

Fourteen host species contained the requisite 10 or more *T. gondii* samples for inclusion in the species analysis. These include pigs, chickens, cats, Canada geese, sheep, goats, red foxes, black bears, raccoons, white-tailed deer, coyotes, bobcats, sea otters and wolves (Table 2.2). Eleven chicken isolates from a farm in Illinois were counted as one isolate due to an outbreak of toxoplasmosis among these chickens, and all 11 isolates belonged to the same genotype (#1) (Dubey et al., 2007b). This sample together with the other 22 isolates from free-range chickens in Ohio and Massachusetts were used as a population (Ying et al., 2017). The pig population comprised 168 isolates from the farm-bound animals and 19 organic pig isolates from the free-roaming group. The cat population comprised nine isolates from farm-bound group and four feral cat isolates from the free-roaming group. The other animal populations are described in Table 2.2.

Data analysis

The frequency of genotypes in each population was represented in pie charts constructed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Multilocus PCR-RFLP typing data was coded for all genetic loci. For a given locus, the presence or absence of DNA restriction fragments was coded with 1 s and 0 s, respectively. Therefore, a given locus was coded with a string of 1 s and 0 s. The genetic differences between and within populations was compared using Arlequin ver 3.5.1.2 (Excoffier and Lischer, 2010; Rajendran et al., 2012). For analysis with Arlequin, the data type was set as “haplotypic” data, and the data form was set as “Standard” which compares the data for their content at each locus without taking into special consideration the mode of mutation of the alleles. Within-population genetic diversity was determined at the haplotype level for each population. A between-population pairwise comparison was performed by genetic structure analysis, with the statistical significance level established by 1000 permutations, and $P = 0.001$ was used as the statistical significance level.

Results

The spatial distribution of *T. gondii* genotypes

The spatial distribution of *T. gondii* genotypes is summarized in Fig. 2.1. For the farm-bound animal group, seven genotypes were identified among the 195 *T. gondii* samples. ToxoDB genotypes #1 and #3 (collectively known as type II) and #2 (type III) were the major genotypes accounting for 56.4% (110/195) and 25.6% (50/195) of the total, respectively. Genotypes #4 and #5 (collectively known as type 12) accounted for 12.3% (24/195) and 0.5% (1/195), respectively. In addition, genotype #8 accounted for 4.6% (9/195), and #42 for 0.5% (1/195).

For the free-roaming animal group, 25 genotypes were identified from 135 *T. gondii* samples. Genotypes #1, #2, #3, #4 and #5 accounted for 32.6% (44/135), 20.7% (28/135), 11.1% (15/135), 8.9% (12/135) and 3.0% (4/135) of the samples, respectively. The remainders of the isolates were represented by 20 atypical genotypes accounting for 23.7% (32/135) of the total (Fig. 2.1).

Within the wildlife group of 293 *T. gondii* samples, a total of 38 genotypes were identified (Fig. 2.1, Table 2.1). Genotypes #1, #2, #3, #4 and #5 accounted for 11.6% (34/293), 9.6%

(28/293), 8.9% (26/293), 5.1% (15/293) and 43.7% (128/293) of the samples, respectively. The remaining isolates were represented by 33 genotypes, each of which accounted for a small proportion of the total. Collectively, these accounted for 21.1% (62/293) of samples in the group (Fig. 2.1).

Within-population diversity and pairwise comparison of genetic makeup of the three spatial groups was performed using Arlequin v. 3.5.1.2. Within-population diversities for farm-bound animals, free-roaming animals and wildlife were relatively high in each case (0.75 ± 0.01 , 0.83 ± 0.02 , and 0.78 ± 0.02 , respectively) (Table 2.1). A pairwise comparison of genetic makeup showed that *T. gondii* in the wildlife population was significantly different from that in the farm-bound and free-roaming animal populations ($P < 0.001$) (Table 2.1). There was no significant difference between farm-bound and free-roaming animal populations. In addition, there were 1, 11 and 23 unique genotypes for farm-bound, free-roaming and wildlife populations, respectively.

***Toxoplasma gondii* genotype distribution in animal species**

The genotypic composition of each animal population with 10 or more isolates is shown in Table 2.2. Within-population genetic diversity of samples in bobcats (0.21 ± 0.10), wolves (0.31 ± 0.11), sea otters (0.65 ± 0.05) and white-tailed deer (0.70 ± 0.07) encompassed notably low diversity. However, between-population comparisons found these animal populations to be significantly different from at least five other animal populations (Fig. 2.2). In particular, parasites from sea otters significantly differed from those infecting 11 other species. Domestic animals (pigs, cats, chickens, sheep, goats) had similar genotypic compositions, whereas wild animals tended to have diverse populations. Genotype #5 dominated in a few wildlife species such as white-tailed deer, coyotes, bobcats, sea otters and wolves (Fig. 2.2).

Discussion

We have compared the composition of *T. gondii* genotypes in animals with varying proximities to areas of human settlement. There are several important findings. First, we found genotypes #1–#5 in all three habitats. Genotypes #1 and #2 dominated farm-bound and free-roaming animal groups, while genotype #5 dominated wildlife (Fig. 2.1, Table 2.1). The frequencies of #1, #2, #3 and #4 decreased along the gradient from the farm-bound group to the wild. In contrast, the frequency of

#5 increases along the same gradient. These results indicate a spatial partitioning of *T. gondii* genotypes across the habitat. Second, the within-population diversity in the free-roaming animals was similar to that in wildlife, but significantly higher than that of the farm-bound animals (Fig. 2.1, Table 2.1), suggesting a lower genetic diversity of *T. gondii* in areas of human settlement than in the wild. Third, comparison of between-population genetic compositions showed that the wildlife group is significantly different from the farm-bound and the free-roaming groups (Fig. 2.1, Table 2.1), and there were increasing numbers of unique genotypes along the gradient from farm-bound, to free-roaming, then to wildlife, suggesting different *T. gondii* genotypes are circulating in different spatial habitats. Finally, genotypes of *T. gondii* were associated with animal species (Fig. 2.2, Table 2.2). Farm animals (pigs, chickens, cats, sheep and goats) tended to harbor similar *T. gondii* genotypes, with genotypes #1, #2, and #3 being the major ones. However, parasite genotypes were more diverse in wildlife, with a clear dominance of genotype #5 in a few wildlife species including white-tailed deer, coyotes, sea otters and wolves.

The low genetic diversity of *T. gondii* in areas of human settlement may be the result of a reduced biological diversity of animal hosts. Intensive animal rearing results in a reduction of local animal diversity and a dramatic increase in animal numbers, which in turn leads to a reduced diversity of animal pathogens and influences their transmission dynamic (Crosby, 2004; Mcdonalda et al. (2008); Pongsiri et al., 2009; Rosenthal 2009; Cincotta and Gorenflo, 2011). Under this uniform selective pressure, a small number of parasite strains with increased fitness would be expected to circulate in the population and to become dominant (Khan et al., 2014). In our analysis, 56.4% of the genotypes identified from animal species with close human association belong to the genotypes #1 and #3 (collectively known as type II), which suggests a selective advantage for these genotypes within the domestic animal population. Given that these genotypes also predominate in Europe, they may have established themselves in North America through maritime animal transportation, especially in well-adapted hosts such as domestic cats and rodents (Lehmann et al., 2006). The flourishing of agriculture and intensive animal farming may then have facilitated their spread in the domestic population. Notably, these two genotypes were also found in free-roaming animal populations and wild animals, accounting for 45.1% and 24.6% of genotypes, respectively. This suggests that genotypes #1 and #3 may be overflowing from

domestic animals to wild animals. Alternatively, these genotypes may enjoy especially efficient transmission on farms.

In contrast to the agricultural environment, natural ecosystems such as forests support a much greater biodiversity, including a high diversity of both hosts and pathogens. Consistent with this trend, we showed that *T. gondii* genetic diversity is higher in wildlife species. Thirty-eight genotypes were observed wild animals (more than five times the number observed in a sample of nearly 10 times as many domesticated hosts). However, these 38 genotypes identified from wild animals were not equally represented (Fig. 2.2). Genotype #5 dominated in a few wild animal species, whereas #1, #2 and #3 dominated in domestic animals. It is particularly interesting that bobcats have a limited diversity of *T. gondii* genotypes; #5 is overly dominant in this definitive host. This result is in agreement with a previous study in which wild felids were 14 times more likely to be infected with #5 (Type X) than feral domestic cats, and animals in less developed landscapes were more likely be infected with #5 than developed landscape (VanWormer et al., 2014). Taken together, it suggests a potential fitness advantage of particular genotypes in domestic and wild animals, respectively. This phenomenon also lends support to the idea of distinct, geographically separated domestic and sylvatic transmission cycles. Notably, the sea otter population is extraordinarily unique (Fig. 2.2) as its genotypes comprised of approximately equal numbers of #5 (n = 24), and #1 and #3 (type II, n = 18). This suggests that the genotypic composition of sea otters may have two overlapping transmission cycles (VanWormer et al., 2014).

We grouped domestic animals with wider home ranges into an independent group (free-roaming). These animals included goats, sheep and feral cats. From this group, 135 isolates were collected, among which 25 different genotypes were identified. Parasites isolated from these animals showed a higher diversity compared with those from the farm-bound group (seven genotypes). Genotypes #1, #2, #3 and #4 were found to be the major genotypes in this group of animals, which is most likely the result of the overlapping phenomenon of the domestic and sylvatic transmission cycles. Moreover, the other genotypes found in this group are mostly different from those identified in wild animals, which suggests genotypic specificity in different animal hosts, or spatial separation of *T. gondii* genotypes. Due to the limited available data in this

group, the genetic profile of this population may be incomplete. More isolates of this group from a wider geographic region will be needed to draw firm conclusions.

In addition to the geographical location from which parasites were isolated, our findings suggest that host animal species may shape the genetic structure of *T. gondii* populations in North America. Strong genetic differences characterized populations derived from various host species, perhaps owing to some degree of species-specific host adaptation. However, regional sampling biases may contribute to the appearance of such differences. Thus, the degree to which each of these two factors contributes to the genetic structure of *T. gondii* in North America remains unclear. To more precisely define these contributions, future studies should examine the genetic structure of sympatric parasites isolated from different host species, and others should examine a given host species across its geographic range.

Host-specificity of *T. gondii* genotypes may have ramifications for human infection. The eighty-four sheep and goat samples intended for human consumption, obtained from retail meat stores or slaughterhouses, yielded twenty-four genotypes of *T. gondii*. This high level of diversity, coupled with the established potential for parasite transmission to humans from these hosts, raises concerns about the potential for the transmission, from ovine samples, of atypical parasite lineages that may include especially virulent types. Future studies focusing on surveillance of parasite infections in goats and sheep would not only help identify *T. gondii* strains present in the environment but would also shed light on parasite-to-human transmission routes.

Taken together, our findings demonstrate an inverse relationship between parasite diversity and the proximity of host habitat to areas of human settlement, indicating the dramatic impact of human modification of habitat on the distribution of *T. gondii* strains in the environment. The prevalence of genotype #5 strains in wild hosts suggests that they may have originated in wild animals and have successfully circulated in the wilderness. However, difficulties in isolating parasites from asymptomatic, chronically infected animals cloud our understanding of the true prevalence and genotypic composition in many host species. For example, pigs contribute disproportionately to our current description of genetic diversity in domestic animals. In contrast, isolates from domestic cats, the definitive host that is the core of the domestic cycle, are scarce in our study. A previous study showed that, due to its large population size, domestic feral cats contribute more oocysts to contaminate areas of human settlement than wild felids, and therefore

likely played a more important role in transmission of *T. gondii* in domestic animals and humans (VanWormer et al., 2013). Further study focusing on *T. gondii* strains from domestic cats and a broader host spectrum from more geographically diverse regions is imperative. These data underscore the impact of host proximity to human society on parasite population structure. A greater understanding of this relationship may be achieved through increased sampling from a greater diversity of host species and geographic areas and should provide invaluable insights into the dynamics of the transmission of *T. gondii* and potentially other important zoonotic human pathogens.

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References

- Ajzenberg, D., Bañulsbm, A.L., Su, C., Dumètre, A., Demard, M., Carmed, B., Dardéa, M.L., 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *Int J Parasitol*, 34: 1185–1196.
- Cincotta, R.P., Gorenflo, L.J., 2011. Human Population: Its Influences on Biological Diversity: 214 (Ecological studies). Springer, Heidelberg, Dordrecht, London and New York.
- Crosby, A.W., 2004. Ecological Imperialism: The Biological Expansion of Europe, 900–1900. Cambridge University Press, New York NY, USA.
- de-la-Torre, A., Sauer, A., Pfaff, A.W., Bourcier, T., Brunet, J., Speeg-Schatz, C., Ballonzoli, L., Villard, O., Ajzenberg, D., Sundar, N., Grigg, M.E., Gomez-Marin, J. E., Candolfi, E., 2013. Severe South American ocular toxoplasmosis is associated with decreased IFN- γ /IL-17a and increased IL-6/IL-13 intraocular levels. *PLoS Negl Trop Dis*, 7: e2541.
- Demar, M., Ajzenberg, D., Maubon, D., Djossou, F., Panchoe, D., Punwasi, W., Valery, N., Peneau, C., Daigre, J.L., Aznar, C., Cottrelle, B., Terzan, L., Darde, M.L., Carne, B., 2007. Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical, and parasitological aspects. *Clin Infect Dis*, 45: 88–95.
- Dubey, J.P., Thulliez, P., Powell, E.C., 1995a. *Toxoplasma gondii* in Iowa sows: comparison of antibody titers to isolation of *T. gondii* by bioassays in mice and cats. *J Parasitol*, 81: 48–53.
- Dubey, J.P., Weigel, R.M., Siegel, A.M., Thulliez, P., Kitron, U.D., Mitchell, M.A., Mannelli, A., Mateus-Pinilla, N.E., Shen, S.K., Kwok, O.C.H., Todd, K.S., 1995b. Sources and reservoirs of *Toxoplasma gondii* infection on 47 swine farms in Illinois. *J Parasitol*, 81: 723–729.
- Dubey, J.P., Gamble, H.R., Hill, D.E., Sreekumar, C., Romand, S., Thulliez, P., 2002. High prevalence of viable *Toxoplasma gondii* infection in market weight pigs from a farm in Massachusetts. *J Parasitol*, 88: 1234–1238.
- Dubey, J.P., Hill, D.E., Jones, J.L., Hightower, A.W., Kirkland, E., Roberts, J.M., Marcet, P.L., Lehmann, T., Vianna, M.C.B., Miska, K., Sreekumar, C., Kwok, O.C.H., Shen, S.K., Gamble, H.R., Roaberts, J.M., 2005. Prevalence of viable *Toxoplasma gondii* in beef,

- chicken, and pork from retail meat stores in the United States: Risk assessment to consumers. *J Parasitol*, 91: 1082–1093.
- Dubey, J.P., Sundar, N., Nolden, C.A., Samuel, M.D., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Bodenstein, B., Su, C., 2007a. Characterization of *Toxoplasma gondii* from raccoons (*Procyon lotor*), coyotes (*Canis latrans*), and striped skunks (*Mephitis mephitis*) in Wisconsin identified several atypical genotypes. *J Parasitol*, 93: 1524–1527.
- Dubey, J.P., Webb, D.M., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Su, C., 2007b. Endemic avian toxoplasmosis on a farm in Illinois: clinical disease, diagnosis, biologic and genetic characteristics of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*), and a goose (*Anser anser*). *Vet Parasitol*, 148: 207–212.
- Dubey, J.P., Hill, D.E., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Pierce, V., Kelly, K., Dulin, M., Thulliez, P., Iwueke, C., Su, C., 2008a. Endemic toxoplasmosis in pigs on a farm in Maryland: isolation and genetic characterization of *Toxoplasma gondii*. *J Parasitol*, 94: 36–41.
- Dubey, J.P., Quirk, T., Pitt, J.A., Sundar, N., Velmurugan, G.V., Kwok, O.C.H., Leclair, D., Hill, R., Su, C., 2008b. Isolation and genetic characterization of *Toxoplasma gondii* from raccoons (*Procyon lotor*), cats (*Felis domesticus*), striped skunk (*Mephitis mephitis*), black bear (*Ursus americanus*), and cougar (*Puma concolor*) from Canada. *J Parasitol*, 94: 42–45.
- Dubey, J.P., Sundar, N., Hill, D., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Majumdar, D., Su, C., 2008c. High prevalence and abundant atypical genotypes of *Toxoplasma gondii* isolated from lambs destined for human consumption in the USA. *Int J Parasitol*, 38: 999–1006.
- Dubey, J.P., Mergl, J., Gehring, E., Sundar, N., Velmurugan, G.V., Kwok, O.C.H., Grigg, M.E., Su, C., Martineau, D., 2009. Toxoplasmosis in Captive Dolphins (*Tursiops truncatus*) and Walrus (*Odobenus rosmarus*). *J Parasitol*, 95: 82–85.
- Dubey, J.P., 2010. Toxoplasmosis of animals and humans. CRC Press, Boca Raton, FL, USA.
- Dubey, J.P., Rajendran, C., Ferreira, L.R., Kwok, O.C.H., Sinnett, D., Majumdar, D., Su, C., 2010. A new atypical highly mouse virulent *Toxoplasma gondii* genotype isolated from a wild black bear in Alaska. *J Parasitol*, 96: 713–716.

- Dubey, J.P., Rajendran, C., Ferreira, L.R., Martins, J., Kwok, C.H., Hill, D.E., Villena, I., Zhou, H., Su, C., Jones, J.L., 2011a. High prevalence and genotypes of *Toxoplasma gondii* isolated from goats, from a retail meat store, destined for human consumption in the USA. *Int J Parasitol*, 41: 827–833.
- Dubey, J.P., Velmurugan, G.V., Rajendran, C., Yabsley, M.J., Thomas, N.J., Beckmen, K. B., Sinnott, D., Ruid, D., Hart, J., Fair, P.A., McFee, W.E., Shearn-Bochsler, V., Kwok, O.C.H., Ferreira, L.R., Choudhary, S., Faria, E.B., Zhou, H., Felix, T.A., Su, C., 2011b. Genetic characterization of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. *Int J Parasitol*, 41: 1139–1147.
- Dubey, J.P., Hill, D.E., Rozeboom, D.W., Rajendran, C., Choudhary, S., Ferreira, L.R., Kwok, O.C.H., Su, C., 2012. High prevalence and genotypes of *Toxoplasma gondii* isolated from organic pigs in northern USA. *Vet Parasitol*, 188: 14–18.
- Dubey, J.P., Prowell, M., 2012. Ante-mortem diagnosis, diarrhea, oocyst shedding, treatment, isolation, and genetic typing of *Toxoplasma gondii* associated with clinical toxoplasmosis in a naturally infected cat. *Clin Microbiol Infect*, 18: E221–E231.
- Dubey, J.P., Choudhary, S., Ferreira, L.R., Kwok, O.C.H., Butler, E., Carstensen, M., Yu, L., Su, C., 2013a. Isolation and RFLP genotyping of *Toxoplasma gondii* from the gray wolf (*Canis lupus*). *Vet Parasitol*, 197: 685–690.
- Dubey, J.P., Hill, D., Zarlenga, D., Choudhary, S., Ferreira, L.R., Oliveira, S., Verma, S.K., Kwok, O.C.H., Driscoll, C.P., Spiker, H., Su, C., 2013b. Isolation and characterization of new genetic types of *Toxoplasma gondii* and prevalence of *Trichinella murrelli* from black bear (*Ursus americanus*). *Vet Parasitol*, 196: 24–30.
- Dubey, J.P., Randall, A.R., Choudhary, S., Ferreira, L.R., Verma, S.K., Oliveira, S., Kwok, O.C.H., Su, C., 2013c. Occurrence, isolation, and genetic characterization of *Toxoplasma gondii* from white-tailed deer (*Odocoileus Virginianus*) in New Jersey. *J Parasitol*, 99: 763–769.
- Dubey, J.P., Casey, S.J., Zajac, A.M., Wildeus, S.A., Lindsay, D.S., Verma, S.K., Oliveira, S., Kwok, O.C.H., Su, C., 2014a. Isolation and genetic characterization of *Toxoplasma gondii* from alpaca (*Vicugna pacos*) and sheep (*Ovis aries*). *Trop Anim Health Prod*, 46: 1503–1507.

- Dubey, J.P., Dennis, P.M., Verma, S.K., Choudhary, S., Ferreira, L.R., Oliveira, S., Kwok, O.C.H., Butler, E., Carstensen, M., Su, C., 2014b. Epidemiology of toxoplasmosis in white tailed deer (*Odocoileus virginianus*): occurrence, congenital transmission, correlates of infection, isolation, and genetic characterization of *Toxoplasma gondii*. *Vet Parasitol*, 202: 270–275.
- Dubey, J.P., Why, K.V., Verma, S.K., Choudhary, S., Kwok, O.C.H., Khan, A., Behinke, M. S., Sibley, L.D., Ferreira, L.R., Oliveira, S., Weaver, M., Stewart, R., Su, C., 2014c. Genotyping *Toxoplasma gondii* from wildlife in Pennsylvania and identification of natural recombinants virulent to mice. *Vet Parasitol*, 200: 74–84.
- Dubey, J.P., Verma, S.K., Calero-Bernal, R., Cassinelli, A.B., Kwok, O.H.C., Van, W.K., Su, C., Humphreys, J.G., 2015. Isolation and Genetic Characterization of *Toxoplasma gondii* from black bears (*Ursus americanus*), bobcats (*Lynx rufus*), and feral cats (*Felis catus*) from Pennsylvania. *J Eukaryot Microbiol*, 62: 410–415.
- Dubey, J.P., Brown, J., Verma, S.K., Cerqueira-Cézar, C.K., Banfield, J., Kwok, O.C.H., Ying, Y., Murata, F.H.A., Pradhan, A.K., Su, C., 2017. Isolation of viable *Toxoplasma gondii*, molecular characterization, and seroprevalence in elk (*Cervus canadensis*) in Pennsylvania, USA. *Vet Parasitol*, 243: 1–5.
- Edwards, J.F., Dubey, J.P., 2013. *Toxoplasma gondii* abortion storm in sheep on a Texas farm and isolation of mouse virulent atypical genotype *T. gondii* from an aborted lamb from a chronically infected ewe. *Vet Parasitol*, 192: 129–136.
- Elbez-Rubinstein, A., Ajzenberg, D., Darde, M.L., Cohen, R., Dumetre, A., Yera, H., Gondon, E., Janaud, J.C., Thulliez, P., 2009. Congenital toxoplasmosis and reinfection during pregnancy: case report, strain characterization, experimental model of reinfection, and review. *J Infect Dis*, 199: 280–285.
- Excoffier, L., Lischer, H.E., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour*, 10: 564–567.
- Gerhold, R.W., Saraf, P., Chapman, A., Zou, X., Hickling, G., Stiver, W.H., Houston, A., Souza, M., Su, C., 2017. *Toxoplasma gondii* seroprevalence and genotype diversity in select wildlife species from the southeastern United States. *Parasit Vectors*, 10: 508.
- Grigg, M.E., Sundar, N., 2009. Sexual recombination punctuated by outbreaks and clonal expansions predicts *Toxoplasma gondii* population genetics. *Int J Parasitol*, 39: 925–933.

- Guthrie, A., Rooker, L., Tan, R., Gerhold, R., Trainor, K., Jiang, T., Su, C., 2017. Newly described *Toxoplasma gondii* strain causes high mortality in red necked wallabies (*Macropus rufogriseus*) in a zoo. *J Zoo Wildl Med*, 48: 694–702.
- Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis*, 172: 1561–1566.
- Jones, J.L., Kruszon-Moran, D., Rivera, H.N., Price, C., Wilkins, P.P., 2014. *Toxoplasma gondii* seroprevalence in the United States 2009–2010 and comparison with the past two decades. *Am J Trop Med Hyg*, 90: 1135–1139.
- Khan, A., Dubey, J.P., Su, C., Ajioka, J.W., Rosenthal, B.M., Sibley, L.D., 2011. Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. *Int J Parasitol*, 41: 645–655.
- Khan, A., Ajzenberg, D., Mercier, A., Demar, M., Simon, S., Dardé, M.L., Wang, Q., Verma, S.K., Rosenthal, B.M., Dubey, J.P., Sibley, L.D., 2014. Geographic separation of domestic and wild strains of *Toxoplasma gondii* in French Guiana correlates with a monomorphic version of chromosome 1a. *PLoS Negl Trop Dis*, 8: e3182.
- Lehmann, T., Marcet, P.L., Graham, D.H., Dahl, E.R., Dubey, J.P., 2006. Globalization and the population structure of *Toxoplasma gondii*. *PNAS* 103, 11423–11428.
- McDonalda, R.I., Kareivab, P., Formana, R., 2008. The implications of current and future urbanization for global protected areas and biodiversity conservation. *Biol Cons*, 141: 1695–1703.
- Mercier, A., Ajzenberg, D., Devillard, S., Demar, M.P., de Thoisy, B., Bonnabau, H., Collinet, F., Boukhari, R., Blanchet, D., Simon, S., Carme, B., Dardé, M.L., 2011. Human impact on genetic diversity of *Toxoplasma gondii*: example of the anthropized environment from French Guiana. *Infect Genet Evol*, 11: 1378–1387.
- Mondragon, R., Howe, D.K., Dubey, J.P., Sibley, L.D., 1998. Genotypic Analysis of *Toxoplasma gondii* Isolates from Pigs. *J Parasitol*, 84: 639–641.
- Pongsiri, M.J., Roman, J., Ezenwa, V.O., Goldberg, T.L., Koren, H.S., Newbold, S.C., Ostfeld, R.S., Pattanayak, S.K., Salkeld, D.J., 2009. Biodiversity loss affects global disease ecology. *Bioscience*, 59: 945–954.
- Rajendran, C., Su, C., Dubey, J.P., 2012. Molecular genotyping of *Toxoplasma gondii* from Central and South America revealed high diversity within and between populations. *Infect*

- Genet Evol, 12: 359–368.
- Rosenthal, B. M., 2009. How has agriculture influenced the geography and genetics of animal parasites? Trends Parasitol, 25: 67–70.
- Shwab, E. K., Zhu, X. Q., Majumdar, D., Pena, H.F., Gennari, S. M., Dubey, J. P., Su, C., 2014. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. Parasitology, 141: 453–461.
- Su, C., Schwab, E.K., Zhou, P., Zhu, X.Q., Dubey, J.P., 2010. Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology, 137: 1–11.
- Sundar, N., Cole, R.A., Thomas, N.J., Majumdar, D., Dubey, J.P., Su, C., 2008. Genetic diversity among sea otter isolates of *Toxoplasma gondii*. Vet Parasitol, 151: 125–132.
- Vanwormer, E., Conrad, P.A., Miller, M. A., Melli, A. C., Carpenter, T. E., Mazet, J. A., 2013. *Toxoplasma gondii*, source to sea: higher contribution of domestic felids to terrestrial parasite loading despite lower infection prevalence. Eco Health, 10: 277–289.
- VanWormer, E., Miller, M.A., Conrad, P.A., Grigg, M.E., Rejmanek, D., Carpenter, T.E., Mazet, J.A.K., 2014. Using molecular epidemiology to track *Toxoplasma gondii* from terrestrial carnivores to marine hosts: implications for public health and conservation. PLoS Negl Trop Dis, 8: e2852.
- Velmurugan, G.V., Su, C., Dubey, J.P., 2009. Isolate designation and characterization of *Toxoplasma gondii* isolates from pigs in the United States. J Parasitol, 95: 95–99.
- Verma, S. K., Su, C., Dubey, J.P., 2015. *Toxoplasma gondii* isolates from mouflon sheep (*Ovis ammon*) from Hawaii, USA. J Eukaryot Microbiol, 62: 141–143.
- Verma, S. K., Calero-Bernal, R., Cerqueira-Cézar, C. K., Kwok, O. C. H., Dubey, M., Jiang, T., Su, C., Hill, D., Dubey, J.P., 2016a. Toxoplasmosis in geese and detection of two new atypical *Toxoplasma gondii* strains from naturally infected Canada geese (*Branta canadensis*). Parasitol Res, 115: 1767–1772.
- Verma, S.K., Minicucci, L., Murphey, D., Carstensen, M., Humpal, C., Wolf, P., Calero- Bernal, R., Cerqueira-Cezar, C.K., Kwok, O., Su, C., Hill, D., Dubey, J.P., 2016b. Antibody detection, and molecular characterization of *Toxoplasma gondii* from bobcats (*Lynx rufus*), domestic cats (*Felis catus*), and wildlife from Minnesota, USA. J Eukaryot Microbiol, 63:

567–571.

- Verma, S.K., Sweeny, A.R., Lovallo, M.J., Calero-Bernal, R., Kwok, O.C., Jiang, T., Su, C., Grigg, M.E., Dubey, J.P., 2017. Seroprevalence, isolation and co-infection of multiple *Toxoplasma gondii* strains in individual bobcats (*Lynx rufus*) from Mississippi, USA. *Int J Parasitol*, 47: 297–303.
- Weiss, M.L., Kim, K., 2013. *Toxoplasma gondii*: The Model Apicomplexan- Perspectives and Methods. Elsevier Science, Amsterdam, Boston, Heidelberg and London.
- Ying, Y., Verma, S.K., Kwok, O.C.H., Alibana, F., Mcleod, R., Su, C., Dubey, J.P., Pradhan, A.K., 2017. Prevalence and genetic characterization of *Toxoplasma gondii* in free-range chickens from grocery stores and farms in Maryland, Ohio and Massachusetts, USA. *Parasitol Res*, 116: 1591–1595.
- Yu, L., Shen, J., Su, C., Sundermann, C.A., 2013. Genetic characterization of *Toxoplasma gondii* in wildlife from Alabama, USA. *Parasitol Res*, 112: 1333– 1336.

Chapter 2 Appendix

Table 2. 1 *Toxoplasma gondii* isolates from animals in different environments and geographical locations in North America

Spatial groups	Animal species	Locations ^a	Genotypes	Unique genotypes	Within-population haplotype diversity ^b	Between-population genetic composition ^c	References
Farm-bound (n=195)	Pigs (n=168), chickens (n=11), cats (n=9), house mice (n=6), goose (n=1)	Iowa (n=85), Massachusetts (n=53), Pennsylvania (n=29), Illinois (n=26), Maryland (n=1), New Jersey (n=1)	#1 (n=70), #2 (n=50), #3 (n= 40), #4 (n=24), #5 (n=1), #8 (n=9), #42 (n=1). <u>Total: 7 genotypes</u>	#42	0.75 +/- 0.01; 95% C.I. (0.72 - 0.78)	Not different from free-roaming, but different from wildlife.	Dubey et al. (1995a, b, 2002, 2005, 2007b), Dubey and Prowell (2012), Mondragon et al. (1998), Velmurugan et al. (2009)
Free-roaming (n=135)	Sheep (n=59), goats (n=29), free-range chickens (n=22), organic pigs (n=19), feral cats (n=4), alpacas (n=2)	Maryland, Virginia and West Virginia (n=55), Maryland, Virginia and Pennsylvania (n=29), Ohio (n=15), Michigan (n=15), Massachusetts (n=7), Virginia (n=5), Maryland (n=4), Manitoba (Canada) (n=2), Pennsylvania (n=2), Texas (n=1)	#1 (n=44), #2 (n=28), #3 (n=15), #4 (n=12), #5 (n=4), #7 (n=4), #9 (n=1), #32 (n=1), #39 (n=3), #54 (n=1), #72 (n=1), #73 (n=2), #74 (n=1), #110 (n=1), #113(n=1), #118 (n=1), #130 (n=1), #131 (n=1), #143 (n=1), #154 (n=1), #156 (n=1), #167 (n=1), #170 (n=5), #216 (n=2), #230 (n=2). <u>Total: 25 genotypes</u>	#72, 73, 74, 110, 113, 118, 130, 131, 156, 170, 230.	0.83 +/- 0.02; 95% C.I. (0.79 - 0.87)	Not different from farm-bound but different from wildlife.	Dubey et al. (2008a,b,c, 2011a, 2012, 2014a, 2015), Edwards, et al. (2013), Ying et al. (2017)

Table 2.1 Continued

Spatial groups	Animal species	Locations ^a	Genotypes	Unique genotypes	Within-population haplotype diversity ^b	Between-population genetic composition ^c	References
Wildlife (n=293)	White-tailed deer (n=54), sea otters (n=46), wolves (n=30), bobcats (n=27), coyotes (n=21), red foxes (n=13), Canadian geese (n=11), black bears (n=13), raccoons (n=12), dolphins (n=7), hawks (n=7), Bennett's wallabies (n=6), arctic foxes (n=5), geese (n=4), wallabies (n=4), skunks (n=4), swans (n=3), feral pigs (n=3), white-footed mice (n=2), Hawaiian crows (n=2), moose (n=2), badgers (n=2), opossums (n=2), mouflon sheep (n=2), bald eagles (n=2), elks (n=2), brown bear (n=1), woodrat (n=1), cougar (n=1), European starling (n=1), barn owl (n=1), American kestrel (n=1), mink (n=1)	Pennsylvania (n=43), Mississippi (n=42), Minnesota (n=37), California (n=30), Washington (n=17), Wisconsin (n=15), South Carolina (n=15), Maryland (n=12), Alaska (n=10), Georgia (n=9), New Jersey (n=9), Hawaii (n=8), Colorado (n=7), Tennessee (n=7), Virginia (n=6), Alabama (n=5), Manitoba (Canada) (n=2), British Columbia (Canada) (n=1), Quebec (Canada) (n=1), unknown location (Canada) (n=1), Ohio (n=4), Massachusetts (n=3), North Carolina (n=3), Michigan (n=2), Texas (n=2), Washington D.C. (n=1), Rhode Island (n=1)	#1 (n=34), #2 (n=28), #3 (n=26), #4 (n=15), #5 (n=128), #7 (n=3), #8 (n=1), #9 (n=1), #10 (n=1), #11 (n=1), #14 (n=1), #15 (n=2), #24 (n=2), #32 (n=2), #39 (n=2), #54 (n=2), #66 (n=1), #90 (n=1), #141 (n=1), #143 (n=1), #146 (n=1), #147 (n=1), #154 (n=1), #157 (n=1), #167 (n=3), #177 (n=2), #180 (n=1), #186 (n=1), #216 (n=11), #219 (n=1), #220 (n=1), #221 (n=3), #249 (n=1), #261 (n=3), #262 (n=1), #263 (n=6), #266 (n=1), #267 (n=1). <u>Total: 38 genotypes</u>	#10, 11, 14, 15, 24, 66, 90, 141, 146, 147, 157, 177, 180, 186, 219, 220, 221, 249, 261, 262, 263, 266, 267	0.78 +/- 0.02; 95% C.I. (0.73 - 0.82)	Different from farm-bound and free-roaming animals.	Howe and Sibley (1995), Dubey et al. (2007a, 2008b, 2009, 2010, 2011b, 2013a, b, c, 2014b, c, 2015, 2017), Sundar et al. (2008), Yu et al. (2013), Verma et al. (2015, 2016 a,b, 2017), Guthrie et al. (2017), Gerhold et al. (2017)

C.I., Confidence interval.

^a Locations are states of the USA except where otherwise indicated in parentheses.^b Within-population diversity: the diversity in increasing order is free roaming animals > wildlife > farm-bound animals. The diversity of the free

roaming animals is significantly higher than the farm-bound animals, $P < 0.05$.

^c Between-population pairwise genetic diversity analysis of three *T. gondii* populations. Statistical significance is set at $P < 0.001$.

Table 2. 2 *Toxoplasma gondii* populations of 14 animal species from North America.

Animal hosts	Genotypes	Locations ^a	Within-population diversity	References
Pigs (<i>Sus domesticus</i>) n=187	#1 (n=67), #2 (n=53), #3 (n=36), #4 (n=17), #5 (n=1), #7 (n=3), #8 (n=8), #42 (n=1), #113 (n=1). <u>Total: 9 Genotypes</u>	Iowa (n=85), Massachusetts(n=53), Pennsylvania (n=29), Michigan (n=15), Maryland (n=4), New Jersey (n=1),	0.75 +/- 0.02, 95% C.I. (0.72, 0.78)	Dubey et al. (1995a, 2002, 2005, 2008a, 2012); Mondragon et al. (1998), Velmurugan et al. (2009)
Chickens (<i>Gallus gallus domesticus</i>) n=23	#1 (n=8), #2 (n=11), #3 (n=1), #170 (n=3). <u>Total: 4 Genotypes</u>	Ohio (n=15), Massachusetts (n=7), Illinois (n=1)	0.66 +/- 0.06, 95% C.I. (0.54, 0.78)	Dubey et al. (2007b), Ying et al., (2017)
Cats (<i>Felis catus</i>) n=13	#1 (n=1), #3 (n=1), #4 (n=6), #5 (n=1), #8 (n=1), #130 (n=1), #216 (n=2). <u>Total: 7 Genotypes</u>	Illinois (n=8), Pennsylvania (n=2), Manitoba (Canada) (n=2), Maryland (n=1)	0.79 +/- 0.11, 95% C.I. (0.58, 1.01)	Dubey et al. (2008b, 2015), Dubey and Prowell (2012)
Canada geese (<i>Branta canadensis</i>) n=11	#1 (n=1), #2 (n=5), #4 (n=2), #143 (n=1), #266 (n=1), #267 (n=1). <u>Total: 6 Genotypes</u>	Maryland (n=9), Mississippi (n=1), Pennsylvania (n=1)	0.80 +/- 0.11, 95% C.I. (0.58, 1.02)	Dubey et al. (2014c), Verma et al. (2016a)
Sheep (<i>Ovis aries</i>) n=59	#1 (n=17), #2 (n=9), #3 (n=9), #4 (n=9), #5 (n=1), #7 (n=1), #9 (n=1), #32 (n=1), #39 (n=2), #72 (n=1), #73 (n=2), #74 (n=1), #54 (n=1), #110 (n=1), #131 (n=1), #230 (n=2). <u>Total: 16 Genotypes</u>	Maryland, Virginia and West Virginia (n=55), Virginia (n=3), Texas (n=1)	0.86 +/- 0.03, 95% C.I. (0.80, 0.91)	Dubey et al. (2008c, 2014a), Edwards and Dubey (2013)

Table 2.2 Continued

Animal hosts	Genotypes	Locations ^a	Within-population diversity	References
Goats (<i>Capra aegagrus hircus</i>) <i>n</i> =29	#1 (<i>n</i> =9), #2 (<i>n</i> =4), #3 (<i>n</i> =4), #4 (<i>n</i> =3), #5 (<i>n</i> =2), #39 (<i>n</i> =1), #118 (<i>n</i> =1), #143 (<i>n</i> =1), #154 (<i>n</i> =1), #156 (<i>n</i> =1), #167 (<i>n</i> =1), #170 (<i>n</i> =1). <u>Total: 12 Genotypes</u>	Maryland, Virginia and Pennsylvania (<i>n</i> =29)	0.87 +/- 0.04, 95% C.I. (0.79, 0.96)	Dubey et al. (2011a)
Red foxes (<i>Vulpes vulpes</i>) <i>n</i> =13	#1 (<i>n</i> =1), #2 (<i>n</i> =2), #3 (<i>n</i> =3), #4 (<i>n</i> =1), #54 (<i>n</i> =1), #141 (<i>n</i> =1), #216 (<i>n</i> =4). <u>Total: 7 Genotypes</u>	Pennsylvania (<i>n</i> =11), Alaska (<i>n</i> =2)	0.87 +/- 0.07, 95% C.I. (0.74, 1.00)	Dubey et al. (2011b, 2014c)
Black bears (<i>Ursus americanus</i>) <i>n</i> =13	#1 (<i>n</i> =3), #2 (<i>n</i> =2), #4 (<i>n</i> =2), #5 (<i>n</i> =2), #90 (<i>n</i> =1), #147 (<i>n</i> =1), #216 (<i>n</i> =2). <u>Total: 7 Genotypes</u>	Pennsylvania (<i>n</i> =8), Maryland (<i>n</i> =3), Alaska (<i>n</i> =1), Quebec (Canada) (<i>n</i> =1)	0.91 +/- 0.05, 95% C.I. (0.82, 1.01)	Dubey et al. (2008b, 2010, 2011b, 2013b, 2015)
Raccoons (<i>Procyon lotor</i>) <i>n</i> =12	#1 (<i>n</i> =2), #2 (<i>n</i> =1), #4 (<i>n</i> =1), #5 (<i>n</i> =4), #7 (<i>n</i> =2), #32 (<i>n</i> =2). <u>Total: 6 Genotypes</u>	Wisconsin (<i>n</i> =5), Georgia (<i>n</i> =3), Minnesota (<i>n</i> =2), Manitoba (Canada) (<i>n</i> =1), Texas (<i>n</i> =1)	0.86 +/- 0.07, 95% C.I. (0.72, 1.00)	Dubey et al. (2007a, 2008b, 2011b), Verma et al. (2016b)
WT deer (<i>Odocoileus virginianus</i>) <i>n</i> =54	#1 (<i>n</i> =2), #2 (<i>n</i> =4), #3 (<i>n</i> =4), #4 (<i>n</i> =4), #5 (<i>n</i> =29), #146 (<i>n</i> =1), #154 (<i>n</i> =1), #167 (<i>n</i> =1), #216 (<i>n</i> =4), #220 (<i>n</i> =1), #221 (<i>n</i> =3). <u>Total: 11 Genotypes</u>	Mississippi (<i>n</i> =19), South Carolina (<i>n</i> =9), New Jersey (<i>n</i> =9), Pennsylvania (<i>n</i> =7), Ohio (<i>n</i> =4), Tennessee (<i>n</i> =4), Alabama (<i>n</i> =1), Minnesota (<i>n</i> =1)	0.70 +/- 0.07, 95% C.I. (0.57, 0.83)	Dubey et al. (2011b, 2013c, 2014b,c), Yu et al. (2013), Gerhold et al. (2017)
Coyotes (<i>Canis latrans</i>) <i>n</i> =21	#1 (<i>n</i> =7), #3 (<i>n</i> =2), #4 (<i>n</i> =4), #5 (<i>n</i> =7), #14 (<i>n</i> =1). <u>Total: 5 Genotypes</u>	Pennsylvania (<i>n</i> =10), Wisconsin (<i>n</i> =7), Tennessee (<i>n</i> =2), Georgia (<i>n</i> =1), Minnesota (<i>n</i> =1)	0.77 +/- 0.05, 95% C.I. (0.67, 0.87)	Dubey et al. (2007a, 2014c), Verma et al. (2016b), Gerhold et al. (2017)

Table 2.2 Continued

Animal hosts	Genotypes	Locations ^a	Within-population diversity	References
Bobcats (<i>Lynx rufus</i>) <i>n</i> =27	#2 (<i>n</i> =1), #5 (<i>n</i> =24), #24 (<i>n</i> =2). <u>Total: 3 Genotypes</u>	Mississippi (<i>n</i> =20), Georgia (<i>n</i> =4), Alabama (<i>n</i> =2), Pennsylvania (<i>n</i> =1)	0.21 +/- 0.10, 95% C.I. (0.01, 0.41)	Yu et al. (2013), Dubey et al. (2015), Verma et al. (2017)
Sea otters (<i>Enhydra lutris</i>), <i>n</i> =46	#1 (<i>n</i> =6), #3 (<i>n</i> =12), #4 (<i>n</i> =1), #5 (<i>n</i> =24), #39 (<i>n</i> =2), #54 (<i>n</i> =1). <u>Total: 6 Genotypes</u>	California (<i>n</i> =29), Washington (<i>n</i> =17)	0.65 +/- 0.05, 95% C.I. (0.55, 0.76)	Howe and Sibley (1995), Sundar et al. (2008), Dubey et al. (2011b)
Wolves (<i>Canis lupus</i>) <i>n</i> =30	#1 (<i>n</i> =2), #3 (<i>n</i> =1), #5 (<i>n</i> =25), #9 (<i>n</i> =1), #219 (<i>n</i> =1). <u>Total: 5 Genotypes</u>	Minnesota (<i>n</i> =28), Alaska (<i>n</i> =1), Wisconsin (<i>n</i> =1)	0.31 +/- 0.11, 95% C.I. (0.10, 0.52)	Dubey et al. (2011b, 2013a)

C.I., confidence interval.

^a Locations are all states of the USA except where otherwise stated in parentheses.

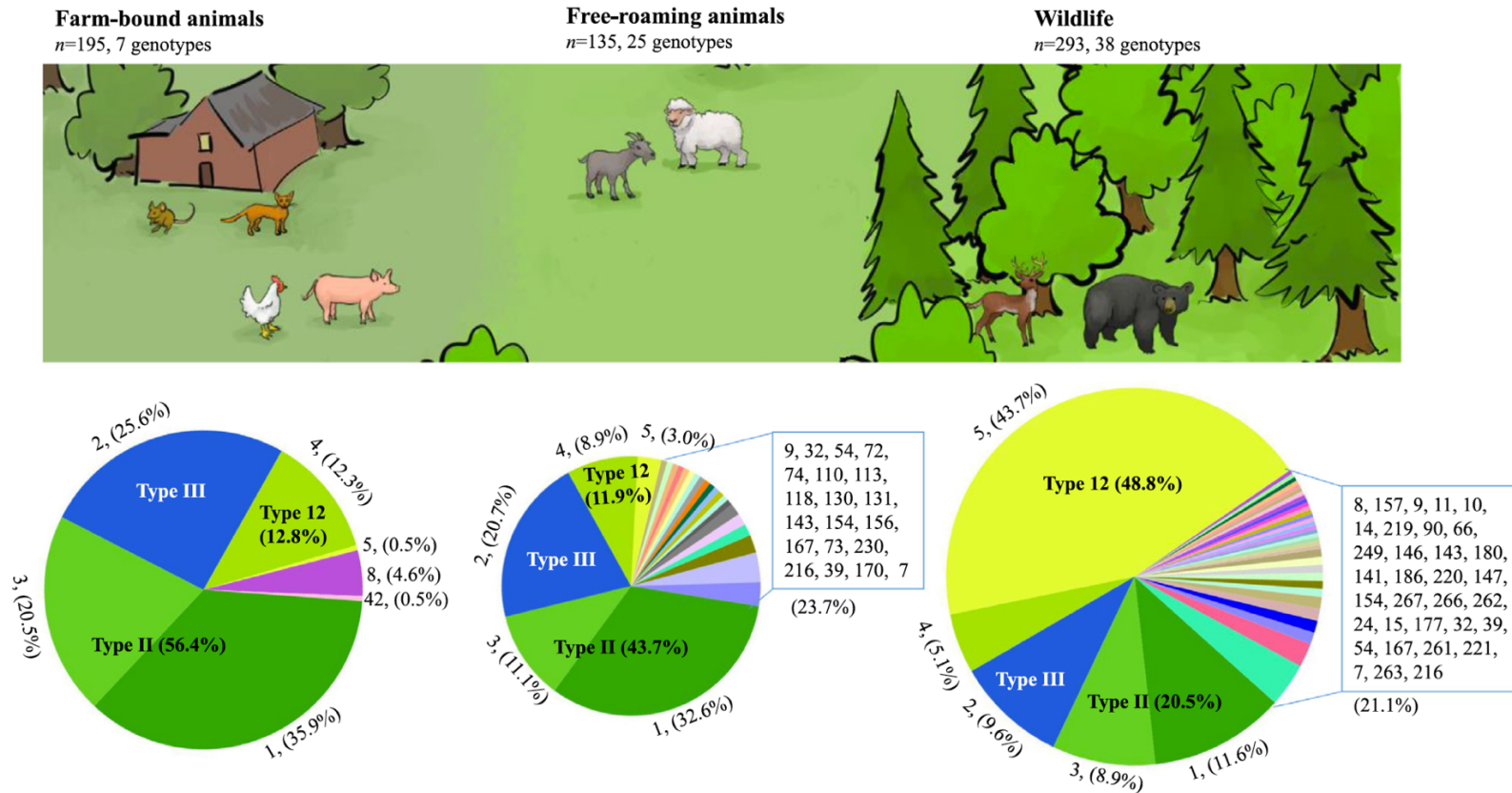


Figure 2. 1 *Toxoplasma gondii* genotype distribution along the spatial gradient from farm to the wild. The cartoon illustrates the spatial gradient and animal populations with varied proximities to human habitat. Representatives of animal species in each population are depicted. The numbers around pie chart edges indicate ToxoDB PCR-RFLP genotypes. The archetypal lineages (Type II, III, 12) are overlaid on pie charts in bold lettering. The sizes of pie charts correlate with the total number (and percentage) of isolates; colors of the slices represent different genotypes.

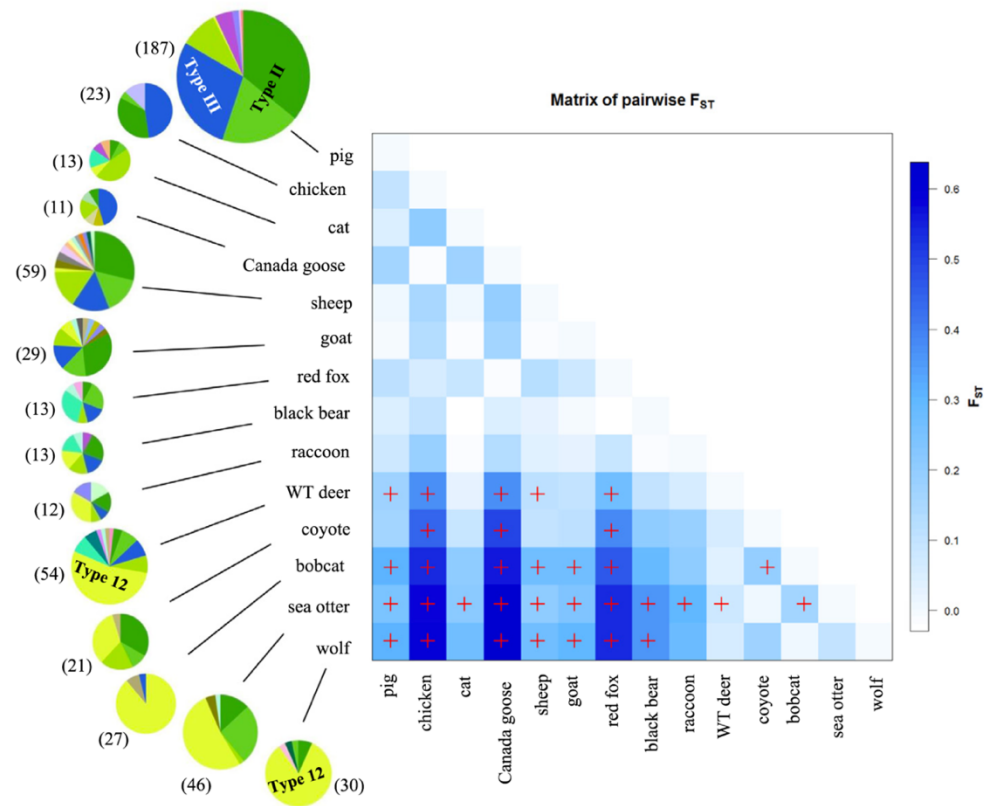


Figure 2. 2 Pairwise comparison of 14 *Toxoplasma gondii* populations for different host species. The number of each species is indicated in parentheses and the colors in the pie chart represent different genotypes. The between population comparison was conducted using Arlequin ver 3.5.1.2. The cut-off value for statistical significance was $P=0.001$. The “+” sign indicates significant difference between two populations. WT deer, white-tailed deer.

CHAPTER 3

**A SYSTEMIC EVALUATION OF *TOXOPLASMA GONDII* INFECTION ON
A FARM THROUGH THE EXAMINATION OF SOIL OOCYST
CONTAMINATION AND ANIMAL INFECTION**

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My contribution to this body of work is collecting samples from rodents, conducting experiments, collecting data, and writing the manuscript. Katherine Kurth trapped all the meso-predators and some rodents. The rest of the co-authors all participated in animal trapping and or manuscript revision.

Abstract

The environmental contamination by *Toxoplasma gondii* contributes to infection in humans and animals. However, *T. gondii* environmental contamination is largely uninvestigated due to the lack of a comprehensive evaluation system. In this study, a systemic evaluation method of *T. gondii* environmental contamination was developed by examining soil oocyst contamination in combination with wild and domestic animal infection on a farm. The study was conducted on a typical dairy farm in East Tennessee from May 2016 to April 2017. Twenty-two soil samples were collected from two cat habitats on the farm. Oocysts in the soil were sucrose-floated and subjected to DNA extraction and PCR-RFLP. Three (13.6%) samples were PCR-RFLP positive; however, *T. gondii* DNA was not consistently detected from these three samples on repeat testing. Rodents and mesopredators were trapped on the farm with blood and or tissue samples obtained. Serological tests via Modified Agglutination Test revealed that at a cutoff of 1:25, 18.5% (5/27) of house mice, 40% (2/5) of white-footed mice, 70.6% (12/17) of raccoons and 50% (1/2) of farm cats, were seropositive for *T. gondii* antibodies; no antibodies were found in one brown rat, six cotton rats, one mole, sixteen opossums, and two skunks. Twenty-nine tissues samples from rodents were also collected and subjected to PCR detection. *T. gondii* was not found in any of the tissues of twenty-nine rodents via PCR-RFLP; however, one house mouse was positive for *Hammondia hammondi* and another house mouse for *Sarcocystis* spp. Sera from thirty heifers from the same farm were seronegative using MAT. In summary, soil sample oocyst contamination may be not sensitive to determine *T. gondii* contamination, considering the low oocyst concentration in soil due to the significant dilution of oocysts by soil. Further, our results indicated that mesopredators such as raccoons and cats are better indicators of *T. gondii* infection.

Keywords: *T. gondii* environmental contamination, oocyst, serological test.

Introduction

Toxoplasma gondii is a coccidian parasite that has a broad spectrum of hosts, including humans, mammals, and birds (Dubey, 2010). *T. gondii* chronically infects one-third of the global human population and 6.7% of U.S. born individuals 12 to 49 years old (Jones et al., 2014). Seronegative pregnant women are susceptible to *T. gondii* infection, creating a significant public health threat due to the potential devastation to the fetus (Dubey, 2010). A fetal infection has a broad spectrum of ramifications, including stillbirth, spontaneous abortion, and various congenital disabilities in newborns (Dubey, 2010). There are an estimated 400-4000 cases of congenital toxoplasmosis each year in the U.S. (Jones et al., 2001). Life-threatening outcomes are seen in individuals with compromised immune systems including HIV or cancer patients (Dubey, 2010). In immune-competent people, infection generally consists of mild symptoms, such as fatigue or malaise; however, infection can result in intensive hospitalization despite the competent immune system of these patients. More serious cases likely result from infection with highly virulent strains of *T. gondii* (Dardé et al., 1998).

T. gondii uses felids as definitive hosts. The central role of cats in *T. gondii* transmission was validated as there was no or less *T. gondii* infection in areas without cats (Wallace, 1969). Upon infection, felines, such as free-roaming domestic cats, shed millions of oocysts into the environment (Dubey, 2010). These oocysts can be dispersed into environmental matrices including water, vegetables, soil, and fruits. Intermediate hosts, such as humans and livestock, contract infection via inadvertently ingesting oocysts disseminated in the environment.

As a companion animal of humans, the cat population increased with the growth of the economy and human society. There are approximately 78 million domestic cats and 73 million feral cats in the U.S. (Dubey, 2010). An average of 30% of cats worldwide is seropositive for *T. gondii*, indicating a current or previous shedding of oocysts and a potential re-shedding upon reinfection (Dubey, 2010). The number of oocysts one cat can shed ranges from 3 to 810 million over the shedding period of approximately one week. In California, the annual average oocyst burden in soil ranges from 94 to 4671 oocysts per square meter (Dabritz et al., 2007). Aside from their abundance, oocysts are hardy and resistant. Outdoors (6°C-36°C), oocysts can survive for 46 days when feces are uncovered, 334 days in covered feces, and 18 months in feces buried under

3-9 cm in soil (Dubey, 1998; Frenkel et al., 1975). The concentration of the disinfectants used to sterilize drinking water such as sodium hypochlorite and ozone are unable to kill oocysts. High infectivity is another attribute of oocysts, which, further, perpetuates the transmission of the parasite. One oocyst alone can experimentally infect a pig (Dubey et al., 1996) or a mouse (Dubey, 2006).

The stability, high infectivity, and abundance of oocyst contribute to the successful transmission of *T. gondii*, posing a threat to the public health. Inadvertently ingesting oocysts is the primary source of human infection of *T. gondii* (Jones and Dubey, 2010). Severe clinical toxoplasmosis outbreaks have been associated with ingesting oocysts in drinking water or food (Bowie et al., 1997; De Moura et al., 2006). Oocysts have been identified as being responsible for 78% of congenital infections in North America (Boyer et al., 2011). Oocysts in the soil can be washed off from terrestrial environments into large water bodies, leading to the infection of marine animals. *T. gondii* infection in aquatic mammals in California and the Amazon river signified oocyst contamination of water (VanWormer et al., 2014).

The surveillance of *T. gondii* contamination in the environment is critical to assess the infection risks in both humans and animals. *T. gondii* infection in farm animals raised for human consumption poses a risk for human infection on account of human consumption of undercooked meat. The soaring demand for meat products from organically raised animals increased the odds of human infection (Weigel et al., 1999). Recent studies have shown that the non-confinement raised pigs have a higher rate of *T. gondii* infection (Dubey et al., 2012; Guo et al., 2016). The evaluation of *T. gondii* environmental contamination is fundamental for the development of methods to reduce the contamination. Controlling *T. gondii* environmental contamination is of great importance to reduce human infection, thus alleviating the social and economic burden of toxoplasmosis. To this end, it is imperative to establish a practical procedure to monitor *T. gondii* contamination in the environment.

Materials and Methods

Sample collection

The twenty-two soil samples were collected from a dairy farm (Figure 3.1A)- the Little River Animal and Environmental Unit. The farm was located in Walland, Tennessee on 529 acres of land. The farm is affiliated with the University of Tennessee and was mainly used for research and education purposes. With a primary emphasis on milk and Holstein cow production, this farm is a representation of the modern dairy farm. There were roughly eight feral cats on the farm according to the farmers, which were kept for rodent control. The cats were fed by the farmers and had the freedom to hunt. Twenty-two soil samples were collected from the farm in May 2016 (Figure 3.1B). Two locations were chosen for soil sample collection where cats were fed, resting, or seen free-roaming (Figure 3.2A). The first location (N 35° 46.005'; W 083° 50.455') was the calf raising area where cats were fed and resting (Figure 3.2A, location # 1); The second location (N 35° 46.142'; W 083° 50.546') was the maintenance shop and silage storage area, where cats were found free-roaming (Figure 3.2A, location # 2). In each location, soil sampling sites were intentionally chosen to greatly disperse over a large area and to represent different vegetative cover. At each site, surface soil with a depth of no more than 2 cm in an area of 10 cm by 10 cm was collected in bags. Approximately 20 g of soil from each site was collected and stored at 4°C before processing.

Sugar floating method for oocyst extraction

Sugar floating method was used to float oocysts in soil (Figure 3.2B) (Lelu et al., 2011). First, soil samples were dried and passed through a sieve with a mesh size of 2×2 mm at room temperature. For each sieved soil sample, 5 grams or 10 grams were taken for testing. In a 50 ml tube, 5 g of soil was mixed with 5 ml of 2% sulfuric acid. The mixture was then incubated at 4°C overnight and diluted with 15 ml ddH₂O the following day. In a new 50 ml tube, 20 ml cold sucrose solution (cane sugar; density: 1.20) was overlaid with the soil sample, which was subsequently centrifuged at $1500 \times g$ for 20 min. The aqueous phase (approximately 10 ml) and interphase (approximately 10 ml) were collected separately or collectively (Figure 3.2B) to test the location where oocysts

concentrate in the solution after centrifugation. The content was washed and finally suspended in 100 µl ddH₂O.

PCR-RFLP for oocyst detection

The concentrated oocysts were subjected to three cycles of freeze (-80°C) and thaw (+20°C) with no fewer than 4 hours at each temperature. DNA was extracted from the oocyst lysates using NucleoSpin soil DNA extraction kit (Macherey-Nagel, Duren, Germany; Cat: #740780.50). 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 able to differentiate *T. gondii* from other closely related apicomplexans (*Hammondia hammondi*, *Neospora caninum*, *Sarcocystis* spp) without sequencing. The 25 µM external primers Tg18s48F/Tg18s 359R and 50 µM the internal primers Tg18s 58F/Tg18s 348R were used for nested PCR amplification (Silva et al., 2009). The PCR product was 290 base pairs in the case of *Sarcocystis neurona*, *N. caninum*, *T. gondii*, and *H. hammondi*; a 310 base pair product was generated in the case of other *Sarcocystis* spp. Restriction enzymes Ddel, 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.

The same PCR-RFLP method was used to detect *T. gondii* in tissues of twenty-nine trapped rodents. Five grams of tissues from brain and heart of trapped rodents were combined and homogenized. DNA was extracted from 100 µl of homogenized tissue (100 grams) using DNeasy Blood & Tissue Kit (Qiagen, Cat. No. 69504) and eluted in 100 µl AE buffer.

The efficiency of oocyst detection from soil

To test the sensitivity of the sugar floating method, *H. hammondi* oocysts (10⁵ or 10⁶) stored in our lab were spiked into 5 grams of *T. gondii*-negative soil. *H. hammondi* was used as a substitute for *T. gondii* due to its inability to infect humans and its close relation to *T. gondii* genetically (Silva et al., 2009). The spiked soil samples were subjected to the same procedure as mentioned above for oocyst detection.

Animal trapping and the detection of *T. gondii* infection

Animals on the farm were trapped to investigate the prevalence of *T. gondii* infections. Two periods of trapping were conducted from September 2016 to April 2017 (Figure 3.3). During the first trapping period from September 2016 to November 2016, only rodents were trapped. The second trapping period from December 2016 to April 2017, both rodents and meso-predators were trapped; however, rodent trapping was terminated after March 5th, 2017 due to low seroprevalence in rodents. Animal trapping and handling were conducted in compliance with the procedures approved by the Institutional Animal Care and Use Committee (IACUC). Sherman traps (50-90) and Tomahawk traps (10) were used to trap small rodents and meso-predators, respectively. Traps were set at dusk and checked before dawn. Oatmeal was used as rodent bait, whereas sardine was used as bait for meso-predator trapping. For rodent anesthesia, cotton balls soaked with isoflurane were placed in traps. Rodents were then euthanized by heart injection of pentobarbital (Fatal-Plus Solution, 390mg/mL) at a dose of 3.9 mg per gram of rodent body weight. Animal carcasses were kept on ice and immediately transferred to the laboratory. Approximately 100 µl of blood sample was collected from each rodent. Heart and brain tissues were also collected from each rodent. Meso-predators were sedated using Ketamine (100mg/ml) and Xylazine (20mg/ml) at doses of 16 mg/kg and 3.2 mg/kg per kilogram of animal body weight, respectively. The weight of the animal, pulse per min, breathing per min, and rectal temperature were recorded for each captured meso-predator. No more than 8% of animal total blood volume was taken during anesthesia.

Trapping was conducted in two major areas with distinct proximities to farm buildings (Figure 3.1A, red and orange circles). Each trapping area had an approximate radius of 500 meters. At the location near human settlements (Figure 3.1A, orange circle), trapping was conducted in two primary locations- hay bales number #1 and number #2 (Figure 3.1B). At the location away from farm buildings (Figure 3.1A, red circle), two brush piles (Figure 3.1C) were places where trapping were densely conducted. Additional trapping locations along the Little River and within the brush pile zone were delineated in yellow lines in Figure 3.4. Trapping locations were adjusted to reach the maximum trapping potential. Both serum and tissues (brain and heart) of trapped rodents were collected, whereas only sera were collected from trapped mesopredators. Tissues of meso-predators were not collected unless animals were previously tested as seropositive for *T. gondii*. Thirty heifer serum samples were obtained from farm veterinarians. Serum samples from

trapped animals or heifers were tested via Modified Agglutination Assay (MAT) (Dubey and Desmonts, 1987). The cutoff value was set at 1:25 which is the commonly used cutoff value (Dubey et al., 1995a and 1995b); however, results using 1:50 as cutoff were also summarized for comparison.

Results

Soil oocyst detection

To test the efficacy of the methodology, namely oocyst extraction in conjunction with PCR-RFLP detection, we spiked 10^5 or 10^6 *H. hammondi* into 5 or 10 grams of *T. gondii*-negative soil samples. We were able to detect *H. hammondi* from 5 or 10 grams of soil with 10^5 or more oocysts, which indicated that the methodology was effective (Figure 3.5). To determine where the oocysts concentrate in the centrifuged sugar solution, the aqueous layer (denoted with a letter “T”) and the interphase (denoted with a letter “I”) were collected separately at the early stage of the study; and the combined aqueous layer and the interphase were taken during the later period of this study. *H. hammondi* was detected in solution from the interphase (Figure 3.5, P3I and P4I) or in mixed solution combining the interphase and the aqueous layer (Figure 3.5, P2TI), but not in top aqueous layer (Figure 3.5, P1T, P3T and P4T), indicating that *H. hammondi* oocysts concentrate in the interphase.

DNA extracted from the concentrated oocysts from 5 grams of soil were subjected to PCR-RFLP. On a 96-well plate, 25 percent of negative controls (sample to negative control ratio equals to 3:1) were incorporated in the PCR-RFLP, for which DNA was replaced by water in PCR reaction mix (Figure 3.5, Table 3.2). Positive controls using DNA from *T. gondii* (PTG strain), *N. caninum*, and *H. hammondi* were also included (Figure 3.5, Table 3.2). The same experiment was repeated the following day (Figure 3.5B). In the first test, *T. gondii* appeared in the positive controls (PTG strain) and in soil samples number # 2 and number # 11 (Figure 3.5A, Table 3.2). In the repeat experiment, number # 21 was positive for *T. gondii*, and earlier positive samples (# 2 and # 11) were not consistently positive (Figure 3.5B, Table 3.2). For validation, another 5 grams of soil from each of the 22 samples were subjected to the same procedure as mentioned above. No positive samples were detected using PCR-RFLP (Table 3.3). We further investigated whether

double the amount of soil would yield positive results. To that end, 10 grams of soil sample were processed and subjected to PCR-RFLP (Table 3.3). No positive samples were detected. The inconsistency of positive results across the independent tests suggests that the oocyst concentration in the 22 soil samples was low relative to the sensitivity of the detection system.

Rodent trapping and T. gondii detection

Total forty rodents, both wild and domestic, were trapped with blood and tissue samples (heart and brain) taken (Figure 3.4; Table 3.1). Serum samples were obtained from these 40 rodents, including twenty-seven house mice, one brown rat, five white-footed mice, six cotton rats, and one mole (Table 3.1). Two major trapping locations (hay bales number #1 and number #2) for domestic house mice were indicated in Figure 3.1B and Figure 3.5. House mice were captured close to human settlements (Figure 3.1A, yellow cycle), whereas the wild rodents were captured in areas away from human settlements (Figure 3.1A, red cycle).

Serological tests via MAT revealed that with a cutoff at 1: 25, 18.5% (5/27) of the house mice, 40% (2/5) of white-footed mice, 0% (0/1) of brown rats, 0% (0/6) of cotton rats and 0% (0/1) of moles were positive for *T. gondii* antibodies. However, the positivity of rodents significantly decreased with a cutoff set at 1:50. With a cutoff at 1:50, 3.7% (1/27) of house mice and 0% (0/5) of white-footed mice were *T. gondii* seropositive. Brain and heart tissues from 29 rodents, including seventeen house mice, five white-footed mice, five cotton rats, one brown rat, and one mole, were subjected to PCR-RFLP. DNA was extracted from combined heart and brain tissues and was subjected to PCR-RFLP for *T. gondii* detection. Four samples were positive for *T. gondii* during the first test. However, these four samples were not consistently positive in the subsequent experimental tests. Two samples, however, were consistently positive with one being *H. hammondi* and the other being *Sarcocystis* spp (Table 3.4). No sample was found consistently positive for *T. gondii*, indicating the absence of *T. gondii* DNA or the extremely low concentration of *T. gondii* DNA in these tissues (Table 3.4).

Mesopredator trapping and T. gondii detection

Eighteen opossums were responsible for the sixty-eight captures (Figure 3.6). Due to the repeated capture of the same animal, the home range of opossum was revealed. Note worthily, opossum

number # 81, # 43 and # 47 were caught 15, 13 and 9 times, respectively at various locations, among which, number # 47 had the largest home range (Figure 3.6). Seventeen raccoons were responsible for the 25 captures (Figure 3.6). One otter was captured, but the attempt for blood sampling was futile. Two skunks and two farm cats were responsible for 4 and 3 captures, respectively (Figure 3.6). Serum samples were obtained from seventeen raccoons, sixteen opossums, two farm cats, and two skunks (Table 3.1). When the cutoff value was set at 1:25, 70.6% (12/17) of raccoons and 50% (1/2) of farm cats, 0% (0/16) of opossums and 0% (0/2) of skunks were serologically positive for *T. gondii* (Table 3.1). When cutoff value was set at 1:50, 52.9% (9/17) of raccoons and 50% (1/2) of farm cats positive for *T. gondii* (Table 3.1). All thirty heifer serum samples were negative for *T. gondii* antibodies. Attempts to isolate parasites from animal tissues was not conducted due to no recapture of *T. gondii* seropositive animals. Raccoons number # 74 and number # 88 were recaptured and were seropositive at titer 1:100 and 1:25, respectively. No parasite isolation was attempted due to their low titer ($\geq 1:50$) based on the first serological test before the final titration.

Discussion

The stability, high infectivity, and abundance of oocysts ensure the perpetuation of the *T. gondii* life cycle, which raises compelling public health concerns. Inadvertently ingesting oocysts disseminated in the environmental matrices, such as soil, water, vegetables, and fruits, is an important source of human infection of *T. gondii*. In Brazil, ingesting oocysts in drinking water or food was the primary source of infection (Bahia-Oliveira et al., 2003). A myriad of studies have substantiated that oocyst-mediated transmission route was more significant than tissue cyst-mediated transmission. First, oocyst induced infections are more severe than those induced by orally ingesting bradyzoites in tissue cysts (Dubey, 1997a; Dubey et al., 1997b). Severe clinical toxoplasmosis outbreaks were reported to be linked with ingesting oocysts in water or food (Boyer et al., 2011; Bowie et al., 1997; Dubey, 2004; Moura et al., 2006). Second, oocysts are implicated in water-related infection, which may lead to the disease of a considerable number of humans and aquatic animals (Bowie et al., 1997; Kreuder et al., 2003). Third, oocysts are also associated with congenital infection. Recent studies demonstrated that oocysts caused 78% of congenital infection

in North America (Boyer et al., 2011); 43% in Southern Chile (Munoz-Zanzi et al., 2010), and 80% in swine in Southern Chile (Munoz-Zanzi et al., 2012).

Among the routes of *T. gondii* transmission, soil oocyst ingestion is the most implicated in animal and human infection. It has been suggested that contacting contaminated soil rather than handling pigs is responsible for *T. gondii* infection in farm workers (Weigel et al., 1999). Children are more likely to be infected on account of frequent soil contact (Dattoli et al., 2011; Stagno et al., 1980). The soil is the primary environmental matrix harboring oocysts, and through the soil, oocysts can be dispersed onto food or into water matrices (Santos et al., 2011; Kreuder et al., 2003). The presence of oocysts in soil heralds the environmental contamination and poses the risk of human and animal infection. However, the environmental contamination by oocyst is largely undefined. Published data had suggested that the concentration of oocysts in the environment was not evenly distributed and that the detection rate of oocysts in soil was low in random areas. However, the soil oocyst contamination rate was shown to be higher in cats' habitats (Gotteland et al., 2014) and their defecation sites (Afonso et al., 2008; Simon et al., 2017). Similarly, a spatial distribution of *T. gondii* oocysts in the soil has been investigated on a farm, in which the soil oocyst contamination rate plummets with distance from cat habitats (Gotteland et al., 2014). However, in our study, we were unable to consistently detect oocyst in the 22 soil samples collected from two cat habitats. The lack of positive sample signifies the absence of oocysts, or the extremely low concentration of oocyst in the soil, which falls below the detection limit of our tests. Our results coincide with the publication by Davis et al., in which no positive sample was detected from the 120 soil samples collected from the University of Hawaii at Mānoa (Davis et al., 2017). However, our study contradicts a report of high oocyst contamination rate (30.3%) in soil; nine thousand soil samples were collected from Harbin, a northeastern city, in China; random locations to represent land use and vegetative cover were chosen for soil collection (Gao et al., 2016).

Sugar floating technique was widely used to isolate oocysts from soil samples to eliminate PCR inhibitors such as humic acid (Lelu et al., 2011). However, the sugar floating process also might lose a certain number of oocysts. A recent study had modified the conventional floating method by underlying a sugar solution with the soil suspension, generating a 10-fold higher yield (Lelu et al., 2011). The detection sensitivity of oocysts in soil depends on 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). With one-year-old or younger oocysts, a minimum detectable concentration of 100-1000 oocysts per gram of soil has been reported (Afonso et al., 2008).

Three cycles of free-thaw were shown to be effective to lyse oocyst for subsequent DNA extraction (Manore et al., 2019). NucleoSpin soil DNA extraction kit (Macherey-Nagel, Cat. No. 740780) outcompeted four other DNA extraction kits in the detection of 50 spiked *T. gondii* oocysts per gram of fecal sample (Herrmann et al., 2011). However, the DNA extraction process also lost DNA as evidenced by a 100-time detection limit difference with or without DNA extraction using lysed oocyst (Gerhold et al., 2015). In our study, attempts were made to detect *T. gondii* directly from lysed oocyst without DNA extraction according to Gerhold et al. However, concerns regarding PCR inhibition (inhibitors in the soil) as well as *T. gondii* contamination strangled the continuation of directly using oocyst lysates for PCR; therefore, DNA extraction was conducted before PCR detection. In our method of PCR-RFLP following sugar floating and DNA extraction, we were able to detect *H. hammondi* DNA from 5 grams of soil experimentally seeded with 105 and 106 *H. hammondi* oocysts.

In addition to using soil contamination to evaluate the contamination rate of the farm, animal infection was investigated. Animals, either domestic animals or wild animals, contract infections via ingesting oocysts disseminated in feeds, water, and soil (Weigel et al., 1995; Lehmann et al., 2003). Oocysts have been found in pig feeds, soils, and water (Weigel et al., 1995). Therefore, the seroprevalence of farm animals can help to assess *T. gondii* contamination on the farm. It has been shown that the infection of farm-associated animals was closely associated with the density of cats and their activities. The infection rate of finishing pigs positively correlates with the number of cats on farms (Weigel et al., 1995). There was a positive correlation between the seroprevalence of wild animals and the proximities of their habitats to pig stiles where cats frequently roamed (Lehmann et al., 2003).

A partition of animal species was observed as wild and domestic rodents were captured in landscapes with distinct proximities to human settlements. Twenty-seven house mice and one brown rat were captured in the areas close to farm buildings, that were hay bale #1 and #2 (Figure 3.1B and 3.4); whereas the wild rodents, including five white-footed mice, seven cotton rats, and one mole, were captured in the woods away from human dwellings (Figure 3.1C and 3.4).

However, one house mouse was caught in the woods away from human households (Figure 3.1C and 3.4).

Several serological tests for *T. gondii* antibody examination have been developed in the laboratory. Sabin-Feldman dye test (DT) used to be the benchmark of the serological test but was replaced due to the safety issues of working with live parasites (Dubey et al., 1988). The indirect fluorescent antibody test is highly specific but is complicated due to the involvement of fluorescent microscope and fluorescent-labeled antibodies (Dubey et al., 1988). ELISA shows high sensitivity and specificity (Gamble et al., 2005) but requires further refinement of procedures (Dubey et al., 1995a). MAT is less sophisticated and shows high sensitivity and specificity, thus being widely used in serological tests (Shaapan et al., 2008; Afonso et al., 2006; Gamble et al., 1999). One caveat of the serological test is that seronegative animals (i.e., rodents and avian) can still harbor parasites. Another drawback of the serological test (i.e., MAT) is that it is difficult to determine the cutoff value for different species without specific testing. The infection rate of *T. gondii* in small rodents has been reported to be low. A large-scale survey revealed that the infection rate in house mice was 0.7% (6/884) (Hejlíček and Literák, 1998). Our MAT results (cutoff: 1:25) showed that the seropositivity of house mice and white-footed mice are 18.5% (5/27) and 40% (2/5), respectively. The results using cutoff at 1:50 were also listed in Table 3.1. With a cutoff at 1:50, the seropositivity in house mice was 3.7% (1/27), and no other rodents trapped was found positive for *T. gondii*. Bioassay in mice or cats is the definitive route for *T. gondii* detection. The bioassay was not conducted in our study because serum samples were either positive at low titer or negative. Taken together, the serological testing of rodent serum sample is not a good indicator of *T. gondii* infection.

It has been known that *T. gondii* infection rate in cattle is low and that cattle are resistant to *T. gondii* infection (Dubey et al., 2005). No positive sample was identified in the thirty heifer sera, which coincided with previous studies. Among the trapped mesopredators, the raccoon had the highest infection rate (70.6%). In addition, one farm cat (50%) was infected, suggesting the previous shedding of oocysts onto the farm. The higher infection rate of *T. gondii* in raccoons is likely because being generalists, they have a broad spectrum of food sources. Further, 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*

(Dubey et al., 2007a). Viable *T. gondii* was isolated from 5 of the 30 seropositive raccoon samples, and 1 of the five skunk samples (Dubey et al., 2007a). In Canada, *T. gondii* was isolated from tissues of 2 raccoons, two feral cats, and one skunk (Dubey et al., 2008b). In our study, the two 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 varies among studies, and very few isolates were obtained from opossums (Jiang et al., 2018). Dubey et al. (1995b) have 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 (Dubey et al., 1995b). Gerhold et al. had reported a 50% (6/12) seroprevalence in opossums from southeastern U.S. (Gerhold et al., 2017). The possible explanations for the current observation are, 1) opossums are resistant to *T. gondii* infection, and or, 2) they are immunotolerant to *T. gondii* in addition to having short life-spans as is the case in house mice.

Conclusions

Further study of *T. gondii* infection rate in these animals is imperative to understand not only the susceptibility of these animals to *T. gondii* infection but also the contamination rate of the environments where these animals inhabit. Taken together, our study showed that mesopredators, especially the raccoon and cat, would be a suitable animal model to monitor *T. gondii* environmental infection. Further research should focus on investigating animal infection as a critical parameter to assess the environmental contamination especially in areas with low soil contamination.

Conflicts of Interest

The authors declare no conflicts of interest.

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References

- Afonso E., Thulliez P., Gilot-Fromont, E., 2006. Transmission of *Toxoplasma gondii* in an urban population of domestic cats (*Felis catus*). International Journal for Parasitology. 36: 1373-1382.
- Afonso, E., Lemoine, M., Poulle, M.L., Ravat, M.C., Romand, S., Thulliez, P., Villena, I., Aubert, D., Rabilloud, M., Riche, B., Gilot-Fromont, E., 2008. Spatial distribution of soil contamination by *Toxoplasma gondii* in relation to cat defecation behavior in an urban area. Int J Parasitol, 38: 1017 – 1023.
- Bahia-Oliveira, L. M. G., Jones J. L., Azevedo-Silva, J., Alves, C. C., Oréface, F., Addiss, D. G., 2003. Highly endemic waterborne toxoplasmosis in north Rio de Janeiro State. Brazil Emerging Infectious Disease. 9: 55-62.
- Bowie, W. R., King, A. S., Werker, D. H., Isaac-Renton, J. L., Bell, A., Eng, S.B., Marion, S.A., 1997. Outbreak of toxoplasmosis associated with municipal drinking water. Lancet, 350: 173 – 177.
- Boyer, K., Hill, D., Mui, E., Wroblewski, K., Karrison, T., Dubey, J.P., Sautter, M., Noble, A.G., Withers, S., Swisher, C., Heydemann, P., Hosten, T., Babiarz, J., Lee, D., Meier, P., McLeod, R., Group., T.S., 2011. Unrecognized ingestion of *Toxoplasma gondii* oocysts leads to congenital toxoplasmosis and causes epidemics in North America. Clin Infect Dis, 53: 1081 – 1089.
- Dabritz, H. A., Miller, M. A., Atwill, E. R., Gardner, I. A., Leutenegger, C. M., Melli, A. C., Conrad, P. A., 2007. Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. JAVMA, 231: 1676 – 1684.
- Dardé, M. L., Villena, I., Pinon, J. M., Beguinot, I., 1998. Severe toxoplasmosis caused by a *Toxoplasma gondii* strain with a new isotype acquired in French Guyana. J. Clin. Microbiol, 36: 324.
- Dattoli, V. C., Veiga, R. V., Cunha, S. S., Pontes-de-Carvalho, L., Barreto, M. L., Alcantara-Neves, N. M., 2011. Oocyst ingestion as an important transmission route of *Toxoplasma gondii* in Brazilian urban children. J Parasitol, 97: 1080– 1084.

- Davis, A.A., Lepczyk, C.A., Haman, K.H., Morden, C.W., Crow, S.E., Fensen, N., Lobr, M.T., 2018. *Toxoplasma gondii* Detection in Fecal Samples from Domestic Cats (*Felis catus*) in Hawaii. *Pac Sci*, 72: 501–511.
- De Moura, L., Bahia-Oliveira, L. M. G., Wada, M. Y., Jones, J. L., Tuboi, S. H., Carmo, E. H., Ramalho, W. M., Camargo, N. J., Trevisan, R., Graça, R. M. T., Silva, A. J., Moura, I., Dubey, J. P., Garret, D. O., 2006. Waterborne toxoplasmosis, Brazil, from field to gene. *Emerg Infect Dis*, 12: 326 – 329.
- Dubey, J. P., 1997a. Bradyzoite-induced murine toxoplasmosis: stage conversion, pathogenesis, and tissue cyst formation in mice fed bradyzoites of different strains of *Toxoplasma gondii*. *Journal of Eukaryotic Microbiology*. 44: 592-602.
- Dubey JP., Speer CA., Shen, S. K., Kwok, O.C., Blixt, J. A., 1997b. Oocyst-induced murine toxoplasmosis: life cycle, pathogenicity, and stage conversion in mice fed *Toxoplasma gondii* oocysts. *Journal of Parasitology*. 83: 870-882.
- Dubey, J. P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. *Equine Vet J*, 19: 337 – 339.
- Dubey, J.P., Beattie, C.P., 1988. *Toxoplasmosis of animals and man*. CRC Press Inc., Boca Raton, FL, USA.
- Dubey, J. P., Thulliez, P., Weigel, R.M., Andrews, C.D., Lind, P., Powell, E.C., 1995a. Sensitivity and specificity of various serological tests for detection of *Toxoplasma gondii* infection in naturally infected sows. *Am J Vet Res*, 56: 1030 – 1036.
- Dubey, J. P., Weigel, R. M., Siegel, A. M., Thulliez, P., Kitron, U. D., Mitchell, M. A., Mannelli, A., Mateus-Pinilla, N. E., Shen, S. K., Kwok, O. C. H., Todd, K. S., 1995b. Sources and reservoirs of *Toxoplasma gondii* infection on 47 swine farms in Illinois. *J Parasitol*. 81: 723–729.
- Dubey, J. P., 1996. Pathogenicity and infectivity of *Toxoplasma gondii* oocysts for rats. *J Parasitol*, 82: 951 – 956.
- Dubey, J. P., 1998. *Toxoplasma gondii* oocyst survival under defined temperatures. *J Parasitol*, 84: 862 – 865.
- Dubey, J. P., 2004. Toxoplasmosis-a waterborne zoonosis. *Veterinary Parasitology*. 126: 57-72.

- Dubey, J. P., Hill, D.E., Jones, J.L., Hightower, A.W., Kirkland, E., Roberts, J.M., Marcet, P.L., Lehmann, T., Vianna, M.C., Miska, K., Sreekumar, C., Kwok, O.C., Shen, S.K., Gamble, H.R., 2005. Prevalence of viable *Toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. *J Parasitol*, 91: 1082 – 1093.
- Dubey, J. P., 2006. Comparative infectivity of oocysts and bradyzoites of *Toxoplasma gondii* for intermediate (mice) and definitive (cats) hosts. *Vet Parasitol*, 140: 69 – 75.
- Dubey, J. P., Sundar, N., Nolden, C. A., Samuel, M. D., Velmurugan, G. V., Bandini, L. A., Kwok, O. C. H., Bodenstein, B., Su, C., 2007. Characterization of *Toxoplasma gondii* from Raccoons (*Procyon lotor*), Coyotes (*Canis latrans*), and Striped Skunks (*Mephitis mephitis*) in Wisconsin Identified Several Atypical Genotypes. *J Parasitol*, 93:1524 – 1527.
- Dubey, J.P., Quirk, T., Pitt, J. A., Sundar, N., Velmurugan, G. V., Kwok, O. C. H., Leclair, D., Hill, R., Su, C., 2008. Isolation and genetic characterization of *Toxoplasma gondii* from raccoons (*Procyon lotor*), cats (*Felis domesticus*), striped skunk (*Mephitis mephitis*), black bear (*Ursus americanus*), and cougar (*Puma concolor*) from Canada. *J Parasitol*, 94: 42 – 45.
- Dubey, J.P., 2010. Toxoplasmosis of animals and humans, 2 Edition. CRC Press, Boca Raton, FL., USA, p336.
- Dubey, J. P., Hill, D. E., Rozeboom, D. W., Rajendran, C., Choudhary, S., Ferreira, L.R., Kwok, O. C. H., Su, C., 2012. High prevalence and genotypes of *Toxoplasma gondii* isolated from organic pigs in northern USA. *Vet Parasitol*, 188: 14-18.
- Frenkel, J. K., Ruiz, A., Chinchilla, M., 1975. Soil survival of *Toxoplasma* oocysts in Kansas and Costa Rica. *Am J Trop Med Hyg*, 24: 439 – 443.
- Gamble, H. R., Brady, R. C., Dubey, J. P., 1999. Prevalence of *Toxoplasma gondii* infection in domestic pigs in the New England states. *Veterinary Parasitology*. 82: 129-136.
- Gamble, H. R., Dubey, J. P., Lambillotte, D. N., 2005. Comparison of a commercial ELISA with the modified agglutination test for detection of *Toxoplasma* infection in the domestic pig. *Veterinary Parasitology*. 128: 177-181.
- Gao, X., Wang, H., Wang, H., Qin, H., Xiao, J., 2016. Land use and soil contamination with *Toxoplasma gondii* oocysts in urban areas. *Sci Total Environ*, 568: 1086–1091.

- Gerhold, R. W., McDougald, L. R., Beckstead, R. B., 2015. A Novel, Simplified Technique to Amplify *Eimeria* (Coccidia: Apicomplexa) DNA from Oocysts. *J Parasitol*, 101: 102–103.
- Gerhold, R.W., Saraf, P., Chapman, A., Zou, X., Hickling, G., Stiver, W.H., Houston, A., Souza, M., Su, C., 2017. *Toxoplasma gondii* seroprevalence and genotype diversity in select wildlife species from the southeastern United States. *Parasit Vectors*, 10: 508.
- Gotteland, C., Gilot-Fromont, E., Aubert, D., Poulle, M. L., Dupuis, E., Dardé, M. L., Forin-Wiart, M. A., Rabilloud, M., Riche, B., Villena, I., 2014. Spatial distribution of *Toxoplasma gondii* oocysts in soil in a rural area: influence of cats and land use. *Vet Parasitol*, 205: 629 – 637.
- Guo, M., Mishra, A., Buchanan, R.L., Dubey, J.P., Hill, D.E., Gamble, H.R., Jones, J.L., Pradhan, A.K., 2016. A systematic meta-analysis of *Toxoplasma gondii* prevalence in food animals in the United States. *Foodborne Path Dis*, 13: 109 – 118.
- Hejlíček, K., Literák, I., 1998. Long-term study of *Toxoplasma gondii* prevalence in small mammals (*Insectivora* and *Rodentia*). *Folia Zool*, 47: 93 – 101.
- Herrmann, D. C., Maksimov, A., Pantchev, N., Vrhovec, M. G., Conraths, F. J., Schares, G., 2011. Comparison of different commercial DNA extraction kits to detect *Toxoplasma gondii* oocysts in cat faeces. *Berl Munch Tierarztl Wochenschr*, 124: 497–502.
- Jiang, T., Shwab, E. K., Martin, R. M., Gerhold, R. W., Rosenthal, B. M., Dubey, J. P., Su, C., 2018. A partition of *Toxoplasma gondii* genotypes across spatial gradients and among host species, and decreased parasite diversity towards areas of human settlement in North America. *Int J Parasitol*, 48: 611–619.
- Jones, J. L., Lopez, A., Wilson, M., Schulkin, J., Gibbs, R., 2001. Congenital toxoplasmosis: a review. *Obstet Gynecol Surv*, 56: 296 – 305.
- Jones, J. L., Dubey, J. P., 2010. Waterborne toxoplasmosis – Recent developments. *Exp Parasitol*, 124: 10 – 25.
- Jones, J. L., Kruszon-Moran, D., Rivera, H. N., Price, C., Wilkins, P. P., 2014. *Toxoplasma gondii* seroprevalence in the United States 2009–2010 and comparison with the past two decades. *Am J Trop Med Hyg*, 90: 1135 – 1139.

- Kreuder, C., Miller, M. A., Jessup, D. A., Lowenstine, L. J., Harris, M. D., Ames, J. A., Carpenter, T. E., Conrad, P. A., Mazet, J. A., 2003. Patterns of mortality in southern sea otters from 1998-2001. *J Wildl Dis*, 39: 495 – 509.
- Lehmann, T., Graham, D. H., Dahl, E., Sreekumar, C., Launer, F., Corn, J. L., Gamble, H. R., Dubey, J. P., 2003. Transmission dynamics of *Toxoplasma gondii* on a pig farm. *Infect Genet Evol*, 3: 135 – 141.
- Lelu, M., Gilot-Fromont, E., Aubert, D., Richaume, A., Afonso, E., Dupuis, E., Gotteland, C., Marnef, F., Poulle, M.L., Dumètre, A., Thulliez, P., Dardé, M.L., Villena, I., 2011. Development of a sensitive method for *Toxoplasma gondii* oocyst extraction in soil. *Vet Parasitol*, 183: 59 – 67.
- Lindsay, D. S., Dubey, J. P., 2009. Long-term survival of *Toxoplasma gondii* sporulated oocysts in seawater. *Journal of Parasitology*. 95: 1019-1020.
- Manore, A. J. W., Harper, S. L., Aguilar, B., Weese, J. S., Shapiro, K., 2019. Comparison of freeze-thaw cycles for nucleic acid extraction and molecular detection of *Cryptosporidium parvum* and *Toxoplasma gondii* oocysts in environmental matrices. *Journal of Microbiological Methods*, 156: 1-4
- Moura, L. D., Bahia-Oliveira, L. M. G., Wada, M.Y., Jones, J.L., Tuboi, S.H., Carmo, E.H., Ramalho, W.M., Camargo, N.J., Trevisan, R., Graça, R.M., da Silva, A.J., Moura, I., Dubey, J.P., Garrett, D.O., 2006. Waterborne toxoplasmosis, Brazil, from field to gene. *Emerging Infectious Diseases*. 12: 326-329.
- Munoz-Zanzi, C. A., Fry, P., Lesina, B., Hill, D., 2010. *Toxoplasma gondii* oocyst-specific antibodies and source of infection. *Emerging Infectious Diseases*. 16: 1591-1593.
- Munoz-Zanzi, C. A., Tamayo, R., Balboa, J., Hill, D., 2012. Detection of oocyst-associated toxoplasmosis in swine from southern Chile. *Zoonoses Public Health*, 59: 389–392
- Parson, W., Pegoraro, K., Niederstätter, H., Föger, M., Steinlechner, M., 2000. Species identification by means of the cytochrome b gene. *Int J Legal Med*, 114: 23 – 28.
- Santos, P. S., Albuquerque, G. R., da Silva, V. M., Martin, A. R., Marvulo, M. F., Souza, S. L., Ragozo, A. M., Nascimento, C. C., Gennari, S. M., Dubey, J. P., Silva, J. C., 2011. Seroprevalence of *Toxoplasma gondii* in free-living Amazon River dolphins from central Amazon, Brazil. *Vet Parasitol*, 183: 171 – 173.

- Shaapan, R. M., El-Nawawi, F. A., Tawfik, M.A., 2008. Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sheep. *Veterinary Parasitology*, 153: 359-362.
- Silva, R. C, Su, C., 2009. First identification of *sarcocystis tenella* by PCR in naturally infected sheep from Brazil. *Vet Parasitol*, 165: 332 – 336.
- Simon, J.A., Kurdzielewicz, S., Jeannot, E., Dupuis, E., Marnef, F., Aubert, D., Villena, I., Poulle, M.L., 2017. Spatial distribution of soil contamination by *Toxoplasma gondii* in relation to cat defecation behavior in an urban area. *Int J Parasitol*, 47: 357-367.
- Stagno, S., Dykes, A. C., Amos, C. S., Head, R. A., Juranek, D. D., Walls, K., 1980. An outbreak of toxoplasmosis linked to cats. *Pediatrics*, 65: 706 – 712.
- VanWormer, E., Miller, M. A., Conrad, P. A., Grigg, M. E., Rejmanek, D., Carpenter, T. E., Mazet, J. A. K., 2014. Using molecular epidemiology to track *Toxoplasma gondii* from terrestrial carnivores to marine hosts: implications for public health and conservation. *PLoS Negl Trop Dis*, 8: e2852.
- Wallace, G. D., 1969. Serologic and epidemiologic observations on Toxoplasmosis on three pacific atolls. *Am J Epidemiol*, 90: 103 – 111.
- Weigel, R. M., Dubey, J. P., Siegel, A. M., Kitron, U. D., Mannelli, A., Mitchell, M. A., Mateus-Pinilla, N. E., Thulliez, P., Shen, S. K., Kwok, O. C., Todd, K. S., 1995. Risk factors for transmission of *Toxoplasma gondii* on swine farms in Illinois. *J Parasitol*, 81: 735 – 741.
- Weigel, R. M., Dubey, J. P., Dyer, D., Siegel, A. M., 1999. Risk factors for infection with *Toxoplasma gondii* for residents and workers on swine farms in Illinois, USA. *Am J Trop Med Hyg*, 60: 793 – 798.

Chapter 3 Appendix

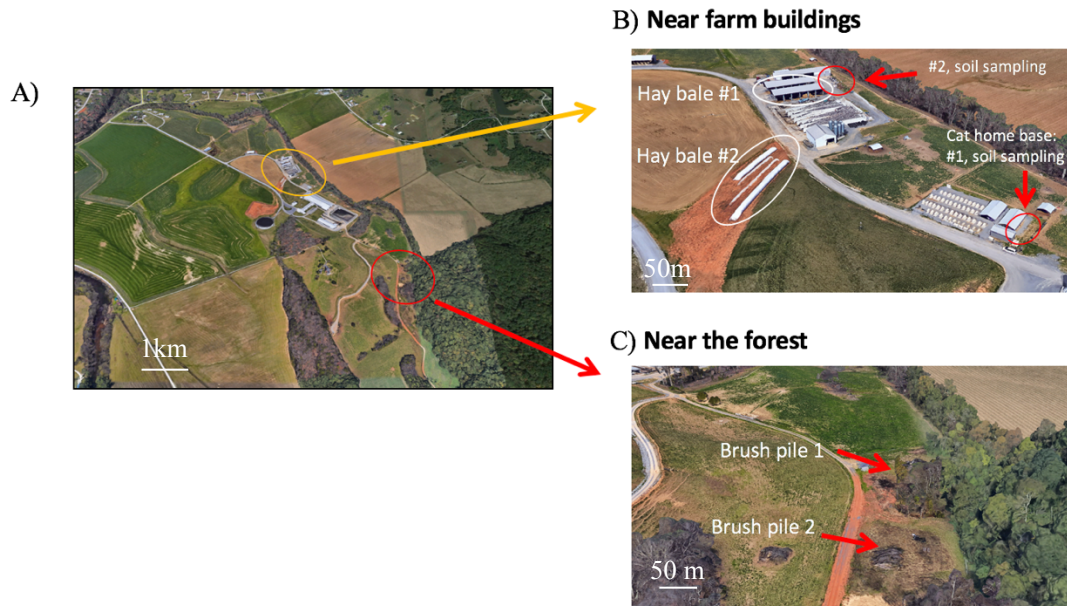


Figure 3. 1 Soil sampling and animal trapping locations on the Little River farm. A) Two trapping locations, near farm buildings and near the forest, were marked in yellow and red circles with different proximities to human settlement. B) Two soil sampling locations were marked in red circles; two trapping locations were marked in white circles. C) The two major trapping locations were marked with red arrows.

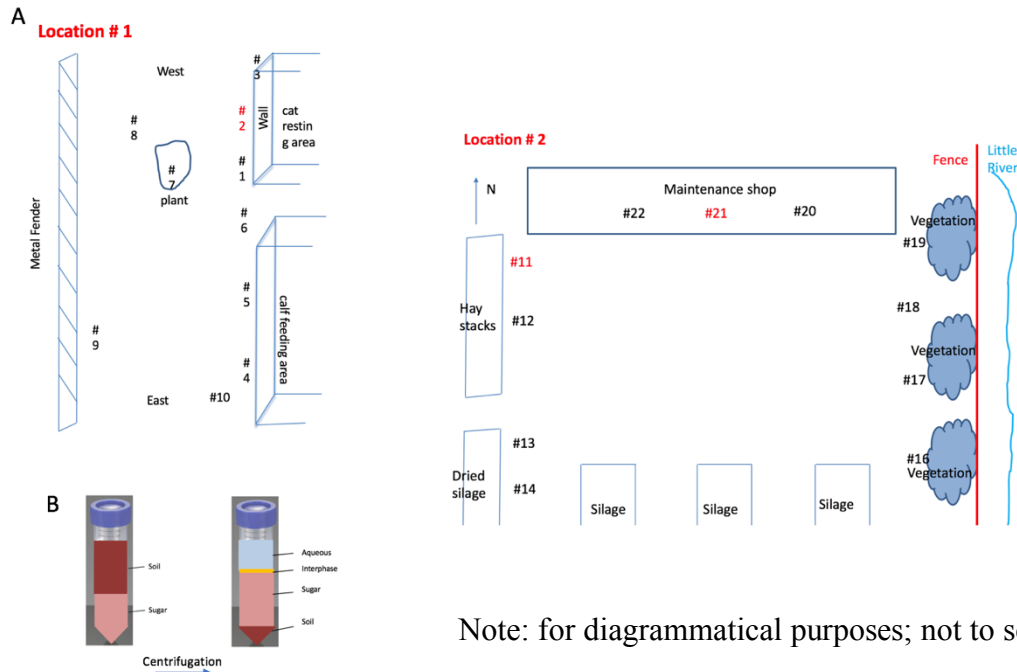


Figure 3. 2 Two locations for soil sample collection. A) location #1 is the calf raising area where cats were found feeding, resting and free-roaming. Location #2 is the maintenance shop and silage storage area where cats were seen free-roaming. B) A schematic presentation of the sugar floating for oocyst enrichment. Stages of before and after centrifugation were shown.

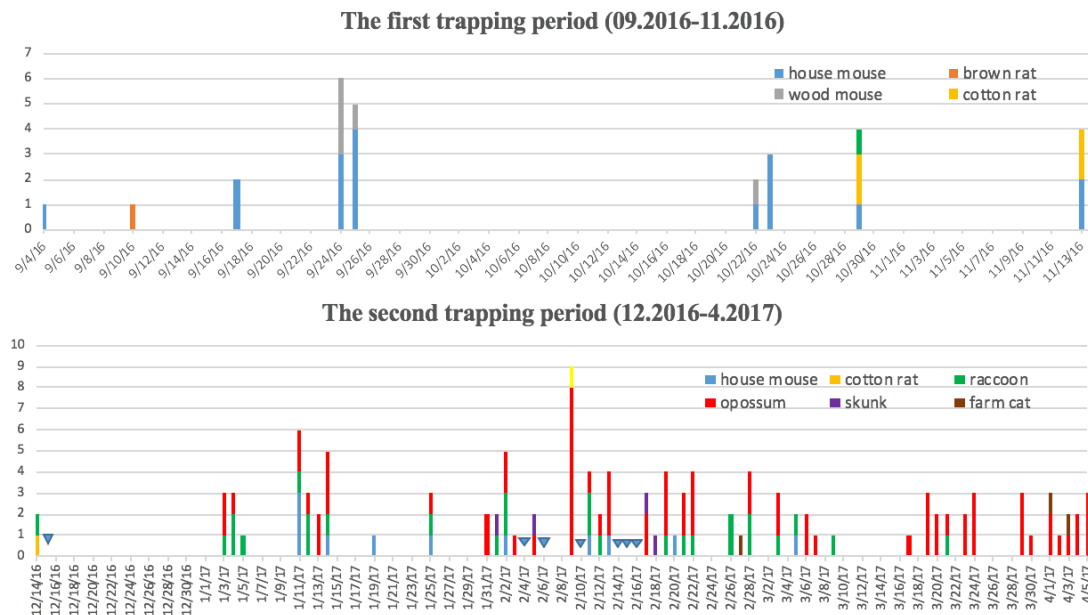


Figure 3. 3 Trapping dates and statistics. (a). The first trapping period (09/2016 – 11/2016). Trapping only targeted small rodents. Sherman traps (50-90) were set on the farm property in the evening and examined the following morning. Sample from one trapped house mouse was not obtained due to handling error (b). The second trapping period (12/2016 – 4/2017). Trapping targeted small rodents and mesopredators during this period, although rodent trapping was terminated after 3/5/2017. Sherman traps (50-90) and Tomahawk traps (10) were set on the farm property in the evening and examined in the following morning. Animals in cages were counted regardless of recapture or the success of blood sampling. Trips with no animal captured were marked with inverted blue triangles.

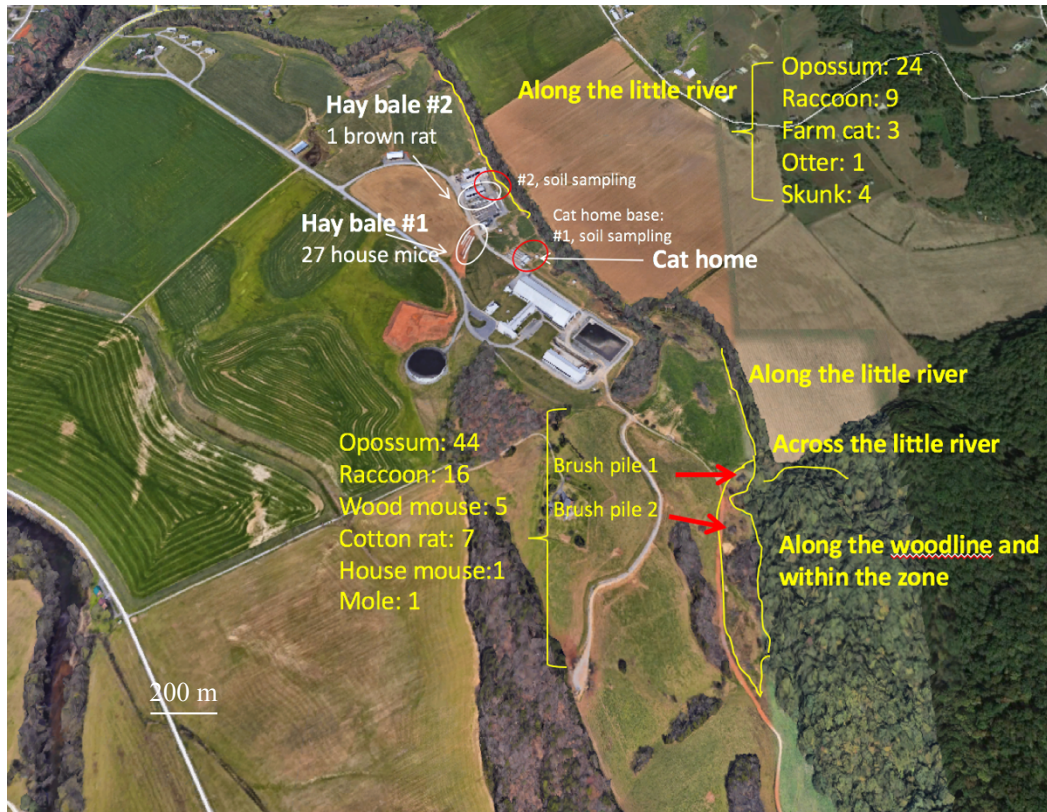


Figure 3. 4 Locations for animal trapping as well as soil sampling on the farm. House mice were trapped in hay bale #1 and #2 (white circles); wild rodents and meso-predators were trapped along/across the river, along the woods, and around two brush piles. Soil was collected from two cat habitats (red circles).

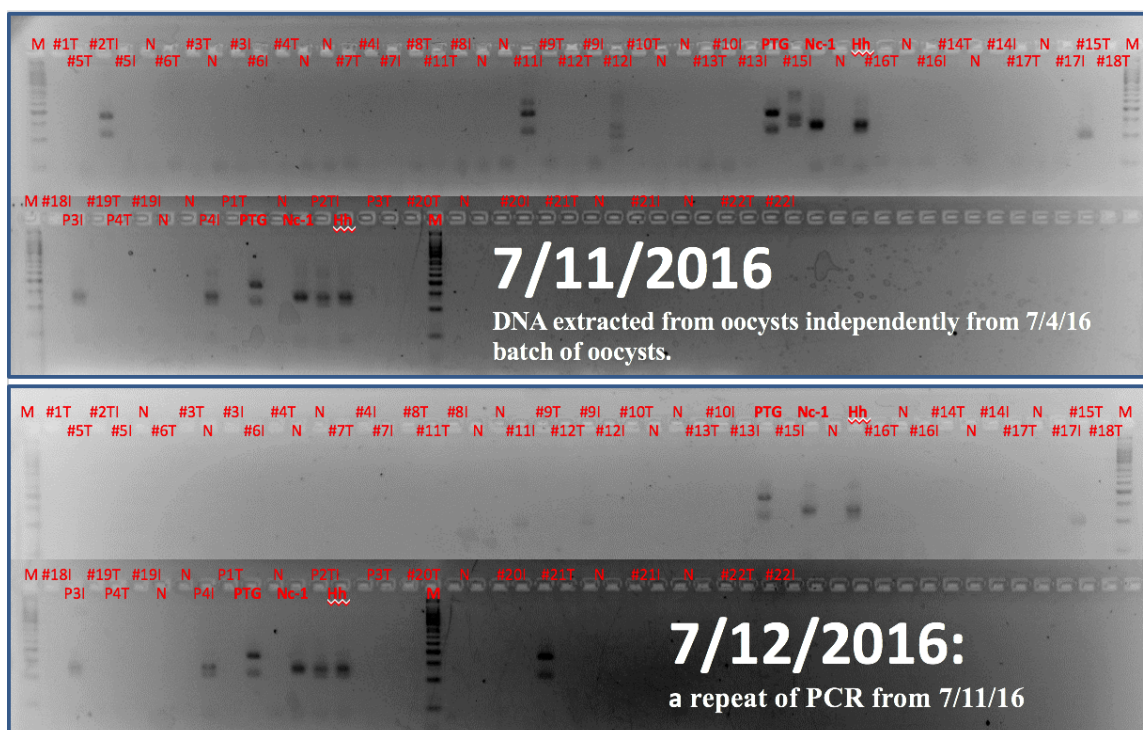


Figure 3. 5 Soil oocyst detection via PCR-RFLP. Either the top aqueous layer (T) or the interphase(I) or both (TI) were collected. PCR-RFLP was performed after DNA extraction. N denotes negative control. M denotes marker. Positive controls are DNA from *T. gondii* (PTG), *N. caninum* (Nc-1), and *H. hammondi* (Hh). 10^5 (in the case of P1T, P3I, P3T) or 10^6 (in the case of P2TI, P4I, P4T) *H. hammondi* oocysts were spiked in 5 grams of soil samples to test the efficiency of the detection method.



Figure 3. 6 The trapping locations of meso-predators on the farm based on GPS coordinates. Each teardrop is one GPS coordinate. At each GPS coordinate, total number of animals captured or recaptured were counted; animal species and ID (ear tag No.) were also indicated. Opossum #43 was marked in red to show its relatively wide home range.

Table 3. 1 Seroprevalence of *Toxoplasma gondii* in captured animals on the farm.

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)	3.7% (1/27)
Brown rat	1	1	0	0	0	0	0	0	0	0	0	0	0
White-footed mouse	5	3	2	0	0	0	0	0	0	0	0	40% (2/5)	0
Cotton rat	6	6	0	0	0	0	0	0	0	0	0	0	0
Mole	1	1	0	0	0	0	0	0	0	0	0	0	0
Raccoon	17	5	3	0	2	0	2	1	2	1	1	70.6% (12/17)	52.9% (9/17)
Opossum	16	16	0	0	0	0	0	0	0	0	0	0	0
Farm cat	2	1	0	0	0	0	0	0	1	0	0	50% (1/2)	50% (1/2)
Skunk	2	2	0	0	0	0	0	0	0	0	0	0	0

Table 3. 2 PCR-RFLP results of 22 soil samples (first five grams of soil) collected from the little river farm.

Sample ID	The first 5g of soil with DNA extraction ^a			
	Test #1 (7/11/16)		Repeat #1 (7/12/16)	
	Top	Interphase	Top	Interphase
#1 ^c	N ^b	Not done	N	No done
#2 ^d	<i>T. gondii</i>		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	<i>T. gondii</i>	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	<i>T. gondii</i>	N
#22	N	N	N	N
<i>T. gondii</i>	<i>T. gondii</i>		<i>T. gondii</i>	
<i>H. hammondi</i>	<i>H. hammondi</i>		<i>H. hammondi</i>	
<i>N. caninum</i>	<i>N. caninum</i>		<i>N. caninum</i>	
Negative controls	Total 18 negative controls were included, and all were negative		Total 18 negative controls were included, and all were negative	
Soil sample spiked with <i>H. hammondi</i>	<i>P1</i>	<i>N</i>	<i>P1</i>	<i>N</i>
	<i>P2</i>	<i>H. hammondi</i>	<i>P2</i>	<i>H. hammondi</i>
	<i>P3T</i>	<i>N</i>	<i>P3T</i>	<i>N</i>
	<i>P3I</i>	<i>H. hammondi</i>	<i>P3I</i>	<i>H. hammondi</i>
	<i>P4T</i>	<i>N</i>	<i>P4T</i>	<i>N</i>
	<i>P4I</i>	<i>H. hammondi</i>	<i>P4I</i>	<i>H. hammondi</i>

^aDNA was extracted from the soil extracts and then used for PCR-RFLP. P1 and P3 were positive controls in which 10⁵ *H. hammondi* oocysts were spiked in 5 grams of soil; P2, P4 were positive controls in which 10⁶ *H. hammondi* oocysts were spiked in 5 grams of soil.

^bN: negative result.

^cOnly top layer was collected for this sample

^dTop and interphase were combined for this sample

Table 3. 3 PCR-RFLP results of 22 soil samples (another five or ten grams of soil) collected from the little river farm.

Sample ID	^a 5g of soil-7/14/2016		^b 10g of soil-9/10/2016	
	Test #1	Repeat #1	Test #1	Repeat #1
#1	Not done	Not done	N	<i>T. gondii</i>
#2	Not done	Not done	N	N
#3	^c N	N	<i>H. hammondi</i>	<i>H. hammondi</i>
#4	Not done	Not done	N	N
#5	N	N	N	<i>H. hammondi</i>
#6	Unknown band pattern	N	<i>H. hammondi</i>	N
#7	N	N	N	N
#8	N	N	<i>H. hammondi</i>	<i>H. hammondi</i>
#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	<i>T. gondii</i>
#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	<i>T. gondii</i>
#22	N	N	Unknown band pattern	<i>T. gondii</i>
<i>T. gondii</i>	<i>T. gondii</i>		<i>T. gondii</i>	
<i>H. hammondi</i>	<i>H. hammondi</i>		<i>H. hammondi</i>	
<i>N. caninum</i>	<i>N. caninum</i>		<i>N. caninum</i>	
Negative controls	Total 15 negative controls were included, and all were negative		Total 18 negative controls were included; one was positive with <i>T. gondii</i> , and one was positive with <i>H. hammondi</i>	
^d Soil sample spiked with <i>H. hammondi</i>	P16-1	<i>H. hammondi</i>	#23-1	N
	P16-2	<i>H. hammondi</i>		
	P19-1	<i>H. hammondi</i>	#23-2	N
	P19-2	<i>H. hammondi</i>	#24-1	<i>H. hammondi</i>
	P20-1	<i>H. hammondi</i>	#24-2	<i>H. hammondi</i>
	P20-2	<i>H. hammondi</i>		

^{a,b}DNA was extracted from another 5 grams or 10 grams of soil from each sample.

^bTo test if double the soil quantity would yield better results, ten grams of soil sample was subjected to sugar flotation, DNA extraction, and PCR-RFLP.

^cN denotes negative result

^dSample 23 and 24 were positive controls in which 10^6 *H. hammondi* oocysts were spiked in 10 grams of soil.

Table 3. 4 PCR-RFLP results of 29 rodent tissue samples collected from the little river farm.

^a Sample ID	^c The first DNA extraction (11/18/2016)			^c The second DNA extraction (12/2/2016)	
	Test #1 (11/28/2016)	Repeat #1 (12/6/2016)	Repeat #2 (12/6/2016)	Test #1 (12/6/2016)	Repeat #1 (12/6/2016)
925HM2	<i>T. gondii</i>	Unknown band pattern	Unknown band pattern	^b N	<i>T. gondii</i>
1023HM1	<i>H. hammondi</i>	<i>H. hammondi</i>	<i>H. hammondi</i>	<i>H. hammondi</i>	<i>H. hammondi</i>
1029HM1	<i>T. gondii</i>	<i>T. gondii</i>	<i>T. gondii</i>	N	N
1029CR1	<i>T. gondii</i>	N	<i>T. gondii</i>	N	N
1113HM1	<i>T. gondii</i>	N	N	N	N
1113HM2	<i>Sarcocystis</i> spp	<i>Sarcocystis</i> spp	<i>Sarcocystis</i> spp	<i>Sarcocystis</i> spp	<i>Sarcocystis</i> 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 positive for <i>T. gondii</i>			
<i>T. gondii</i>	<i>T. gondii</i>			<i>T. gondii</i>	
<i>H. hammondi</i>	<i>H. hammondi</i>			<i>H. hammondi</i>	
<i>N. caninum</i>	<i>N. caninum</i>			<i>N. caninum</i>	

^a Twenty-nine rodent tissues were included for *T. gondii* detection using PCR-RFLP. DNA was extracted from tissue homogenate. Samples that were initially tested negative were not included in this table; these twenty-two samples included 94HM1, 910rat, 925HM3, 925HM4, 917HM1, 924WM1, 917HM2, 924WM2, 924HM1, 924HM2, 924WM3, 924HM3, 925HM1, 1023HM2, 1023HM3, 925WM1, 1022HM1, 1022WM1, 1029CR2, 1029mole, 1113CR2, 1114CR1. The seven samples that were tested positive were subjected to further testing as listed in this table.

^b N: negative result

^d DNA was extracted from two equal amount of tissue homogenates on different dates

CHAPTER 4

**GENOTYPE IDENTIFICATION OF *TOXOPLASMA GONDII* IN
MACROPODS FROM A ZOOLOGICAL PARK IN FLORIDA, USA**

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My contribution to the work presented in this chapter was conducting experiments (blood antibody test, PCR-RFLP genotyping, and mouse bioassay), collecting data, and writing the manuscript draft. Maria Spriggs collected the samples and rewrote the manuscript. Nancy Stedman helped with the histopathology. Natalia López-Orozco helped with PCR-RFLP. The rest of the co-authors contributed to the manuscript revision and coordinating and securing samples.

Abstract

Toxoplasma gondii is a common cause of mortality in macropods, but there are limited reports of the genetic characterization of *T. gondii* infecting captive macropods in North America. A novel genotype, ToxoDB PCR-RFLP genotype #263, was reported from six wallabies at a zoological facility in Virginia, USA, prompting an investigation into the genotypes infecting macropods at a zoological park in Florida, USA. Cardiac samples from an agile wallaby (n = 1), red kangaroos (n = 8), red-necked wallaby (n = 1), and a tammar wallaby (n = 1) that died between 2014 and 2018 were collected. All 11 cases were confirmed to be infected with *T. gondii* via serology, histopathology, or parasite isolation. A mouse bioassay was used for parasite isolation, and isolated parasites were subjected to multilocus PCR-RFLP genotyping of *T. gondii*. Two cases of toxoplasmosis were identified as the reported novel genotype, ToxoDB PCR-RFLP genotype #263, but no common source of exposure could be identified between the two zoos. In addition, five cases were identified as ToxoDB PCR-RFLP genotype #2 (type III strain), and four cases were identified as ToxoDB PCR-RFLP genotype #216, a genotype that has been previously reported in wildlife in North America. In addition, a freckled duck that died of toxoplasmosis at the same park was genotyped as genotype #2. These results suggest that at least three distinct sources and transmission events occurred at this park between 2014 and 2018. Genotype identification can aid in understanding the sources of infective oocysts in a geographic area and determining whether multiple mortalities are due to single or multiple exposure events. Further research is needed to determine whether different genotypes vary in pathogenicity in macropods and to identify effective preventive measures for captive macropods.

Keywords: acute toxoplasmosis, genotype, macropod, *Toxoplasma gondii*

Introduction

Toxoplasma gondii is a zoonotic intracellular parasite with a worldwide distribution and broad host spectrum, including mammals and birds (Dubey, 2010). Felidae are the sole definitive host, with the ability to discharge millions of oocysts into the environment (Dubey et al., 2011). Host infection occurs through ingestion of oocysts dispersed in the environment, consumption of meat containing parasite tissue cysts, or vertical transmission during pregnancy (Dubey et al., 2011). Infection in immunocompetent animals is generally asymptomatic but can cause illness, including respiratory or neurologic clinical signs, decreased reproductive success, and/or mortality (Dubey, 2010). Latent asymptomatic infection with subsequent recrudescence can occur as well (Dubey, 2010; Portas, 2010). Macropods, including kangaroos and wallabies, are particularly susceptible to infection and death in North American zoos due to toxoplasmosis (Guthrie et al., 2017; Portas, 2010).

There are over 180 reported genotypes of *T. gondii*, and different genetic lineages exhibit distinct geographic distribution (Shwab et al., 2014). In North America, the most commonly reported lineages include ToxoDB PCR-RFLP genotypes #1 and #3 (collectively known as type II), genotype #2 (known as type III) in domestic animals, and genotypes #4 and #5 (collectively known as type 12) in wildlife (Dubey et al., 2011; Schwab et al., 2014). Certain genotypes are associated with more severe clinical disease in humans, but the relationship between *T. gondii* genotype and clinical disease in animals, including macropods, is unknown (Calero-Bernal et al., 2015; Dubey et al., 2013; Su et al., 2012). A novel genotype, ToxoDB genotype #263, was reported in six wallabies that died at the Virginia Zoo (Norfolk, Virginia, USA) in 2014 (Guthrie et al., 2017). Three other wallabies from this zoo were infected with the genotype #4 strain, which was previously reported in North American wildlife (Guthrie et al., 2017). By identifying the *T. gondii* genotypes infecting captive macropods at a particular facility, one may better infer the number of sources of infective oocysts during toxoplasmosis outbreaks and the pathogenicity of different strains. Here, we attempted to isolate and/or genotype *T. gondii* from eleven fatal toxoplasmosis cases from a zoological facility in Florida, USA.

Materials and Methods

Animals, collection of samples, histopathology, and medical record review

Between 2014 and 2018, serum and fresh cardiac muscles were collected from a subset of macropods that died due to suspected or known toxoplasmosis at a single zoological facility in Florida, USA for *T. gondii* isolation and characterization. Species from which parasites were isolated included red kangaroo (*Macropus rufus*, n = 4), agile wallaby (*Macropus agilis*, n = 1), and tammar wallaby (*Macropus eugenii*, n = 1). Cases were selected for parasite isolation opportunistically based upon the availability of personnel to complete the sampling and laboratory mice for the bioassay isolation. The combined frozen heart and lung were used from five additional cases for PCR-RFLP genotyping, including red kangaroo (n = 4) and red-necked wallaby (*Macropus rufogriseus*, n = 1). Additionally, cardiac muscles from a freckled duck (*Stictonetta naevosa*, n = 1) that died of toxoplasmosis at the same park was genotyped following the same methods. The same veterinary pathologist performed a gross necropsy for each case. Tissues were preserved in 10% neutral buffered formalin and stained with hematoxylin and eosin (H & E) for histopathologic examination. Fresh cardiac and serum samples were refrigerated at 1.7-3.3°C, and frozen samples were stored at -20 °C. Samples were shipped overnight to the Department of Microbiology at the University of Tennessee (Knoxville, Tennessee, USA) for *T. gondii* detection, isolation, and genetic characterization.

Medical and necropsy records of macropods that died between 2014 and 2018 at a zoological facility in Florida, USA were retrospectively reviewed for cases of toxoplasmosis, and information was collected regarding the clinical signs and treatment of the 11 cases reported herein (Table 1).

Serologic testing

Serum was collected ante- or postmortem from 7 of the 11 cases. Competitive enzyme-linked fluorescence assay (ELFA, n = 3) or modified agglutination test (MAT, n = 4) was used to detect *T. gondii* antibodies using previously described methods. For ELFA, a positive test value threshold of <1.6 was used (University of Tennessee Veterinary Medical Center Parasitology, 2019). For

MAT, a positive dilution cutoff value of 1:25 was used by the method described previously (Dubey and Desmonts, 1987).

Ethical statement

All procedures of mouse experiment were approved by the Institutional Animal Care and Use Committee (University of Tennessee, IACUC protocol number 1419).

Bioassay to isolate *T. gondii* in mice

Bioassays were conducted in accordance with a previously described method (Dubey, 2010). Briefly, five grams of heart tissues or heart and lung combined were cut into small pieces and transferred into a 20 ml DT-20 dispenser tube (IKA ULTRA-TURRAX, Sigma-Aldrich, USA). Fifteen milliliters of saline (0.85% NaCl) were added to the tube. The tissues were homogenized using the IKA ULTRA TURRAX Tube Drive homogenizer (IKA ULTRA-TURRAX, Sigma-Aldrich, USA). One hundred microliters of homogenized tissue (approximately 100 mg) were taken for DNA extraction using DNeasy Blood and Tissue kit (Qiagen; Cat No. 69504). The homogenized tissues were digested with acid pepsin (Sigma, # P7000-25G) at 37°C for 40-60 min. The digested tissues were gauze-filtered and washed by PBS and resuspended in PBS containing 10mg/ml gentamicin. One milliliter of prepared tissue in PBS, containing 10 µg of gentamicin, was injected intraperitoneal to each of three outbred CD-1 mice to isolate the parasites. Dexamethasone phosphate (15 µg/ml) was added in the drinking water to suppress mouse immune response for better parasite isolation. Mice showing symptoms of *T. gondii* infection (rough fur, lethargy) between day 6 and day 12 were euthanized. Five milliliters of PBS with gentamicin were injected into the peritoneal cavity, and the exudates were collected. One milliliter of peritoneal lavage was spun down and resuspended in 100 µl PBS and subjected to DNA extraction using the Qiagen kit above. DNA was eluted in 100 µl of buffer AE.

Genotyping of *T. gondii* samples

DNA samples directly extracted from heart tissue or combined heart and lung tissues or from peritoneal lavage of infected mice were subjected to PCR-RFLP typing using 10 genetic markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) (Su et al., 2010).

Results

Between 2014 and 2018, there were 27 macropod mortalities identified in which toxoplasmosis was suspected based on histopathology (Fig. 4.1). Species affected included agile wallaby (n = 7), red kangaroo (n = 15), red-necked wallaby (n = 3), tammar wallaby (n = 1), and western gray kangaroo (*Macropus fuliginosus*, n = 1). Of these, 15 were acute or peracute cases of toxoplasmosis, and 12 had another type of concurrent disease or chronic visual impairment possibly due to ocular toxoplasmosis and were euthanized. Mortality dates for the animals described in this reported are as follows: May 2014 (red kangaroo, n = 1; agile wallaby, n = 1), July 2015 (red-necked wallaby, n = 1), September 2015 (red kangaroo, n = 1), April 2017 (red kangaroo, n = 1), September 2017 (red kangaroo, n = 1), October 2017 (tammar wallaby, n = 1; red kangaroo, n = 1), December 2017 (red kangaroo, n = 1), February 2018 (red kangaroo, n = 1) and May 2018 (red kangaroo, n = 1) (Fig. 4.1). Clinical signs, treatment, serology results, and necropsy findings from these 11 cases are summarized in Table 1. Peracute or acute death occurred in 9 of the cases, while two of the cases had clinical signs for 3-4 days in duration. Case #6 was euthanized due to a deteriorating clinical condition. Clinical signs varied in each case but included lethargy, inappetence, tachypnea, recumbency, and seizures. Various treatments were attempted in five cases, including subcutaneous fluid therapy, antibiotics, antiprotozoals, and a diuretic. Six of the seven cases tested were seropositive at the time of death. MAT titers ranged from 1:50 to ≥ 8192 . The agile wallaby in case 2 had a peracute mortality and was seronegative at the time of death.

Toxoplasmosis was determined to be the cause of death in 10 of the cases. Concurrent conditions were identified in some cases, including gastrointestinal ulceration with intralesional *T. gondii* and *Entamoeba* (case 1), chronic ascending cholecystitis (case 2), gastric nematodiasis (cases 3, 4, 10), gastric foreign material (case 4), minimal otitis externa (cases 7 and 10), jugular hematoma (case 8), entamoebiasis of proximal saccular stomach (case 10), and bacterial dermatitis of the pouch (case 10). In case 6, methemoglobinemia was determined to be the cause of death, as it was severe enough to account for the clinical deterioration necessitating euthanasia. Although toxoplasmosis was also present, methemoglobinemia was considered the more severe finding.

Heart tissues from cases 1, 2, 6, 8, and 9 were used for bioassay to isolate *T. gondii*. Three isolates were obtained from three red kangaroos (cases, 1, 6, 8) and designated as TgKgrFL1, TgKgrFL2, and TgKgrFL3 (Table 2). Mice inoculated with heart tissue of case 2 (agile wallaby) did not show obvious symptoms by day 14 post-infection. In this case, *T. gondii* was not isolated, and genotyping was performed using heart tissue DNA. Mice inoculated with tissue of case 9 (red kangaroo) were severely sick on day 2 post-infection, possibly due to toxic products from degradation of the tissue (20 days post collection) and were euthanized. Heart tissue DNA was subsequently used for genotyping of case 9. Case 8 was genotyped using both parasite isolate and heart tissue DNA. The remaining cases were not bioassayed at the time of death, and genotyping was performed using either fresh or frozen heart tissue DNA (Table 2).

Genotyping results revealed ToxoDB genotype #263 (cases 1 and 2), genotype #2 (cases 3, 4, 5, 6, 11), and genotype #216 (cases 7, 8, 9, 10) (Table 2). Additionally, a freckled duck from an aviary adjacent to the macropod exhibit died of toxoplasmosis in July 2017 and was confirmed to be genotype #2 using tissue DNA from combined heart and lung.

Discussion

Toxoplasmosis is commonly reported in captive macropods (Basso et al., 2007; Dubey and Crutchley, 2008; Guthrie et al., 2017), and the outbreak occurred from May-June 2014 in this report is unremarkable. Acute toxoplasmosis progresses rapidly, and sudden death can occur (Miller et al., 1992, Patton et al., 1986). Treatment was initiated for toxoplasmosis when possible based on clinical signs and included antiprotozoals, antibiotics, subcutaneous fluids, and diuretic for treatment of pulmonary edema. Treatment varied depending on the presentation of the animal and preference of the attending veterinarian. In one report, a trimethoprim and sulfadiazine combination was used to treat acute toxoplasmosis in wallabies with marginal or no effect (Miller et al., 1992). Dubey and Crutchley (2008) have reported successful treatment of toxoplasmosis in four Bennett's wallabies using atovaquone. Atovaquone has been shown to have a synergistic effect when administered together with pyrimethamine, clindamycin, or sulfadiazine (Dubey and Crutchley, 2008). Guthrie et al. (2017) reported the death in macropods due to toxoplasmosis,

despite treatment with atovaquone. The effect of treatment varies from case to case (Jensen et al., 1985; Guthrie et al., 2017) but is generally deemed unrewarding (Portas, 2010).

Serologic testing was not clinically useful for antemortem diagnosis in most cases at this facility due to the rapid progression of clinical signs and death and possibility for a negative result as seen in case 2 likely due to insufficient time for antibody production or a positive titer due to chronic or latent infection (Basso et al., 2007; Mayberry et al., 2014). If the clinical course were long enough to allow repeated testing, the demonstration of a rising titer would support the diagnosis of toxoplasmosis (Miller et al., 2003).

We confirm the *T. gondii* genotypes #263, #2, and #216 from the 11 cases of toxoplasmosis from a zoological park in Florida. This is the first report of genotype #216 in captive macropods. Although little is known about the genetic characterization of *T. gondii* infection of wild macropods, there have been several reports of the parasite's genotyping from captive macropods. Genotype #263 (which does not belong to any clonal type) and #4 (clonal type 12) were responsible for the death of six and three red-necked wallabies in Virginia Zoo, respectively (Guthrie et al., 2017). Genotype #263 has only been found in the Virginia wallabies and in cases 1 and 2 reported here to date. It is possible that some or all of the other mortalities that occurred during the May-June 2014 outbreak, along with cases 1 and 2, were due to this same genotype. It is interesting to note that this strain was associated with a larger number of mortalities than typical at this zoo in Florida as well as at the Virginia Zoo as reported by Guthrie et al. (2017). It is unknown if this is coincidental or due to increased pathogenicity of genotype #263 in macropods. In humans, atypical genotypes have been reported to cause severe toxoplasmosis in patients without immune compromise (Dubey et al., 2011). Because genotype #263 is only reported from macropods from these two facilities, the potential for a common source exposure was investigated. None of the affected animals were ever housed at the same facility, and the food sources (both concentrate ration and hay) were confirmed to be different at each facility (M. Spriggs, pers. comm.). Clonal type III (ToxoDB PCR-RFLP genotype #2) was the one of the four clonal genotypes highly prevalent in animals in North America and was isolated from one tammar wallaby and two Bennett's wallabies (*Macropus rufogriseus*) in Pennsylvania (Dubey and Crutchley, 2008; Dubey et al., 2011; Jiang et al., 2018). Two *T. gondii* strains were isolated from one eastern grey kangaroo (*Macropus giganteus*) and one red kangaroo at a zoo in Argentina, identified as clonal type II and

III, respectively (Moré, et al., 2010). Genotype #6 was isolated from a captive Tammar wallaby from National Zoo (District of Columbia, USA) and is also found in Africa and Brazil, while an atypical strain with an undetermined genotype caused the death of a Bennett's wallaby in Spain (Dubey et al., 2011; Fernández-Aguilar et al., 2013). In North America, genotype #216 has been identified in white-tailed deer (*Odocoileus virginianus*, n = 4), red fox (*Vulpes vulpes*, n = 4), black bear (*Ursus americanus*, n = 2), and feral cats (*Felis catus*, n = 2) (Jiang et al., 2018).

From May-June 2014 outbreak of 11 mortalities, two cases were genotype #263, while two cases between July and September 2017 were genotype #2, and three cases between October 2017 and February 2018 were genotype #216, suggesting that at least three different sources of infections or three transmission events occurred. Precipitation was higher than average in May 2014 at this facility. It has been suggested that heavy rains may periodically wash infective oocysts into the macropod and adjacent exhibits, but other sources have not been ruled out. Prevention of toxoplasmosis in captive macropods is challenging given that there is no vaccine, prophylactic therapy, or practical and effective means of environmental decontamination. Prevention of exposure to infective oocysts remains key and includes eliminating feral or wild felids from zoo grounds as well as any place where they have the potential to contaminate food or water sources. It may also be prudent to design macropod exhibits in a way that prevents storm water, a potential source of infective oocysts, from running into the exhibit.

Toxoplasma genotyping in captive macropods can provide insights into exposure and transmission of the parasite within a group and over various timelines. It remains to be determined whether certain strains are more pathogenic than others, and the prevalence of genotype #263 in felids or other wildlife species is unknown. Ongoing research into the prevalence, pathogenicity, and transmission of various genotypes within a geographic area is needed. This information can help inform prevention strategies to reduce the effects of this important disease in captive macropods.

Declaration of Interest

There is no conflict of interest. This study was not published previously and is not under consideration for publication elsewhere. All authors participated in the study and approved to

to publish the results.

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References

- Basso, W., Venturini, M.C., Moré, G., Quiroga, A., Bacigalupe, D., Unzaga, J.M., Larsen, A., Laplace, R., Venturini, L., 2007. Toxoplasmosis in captive Bennett's wallabies (*Macropus rufogriseus*) in Argentina. *Vet Parasitol*, 144: 157–161.
- Calero-Bernal, R., Saugar, J.M., Frontera, E., Pérez-Martín, J.E., Habela, M.A., Serrano, F.J., Reina, D., Fuentes, I., 2015. Prevalence and genotype identification of *Toxoplasma gondii* in wild animals from southwestern Spain. *J Wildl Dis*, 51: 233–238.
- Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. *Equine Vet J*, 19: 337–339.
- Dubey, J.P., Crutchley, C., 2008. Toxoplasmosis in wallabies (*Macropus rufogriseus* and *Macropus eugenii*): blindness, treatment with atovaquone, and isolation of *Toxoplasma gondii*. *J Parasitol*, 94: 929–933.
- Dubey, J.P., 2010. Toxoplasmosis of animals and humans. 2th edition. CRC Press, Boca Raton, FL, USA.
- Dubey, J.P., Velmurugan, G.V., Rajendran, C., Yabsley, M.J., Thomas, N.J., Beckmen, K.B., Sinnett, D., Ruid, D., Hart, J., Fair, P.A., McFee, W.E., Shearn-Bochsler, V., Kwok, O.C.H., Ferreira, L.R., Choudhary, S., Faria, E.B., Zhou, H., Felix, T.A., Su, C., 2011. Genetic characterization of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. *Int J Parasitol*, 41: 1139–1147.
- Dubey, J.P., Choudhary, S., Kwok, O.C.H., Ferreira, L.R., Oliveira, S., Verma, S.K., Marks, D.R., Pedersen, K., Mickley, R.M., Randall, A.R., Arsnoe, D., Su, C., 2013. Isolation and genetic characterization of *Toxoplasma gondii* from mute swan (*Cygnus olor*) from the USA. *Vet Parasitol*, 195: 42–6.
- Fernández-Aguilar, X., Ajzenberg, D., Cabezón, O., Martínez-López, A., Darwich, L., Dubey, J.P., Almería, S., 2013. Fatal toxoplasmosis associated with an atypical *Toxoplasma gondii* strain in a Bennett's wallaby (*Macropus rufogriseus*) in Spain. *Vet Parasitol*, 196: 523–527.

- Guthrie, A., Rooker, L., Tan, R., Gerhold, R., Trainor, K., Jiang, T., Su, C., 2017. Newly described *Toxoplasma gondii* strain causes high mortality in red-necked wallabies (*Macropus rufogriseus*) in a zoo. *J Zoo Wildl Med*, 48: 694–702.
- Jensen, J.M., Patton, S., Wright, B.G., Loeffler, D.G., 1985. Toxoplasmosis in marsupials in a zoological collection. *J Zoo Anim Med*, 16: 129–131.
- Jiang, T., Shwab, E.K., Martin, R.M., Gerhold, R.W., Rosenthal, B.M., Dubey, J.P., Su, C., 2018. A partition of *Toxoplasma gondii* genotypes across spatial gradients and among host species, and decreased parasite diversity towards areas of human settlement in North America. *Int J Parasitol*, 48: 611–619.
- Mayberry, C., Maloney, S.K., Mitchell, J., Mawson, P.R., Bencini, R., 2014. Reproductive implications of exposure to *Toxoplasma gondii* and *Neospora caninum* in Western gray kangaroos (*Macropus fuliginosus ocydromus*). *J Wildl Dis*, 50: 364–368.
- Miller, M.A., Ehlers, K., Dubey, J.P., Van, S. K., 1992. Outbreak of toxoplasmosis in wallabies on an exotic animal farm. *J Vet Diagn Invest*, 4: 480–483.
- Miller, D.S, Faulkner, C., Patton, S., 2003. Detection of *Toxoplasma gondii* IgG antibodies in juvenile great grey kangaroos, *Macropus giganteus giganteus*. *J Zoo Wildl Med*, 34: 189–193.
- Moré, G., Pardini, L., Basso, W., Machuca, M., Bacigalupe, D., Villanueva, M.C., Schares, G., Venturini, M.C., Venturini, L., 2010. Toxoplasmosis and genotyping of *Toxoplasma gondii* in *Macropus rufus* and *Macropus giganteus* in Argentina. *Vet Parasitol*, 169: 57–61.
- Patton, S., Johnson, S.L., Loeffler, D.G., Wright, B.G., Jensen, J.M., 1986. Epizootic of toxoplasmosis in kangaroos, wallabies, and potaroos: possible transmission via domestic cats. *J Am Vet Med Asso*, 189: 1166–1169.
- Portas, T.J., 2010. Toxoplasmosis in macropodids: A review. *J Zoo Wildl Med*, 41: 1–6.
- Shwab, E.K., Zhu, X., Majumdar, D., Pena, H.F.J., Gennari, S.M., Dubey, J.P., Su, C. 2014. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. *Parasit*, 141: 453–461.
- Su, C., Shwab, E.K., Zhou, P., Zhu, X-Q., Dubey, J.P., 2010. Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasit*, 137: 1–11.

Su, C., Khan, A., Zhou, P., Majumdar, D., Ajzenberg, D., Dardé, M-L., Zhu, X-Q., Ajioka, J.W., Rosenthal, B.M., Dubey, J.P., Sibley, L.D., 2012. Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. *Proc Natl Acad Sci USA*, 109: 5844–9.

University of Tennessee Veterinary Medical Center Parasitology website, 2019. New

Toxoplasma serologic test.

https://vetmed.tennessee.edu/vmc/dls/Parasitology/Documents/FactSheet_BDS_Parasitology_ToxoplasmaELFA.pdf

Chapter 4 Appendix

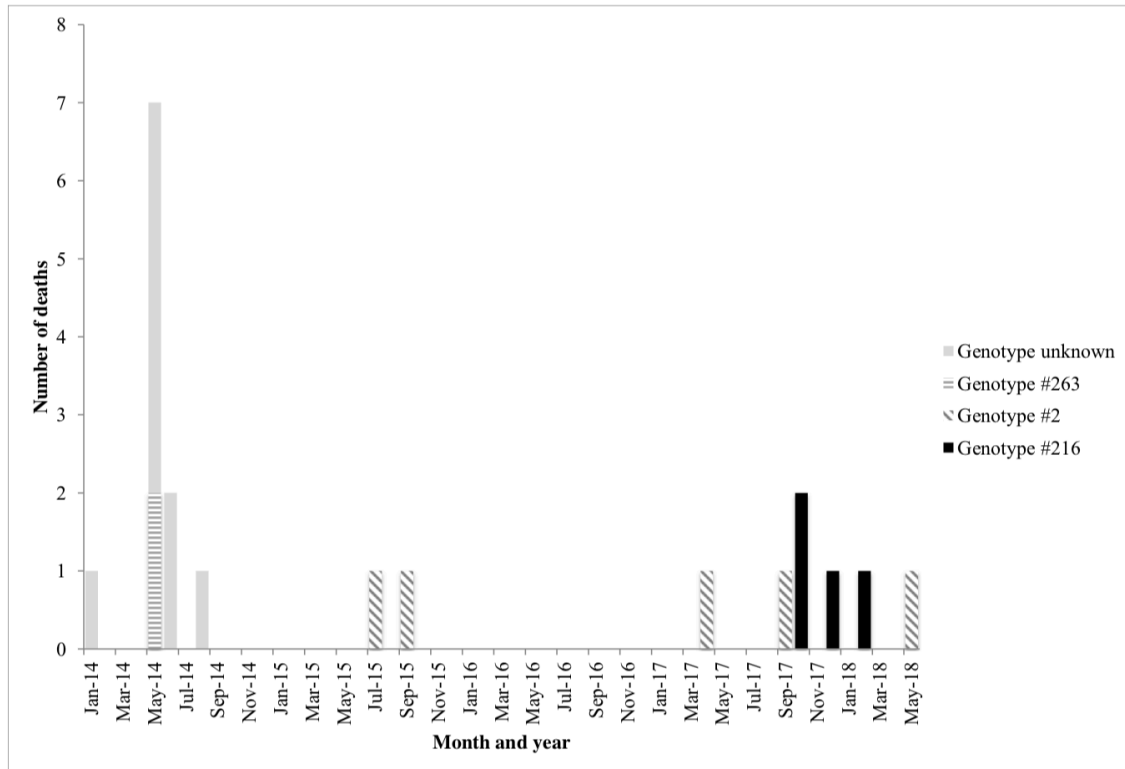


Figure 4. 1 The 20 mortalities of macropods between 2014 and 2018 in Busch Gardens, Florida. The genotypes of the 11 cases reported in this study were indicated in the figure. The mortality dates for the animals described in this reported are as follows: May 2014 (red kangaroo, n = 1; agile wallaby, n = 1), July 2015 (red-necked wallaby, n = 1), September 2015 (red kangaroo, n = 1), April 2017 (red kangaroo, n = 1), September 2017 (red kangaroo, n = 1), October 2017 (tammar wallaby, n = 1; red kangaroo, n = 1), December 2017 (red kangaroo, n = 1), February 2018 (red kangaroo, n = 1) and May 2018 (red kangaroo, n = 1).

Table 4. 1 *Toxoplasma gondii* genotype, signalment, clinical signs, serology results, treatment, and necropsy findings from 11 macropod mortalities diagnosed with toxoplasmosis at a zoological facility in Florida, USA.

Case #	Genotype # (Isolate ID)	Species	Sex	Location of birth	Date of death	Age at time of death (years)	Clinical signs and physical examination findings	Duration of signs prior to death (days)	Serology result at time of death ¹	Treatment	Significant comorbidities
1	#263 (TgKgrFL1)	Red kangaroo (<i>Macropus rufus</i>)	Female	Tampa, FL (BGT)	5/22/2014	0.8	Inappetence, lethargy, dehydration, tachypnea	1	Positive (ELFA)	Fluid therapy ²	None
2	#263	Agile wallaby (<i>Macropus agilis</i>)	Female	Kangaroo conservation center	5/22/2014	8.3	Easily approachable, sudden collapse	Peracute death	Negative (ELFA)	None	Bacterial cholecystitis = (<i>E. coli</i> , <i>Acinetobacter baumannii</i> , and <i>Enterococcus</i>)
3	#2	Red-necked wallaby (<i>Macropus rufogriseus</i>)	Female	Tampa, FL (BGT)	7/24/2015	1.6	Found dead	Peracute death	Not done	None	None
4	#2	Red kangaroo	Male	Chatfield	9/11/2015	6.0	Diarrhea	1	Positive (MAT titer $\geq$ 8192)	Fluid therapy ² , sulfadimethoxine/pyrimethamine ³	None
5	#2	Red kangaroo	Male	Tampa, FL (BGT)	4/6/2017	0.5	Found dead	Peracute death	Not done	None	None

Table 4.1 Continued

Case #	Genotype # (Isolate ID)	Species	Sex	Location of birth	Date of death	Age at time of death (years)	Clinical signs and physical examination findings	Duration of signs prior to death (days)	Serology result at time of death ¹	Treatment	Significant comorbidities
6	#2 (TgKgrFL2)	Red kangaroo	Male	Unknown, acquired from chatfield	9/10/2017	8.3	Weight loss, lethargy, tachypnea, grey mucous membranes	4	Positive (MAT titer 1:1600)	Fluid therapy ² , ceftiofur crystalline free acid ⁴ , ponazuril ⁵ , euthanasia	Idiopathic methemoglobinemia
7	#216	Tammar wallaby (Macropus eugenii)	Female	Wild? Acquired from Lincoln children's zoo	10/3/2017	4.5	Easily approachable, lethargy, tachypnea	3	Positive (MAT titer 1:50)	None	None
8	#216 (TgKgrFL3)	Red kangaroo	Female	Tampa, FL (BGT)	10/19/2017	1	Recumbency, tachypnea, seizures	<1 day	Positive (MAT titer ≥ 1:3200)	Fluid therapy ² , atovaquone ⁶ , furosemide ⁷	None
9	#216	Red kangaroo	Female	Tampa, FL (BGT)	12/20/2017	1.2	Recumbency, seizures	Peracute death	Not done	None	None
10	#216	Red kangaroo	Female	Tampa, FL (BGT)	2/18/2018	1.3	Lethargy, shaking, tachypnea	1	Not done	Atovaquone ⁶ , ponazuril ⁵ , furosemide ⁷ , antibiotic	None
11	#2	Red kangaroo	Female	Zoo Atlanta	5/30/2018	8.3	Found dead	Peracute death	Positive (ELFA 0.4)	None	None

¹ELFA = competitive enzyme linked fluorescence assay; no titer is reported. MAT = modified agglutination test, >1:25 considered positive

²Lactated Ringer's Injection USP (Hospira, Lake Forest, Illinois, USA), 40 ml/kg, SQ (case 1); 32 ml/kg, SQ (case 4); 27 ml/kg, SQ (case 6); 22 ml/kg, SQ (case 8)

³Sulfadimethoxine/pyrimethamine suspension (250 mg/ml and 12.5 mg/ml), 32 mg/kg, PO

⁴Excede[®] ceftiofur crystalline free acid (200 mg/mL, Zoetis, Parsippany, New Jersey, USA), 5.4 mg/kg, SQ (case 8); 6.5 mg/kg, SQ (case 10)

⁵Marquis[®] (15% w/w ponazuril, Merial, Duluth, Georgia, USA), 1.6 mg/kg, PO (case 3); 21.4 mg/kg, PO (case 10)

⁶Mepron[®] Atovaquone suspension (750 mg/5 ml, GlaxoSmithKline, Philadelphia, Pennsylvania, USA), 21.9 mg/kg, PO (case 8); 97.4 mg/kg, PO (case 10)

⁷Salix[®] furosemide (50 mg/ml, Merck, Madison, New Jersey, USA); 2.2 mg/kg, IV (case 8); 1.9 mg/kg, IM (case 10)

Table 4. 2 Results of PCR-RFLP typing of *Toxoplasma gondii* from cases of toxoplasmosis in macropods and a freckled duck at a zoological park in Florida, USA.

Case # (sample ID)	Sample for genotyping	SAG 1	SAG 2	alt-SAG2	SAG 3	BTU B	GRA6	C22-8	C29-2	L35 8	PK 1	Apico	<i>T. gondii</i> isolate ID	ToxoDB Genotype
1	Isolate ¹ and tissue ²	I	I	I	I	I	I	III	I	III	I	III	TgKgrFL 1	#263
2	Tissue ²	I	I	I	I	I	I	III	I	III	I	III		#263
3	Tissue ²	II/III	III	III	III	III	nd ⁴	III	III	III	III	III		#2
4	Tissue ²	II/III	III	III	III	III	III	III	III	III	III	III		#2
5	Tissue ²	II/III	III	III	nd	III	III	III	nd	III	III	III		#2
6	Isolate ¹	II/ III	III	III	III	III	III	III	III	III	III	III	TgKgrFL 2	#2
7	Tissue ³	I	I	I	III	III	I	III	III	III	I	III		#216
8	Isolate ¹ and tissue ³	I	I	I	III	III	I	III	III	III	I	III	TgKgrFL 3	#216
9	Tissue ³	I	I	I	III	III	I	III	nd	III	I	III		#216
10	Tissue ³	I	I	I	III	III	I	III	nd	III	I	III		#216
11	Tissue ³	II/III	III	III	III	III	III	III	III	III	III	III		#2

¹*T. gondii* parasite isolate from a mouse bioassay was used for genotyping.

²DNA extracted from heart tissue was used for genotyping.

³DNA extracted from heart and lung tissues was used for genotyping.

⁴“nd” denotes no data

CHAPTER 5
THE STUDY OF TACHYZOITE TO BRADYZOITE CONVERSION OF
***TOXOPLASMA GONDII*: ATTEMPTS TO PRODUCE A CAT VACCINE**
T263 *IN VITRO*

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Abstract

Felids are the only hosts that can excrete *Toxoplasma gondii* oocysts into the environment. Oocysts are environmentally resistant and highly infectious. Inadvertent ingestion of oocysts disseminated in soil, water, and food accounts for an important route of animal and human infection. Thus far, the most effective way to reduce human and animal infection is to reduce the environmental contamination by oocyst. Controlling oocyst shedding by cats through the development of a vaccine becomes the goal of this study. One dose of an oral live bradyzoite vaccine T263 can prevent oocyst shedding in 84% (Frenkel et al., 1991) and 45% (Mateus-Pinilla et al., 1999) of cats in laboratory and in field, respectively. Due to its high cost resulting from its cumbersome preparation, the vaccine has not been commercialized. To produce the vaccine, T263 is injected into mice; a month later, mice brain and heart tissues are harvested, homogenized, and digested with pepsin. The vaccine was then fed to cats for immunization. To simplify the vaccine production, we investigated two strategies to convert T263 from tachyzoites to bradyzoites *in vitro* to bypass the heretofore required mouse bioassay. We tried vacuum conditions and low temperature at 31°C to switch tachyzoites to bradyzoites in cell culture. Conversion efficacy was tested via acid-pepsin digestion, indirect fluorescent assay (IFA), and mouse bioassay. Vacuum conditions were able to convert TgCkVe6 strain from tachyzoites to bradyzoites as evidenced by the expression of bradyzoite antigen 1 (BAG1) and cyst wall protein (CST1) but was unable to convert T263. However, low temperature at 31°C was able to convert T263 as evidenced by the expression of BAG1 and CST1. Low temperature treated T263 were infectious to mice via oral route and was more prone to produce brain tissue cysts as compared to untreated T263. To conclude, in preliminary experiments, we showed that low temperature (31°C) treatment can produce T263 bradyzoites *in vitro*. Further study should test the whether the *in vitro* produced T263 bradyzoites can induce immunity to oocyst shedding in cats.

Keywords: T263 vaccine, tachyzoite, bradyzoite, *Toxoplasma gondii*.

Introduction

Toxoplasma gondii is a coccidian parasite that infects a broad spectrum of hosts, including humans, animals, and birds (Dubey, 2010). Infection is ubiquitous as one-third of the global human population has been exposed to *T. gondii*. Toxoplasmosis, the disease caused by *T. gondii*, has been shown to cause social and economic loss (Scharff, 2012; Hoffmann et al., 2012). Notably, infection can cause spontaneous abortion, stillbirth or severe congenital disabilities in newborns (Dubey, 2010). Infection can cause fatal outcomes in immunocompromised individuals, such as AIDS patients and those who undergo radiation therapy for cancer treatment. Immunocompetent individuals experience a short period of flu-like symptoms after the initial infection, which is followed by an asymptomatic chronic infection. Severe consequences in immunocompetent patients manifested as ocular toxoplasmosis, systematic toxoplasmosis, or death, have been reported especially in the recent few decades (Holland, 2003; Carme et al., 2009; Demar et al., 2007).

Three parasitological stages exist in the life cycle of *T. gondii*: tachyzoite, bradyzoite, and sporozoite (Dubey, 2010; Weiss et al., 2013). Tachyzoites are the fast-replicating form that can induce an IFN- γ -dependent host immune response. Bradyzoites are the slow-growing, semi-dormant stage and are enclosed by cyst walls which sequester parasites to evade host immune attack. Sporozoites, the environmental form of the parasite, reside in sporulated oocysts. Upon infection, in the pressure of host immune response, tachyzoites are converted to bradyzoites; the latter form cysts that reside in host tissues (Skariah et al., 2010). Reactivation of bradyzoites takes place in immune-compromised people such as *T. gondii*-infected AIDS patients (Grant et al., 1990). During the conversion from tachyzoites to bradyzoites, the gene expression profile altered, resulting in a decrease in immunogenic surface proteins, metabolic enzymes, and an increase in proteins necessary for the entry into G₀ (Blader and Saeij, 2009).

Concerning surface protein expression, bradyzoite expresses bradyzoite antigen 1 (BAG1). BAG1 protein is a characteristic protein solely expressed on the bradyzoite surface and is therefore often used as a marker for bradyzoite identification (Blader and Saeij, 2009). Cells expressing BAG1 were seen after 24 hours of exposure to stress such as pH 8.1 (Weiss and Kim, 2011). Deletion of BAG1 did not prevent cyst formation but only hindered the efficiency of cyst formation

(Bohne et al., 1998). In addition, the formation of cyst wall, often recognized by detecting cyst wall protein (CST1), is another hallmark of bradyzoite formation (Blader and Saeij, 2009). The exact structure and composition of the cyst wall is not clear but was proved to contain polysaccharides and chitin (Boothroyd et al., 1997). The N-acetylgalactosamine contained in a 116 kDa glycoprotein CST1 accounts for the binding of DBL (*Dolichos biflorus* lectin) to cyst wall (Skariah et al., 2010; Lyons et al., 2002; Fux et al., 2007).

T. gondii infection is prevalent in humans and animals. The high infection rate in domestic animals raised for human consumption poses serious public health concerns. The seroprevalence of pigs raised in poorly managed or non-confined facilities is as high as 68% (Dubey et al., 2008). Viable *T. gondii* has been frequently isolated from pork destined for human consumption; isolation rates range from 0.3% to 92.7%, depending on the age of pigs and pig farming conditions (Dubey and Jones, 2008). Worldwide, chickens raised in the backyard are steadily infected with *T. gondii* with infection rate ranging from 27%- 100% (Dubey and Jones, 2008; Dubey et al., 2005b; Dubey et al., 2006). A study conducted in Paraná, Brazil revealed that 12.2% (47/386) of serum samples from free-range chickens were *T. gondii* positive (Vieira et al., 2018). Sheep and goats are susceptible to *T. gondii* infection evidenced by frequent reports of abortion and neonatal mortality. Congenitally infected lambs that survived can remain asymptomatic, which could be a source of human infection (Dubey and Kirkbride, 1989). Clearly, the high prevalence of *T. gondii* and its concomitant health risk call for methods to intervene its transmission.

T. gondii is transmitted through three major routes, including ingesting oocysts disseminated in the environment, eating undercooked meat that contains tissue cysts, and congenital transmission. As the sole definitive hosts able to shed oocysts into the environment, cats play a central role in the epidemiology and transmission of *T. gondii*. Studies have shown that *T. gondii* infection is scarce or absent in areas without cats (Dubey, 1997; Munday et al., 1972; Wallace et al., 1969). The facts that cats are widespread and that they are predisposed to *T. gondii* infection pose a risk to human health. Approximately, one-third of households in the United States own cats. There are an estimated 78 million domestic cats and 73 million feral cats in the U.S. (Dubey, 2010). An average of 30% of cats worldwide have been infected with *T. gondii* (Dubey, 2010). Cats are also the common animals on farms. A 1999 study demonstrated that a mean of 8.5

cats was present on each pig farm in Illinois, of which six were seropositive for *T. gondii* (Weigel et al., 1999).

Thus far, using vaccines to prevent infection has been mostly ineffective. The only commercialized vaccine is for sheep, which is a strain (S48) that does not form tissue cysts in sheep, used during lambing season to prevent abortion in Europe and New Zealand (Buxton et al., 1993). A vaccine to directly immunize humans has not yet been available. Many obstacles posed in human vaccine production as killed strain provides at best marginal protection and attenuated or mutant strains pose the risk of infection especially to fetuses (Dubey, 2010).

The most effective and rewarding vaccination strategy leans toward the immunization of definitive hosts. A 3-year field trial revealed that vaccination of cats on eight commercial farms using T263 showed promise to reduce the infection in farm animals (finishing pigs and mice) (Mateus-Pinilla et al., 1999). T263 was a mutant produced by exposing tachyzoite to the alkylating agent, N-methyl-N'-nitro-N-nitroso guanidine (Frenkel et al., 1991; Popie et al., 1994) and was selected for its resistance to adenine arabinoside. T263 is genetically mutated and could not form oocyst in the intestinal epithelial cells of the cat (Dubey, 2010). Therefore, oral administration of T263 cannot induce oocyst shedding in cats but can induce immunity to oocyst shedding upon challenge with a wild strain. The oral administration to cats of the bradyzoite/tissue cyst form of T263, not the tachyzoite form, is required to produce immunity to oocyst shedding (Freyre et al., 1993; Freyre et al., 1993).

The application of this cat vaccine had been unsuccessful due to several factors, principally the cumbersome procedure for preparation and the resulting high cost per dose (Frenkel et al., 1991; Freyre et al., 1993; Mateus-Pinilla et al., 1999). Mice are required for vaccine production, whereby brain tissue cysts are allowed to develop for a month. A month later, mice are euthanized, and the brain tissues are harvested and homogenized, digested with pepsin, and preserved in liquid nitrogen (Choromanski et al., 1995). At the time of immunization, vaccine is thawed and orally administered to cats.

To simplify vaccine production and to reduce its price, we sought to develop a methodology to produce live bradyzoites *in vitro*. To achieve that, we aimed to develop a technique to convert T263 tachyzoites to bradyzoites *in vitro*. Previously, low or high pH and heat shock (43°C for 12-48 h) were used to induce the conversion (Lyons et al., 2002). However, in our

attempts, these methods produced significant damage to host cells, which rendered the production of bradyzoites inadequate. In this study, we investigated two conversion strategies, namely, applying vacuum conditions and low temperature at 31°C. We first tested the effect of vacuum conditions on a *T. gondii* strain isolated from a chicken in Venezuela (Rajendrana et al., 2012). The conversion potential of vacuum conditions was inadvertently discovered in TgCKVe6 in an event of developing a method to ship cell culture between labs. We were able to slow down the growth of *T. gondii* strains TgCkVe6 and T263 using vacuum conditions and low temperature at 31°C, respectively. Three approaches were used to evaluate the *in vitro* produced bradyzoites: Indirect fluorescent assay (IFA), pepsin digestion assay, and mouse oral infectivity assay.

Materials and Methods

Cell culturing and treatment conditions

Toxoplasma gondii was routinely cultured in Human Foreskin Fibroblasts (HFFs) in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS). HFFs were grown in either T25 flasks for pepsin digestion assay and mouse bioassay or on glass coverslips inserted into 6-well plates for IFA.

For vacuum conversion treatments, HFFs were infected with TgCkVe6 or T263 in either T25 flasks or 6-well plates at 37°C, 5% CO₂ overnight for 12h. The next day, media was changed to remove cell debris; the T25 flask or 6-well plate with infected HFFs was transferred into a vacuum bag. Air in the bag was depleted using handheld rechargeable vacuum sealing system (FoodSaver FreshSaver, Walmart # 007428735). The cell culture in flask or plate in a vacuumed bag was then incubated at room temperature (20-25°C) for three or four days. Afterward, cell culture was transferred back to 37°C, 5% CO₂ incubator and kept for another seven to ten days.

For low temperature conversion treatment, HFFs were infected with T263 at 31°C in a T25 flask or a 6-well plate. Cell culture was then kept at 31°C for seven to eight days.

For IFA, 10 to 15 drops of media containing parasites ($3-5 \times 10^5$ parasites) were inoculated into each well of a 6-well plate with confluent HFFs on coverslips. For pepsin digestion assay and mouse bioassay, approximately $6-8 \times 10^5$ parasites were inoculated into a T25 flask with confluent HFFs.

Detection of protein expression via IFA

The presence of BAG1 and CST1 in *in vitro* produced bradyzoites were detected using IFA. After the conversion treatment, cell culture infected with TgCkVe6 or T263 in a 6-well plate was fixed in 4% formaldehyde at RT (room temperature) for 30 mins, permeated with 0.5% Triton at RT for 20 mins, and blocked with blocking buffer (PBS with 0.1% Triton and 10% BSA) at RT for 1 h. Each step was followed by three times of washing with ice-cold PBS. For primary antibody staining, monoclonal rabbit anti-BAG1 antibodies (1:100) (Weiss et al., 1992) was added and incubated at 4°C overnight. The next day, upon washing, secondary antibodies Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, A11012) at 1:100 were added and incubated at RT for 1 hour. Finally, after washing, FITC-conjugated *Dolichos biflorus* agglutinin (Fisherbrand, FL-1031) at 1:300 was added for CST1 staining at RT for 1 hour. After incubation, cells on coverslips were washed with PBS; coverslips were taken out, air dried, and mounted onto glass slides. Fluorescent microscopy (Leica DMRX, Leica Microsystems) was used to detect protein expression. As controls, the non-treated parasites (presumably tachyzoites) were subjected to the same IFA procedure.

Pepsin digestion assay

We utilized an acid-pepsin digestion assay to evaluate the treated parasites by comparing the survival rates of treated and non-treated parasites upon pepsin digestion. This method relies on the fact that bradyzoites, cell culture-derived or animal tissue-derived, are more resistant to acid-pepsin (Lyons et al., 2002; Weiss et al., 2011; Popie et al., 1994). Viable parasites of treated and non-treated TgCkVe6 or T263 in a T25 flask were enumerated via plaque assay before pepsin digestion. To release parasites from host cells and parasitophorous vacuoles (PVs), we scraped host cells from the bottom of the flask and pressed them through a 22-gauge needle in a 10 ml syringe four times. Upon washing, parasites from one T25 flask (either treated or non-treated) were equally split into two portions: one was subjected to pepsin digestion (0.26 mg/ml pepsin in 0.170M NaCl, pH1.4), and the other served as negative control in which the base solution (0.170M NaCl) was added. Digestion was conducted for 15 or 30 mins at 37°C. The pepsin digestion was followed by addition of 1.2% sodium bicarbonate (pH 8.3) to bring the solution to neutral pH. The neutralized pepsin solution or base solution was then removed via centrifugation. Finally, parasites

in each of the four groups (treated parasites with pepsin solution, treated parasites with base solution, non-treated parasites with pepsin solution, non-treated parasites with base solution) were enumerated again via plaque assay. The survival rates of treated and non-treated parasites with or without pepsin digestion were compared.

For plaque assay, cell suspension was serially diluted across a range of 10^{-1} to 10^{-4} ; inoculum of 100 μ l at each concentration (10^{-1} to 10^{-4}) was transferred into each well of a 6-well plate; plate was incubated at 37 °C for seven days. After incubation, cell medium was removed, and cells were fixed in 70% ethanol. The plaque number was enumerated under bright light microscope.

Mouse bioassay

We intended to test whether the *in vitro* produced T263 bradyzoites can be liquid nitrogen (LN) preserved. The 31°C treated T263 were preserved at seven days post-infection to simulate the vaccine preservation. After seven days at 31°C, cell culture was scratched from the flask and mixed with 50% FBS in DMEM and was subsequently mixed with 20% dimethyl sulfoxide (DMSO) in DMEM. The mixture was then transferred to LN upon overnight on dry ice. After four months, on the day of mice infection, *in vitro* produced bradyzoites were thawed, washed, and resuspended in PBS. The number of cysts was enumerated using hemocytometer to prepare 2.5×10^4 cysts/ml in 5ml PBS.

We also tested the infectivity of freshly prepared T263 bradyzoites. Seven days post infection at 31°C, cells were scraped off from three T25 flasks, washed and resuspended in PBS. The number of cysts was counted using hemocytometer to prepare 2.5×10^4 cysts/ml in 5ml PBS.

Tachyzoites of T263 were included as control. The fast-growing T263 was cultured at 37 °C and reached a growth speed of 2-day passage. Two days after infection, parasites were bursting from host cells. Cell culture was pressed through a 22-gauge needle to further separate tachyzoites from host cells. The number of tachyzoites was enumerated using hemocytometer to prepare 2.5×10^4 tachys/ml in 5 ml PBS.

Fifteen mice were evenly divided into three groups of five. Each group of mice were infected with parasites of a different preparation, namely, LN preserved bradyzoites, freshly prepared bradyzoites, and fresh tachyzoites. Each mouse in a group was orally fed 1ml of prepared

parasites which contained 2.5×10^4 LN preserved cysts, 2.5×10^4 fresh cysts, and 2.5×10^4 fresh tachyzoites. Mice were monitored for three weeks. After three weeks, mice were euthanized; brain, heart, and lung tissues were taken for PCR detection of *T. gondii*; the brain was also used for DBL staining of tissue cysts. Blood was taken for Modified Agglutination Assay (MAT) (Dubey and Desmonts, 1987); the cutoff value was set at 1:25 (Dubey et al., 1995a and 1995b).

For tissue DNA examination, brain, heart, and lungs were pooled and homogenized; DNA was extracted using DNeasy Blood and Tissue kit (Qiagen; Cat No. 69504). PCR targeting the 18S ribosomal RNA (18S rRNA) gene was used for *T. gondii* detection (Silva et al., 2009). The primer pair Tg18s 58F/Tg18s 348R at a concentration of 50 μ M were used for PCR amplification (Silva et al., 2009). The expected DNA fragments with a size of 290 base pairs (bp) was examined on a 2% agarose gel.

For brain tissue cyst examination, one quarter of the brain tissue was taken and homogenized by sequentially syringing through gauge 16 and 20 needles. The tissue homogenate was fixed and permeated in fixing/permeabilizing solution (6% formaldehyde, 0.2% TritonX-100 in PBS) for 20 mins at 4°C. Cells were washed with 10% goat serum in PBS. DBL at 1:300 final concentration (10 μ l DBL in 3ml 10% goat serum) was used to stain brain tissue cysts for one hour at RT in the dark. After two washings in 10% goat serum, the cysts were resuspended in 2 ml PBS. Ten microliters of cysts in PBS was mounted onto a glass slide and examined for tissue cysts under a fluorescent microscope (Leica DMRX, Leica Microsystems).

Results

Detection of protein expression of vacuum treated TgCkVe6 via IFA

Four days in a vacuum bag at room temperature (RT), the morphology of TgCkVe6 became unrecognizable, and parasites in PVs deformed and merged into dotted smudges. Recover slowly took place in 37 °C, 5% CO₂ incubator, and eventually after 7 -10 days, parasites formed large persisting cysts.

The staining of BAG1 was indicated by the green fluorescence (Figure 5.1). The monoclonal rabbit anti-BAG1 antibodies (1:100) was used as primary antibody, and FITC-conjugated goat anti-rabbit IgG (1:100) was added as secondary antibody.

In another independent experiment (Figure 5.2), CST1 was stained with FITC conjugated DBL (1:300). A significant number of cysts were stained as indicated by the green fluorescence (Figure 5.2).

To compare the protein expression of treated TgCkVe6 and non-treated TgCkVe6, we simultaneously stained BAG1 and CST1 (Figure 5.3) of treated and non-treated parasites. The nuclei were also stained with DAPI. For BAG1 staining, the secondary antibodies Alexa Fluor 594 goat anti-rabbit IgG at 1:100 was used. In Figure 5.3, the staining of BAG1 was observed in both treated and non-treated groups; however, CST1 expression was only observed in treated TgCkVe6. The cross-emission of FITC and DAPI was pronounced in Figure 5.3 as FITC emission extended to DAPI channel and vice versa.

Acid-pepsin digestion of vacuumtreated TgCkVe6

All TgCkVe6, vacuum treated and non-treated, succumbed after 30mins acid-pepsin digestion at 37°C. Therefore, a shorter digestion period, namely 15 mins, was chosen for further testing. The results of the acid-pepsin digestion assay were summarized in Table 5.1. A small proportion (0.24%) of treated TgCkVe6 were pepsin resistant, whereas all non-treated parasites succumbed after 15min acid-pepsin digestion at 37°C. Results of a repeat testing of the pepsin resistance were summarized in Table 5.2. No pepsin resistant parasite was identified regardless of whether the conversion treatment was applied (Table 5.2). In addition, the survival rate (150%) for those kept in the 0.9% NaCl for 15 mins at 37°C was rather implausible as it exceeded 100% (Table 5.2). The problem lay in the enumeration process. The incomplete disruption of tissue cysts likely skewed the initial enumeration via plaque assay; those tissue cysts later ruptured and released a larger number of parasites during incubation in 0.9% NaCl.

Detection of protein expression of 31°C treated T263 via IFA

T263 produced notable large, persistent cysts at 31°C six days post-infection (Figure 5.4). Staining was done on BAG1 and CST1. The staining protocol was described in 2.2. DAPI was not used due to cross-emission with FITC. Although both treated and non-treated T263 express a certain degree of BAG1, indicating spontaneous conversion in the case of non-treated T263 (Figure 5.5). However, the cyst wall staining was only seen in treated T263 (Figure 5.5). The repeat testing of

cyst wall expression of 31°C treated T263 was done seven days post-infection (Figure 5.6). Two different doses of T263, namely 10^5 (Figure 5.6 A) and 10^4 (Figure 5.6 B), were used to infect HFFs in each well of a 6-well plate. As shown in Figure 5.6, the infection rate of panel A was much higher than that of panel B. The CST1 expression was observed but with less intensity of fluorescence (Figure 5.6 A and B) as compared to that of *in vitro* produced TgCkVe6 (Figure 5.2).

Oral infectivity of 31°C treated T263

The three groups of total 15 mice were observed on a daily basis for three weeks. One mouse to which 2.5×10^4 tachyzoites were orally fed suffered from pain (hunched back, dehydration) two days after infection and succumbed five days post infection. The cause of death is unclear but could be the accidental damage of stomach tissue during the oral inoculation. As a result, no tissue or blood sample was taken from this mouse. One mouse to which 2.5×10^4 LN preserved bradyzoites were fed showed symptoms 17 days post-infection and was euthanized with tissues and blood collected. The remaining 13 mice survived 21 days post-infection and were euthanized with blood and tissue samples obtained.

T. gondii antibody detection in blood showed that two out of five mice infected with LN preserved bradyzoites were *T. gondii* antibody positive with a titer of 1:400 (Table 5.3). Two out of four mice infected with tachyzoites were seropositive for *T. gondii* with titers of no less than 1:1600 and 1:3200, respectively (Table 5.3). No seropositive sample was identified in the group of mice infected with fresh *in vitro* produced bradyzoites.

PCR detection of tissue DNA confirmed serological results. *T. gondii* DNA was amplified from the tissues of four seropositive mice (Figure 5.7).

DBL staining of tissue cysts were consistent with serological and PCR results with the exception of LNB No.5. LNB No.5 was from a mouse that showed symptoms 17 days post-infection and was euthanized. Tissue cysts appear in mouse brain tissue at around two weeks post-infection (Cristina et al., 2008), and requires a couple of weeks to grow large enough to be clearly observed under microscope. This may explain why no tissue cysts were seen in LNB No.5. More tissue cysts were identified in 10µl of brain suspension (one-quarter of brain in 2 ml PBS) in LNB No.1 than the other two samples (Tachy No. 1 and No.2) (Figure 5. 8). A total of seven cysts were

found in LNB No.1 in 10µl of brain suspension, whereas only two were found in Tachy No. 2 and one in Tachy No. 1.

Discussion

Vaccines to control *T. gondii* infection have been up to now unsuccessful. Immunizing intermediate hosts have at best limited benefits, and the fact that there is rarely any commercialized vaccine available for intermediate hosts compounds the problem. A cystless strain S48, the only commercialized vaccine thus far, has been used to vaccinate sheep to reduce neonatal mortality in New Zealand and Europe (Buxton et al., 1993). With limited propagation in sheep, this vaccine can protect animals against *T. gondii* induced abortion for up to 18 months with one dose (Buxton et al., 1993; Innes et al., 1995). Vaccine candidates for pigs have been tested to have promising outcomes in laboratory. RH strain, a non-persistent strain, has been shown to protect pigs and to prevent tissue cyst formation in pig tissues (Dubey et al., 1991). CpG Oligodeoxynucleotide has been used in conjunction with RH strain to enhance protective immunity in pigs (Kringel et al., 2004). In addition, a DNA vaccine cocktail (GRA1-GRA7) had shown promises in eliciting strong humoral and Th1-mediated immune response in pigs (Jongert et al., 2008).

A safe and effective vaccine for human use has not been developed. As *T. gondii* is an obligate intracellular parasite, the cell-mediated immune response induced by active parasite invasion is essential, which explains why vaccines using live parasites are necessary to stimulate protective immunity. However, live parasites pose a risk to human vaccine recipients as well as to those who handle vaccines. Obstacles and risks associated with human *T. gondii* vaccination highlight the importance of developing vaccine to immunize cats, its definitive hosts, to interrupt the human infection cycle.

The mutant T263 demonstrated great promise in reducing *T. gondii* contamination by preventing cats from shedding oocyst (Mateus-Pinilla et al., 1999). Under laboratory conditions, one dose of T263 can prevent 84% of cats from shedding oocysts (Frenkel et al., 1991); two doses can prevent 100% of cats from shedding oocysts (Freyre et al., 1993). The corresponding numbers are lower, however, under field conditions. The seroconversion rate of cats in field experiment is 45% with one vaccination and 67% with two vaccinations (Mateus-Pinilla et al., 1999).

The short shelf-life, demanding storage requirement, and cumbersome procedures for preparation hindered the application of vaccine T263. To simplify the vaccine production, an *in vitro* method to convert tachyzoite to bradyzoite is essential. It has been known that tachyzoite to bradyzoite conversion is a stress-related phenomenon. Conventionally, high temperature (43 °C), high (8.0-8.2) or low (6.6-6.8) pH, chemical stresses (i.e., sodium arsenite) can induce tachyzoite to bradyzoite conversion (Soete et al., 1994). However, the use of these methods in our attempts caused host cells damage severe enough to render them unable to support parasites growth.

We investigated two methods to convert T263 from tachyzoites to bradyzoites to bypass the mouse bioassay. We showed that vacuum conditions can successfully convert TgCkVe6 to bradyzoites. We fortuitously stumbled upon this method from attempts to develop a technique for shipping of cell cultures. Obstacles posed in cell culture shipping is that the media often become acid, which become detrimental to host cells and parasites. To maintain a neutral pH, we put the cell cultures (HFFs infected with TgCkVe6) into a vacuum bag with air depleted. The cell cultures in a vacuum bag was incubated at RT for four days to simulate the duration of shipping. To test the duration of recovery of the cell culture once delivered, we transferred the cell culture to 37 °C, 5% CO₂ incubator. After seven to eight days, large persisting cysts were unexpectedly observed, which prompted us to utilize the vacuum for conversion.

The *in vitro* produced TgCkVe6 bradyzoites expressed BAG1 and formed tissue cysts as evidenced by CST1 detection via IFA. Pepsin digestion assay showed that a small percentage of treated parasite TgCkVe6 were able to survive acid pepsin treatment for 15 min. However, the pepsin digestion assay was not repeatable as no resistant parasites were identified on repeat testing. It has been shown that the *in vitro* produced bradyzoites were acid-pepsin resistant (Dubey et al., 1998; Popiel et al., 1996) and can orally infect both mice (Fux et al., 2007) and cats (Hoff et al., 1977). The pepsin resistance rate of the *in vitro* alkaline-induced bradyzoite (9 days post-infection) ranges from 0.03% to 9.4%, depending on the strain (Fux et al., 2007). The discrepancy seen between our results and those in publications may be attributable to the preparation procedure and the different methods used to estimate bradyzoite numbers before pepsin digestion. In our experiment, in order to precisely measure the quantity of bradyzoites, we completely released parasites from PVs and host cells and enumerated bradyzoites using plaque assay. In published

studies, cysts were enumerated using hemocytometer under microscope, and the number of bradyzoites in cysts were roughly estimated as 10 to 20 bradyzoite per cyst (Fux et al., 2007).

In contrast, when the vacuum was applied to T263, no large persisting cysts were formed. Instead, due to the harsh vacuum conditions, T263 disintegrated after treatment, leaving numerous empty vacuoles in host cells. The discrepancy seen between TgCkVe6 and T263 reflects *T. gondii* strain variation. A wide array of factors account for the observed discrepancy of conversion ability under vacuum conditions. It's known that strains with a slower rate of replication and lower virulence are more likely to develop cysts *in vitro* (Matsukayashi et al., 1963; Weiss and Kim, 2011). This holds true in the case of TgCkVe6 which is slow-growing and prone to form large vacuoles even at 37 °C with 5% CO₂.

Luckily, we also tested the temperature effect on the growth of T263, including high temperature at 43°C and 41°C, low temperature at 33°C and 31°C. At 43°C, both host cells and T263 were unable to survive. At 41°C, parasites invade and lyse host cells as efficiently as those at 37°C. At 33°C, the growth of the parasite slowed down but not enough to form large persisting cysts. However, at 31°C, parasites were able to develop large persisting cysts. We used IFA and mouse bioassay to validate the conversion from tachyzoites to bradyzoites. Pepsin digestion was not conducted on this strain due to its inconsistency when used on vacuum-treated TgCkVe6. The expression of BAG1 and CST1 were evident in converted T263, indicating successful conversion. However, when compared to the vacuum-treated TgCkVe6, less CST1 expression was found in 31°C treated T263.

Through oral infection of mice, we demonstrated that the LN preserved bradyzoites of T263 at a dose of 2×10^4 cysts were able to infect 40% (2/5) of mice and form a significant number ($7 \times 200 \times 4 = 5,600$) of tissue cysts in a mouse brain. This proved that our *in vitro* produced T263 bradyzoites were infectious after four months of preservation in liquid nitrogen, indicating the feasibility of preserving *in vitro* produced bradyzoites in liquid nitrogen. Although two (2/4=50%) mice in the tachyzoite group were also infected, we found less tissue cysts (1,600 or 800) in their brains. This preliminarily suggests that our *in vitro* produced T263 bradyzoites are more prone to form tissue cysts in mice. Dubey has shown that the number of bradyzoites required to orally infect a mouse is 1000, whereas the corresponding figure for tachyzoite is $>10^6$ (Dubey, 1998). In our study, 2×10^4 T263 tachyzoites were able to establish infection in mice. The discrepancy seen

between Dubey's results and ours indicates strain variation. Fux et al. have shown that 1000 pepsin resistant bradyzoite were able to infect 50% of mice (Fux et al., 2007). In our study, we presumably used much more than 1,000 bradyzoites as one cyst contains more than one bradyzoite. It is possible that the conversion rate in our study is low resulting in few mature bradyzoites in each tissue cysts. Alternatively, we have used a much higher dose of bradyzoites in mice infection. Further study should increase the conversion efficiency using 31°C. To increase the conversion efficiency, no more than two passages of T263 at 37 °C after revived from liquid nitrogen should be allowed before the conversion treatment. Observation has been made that frequent passage of T263 37 °C resulted in difficulties in conversion. The growth of T263 could not be easily slowed down by the sole treatment of 31°C once parasites were in burgeoning state.

Counterintuitively, our freshly prepared T263 bradyzoites failed to infect mice. The problem lay in the inadequate number of mature bradyzoites fed to each mouse. Although the same number of parasites (6×10^5 to 8×10^5 parasites) were initially used to infect HFFs in a T25 flask, these parasites were not in their most active state at the time of infection. CO₂ supply was briefly disrupted during the course of the treatment. Cell culture likely experienced a shock due to CO₂ outage, which may account for the compromised growth of the parasites.

The signaling pathways and the molecular mechanisms of interconversion are unknown. However, as a stress-induced response, the common stress-related pathways in eukaryotic organisms are implicated. Cyclic nucleotide kinase is believed to partly mediate the differentiation (Eaton et al., 2006). A study has determined that inhibition of cAMP-dependent kinase and cGMP-dependent kinase reduced parasite replication and induced differentiation (Eaton et al., 2006). Parasite-specific eukaryotic initiation factor-2 (eIF2) kinase was also implicated in the differentiation process (Sullivan et al., 2004). As both protein chaperones and regulators of transcription, the heat shock proteins (HSPs) induced during stress response have gained significant research interests. After alkaline stress at pH 8.1, hsp70 was induced three to four folds more than that of at pH 7.1, consistent with the induction seen in other Apicomplexans (Weiss et al., 1998; Shiels et al., 1997). Inhibition of HSPs synthesis using quercetin hindered the *in vitro* bradyzoite development (Weiss et al., 1998).

The molecular mechanisms underlying the induced conversion using vacuum and 31 °C are unknown. Vacuum conditions implicate various cellular stresses, including deprivation of CO₂,

O₂, and low temperature at RT, for both the host and the parasite. Temperature change has been known to induce differentiation. Heat shock (43°C) host cells for two hours with or without heat shock of infected cells for 12-48 h has been used for bradyzoite differentiation (Skariah et al., 2010). Low temperature (RT or 31 °C) likely reduces the growth rate of both host cells and parasites, which contributes to the conversion (Weilhammer et al., 2012).

Secondly, hypoxia likely alters host and parasite metabolisms and contributed to the conversion. The hypoxia-inducible transcription factor (HIF-1 α), a transcription factor that is activated by hypoxia, is likely activated under vacuum conditions. HIF-1 α is critical in preserving the health of both host cells and parasites, especially under hypoxia. It has been shown that HIF-1 α and its downstream signaling pathways (glycolysis, growth-factor signaling, and angiogenesis) were upregulated in *T. gondii* infected fibroblasts (Gail et al., 2001; Blader et al., 2001). In addition, HIF-1 α also plays an essential role in *T. gondii* growth and organelle maintenance at 3% oxygen (Spear et al., 2006).

Lastly, low CO₂ may also be a trigger of conversion under vacuum conditions. Previously, low CO₂ (0.03%) has been used to induce tachyzoites to bradyzoites conversion (Bohne and Roos, 1997). Depleting of CO₂ or low CO₂ was thought to cause pyrimidine starvation as CO₂ is the substrate for *de novo* pyrimidine biosynthesis.

The successful development and large-scale application of a cat vaccine based on T263 are only possible when the obstacles involved in vaccine production are overcome, among which is the cumbersome, time-consuming, and expensive procedure for bradyzoite production. Our study identified a new technique to produce bradyzoites *in vitro*. Further refinement of the technique, followed by tests in cats, is needed to validate the efficacy of the method. In particular, it's imperative to test whether the *in vitro* produced T263 bradyzoite can induce protective immunity to oocyst shedding in cats upon challenge with a wild strain.

References

- Blader, I. J., Saeij, J. P., 2009. Communication between *Toxoplasma gondii* and its host: impact on parasite growth, development, immune evasion, and virulence. *APMIS*, 117: 458–476.
- Blader, I.J., Manger, I.D., Boothroyd, J.C., 2001. Microarray analysis reveals previously unknown changes in *Toxoplasma gondii* infected human cells. *J Biol Chem*, 276: 24223–24231.
- Bohne, W., Roos, D., 1997. Stage-specific expression of a selectable marker in *Toxoplasma gondii* permits selective inhibition of either tachyzoites or bradyzoites. *Mol Biochem Parasitol*, 88: 115–126.
- Bohne, W., Hunter, C. A., White, M. W., Ferguson, D. J., Gross, U., Roos, D. S., 1998. Targeted disruption of the bradyzoite-specific gene BAG1 does not prevent tissue cyst formation in *Toxoplasma gondii*. *Mol Biochem Parasitol*, 92: 291–301.
- Boothroyd, J.C., Black, M., Bonnefoy, S., Hehl, A., Knoll, L.J., Manger, I.D., Ortega-Barria, E., Tomavo, S., 1997. Genetic and biochemical analysis of development in *Toxoplasma gondii*. *Philos Trans R Soc Lond B Biol Sci.*; 352:1347–54.
- Buxton D., Thomson KM., Maley, S., Wright, S., Bos, H.J., 1993. Experimental challenge of sheep 18 months after vaccination with a live (S48) *Toxoplasma gondii* vaccine. *Vet Res.* 133: 310–312.
- Carme, B., Demar, M., Ajzenberg, D., Dardé, M. L., 2009. Severe acquired toxoplasmosis caused by wild cycle of *Toxoplasma gondii*, French Guiana. *Emerg Infect Dis*, 15: 656–8.
- Choromanski, L. A., Freyre, R., Popiel, K., Brown, R. G., Shibley, G., 1995. Safety and efficacy of modified live feline *Toxoplasma gondii* vaccine. *Dev Biol Stand*, 84:269–281.
- Cristina, M.D., Marocco, D., Galizi, R., Proietti, C., Spaccapelo, R., Crisanti, A., 2008. Temporal and Spatial Distribution of *Toxoplasma gondii* Differentiation into Bradyzoites and Tissue Cyst Formation *In Vivo*. *Infect Immun*, 76: 3491-3501.
- Demar, M., Ajzenberg, D., Maubon, D., Djossou, F., Panchoe, D., Punwasi, W., Valery, N., Peneau, C., Daigre, J. L., Aznar, C., Cottrelle, B., Terzan, L., DardeÅL, M. L., Carme, B., 2007. Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical, and parasitological aspects. *Clin Infect Dis*, 45: e88–e95.

- Dubey, J. P., Kirkbride, C. A., 1989. Economic and public health considerations of congenital toxoplasmosis in lambs. *J Am Vet Med Assoc*, 195: 1715–1716.
- Dubey, J.P., Urban, J.F. Jr., Davis, S.W., 1991. Protective immunity to toxoplasmosis in pigs vaccinated with a non-persistent strain of *Toxoplasma gondii*. *Am J Vet Res*, 52: 1316–1319
- Dubey, J.P., Thulliez, P., Weigel, R.M., Andrews, C.D., Lind, P., Powell, E.C., 1995. Sensitivity and specificity of various serologic tests for detection of *Toxoplasma gondii* infection in naturally infected sows. *Am J Vet Res*, 56: 1030–1036.
- Dubey, J.P., 1997. Validation of the specificity of the modified agglutination test for toxoplasmosis in pigs. *Vet Parasitol*, 71: 307–310.
- Dubey, J. P., 1998. Re-examination of resistance of *Toxoplasma gondii* tachyzoites and bradyzoites to pepsin and trypsin digestion. *Parasitology*, 116: 43–50.
- Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* Tachyzoites, Bradyzoites, and Sporozoites and Biology and Development of Tissue Cysts. *Clin Microbiol Rev*, 11: 267–299.
- Dubey, J.P., Gomez-Marin, J.E., Bedoya, A., Lora, F., Vianna, M.C.B., Hill, D., Kwok, O.C.H., Shen, S.K., Marcet, P.L., Lehmann, T., 2005b. Genetic and biologic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Colombia, South America. *Vet Parasitol*, 134: 67–72.
- Dubey, J.P., Patitucci, A.N., Su, C., Sundar, N., Kwok, O.C.H., Shen, S.K., 2006. Characterization of *Toxoplasma gondii* isolates in free-range chickens from Chile, South America. *Vet Parasitol*, 140: 76–82.
- Dubey, J.P., Hill, D.E., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Pierce, V., Kelly, K., Dulin, M., Thulliez, P., Iwueke, C., Su, C., 2008. Endemic toxoplasmosis in pigs on a farm in Maryland: isolation and genetic characterization of *Toxoplasma gondii*. *J Parasitol*, 94: 36–41.
- Dubey, J. P. and Jones, J. L., 2008. *Toxoplasma gondii* infection in humans and animals in the United States. *Int J Parasitol*, 38: 1257–1278.
- Dubey, J.P., 2010. Toxoplasmosis of animals and humans. 2th edition. CRC Press, Boca Raton, FL, USA.

- Eaton, M. S., Weiss, L. M., Kim, K., 2006. Cyclic nucleotide kinases and tachyzoite-bradyzoite transition in *Toxoplasma gondii*. *Int J Parasitol*, 36: 107–114.
- Frenkel, J. K., Pfefferkorn ER., Smith, D. D., Fishback, J. L., 1991. Prospective vaccine prepared from a new mutant of *Toxoplasma gondii* for use in cats. *Am J Vet Res*, 52: 759–763.
- Freyre, A. L., Choromanski, J. L., Fishback, J. L., Popiel, I., 1993. Immunization of cats with tissue cysts, bradyzoites, and tachyzoites of the T-263 strain of *Toxoplasma gondii*. *J Parasitol*, 79: 716–719.
- Fux, B., Nawas, J., Khan, A., Gill, D. B., Su, C., Sibley, L. D., 2007. *Toxoplasma gondii* strains defective in oral transmission are also defective in developmental stage differentiation. *Infect Immun*, 75: 2580–2590.
- Gail, M., Gross, U., Bohne, W., 2001. Transcriptional profile of *Toxoplasma gondii*-infected human fibroblasts as revealed by gene-array hybridization. *Mol Genet Genomics*, 265: 905–912
- Grant, I. H., Gold, J. W., Rosenblum, M., Niedzwiecki, D., Armstrong, D., 1990. *Toxoplasma gondii* serology in HIV-infected patients: the development of central nervous system toxoplasmosis in AIDS. *AIDS*, 4: 519–522.
- Hoff, R.L., Dubey, J.P., Behbehani, A.M., Frenkel, J.K., 1977. *Toxoplasma gondii* cysts in cell culture: new biologic evidence. *J Parasitol*, 63: 1121–4.
- Hoffmann, S., Batz, M. B., Morris, J. G. Jr., 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *J Food Prot*, 75: 1292–1302.
- Holland, G.N., 2003. Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease. *Am J Ophthalmol*. 136: 973–988.
- Innes, E. A., Panton, W. R., Sanderson, A., Thomson, K. M., Wastling, J. M., Maley, S. W., Buxton, D., 1995. Induction of CD4⁺ and CD8⁺ T cell responses in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function. *Parasite Immunol*, 17: 151–160.
- Jongert, E., Melkebeek, V., De Craeye, S., Dewit, J., Verhelst, D., Cox, E., 2008. An enhanced GRA1–GRA7 cocktail DNA vaccine primes anti-Toxoplasma immune responses in pigs, *Vaccine*, 26: 1025–1031

- Kringel, H., Dubey, J. P., Beshah, E., Hecker, R., Urban, J. F. Jr., 2004. CpG oligodeoxynucleotides enhance porcine immunity to *Toxoplasma gondii*. *Vet Parasitol*, 123: 55–66.
- Lyons, R. E., Mcleod, R., Roberts, C. W., 2002. *Toxoplasma gondii* tachyzoite-bradyzoite interconversion. *Trends Parasitol*, 18: 198–201.
- Mateus-Pinilla N. E., Dubey, J. P., Choromanski, L., Weigel, R. M., 1999. A field trial of the effectiveness of a feline *Toxoplasma gondii* vaccine in reducing *T. gondii* exposure for swine. *J Parasitol*, 85: 855–860.
- Matsukayashi H., Akao S., 1963. Morphological studies on the development of the toxoplasma cyst. *Am J Trop Med Hyg*, 12: 321–333.
- Munday, B. L., 1972. Serological evidence of *Toxoplasma* infection in isolated groups of sheep. *Res Vet Sci*, 13: 100–102.
- Popie I., Gold M., Choromanski L., 1994. Tissue cyst formation of *Toxoplasma gondii* T-263 in cell culture. *J Eukaryot Microbiol*, 41: 17S.
- Popiel, I., Gold, M.C., Booth, K.S., 1996. Quantification of *Toxoplasma gondii* bradyzoites. *J Parasitol*, 82: 330–2.
- Rajendrana, C., Su, C., Dubey, J. P., 2012. Molecular genotyping of *Toxoplasma gondii* from Central and South America revealed high diversity within and between populations. *Infect Genet Evol*, 12: 359–368.
- Scharff, R.L., 2012. Economic burden from health losses due to foodborne illness in the United States. *J Food Prot*, 75: 123–131.
- Shiels, B., Aslam, N., McKellar, S., Smyth, A., Kinnaird, J., 1997. Modulation of protein synthesis relative to DNA synthesis alters the timing of differentiation in the protozoan parasite *Theileria annulata*. *J Cell Sci*, 110: 1441–51.
- Skariah, S., McIntyre, M.K., Mordue, D. G., 2010. *Toxoplasma gondii*: determinants of tachyzoite to bradyzoite conversion. *Parasitol Res*, 107: 253–260.
- Soete, M., Camus, D., Dubremetz, J.F., 1994. Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of *Toxoplasma gondii* in vitro. *Exp Parasitol*, 78: 361–70.

- Spear, W., Chan, D., Coppens, I., Johnson, R.S., Giaccia, A., Blader, I.J., 2006. The host cell transcription factor hypoxia-inducible factor 1 is required for *Toxoplasma gondii* growth and survival at physiological oxygen levels. *Cell Microbiol*, 8: 339–352.
- Sullivan, W. J., Narasimhan, J. J., Bhatti, M. M., Wek, R. C., 2004. Parasite-specific eIF2 (eukaryotic initiation factor-2) kinase required for stress-induced translation control. *Biochem J*, 380: 523–531.
- Vieira, F.E.G., Sasse, J.P., Minutti, A.F., Miura, A. C., Barros, L.D., Cardim, S.T., Martins, T.A., Seixas, M., Yamamura, M.I., Su, C., Garcia, J.L., 2018. *Toxoplasma gondii*: prevalence and characterization of new genotypes in free-range chickens from south Brazil. *Parasitol Res*, 117: 681–688.
- Wallace, G.D. 1969. Serologic and epidemiologic observations on toxoplasmosis on three Pacific Atolls. *Am J Epidemiol*, 90: 103–111.
- Weilhammer, D. R., Iavarone, A. T., Villegas, E. N., Brooks, G. A., Sinai, A. P., Sha, W. C., 2012. Host metabolism regulates growth and differentiation of *Toxoplasma gondii*, *Int J Parasitol*, 42: 947–959
- Weiss, L. M., Ma, Y. F., Takvorian, P. M., Tanowitz, H. B., Wittner, M., 1998. Bradyzoite development in *Toxoplasma gondii* and the hsp70 stress response. *Infect Immun*, 66: 3295–3302.
- Weigel, R. M., Dubey, J. P., Dyer, D., Siegel, A. M., 1999. Risk factors for infection with *Toxoplasma gondii* for residents and workers on swine farms in Illinois, USA. *Am J Trop Med Hyg*, 60: 793–798.
- Weiss, L. M., LaPlace, D., Tanowitz, H. B., Wittner, M., 1992. Identification of *Toxoplasma gondii* Bradyzoite-Specific Monoclonal Antibodies. *J Infect Dis*, 166: 213–215.
- Weiss, L. M. and Kim, K., 2011. The development and biology of bradyzoites of *Toxoplasma gondii*. *Front Biosci*, 5: D391–D405.
- Weiss, M. L., Kim, K., 2013. *Toxoplasma gondii*: The model apicomplexan-perspectives and Methods. 2th edition. Amsterdam, Boston, Heidelberg, London.

Chapter 5 Appendix

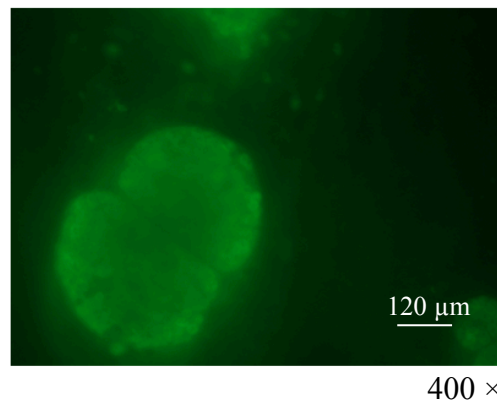
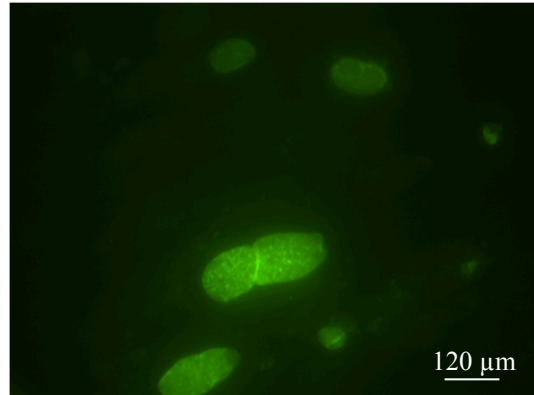


Figure 5. 1 The examination of BAG1 expression of *T. gondii* TgCkVe6 using IFA. TgCkVe6 was vacuum treated as described in 2.1. Seven days post incubation at 37 °C, 5% CO₂, TgCkVe6 formed large persisting cysts under bright light microscope. The primary antibody, monoclonal anti-BAG1 antibodies (1:100), was added and incubated at 4°C overnight. The next day, FITC-conjugated goat anti-rabbit IgG (1:100) were added as secondary antibodies. Fluorescent microscope (Leica) was used for observation at 1000×. The green fluorescence indicated the expression of BAG1 on parasite surface. The above image illustrates two large adjacent tissue cysts with BAG1-expressing parasites enclosed.



400 ×

Figure 5. 2 The examination of CST1 expression of *T. gondii* TgCkVe6 using IFA. TgCkVe6 was vacuum treated as described in 2.1. Seven days post incubation at 37 °C 5% CO₂, TgCkVe6 formed big persisting cysts. The FITC conjugated DBL (1:300) was used to stain CST1 for one hour at RT. Fluorescent microscope (Leica) was used for observation at 400×. The above image indicates the expression of CST1 on cyst wall surface in green. The overlapping area of two tissue cysts was brighter in green, indicating the staining of tissue cysts walls.

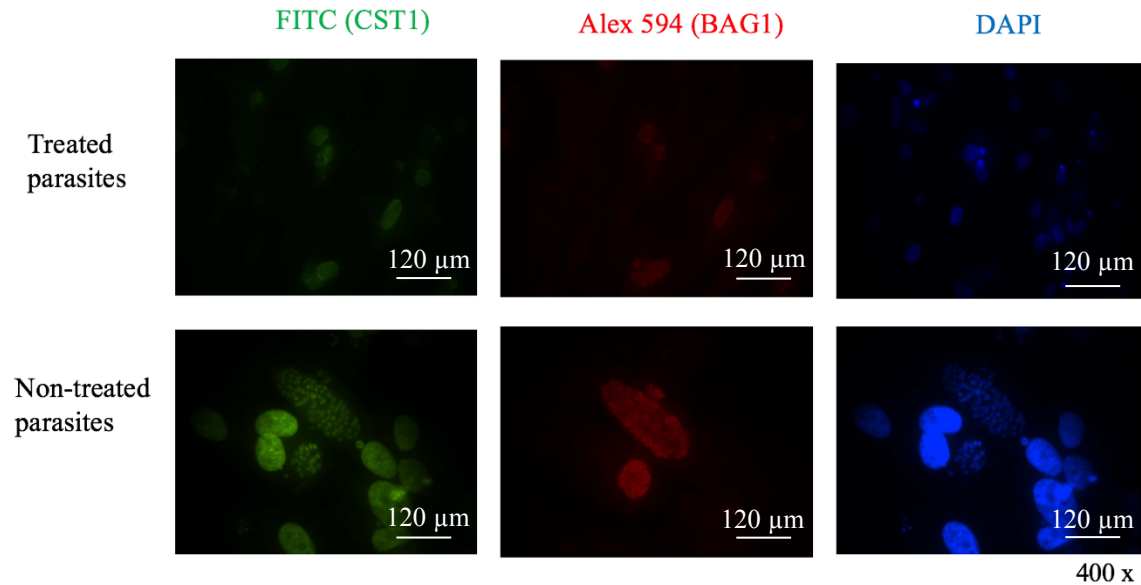
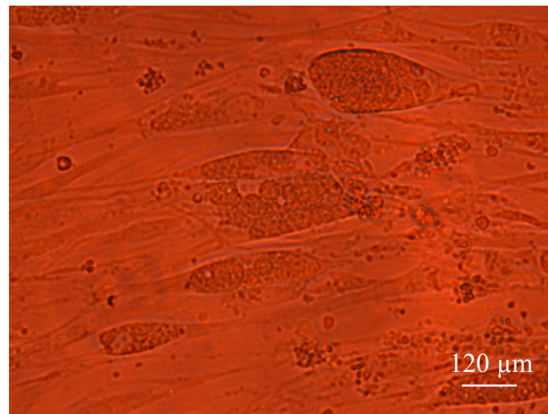


Figure 5. 3 Simultaneous examination of BAG1 and CST1 in treated or non-treated *T. gondii* TgCkVe6 using IFA. The monoclonal rabbit anti-BAG1 antibodies (1:100) was used as primary antibodies, and secondary antibodies were Alexa Fluor 594 goat anti-rabbit IgG at 1:100. Upon washing, FITC-conjugated DBL at 1:300 was added for CST1 staining at RT for one hour. Nuclei were also stained with DAPI for five minutes at RT. Fluorescent microscope (Leica) was used for observation at 400×. The expression of BAG1 was indicated in green fluorescence, whereas the expression of CST1 was shown in red fluorescence. The staining of nuclei was in blue. The cross-emission of FITC and DAPI was pronounced, especially in the non-treated group.



400 ×

Figure 5. 4 The large vacuoles of T263 at six days post-infection at 31 °C. T263 was allowed to infect HFFs at 31 °C and kept at 31 °C 5% CO₂ incubator for seven to eight days. The big persisting cysts were seen under bright light microscope at 400 ×.

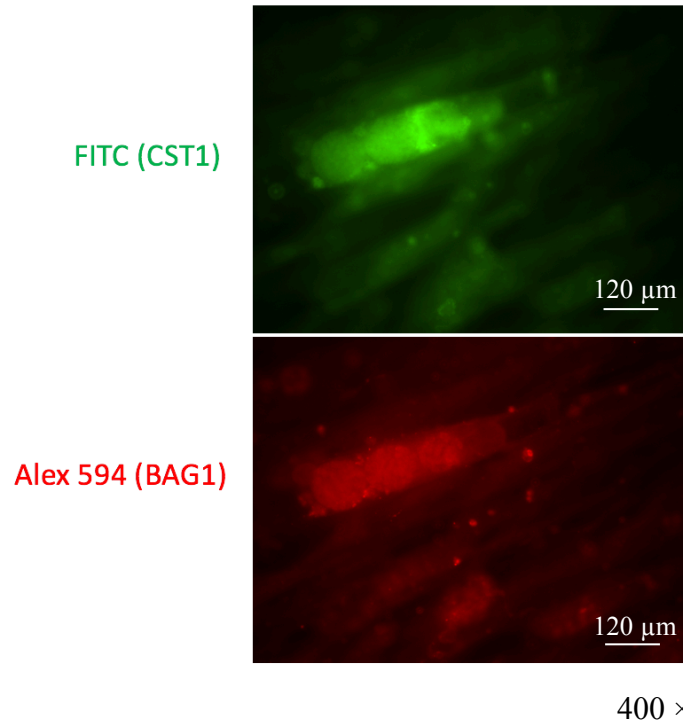


Figure 5. 5 Simultaneous staining of BAG1 and CST1 of T263 (six days post-infection at 31 °C) using IFA. T263 was allowed to infect HFFs at 31 °C and kept at 31 °C, 5% CO₂ incubator for six days. The monoclonal rabbit anti-BAG1 antibodies (1:100) and Alexa Fluor 594 goat anti-rabbit IgG (1:100) were used as primary and secondary antibodies, respectively. FITC-conjugated DBL at 1:300 was used for CST1 staining. Images were taken at 400× magnification.

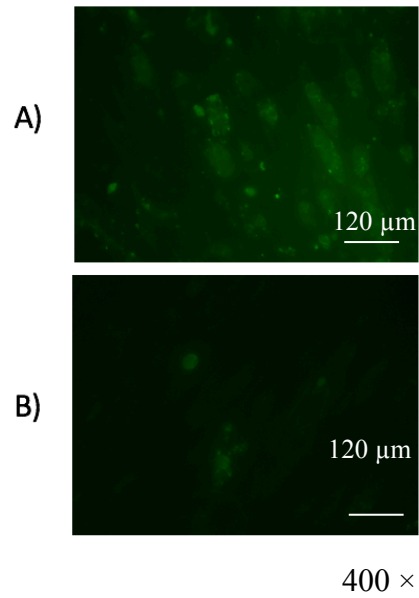


Figure 5. 6 DBL staining of T263 seven days post-infection at 31°C using IFA. A) 3×10^5 T263 tachyzoites were used to infect HFFs in each well of a 6-well plate. FITC-conjugated DBL at 1:400 was added for CST1 staining; **B)** 3×10^4 T263 tachyzoites were inoculated into each well of a 6-well plate. FITC-conjugated DBL at 1:400 was added for CST1 staining.

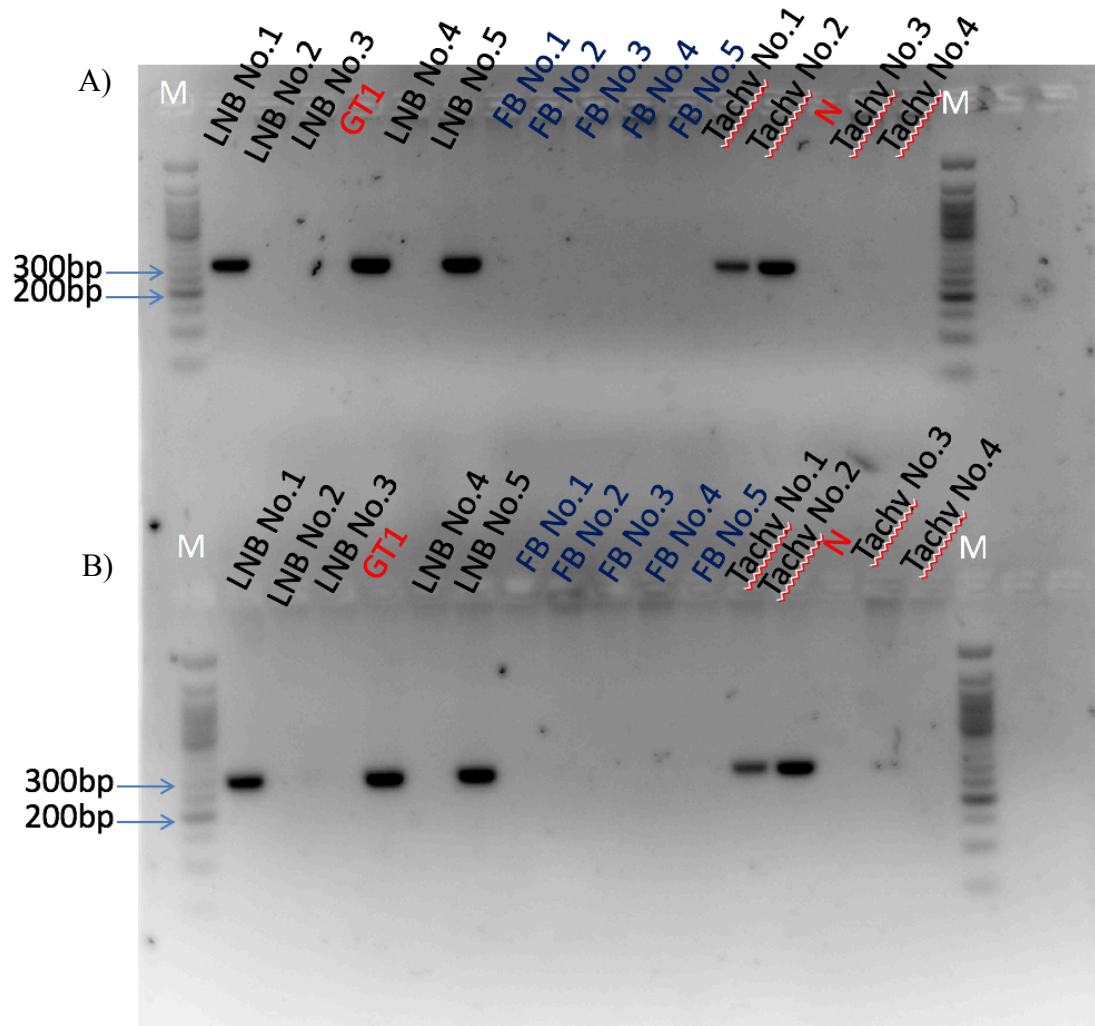


Figure 5. 7 PCR detection of *T. gondii* DNA from tissues of experimentally infected mice. A) Two out of five mice (LNB No.1–No.5) infected with liquid nitrogen preserved bradyzoites (LNB) were positive for *T. gondii* in their tissues (heart, brain, and lungs); two out of four mice (Tachy No. 1–No.4) that were infected with fresh tachys were positive for *T. gondii* in their tissues (heart, brain, and lungs). No positive sample was detected in a group of five mice (FB No.1–No.5) infected with fresh bradyzoites (FB); GT1 was a *T. gondii* strain used as positive control. N denotes negative control in which DNA was not added. **B)** A parallel replicate of panel A. PCR was done on the same 96-well plate.

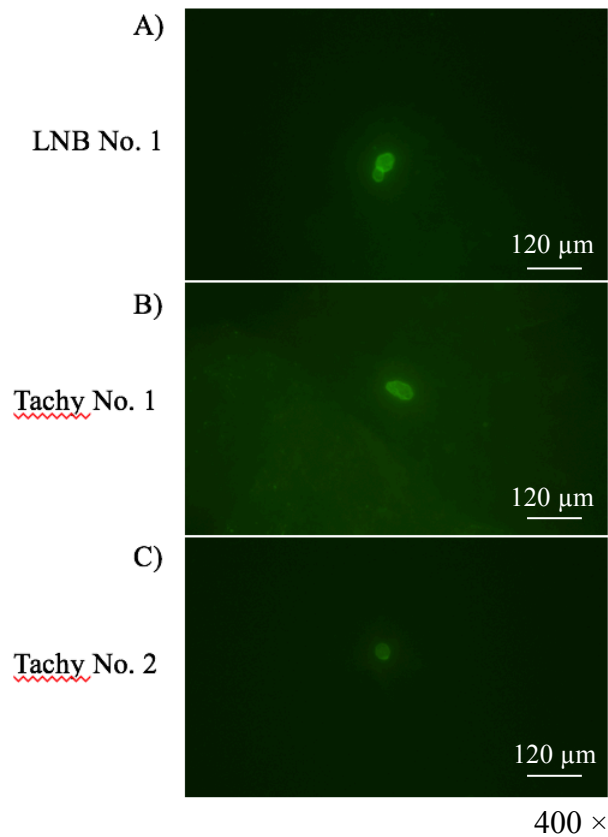


Figure 5. 8 DBL staining of brain tissue cysts. A quarter of brain tissue was homogenized and stained with FITC-conjugated DBL. A total of three positive samples were identified, namely LNB No.1, Tachy No.2, and Tachy No.1. Seven cysts were found on a glass slide with 10 μ l of brain suspension in LNB No. 1, whereas two were found in Tachy No. 2, and one was found in Tachy No.1. **A)** Two cysts were shown from sample LNB No.1 which was from a mouse that were orally infected with liquid nitrogen preserved bradyzoites; **B)** One image from Tachy No. 1 which was obtained from a mouse that was orally infected with *in vitro* produced fresh tachyzoites. C) One representative image from sample Tachy No. 2.

Table 5. 1 Acid-pepsin digestion of vacuum-treated and non-treated TgCkVe6 for 15 mins.

Treated parasites			
	Initial	After treatment	Survival rate
0.9% NaCl, 15 mins	5×10^5	1.36×10^5	27 %
Pepsin, 15 mins	5×10^5	1.2×10^3	0.24 %
Non-treated parasites			
	Initial	After treatment	Survival rate
0.9% NaCl, 15 mins	2.05×10^5	1.52×10^5	74 %
Pepsin, 15 mins	2.05×10^5	0	0 %

Table 5. 2 A repeat of acid-pepsin digestion of vacuum-treated and non-treated TgCkVe6 for 15 mins.

Treated parasites			
	Initial	After treatment	Survival rate
0.9% NaCl, 15 min	2.25×10^4	2.24×10^4	99.6 %
Pepsin, 15 min	2.25×10^4	0	0 %
Non-treated parasites			
	Initial	After treatment	Survival rate
0.9% NaCl, 15 min	8×10^4	1.2×10^5	150 %
Pepsin, 15 min	8×10^4	0	0 %

Table 5. 3 Seroprevalence of 14 mice orally infected with 31°C treated T263.

Sample ID	No. of sera tested	Titer									% positive sera (cutoff at 1:25)
		<25	25	50	100	200	400	800	>=1600	>=3200	
LNB ^a	5	3	0	0	0	0	2	0	0	0	40% (2/5)
Tachys ^b	4	2	0	0	0	0	0	0	1	1	50% (2/4)
Fresh bradys ^c	5	5	0	0	0	0	0	0	0	0	0

^aA group of five mice were orally infected with liquid nitrogen preserved bradyzoites (LNB);

^bA group of five mice were orally infected with *in vitro* produced fresh tachys; one mouse died due to accidental puncture of lung whose carcass was simply discarded without collecting samples.

^cA group of five mice were orally infected with fresh bradyzoites produced *in vitro*.

CHAPTER 6

CONCLUSION

Conclusion

The research conducted in this dissertation represents four of the many aspects of current *T. gondii* study. My first project continued the interest of our lab and investigated the genetic structure of *T. gondii* in North America. We were able to summarize the genotyping data generated in the past decade and examine the underlying factors in shaping the population structure of *T. gondii* in North America. My second project aimed to evaluate the environmental contamination by *T. gondii* on a farm with cats. The evaluation of environmental contamination is fundamental to the development of control measures to reduce *T. gondii* infection in humans and animals. My third project focused on the isolation and characterization of *T. gondii* from clinal fatal toxoplasmosis from macropods. *T. gondii* infection in captive macropods continues to be a problem as zoos and theme parks suffer from the enormous economic loss. In my last project, I strived to convert tachyzoites to bradyzoites *in vitro* in hope to simplify the preparation of a cat vaccine T263 to reduce its costs.

Investigating factors shaping T. gondii genotype landscape in North America

Our lab has been interested in the population genetics of *T. gondii*. The genotypes of *T. gondii* were defined by the multilocus PCR-RFLP genetic markers; the method was initiated by Howe and Sibley (Howe and Sibley, 1995) and refined later (Su et al., 2006 and 2010). Our lab has worked to expand the application of multilocus PCR-RFLP to make data comparable across labs worldwide. With the extensive use of this methodology, a collection of 1400 samples from across the world has been genotyped by 2014 (Shwab et al., 2014). The genetic structure of *T. gondii* was revealed on a global level. My first project built upon the publication of Schwab et al. in 2014 and focused on the genotype landscape in North America. I particularly researched the human influence on *T. gondii* genotype distribution, and whether *T. gondii* genotype had host species specificity. We concluded that *T. gondii* genotype diversity and the host proximity to human settlement had an inverse relationship. Moreover, the dominant genotypes in groups of animals with different association with human activities were different. Type II and type III were found predominant in farm-bound animals, whereas type 12 dominated in wildlife. The dominant genotypes in farm-bound animals (Type II and III) and that in wildlife (Type 12) were found almost equally prevalent in the free-ranging domestic animal group. The observed phenomena signified

human influence on *T. gondii* transmission. A between-population comparison was conducted to study the effect of host specificity on *T. gondii* genotype distribution. Fourteen populations based on animal species with more than ten isolates were selected. The significant differences were found between certain animal populations, indicating that host specificity affected *T. gondii* genetic structure. In the future, more samples from the definitive host especially cats and bobcats, are necessary to understand the transmission dynamics of *T. gondii* in the environment.

The assessment of T. gondii environmental contamination on a dairy farm

This body of research aimed to develop a feasible method to evaluate environmental contamination by *T. gondii*. A typical farm affiliated with the University of Tennessee at Knoxville was chosen to perform the research. We affirmed that the soil oocyst contamination is marginal to the sensitivity of the current detection system. The sugar floating method (Lelu et al., 2011) in conjunction with PCR-RFLP following DNA extraction method (Silva et al., 2009) was used in our study. As we are aware of the potential loss of oocyst during the process of DNA extraction, we made attempts to skip the DNA extraction process. However, when DNA extraction was omitted, issues arose from PCR contamination or PCR inhibition by soil substances (e.g., humic acid). The low concentration of oocyst in soil is likely due to a few factors. First, the massive nature of soil dictates that oocysts excreted by cats will be extremely diluted in the soil. In addition, the unpredictable, harsh environmental conditions, such as desiccation and radiation from sunlight, likely disintegrated oocyst over time. Above all, soil oocyst DNA detection offered no information concerning the viability of oocyst, and thus bore little or no implicative values to the infection in animals and humans. Therefore, results generated based on soil oocyst detection may be difficult to interpret due to potential contamination. In summary, soil oocyst detection may not be a suitable parameter for future contamination evaluation.

The focus of the research then switched to the evaluation of infection in farm animals, both domestic and wildlife. Our study herein demonstrated that rodents had low infection rate. However, a partition of rodent habitats was discovered as human household associated house mice were geographically captured away from those of wild rodents. This supports the idea of two geographically separated *T. gondii* transmission cycles, namely the domestic cycle and the sylvatic cycle. The meso-predators were found to be good targets for evaluation. Raccoons and cats

particularly have higher *T. gondii* infection and thus are better targets for contamination assessment. The field trapping data had revealed that opossums were easy to trap and had high recapture rate and thus would be a good target for assessment. However, the serological results from opossums showed that they are all negative for *T. gondii* antibodies. It was hard to comprehend as to the reasons behind the negative results although plausible speculations were raised. Further study to isolate parasites from opossums could hopefully unravel the mystery.

In conclusion, our current study suggested that future evaluation of *T. gondii* environmental contamination should target animals in the higher trophic level instead of focusing on detecting soil oocyst and rodent infection.

The investigation of eleven fatal toxoplasmosis cases in captive macropods

Fatal toxoplasmosis occurred in 11 captive macropods in a theme park in Tampa, Florida. The 11 macropods successively succumbed to *T. gondii* infection over four years from 2014 to 2017. Infection was confirmed via serology, histopathology, parasite isolation as well as PCR-RFLP genotyping. Genotyping of these parasites either directly from animal tissues or from isolated parasites revealed three genotypes, #2, #216 and #263. Notably, animals died in the same month had the identical genotypes, suggesting that these animals were infected during the same transmission event. The three distinct genotypes indicate that at least three distinct transmission events were responsible for these intermittent incidences.

Macropods are among the most susceptible hosts of *T. gondii*. The infection of macropods in the wild is unknown as there are no wild macropods in the USA. Of the ten wallabies imported from New Zealand, nine wallabies succumbed to acute *T. gondii* infection at a zoological facility in Virginia, USA (Guthrie et al., 2017). Two genotypes were revealed, namely #4 (n=3) and #263 (n=6). The study presented herein reinforced the high mortality of acute toxoplasmosis in these susceptible animals and accentuated oocyst-mediated transmission. It was suspected that the seasonal heavy rainfall in the theme park likely washed oocyst down to the animal exhibit. The exact source of infection has not been investigated. Further study to isolate oocyst from exhibit soil for mice bioassay is likely helpful to identify the source of infection. In addition, the isolation of parasite from feral cats (tissue or feces) roaming on the theme park premises can also aid in the

identificating of infection source. This study also highlights the urgency of control measures to reduce *T. gondii* contamination in zoos and theme parks with the presence of macropods.

The in vitro conversion of T263 from tachyzoite to bradyzoite

In this chapter, we attempted to develop a conversion approach to produce cat vaccine T263 *in vitro* in hope to simplify the vaccine preparation and thus reduce its cost. The strategies we tested included vacuum conditions and low temperature at 31°C. To evaluate the quality of treated parasites, we used three assays, namely IFA, pepsin digestion, and mice oral infection. We concluded that vacuum conditions were able to convert TgCkVe6 from tachyzoites to bradyzoites but was unable to convert T263. Vacuum treated TgCkVe6 expressed BAG1 and CST1; our first test showed that a small percentage (0.24%) of vacuum treated TgCkVe6 were pepsin resistant, although the results were not reproductive. Low temperate at 31 °C was able to convert T263 from tachyzoites to bradyzoites. The expression of BAG1 and CST1 were observed in converted T263. However, the cyst walls were less intensely stained when compared to those of converted TgCkVe6. The converted bradyzoites T263 were able to orally infect mice which demonstrate promise for using this approach to produce T263 vaccine *in vitro*. Although preserved in liquid nitrogen for four months, parasites at a dose of 2.5×10^4 cysts were still viable and infectious to mice. The counterintuitive results were observed in freshly prepared bradyzoites. The explanation was that the initial infection rate is low, which resulted in an inadequate number of mature bradyzoites. A short period of CO₂ outage likely adversely affected the development of bradyzoites. Further studies should aim to optimize the protocol to minimize between-batch inconsistency. A repeat of mice orally infection assay is required to further evaluate *in vitro* produced T263 bradyzoites. Finally, a bioassay in cats is necessary to test the efficacy of the *in vitro* generated T263 as a vaccine to induce immunity to oocyst shedding.

Future directions

The overarching objective of our current research to break the infection cycle of *T. gondii* to reduce infection rates in humans and animals. To accomplish this, much more information of the nature of *T. gondii* distribution and transmission is needed. Our contribution to this overarching effort was to describe the genotypic distribution of *T. gondii* in host species in North America. Future

study to estimate a more accurate animal home range is needed for a more precise classification of animals into the aforementioned three categories. With the understanding of *T. gondii* transmission and genetic diversity, we assessed the farm contaminant of *T. gondii* in attempt to establish the baseline for contamination. Future study on a pig farm with more concentrated cats is needed to elucidate the contamination rates in both soil and animals. For that purpose, the home range of meso-predators need to be determined to ensure their sole association with the study farm. It's also imperative to re-assess the soil contamination of *T. gondii*, especially in areas where the presence of cats is frequent. In addition, we developed a method to simplify a cat vaccine production. Future study should focus on increasing the efficiency of tachyzoites to bradyzoites conversion of T263 to increase mice infection rate. Moreover, the oral infectivity of *in vitro* produced bradyzoites T263 needs to be tested in cats to see whether these parasites can induce immunity to oocyst shedding in cats.

References

- Howe, D. K. and Sibley, L. D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis*, 172: 1561–1566.
- Guthrie, A., Rooker, L., Tan, R., Gerhold, R., Trainor, K., Jiang, T., Su, C., 2017. Newly described *Toxoplasma gondii* strain causes high mortality in red necked wallabies (*Macropus rufogriseus*) in a zoo. *J Zoo Wildl Med*, 48: 694–702.
- Lelu, M., Gilot-Fromont, E., Aubert, D., Richaume, A., Afonso, E., Dupuis, E., Gotteland, C., Marnef, F., Poulle, M.L., Dumètre, A., Thulliez, P., Dardé, M.L., Villena, I., 2011. Development of a sensitive method for *Toxoplasma gondii* oocyst extraction in soil. *Vet Parasitol*, 183: 59 – 67.
- Shwab, E., Zhu, X., Majumdar, D., Pena, H., Gennari, S., Dubey, J., and Su, C., 2014. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. *Parasitology*, 141: 453–461.
- Silva, R.C, Su, C., 2009. First identification of *sarcocystis tenella* by PCR in naturally infected sheep from Brazil. *Vet Parasitol*, 165: 332 – 336.
- Su, C., Zhang, X. and Dubey, J. P., 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int J Parasitol*, 36: 841–848.
- Su, C., Schwab, E. K., Zhou, P., Zhu, X. Q. and Dubey, J. P., 2010. Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. *Parasitology*, 137: 1–11.

APPENDICES

Appendix A: IFA—the Staining of BAG1 and CST1

Reagents

1. 4% formaldehyde
2. Permeabilizing buffer: 0.5% triton: 50 μ l triton in 9950 μ l PBS
3. Blocking buffer: 0.1% triton & 10% BSA in PBS

{	1g of BSA powder
	10 μ l triton ($\times$ 100)
	9990 μ l PBS
4. BAG1 antibodies (1:100): 50 μ l BAG1 in 5ml blocking buffer
5. Alexa Fluor 594 conjugated goat anti-rabbit IgG (1:100): 60 μ l Alexa Fluor 5945 in 6 ml blocking buffer
6. FITC conjugated *Dolichos biflorus* lectin (DBL) (1:300): 20 μ l DBL in 6 ml blocking buffer
7. Glycerol

Procedure

1. Remove medium and wash cells with ice-cold PBS for three times
2. Add 1ml 4% formaldehyde into each well of a 6-well plate; incubate at RT for 30 min.
3. Wash with ice-cold PBS for three times
4. Permeate cells by adding 1ml of permeabilizing buffer into each well; incubate at RT for 20 min.
5. Wash with ice-cold PBS for three times.
6. Add blocking buffer (1ml for each well); incubate at 37 °C for 1 hour
7. Remove blocking buffer but no need for washing; add BAG1 antibodies (1:100) and incubate at 4°C overnight.
8. The next day, wash with blocking buffer for three times.

9. Add secondary antibodies (Alexa Fluor 594 anti-rabbit IgG) at 1:100; incubate at RT for 1 hour.
10. Wash with blocking buffer for three times
11. In each well, add 1ml DBL at 1:300 into each well and incubate for 45 min to 1 hour.
12. wash three times with blocking buffer
13. Take out coverslips, air dry, and mount them onto glass slides using glycerol

Appendix B: Acid-pepsin Digestion of Treated and Non-treated Parasites

Reagents

1. One T25 flask of HFFs infected with treated parasites; another one with non-treated parasites
2. 0.17 M NaCl (pH=7)
3. Syringes
4. HFFs confluent in 12-well plates
5. 0.26 mg/ml pepsin in 0.17M NaCl (pH=1.4)
6. 1.2% sodium bicarbonate (pH=8.3)
7. 70% ethanol
8. pH paper
9. 15 ml and 50 ml conical tubes

Procedure

1. Take a T25 flask of HFFs infected with either tachyzoites or *in vitro* produced bradyzoites; transfer parasites into one 50-ml tube and spin down at 1500 rpm for 10 min.
2. Remove supernatant and resuspend parasites in 5 ml PBS. Syringe parasites through a 22-gauge needle for four times to release parasites from PVs or host cells.
3. Enumerate viable parasites by inoculating parasites into confluent HFFs in a 12-well plate. To do that, make a ten times serial dilution (10^{-1} to 10^{-4}) of the cell suspension. Inoculate 100 μ l of diluted parasites at each concentration into each well of a 12-well plate.
4. Equally split parasites from one T25 into two 15-ml tubes: one will be subjected to pepsin digestion; one will be negative control.
5. Spin down parasites to remove supernatant. Add 5ml 0.26 mg/ml pepsin in 0.170 M NaCl (pH=1.4) into one tube and add 5ml PBS into the other tube. Incubate for 30 min at 37°C.
6. Neutralize with 1.2% sodium bicarbonate (pH=8.3). Spin down to remove supernatant.
7. Resuspend in 2 ml PBS; enumerate viable parasites as described in step 3.

8. Change media of the 12-well plates the next day and incubate for seven days at 37°C in 5% CO₂ incubator.
9. Fix cells with 70% ethanol and allow cells to air dry. Enumerate viable parasites to compare viability.

Appendix C: Mouse Bioassay of 31 °C Treated T263

Reagents:

1. HFFs in T25 flasks
2. T263 tachyzoites grown in HFFs
3. 15 outbred female mice (CD1) for three treatments (five per treatment)
4. Feeding tubes, 3cc syringes, marker
5. PBS

Procedure:

1. Infect two sets of three T25 flasks of HFFs with approximately $6-8 \times 10^6$ T263 tachyzoites grown at 37 °C.
2. Place one set of three flasks into 31 °C, 5% CO₂ incubator and culture for 7 to 8 days. For the other set of three flasks of T263, keep at 37°C, 5% CO₂ and passage every two days.
3. After 7 to 8 days, in 31°C treated cell culture, cysts (PVs) should be visible under microscope, take pictures of treated T263.
4. Preserved two T25 flasks into four cryovials in liquid nitrogen (LN): scrape cells off from each flask and collect cell suspension into a 15 ml tube. Spin down cells at 1800 rpm for 10 mins; remove the supernatant and add 1ml DMEM with 50% FBS to resuspend the pellet, then add 1ml 20% DMSO in DMEM. Aliquot into two cryovials for each T25 flask and put in a box with dry ice. The next day, transfer into LN. These are cryopreserved *in vitro* produced bradyzoites.
5. For fresh 31°C treated cell culture, 7 to 8 days post infection, scrape cells from three flasks. Combine, wash, and resuspend in 5 ml PBS. Count cells (a mixed population of HFF cells with or without PVs enclosed) using hemocytometer. These are fresh *in vitro* produced bradyzoites. Prepare 2.5×10^4 cysts/ml in 5ml PBS for a group of 5 mice.
6. The non-treated cell culture is passed every two days at 37 °C; Collect cell suspension and syringe through a 22-guage needle to completely separate host cells from tachyzoites. Enumerate tachyzoites using hemocytometer. Prepare 2.5×10^4 tachys/ml in 5 ml PBS for

a group of 5 mice. It's reported that at least 10^6 tachyzoites were required to infect a mouse (Dubey, 1998).

7. Four vials of LN preserved *in vitro* produced bradyzoites are thawed at 37 °C in water bath. Wash and resuspend in 5ml PBS. Enumerate parasites using hemocytometer. Prepare 2.5×10^4 cysts/ml in 5ml PBS for a group of 5 mice. Based on published paper, 1000 mature bradyzoites are required to infect a mouse (Fux et al., 2007).
8. Three groups of five mice are orally infected with three treatments (fresh tachyzoites, LN preserved *in vitro* produced bradyzoites, and fresh *in vitro* produced bradyzoites) (1ml for each mouse). Be sure to ease the feeding tube (the end tip of the feeding tube facing toward the experimenter) into mouse digestive tube and deliver all the way down to mouse stomach. Be careful not to puncture lung as doing so will cause immediate death of the mouse.
9. Monitor for three weeks. The survivors are euthanized. Blood samples are taken for MAT; brain, heart, and lung tissues are taken for PCR detection and cyst examination.
10. For cyst examination, FITC-conjugated DBL is used to stain brain homogenate. Fluorescent microscope is used to examine cyst wall staining with DBL. Refer to DBL staining for detailed procedure.

References

- Fux, B., Nawas, J., Khan, A., Gill, D.B., Su, C., Sibley, L.D., 2007. *Toxoplasma gondii* strains defective in oral transmission are also defective in developmental stage differentiation. Infect Immun, 75: 2580–2590.
- Dubey, J. P., 1998. Re-examination of resistance of *Toxoplasma gondii* tachyzoites and bradyzoites to pepsin and trypsin digestion. Parasitology, 116: 43–50.

Appendix D: Staining Toxo Cysts in Brain Lysates by FITC-conjugated DBL

Reagents:

1. Fixing and Permeabilizing solution- 6% formaldehyde, 0.2% triton- $\times 100$ in PBS.

H ₂ O	3
10 $\times$ PBS	1
10% formaldehyde	6
<u>Triton-x 100</u>	<u>0.02 ml</u>
Total	10 ml

2. 10% goat serum in PBS

H ₂ O	40
10 $\times$ PBS	5
<u>Goat serum</u>	<u>5 ml</u>
Total	10 ml

3. FITC-conjugated DBL (Vector) stock solution (1:3 in PBS)

Procedure:

1. Prepare brain suspension in 2ml PBS by syringe pass through gauge 16 and 20 needles.
2. Add 1ml brain lysate to 1ml fixing and permeabilizing solution using a 15 ml polystyrene centrifuge tube. Incubate for 20 min at 4 °C.
3. Spin for 10min at 1700 rpm in TC centrifuge at 4 °C.
4. Wash once with 4ml PBS/10% goat serum. Spin down tissue cysts and remove supernatant.
5. Add 2 ml 10% goat serum in PBS, 20 μ l FITC-conjugated lectin stock solution (final concentration is 1:300; 1: 1000 is okay too!) for 45 min to 1 hour.
6. Wash twice by centrifugation at 1700 rpm, 15 °C in 4 ml 10% goat serum.

7. Resuspend cysts in 2ml PBS.
8. Place 10 μ l brain suspension onto glass slide and screen under microscope.

VITA

Tiantian Jiang was born in a small city in Yantai, Shandong, China. She obtained her bachelor's degree from Qingdao Agricultural University majoring in veterinary medicine in 2010. The same year she was accepted to China Agricultural University to study viral diseases in waterfowl using molecular tools. After two years, she finished her master's degree in veterinary and had a brief work experience in a small biotechnology company in Beijing, China. From there, she came to the USA to pursue her Ph.D. degree concentrating on *Toxoplasma gondii* pathogenesis, transmission, and genetic diversity in 2013.