Genetic Diversity, Population Structure, and Evolution of Virulence in *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is among the most widespread eukaryotic pathogens known. It chronically infects approximately one third of the world's human population and has been isolated from an extremely diverse array of globally distributed mammals and birds. Understanding the structure of the worldwide *T. gondii* population enhances our understanding of the factors that have shaped that structure and led to the proliferation of one of the most evolutionarily successful pathogens on Earth. Herein we collate genotypic data from global isolates, demonstrating that *T. gondii* possesses a unique population structure in which only a small number of genotypes dominate throughout the northern hemisphere, but myriad types coexist in South and Central America with none being notably dominant. On average, South American strains were also found to be more virulent to mice than their northern counterparts. We propose that the clonal population structure in the northern hemisphere is the consequence of the domestic cat-house mouse transmission cycle, which has been greatly enhanced by the spread of human agriculture in the past 11,000 years, and that this transmission cycle plays a significant role in the selection of intermediately virulent *T. gondii* genotypes. To test this idea, we compiled strain virulence and sequential reinfection data. Simulations based on the composite dataset were used to predict patterns of mouse virulence evolution in *T. gondii* populations. Our results indicate an advantage relating to the mouse virulence of particular strain types within the domestic cat–house mouse transmission cycle. However, such selective pressure is alleviated in the context of a sylvatic cycle, in which most wild intermediate hosts are resistant to *T. gondii* infection. This suggests that highly mouse-virulent parasite types have an adaptive advantage during transmission in environments that are rich in wildlife species, such as the tropical rainforests of South America. Mouse virulence was found to be closely associated with particular alleles encoding parasite proteins ROP5 and ROP18, making these markers valuable tools for predicting virulence of future environmental isolates. The results of this study have significant implications concerning the evolution of *T. gondii* in agrarian and urbanized environments and transmission of *T. gondii* to humans.

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Chapter I. Literature Review

Overview

Toxoplasma gondii is a protozoan parasite that appears to be capable of infecting all mammals and birds. It has been isolated from a diverse array of host species from throughout the world, making it possibly the most ubiquitous eukaryotic parasite on Earth (Dubey 2010). Though the parasite is able to infect many animal species, felines are the definitive hosts and sexual reproduction occurs only in the feline intestine. This results in the production of infectious oocysts which are shed into the environment and may be transmitted to new hosts orally. In the majority of hosts, ingestion results in the development of asymptomatic chronic infections which persist throughout the host's lifespan (Dubey 2010, Pusch 2009). Chronic infections are characterized by the production of parasite cysts in many tissues of the host. The second major means of transmission involves the ingestion of these cysts during carnivory of infected hosts by predators or scavengers. In humans, such transmission often occurs through ingestion of undercooked meat. Humans are largely resistant to the parasite, experiencing mild symptoms for a brief period after initial infection, followed by the development of asympomatic chronic infection. In immunocompromised adults and developing fetuses, the parasite may cause more severe illness and potential death (Dubey 2010). Congenital infection in general is thought to occur only in cases where the mother becomes infected for the first time during pregnancy, though very rare exceptions have been noted (Dubey *et al.* 2012). In some cases, immunocompetent adults may also experience more severe symptoms from infection. This manifests most commonly as ocular toxoplasmosis, which may cause vision impairment or blindness in some cases (Holland 2003). Severe systemic toxoplasmosis has also been recorded in immunocompetent adults, most notably in a number of recent cases in French Guiana which required intensive medical care and resulted in several deaths due to organ failure (Darde *et al.* 1998, Demar *et al.* 2007, Carme *et al.* 2009). Both severe ocular and systemic toxoplasmosis have been tentatively linked to atypical parasite strains with high virulence in mice (Grigg *et al.* 2001, Bossi and Bricaire 2004, Delhaes 2010).

Mice are particularly susceptible to the parasite and different strains of *T. gondii* display a wide range of virulence in this host. Early genotyping studies in North America and Europe divided strains into three archetypal lineages referred to as Type I, Type II and Type III, which could be chiefly distinguished phenotypically based on their virulence to mice (Sibley and Boothroyd 1992, Howe and Sibley 1995). Type I strains are highly virulent and do not typically cause chronic infections in mice, rather killing them during the acute phase of infection even at very low dosages. Type II and III strains are much less virulent, resulting in chronic infections at lower dosages, though Type II strains may also cause mortality at moderate dosages whereas Type III strains typically require higher dosages in order to be lethal (Sibley and Boothroyd 1992, Khan *et al.* 2009). Type II and III strains have been found to make up the majority of North American and European parasite populations, with Type I strains being relatively rare (Howe and Sibley 1995). More recent genotyping studies have identified parasite populations in the northern hemisphere, including North America, Europe, the Middle East and southeast Asia, to be largely clonal in structure, chiefly comprising a small number of highly dominant genotypes, most of which are closely related to the Type II lineage (Shwab *et al.* 2014).

Populations in South and Central America, by contrast, display much higher diversity, being composed of a wide variety of atypical strain types, none of which are particularly dominant. Strains from these regions also have a much greater tendency towards mouse virulence than those in the northern hemisphere (Gilbert *et al.* 2008). In this work, we attempt to comprehensively catalogue the global diversity of *T. gondii* and to understand the evolutionary pressures that have shaped parasite populations throughout the world and the implications these findings may have for the future of human disease.

Taxonomy

Toxoplasma gondii belongs to the phylum *Apicomplexa*, a diverse group of eukaryotic microorganisms which share the traits of having complex, intracellularly parasitic life cycles and possessing a number of unique organelles at their apical ends that aid in host invasion by facilitating secretion of an array of effector proteins into occupied cells (Morrison 2009). Other medically significant members of this phylum include species of *Plasmodium* -the causal agents of malaria, *Cryptosporidium*, and *Babesia*. In addition to these human pathogens, there are numerous apicomplexan genera responsible for important veterinary diseases, such as *Neospora* and *Sarcocystis*. (Perkins 2000).

Host range

Unlike other members of the *Apicomplexa*, which tend to have very narrow host ranges comprising only one or a few closely related species, *T. gondii* has an extremely broad host range, apparently extending to all endotherms, or in other words to members of the classes *Mammalia* and *Aves* (Dubey 2010). Since *T. gondii* was first isolated from and named for an African rodent known as the gundi in 1908 (Kim and Weiss 2008), it has subsequently been identified in the tissues of an extremely diverse assortment of animals ranging from the white-tailed deer to the striped dolphin to the keel-billed toucan (Shwab *et al.* 2014). It was not until 1970 however that members of the family *Felidae* were identified as the definitive hosts of the parasite (Dubey 2009). It is only within the intestinal epithelia of cat species that *T. gondii* is able to undergo sexual reproduction, a strong indication that cats represent the ancestral host in which differentiation of *T. gondii* as a species occurred (Dubey 2010).

Life cycle

Felids serve as the definitive host for *T. gondii* in which sexual reproduction occurs. Oocyst shedding has been observed in a wide range of feline species, from domestic cats to Siberian tigers (Dubey 2010). Transmission occurs by two primary means, namely ingestion of oocysts from environmental sources, or ingestion of tissue cysts in infected meat (Figure I-1). The latter method allows for transmission between intermediate hosts without necessitating the involvement of feline species. The range of intermediate hosts in which *T. gondii* has been identified - including animals as diverse as rats, dogs, sheep, crows, toucans, kangaroos and dolphins – is broad enough to suggest that all or nearly all mammals and birds may serve as hosts

(Dubey 2010). Within the feline intestine, the parasite differentiates into male and female gametocytes, which may fuse to form a zygote, followed by maturation into an oocyst containing infectious sporozoites. Oocysts are subsequently released into the intestinal lumen and shed into the surrounding environment along with the host's fecal matter. Oocysts have highly durable structures which may survive and remain infectious for long periods of time, on the order of many months, under adverse conditions of temperature and aridity (Dubey 2010). One of the primary means of infection is through the ingestion of oocysts contaminating food or water sources. If ingested by a suitable host, the cyst dissolves upon reaching the small intestine, releasing sporozoites which then differentiate into the rapidly multiplying tachyzoite stage upon infection of epithelial cells. Infection occurs as the parasite burrows into the interior of the host cell via indentation and eventual vesiculation of the plasma membrane. This vesicle is modified by secretions from the parasite to generate a protective parasitophorous vacuole (PV). During this acute phase of infection, the tachyzoites spread throughout the host's body via the circulatory system, infecting numerous tissues (Dubey 2010). Depending on the immune response of the host and the strain of infecting parasite, this expansion may continue until death of the host occurs or, more commonly, suppression of parasite expansion will result in a shift to the semi-dormant bradyzoite stage. Bradyzoites form intracellular tissue cysts which may persist in a state of equilibrium with the host's immune response for the duration of the host's life span with minimal turnover, thus establishing a long-term chronic infection (Remington 1965, Pusch 2009). Tissue cysts represent the second major mode of *T. gondii* transmission, as ingestion of these cysts by a new host via carnivory may result in initiation of a new round of acute infection in the same manner as described for oocyst ingestion. With regard to human infection, this generally occurs through the consumption of undercooked meat from chronically infected livestock. In other animals, such transmission would be the result of predation or scavenging.

Human toxoplasmosis

For most types of hosts, infection by *T. gondii* does not result in death, but instead proceeds from the acute to the chronic phase of infection. This is generally the case in immunocompetent humans, wherein flu-like symptoms including fatigue, muscle and joint pain and slight fever often persist for one to several weeks before subsiding. In some cases lymphadenopathy may occur which may last for several months (Dubey 2010). After dissipation of such initial symptoms, the chronic phase of infection appears to be capable of persisting indefinitely, most likely throughout the life span of the host (Remington 1965, Pusch 2009). Chronic toxoplasmosis of humans is common in all regions of the world, with some estimates placing the global rate of infection as high as one third of the population (Dubey and Beattie 1998). Infection rates vary considerably geographically, however. In the United States, seropositivity has been estimated to be between approximately 10 to 38 percent in various studies conducted since the 1960's. Lower rates have been estimated in the UK, China and Korea at around 8, 11 and 4 percent, respectively. By contrast, seropositivity among pregnant women was found to be approximately 61 percent in France and 84 percent in Madagascar, and between 61 and 92 percent overall in Brazil, with prevalence among adolescents in some southern regions of the country as high as 98 percent (Holland 2003, Dubey 2010). While the majority of infections in immunocompetent adults are asymptomatic, in immunocompromised individuals such those with AIDS or those undergoing immunosuppressive drug treatment, symptoms may be much more severe. Indeed,

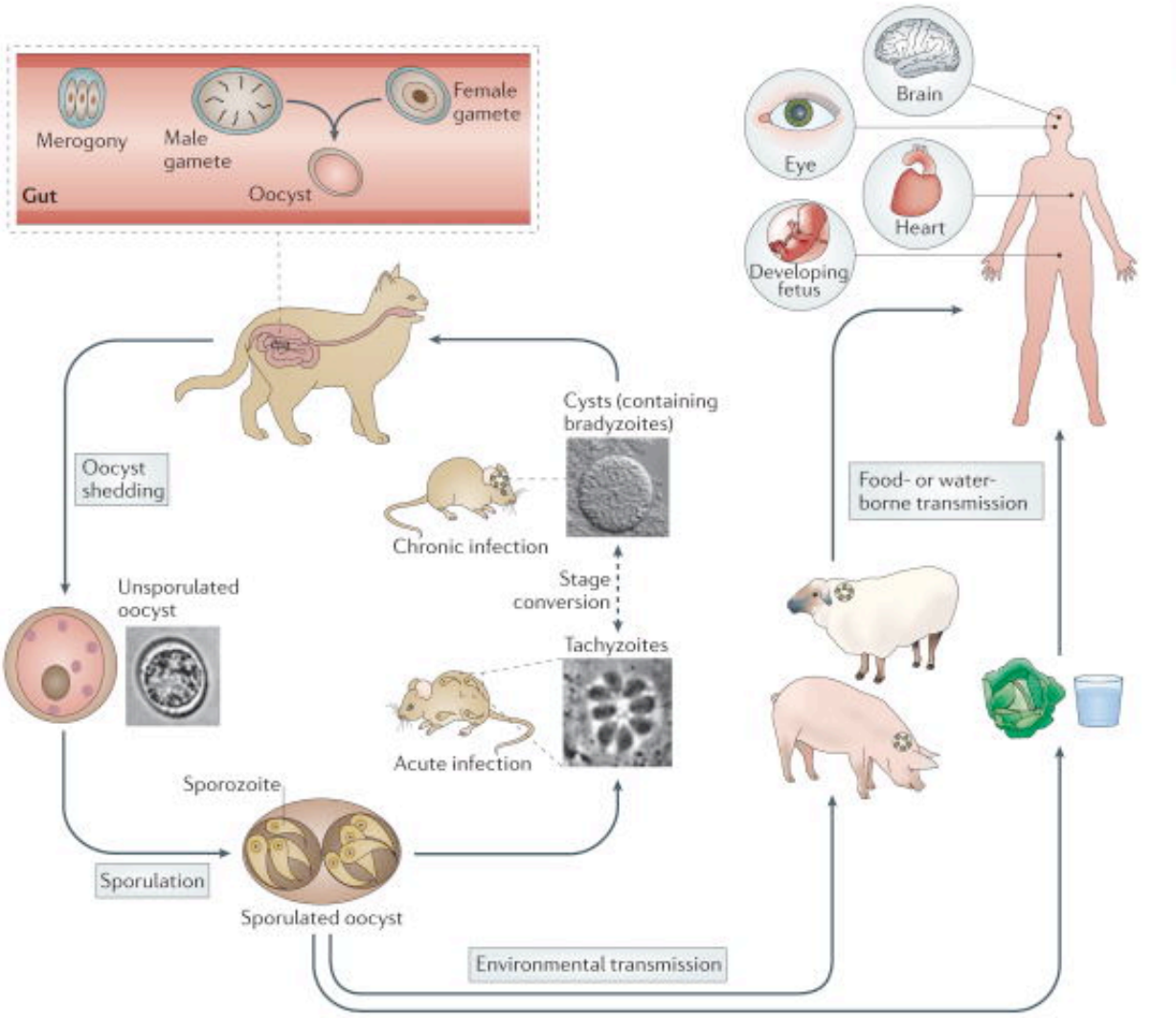


Figure I-1 Life cycle of *T. gondii*. Source: Hunter and Sibley (2012).

toxoplasmosis is among the leading causes of death in AIDS patients, most commonly due to the formation of necrotic brain lesions associated with encephalitis, though pneumonia and other systemic forms of the disease may also occur. In these cases, disease is usually a result of reactivation of latent infections upon the onset of immune dysfunction (Dubey 2010).

The other major human health concern associated with *T. gondii* relates to vertical transmission of the parasite from mother to developing fetus *in utero*. This phenomenon is believed in general to occur only in cases where the mother becomes infected with *T. gondii* for the first time during pregnancy and the parasite is able to spread transplacentally to the fetus during the acute phase of the infection, though cases have been identified in which transmission occurred in women who became infected shortly before pregnancy or, in only a few known cases, during chronic infection (Dubey *et al.* 2012). Broadly speaking, a woman chronically infected with *T. gondii* prior to becoming pregnant is not considered at significant risk for vertical transmission of the parasite, however (Dubey 2010). In cases where vertical transmission does occur, the severity of disease is generally inversely proportional to the developmental stage of the fetus, i.e. more mature fetuses generally experience less severe symptoms than those in earlier stages. However, it also appears to be the case that congenital infection becomes more likely in the later stages of pregnancy. In the most severe cases, fetal toxoplasmosis may result in deformation, severe mental impairment or abortion, but the most common manifestation is ocular disease, and the majority of children with infected mothers are born without symptoms. In some cases, ocular symptoms from congenital infections may not manifest until later childhood or even adulthood (Dubey 2010).

Although the immunocompromised are at highest risk for developing severe acute toxoplasmosis, many instances of severe disease have also been recorded in immunocompetent adults. These most frequently manifest as cases of ocular toxoplasmosis. In this situation, the parasite may cause damage to ocular tissue that can result in vision impairment or blindness if untreated (Holland 2003). Ocular toxoplasmosis is estimated to occur in about 2 percent of infections, and to affect about 1.26 million people in the United States as of the year 2000 (Holland 2003). In addition to ocular toxoplasmosis, severe systemic toxoplasmosis may also occur in healthy adults. Thus far, no definitive link has been demonstrated between parasite genotype and disease severity in humans, though circumstantial evidence provides support for such an association. In a 2011 study, of 20 tissue samples from a diverse assortment of toxoplasmosis patients from multiple hospitals and clinics in Sao Paulo state, Brazil, 18 were determined to be infected with the mouse-virulent ToxoDB genotype #65, which makes up only a small fraction of Brazilian *T. gondii* isolates, suggesting a possible association of this genotype with human disease. Furthermore, in Brazil severe symptoms were found to be associated with congenital ocular toxoplasmosis at a rate five times higher than that of Europe, with statistically significant differences in severity and recurrence of lesions observed (Gilbert *et al.* 2008). This was hypothesized to be due to the prevalence of highly virulent atypical parasite strains in Brazil that are rarely found in Europe. More than 50 particularly severe cases of systemic toxoplasmosis in immunocompetent adults have been recorded in French Guiana since 1998. This form of the disease, often termed “Amazonian toxoplasmosis” appears to be associated with a sylvatic cycle of parasite transmission, through wild definitive and intermediate hosts as opposed to domesticated animals, as patients generally reported recent rainforest-related activities such as consumption of undercooked wild game or surface water. In cases where

parasites have been isolated from patient tissues, isolates were found to possess atypical genotypes that were virulent in mice (Darde *et al.* 1998, Demar *et al.* 2007, Carme *et al.* 2009). This form of the disease often requires intensive medical care and has resulted in deaths due to multiple organ failure (Demar *et al.* 2077, 2012). Though not yet identified outside of French Guiana, it is expected that the strain types responsible may be present in adjacent areas inhabited by wild felids (Carme *et al.* 2009). In a study performed in the United States, two thirds of genotyped isolates from patients with ocular toxoplasmosis were found to belong to the virulent archetypal Type I or atypical genotypes despite the overwhelming dominance of avirulent Type II and III strains in North America (Grigg *et al.* 2001). Disproportionate association of atypical genotypes with severe clinical symptoms has also been observed in Europe (Bossi and Bricaire 2004, Delhaes 2010).

Mouse virulence

Disease presentation in humans appears to be fairly representative of the majority of host species for *T. gondii*, though a wide range of susceptibility is observed, even among closely related species. For example, rats (genus *Rattus*) are generally resistant to toxoplasmosis, whereas house mice (*Mus musculus*) are highly susceptible compared to most other hosts. In this case, evidence suggests that the more effective parasite control in rats compared to mice is dependent on the ability of the former species to efficiently respond to infection through caspase-mediated apoptosis of infected macrophages (Cavailles *et al*. 2014). For resistant hosts, the infecting strain type of *T. gondii* does not make an obvious difference with regard to disease outcome. This is not the case for mice, for which different parasite strains produce dramatically different levels of disease severity. This phenomenon was observed early in the history of *T. gondii* research, and strains were classified as being either virulent or avirulent in mice, virulent strains producing one hundred percent mortality from inocula as low as a single parasite, while avirulent strains required dosages of thousands to tens of thousands of parasites to induce mortality (Sibley and Boothroyd 1992, Khan *et al.* 2009). When PCR-restriction fragment length polymorphism (PCR-RFLP)-based genotyping of *T. gondii* began to be performed in the 1990's, it was discovered that the virulent strains studied all appeared to belong to a single lineage (Sibley and Boothroyd 1992). Soon thereafter, the avirulent strains were found to segregate into two distinct lineages. The virulent lineage was referred to as Type I, while the avirulent lineages were called Types II and III (Howe and Sibley 1995). It was also determined that the Type II lineage was generally more virulent than the Type III lineage (Khan *et al.* 2009). Based on these early genotyping studies of global *T. gondii* isolates, it appeared that the overall population structure of the parasite was dominated by these three lineages that appeared to have expanded clonally throughout the world, with minimal sexual recombination (Howe and Sibley 1995). Upon identification of these major clonal lineages, intensive research was carried out with the intention of determining the genetic basis for the observed differences in virulence.

Genetic basis for mouse virulence differences among archetypal lineages

The key findings relating to virulence differences of the clonal lineages mainly stem from research involving quantitative trait locus (QTL) mapping of progeny from sexual crosses among

Type I, II and III strains (Saeij *et al.* 2006, Taylor *et al.* 2006, Behnke *et al.* 2011). Using this forward genetics approach, a few distinct loci were identified as key determinants of mouse virulence. The first of these to be extensively studied, though not the locus most highly correlated with virulence phenotype, was determined to encode the rhoptry-secreted kinase ROP18 (Taylor *et al.* 2006). In the crosses described above, the Type I and II alleles of ROP18 were found to segregate with virulence, while the Type III allele segregated with nonvirulence. This was determined to be the result of an insertion sequence in the Type III promoter that attenuates expression of the gene (Boyle *et al.* 2008). In Type I and II strains, the protein is expressed, and is secreted into host cells upon invasion.

The chief function of ROP18 appears to involve the phosphorylation and resultant inactivation of a family of host proteins known as the immunity-related p47 GTPases (IRGs) and p65 guanylate-binding proteins (GBPs) which normally bind to the parasitophorous vacuole membrane (PVM) and cause its disruption either directly (in the case of IRGs) by mechanical constriction of the membrane or indirectly (in the case of GBPs) by recruitment of other immunity-related proteins and complexes (Fentress *et al.* 2010, Steinfeldt *et al.* 2010, Howard *et al.* 2011, Winter *et al.* 2011). Expression of functional ROP18 is thus necessary for tachyzoites to evade these immune responses and thereby prevent clearance by the host.

ROP18 alone is insufficient for parasite resistance to the host response. A second locus was identified via the same forward genetics approach that correlated much more strongly with virulence than even ROP18 (Taylor *et al.* 2006). This locus was found to contain a tandem array of paralogous genes encoding variants of the ROP5 rhoptry-secreted pseudokinase (Reese *et al.* 2011). Despite being nonfunctional as a kinase due to mutations of the catalytic site, ROP5 plays an important role in suppression of the host immune response, in part through regulation of ROP18 activity. ROP5 has been shown to form a complex with ROP18 and with murine IRGs, facilitating their phosphorylation and inactivation by ROP18 (Etheridge *et al.* 2014, Reese *et al.* 2014). The fact that it is primarily the most highly polymorphic regions of ROP5 proteins that interact with mouse IRGs appears to indicate a sort of evolutionary arms race between the two classes of proteins, in which mouse IRGs have diversified under the pressure to escape recognition by ROP5, while ROP5 proteins have likewise diversified in order to enhance binding to IRGs (Reese et al 2014). ROP5 was also found to interact with another rhoptry kinase, ROP17, which likely contributes to the stronger influence of ROP5 on parasite virulence compared to ROP18, though other interactions may be identified as well (Etheridge *et al.* 2014). Like ROP18, the functionality of ROP5 varies with strain genotype. In this case, the ROP5 alleles expressed by Types I and III are sufficient to produce a virulent strain in combination with expression of functional ROP18, while the Type II ROP5 alleles are associated with avirulence regardless of the ROP18 genotype (Reese *et al.* 2011, Behnke *et al.* 2011).

In addition to ROP5 and ROP18, two additional polymorphic loci have been identified as playing roles in determining virulence differences between the archetypal strains. These are the rhoptry kinase ROP16 and the dense granule protein GRA15 (Saeij *et al.* 2006, Rosowski *et al.* 2011). In the case of ROP16, virulence is associated with a loss-of-function mutation in the catalytic site of the Type II allele. The functional Type I and III versions of the protein act as transcriptional regulators, phosphorylating and inactivating the pro-inflammatory transcription factors STAT3 and 6, thereby suppressing the host inflammatory immune response. In Type II

strains, expression of nonfunctional ROP16 results in increased production of pro-inflammatory cytokines by the host and a hyperactive inflammatory response, which appear to contribute to host mortality (Saeij *et al.* 2007, Yamamoto *et al.* 2009, Ong *et al.* 2010). The Type II allele of GRA15 appears to exacerbate this condition through suppression of the anti-inflammatory NF-kB signaling pathway, whereas the Type I and III alleles are nonfunctional in this case and therefore NF-kB signaling is not suppressed (Rosowski *et al.* 2011, Jensen *et al.* 2011). The contributions of ROP16 and GRA15 to strain virulence appear to be much less significant than those of ROP5 and ROP18, however.

As noted previously, the vast majority of host species for *T. gondii* are resistant to infection except in special cases. The virulence differences described above appear to be largely specific to house mice and some closely related species. For other host species such as rats or humans, infection with any of the three archetypal lineages typically results in progression to an asymptomatic, chronic infection (Dubey 2010). Mouse resistance to *T. gondii* infection appears to be heavily dependent on the IRG defensive response, as evidenced by the presence of 22 different IRG genes in the mouse genome, located in two chromosomal clusters (Howard *et al.* 2011, Gazzinelli *et al.* 2014). By contrast, humans possess only one functional IRG gene, and this is expressed only in the testes. Furthermore, the apparent paralogous expansion of the IRG genes appears likely to have evolved specifically as a defense mechanism against *T. gondii*. While resistance to several other intracellular pathogens including *Chlamydia trachomatis* has been shown to be dependent on certain IRGs, only resistance to *T. gondii* has been found to require the full complement of these genes (Howard *et al.* 2011). Thus, the importance of ROP5 and ROP18 in strain virulence are emphasized in mice compared to other host species. It seems likely that mice are deficient in innate immune responses that confer resistance in other host species, such as caspase-mediated apoptosis of infected macrophages, as identified in rats (Cavailles *et al.* 2014) and thus have developed this alternative method of parasite control.

Immune response

The Th1, cell-mediated immune response appears to be of primary importance in the control of *T. gondii* in both mice and humans. This response is characterized in part by upregulation of the cytokine IL-12, which stimulates the production of IFNg by T cells and Natural Killer (NK) cells (Gazzinelli *et al.* 1993, Hunter *et al.* 1994). IFNg in turn serves as the primary mediator of *T. gondii* control mechanisms (Suzuki *et al.* 1998). Detection of *T. gondii* is known to involve specific Toll-Like Receptors (TLRs) as well as the TLR-associated adapter molecule MyD88, though it also appears that other methods of detection are involved as well (Scanga *et al.* 2002). Monocytes, neutrophils and dendritic cells (DCs) are recruited early to the site of infection in mice and all three cell types appear to be important producers of IL-12 (Dupont *et al.* 2012). In addition to IL-12 production, neutrophils are also implicated in several direct killing mechanisms, including phagocytosis, production of reactive chemical species, and formation of extracellular DNA nets (Bliss *et al.* 1999, 2000). Inflammatory monocytes also produce IL-12, though for this cell type a more essential role appears to involve the generation of nitric oxide (NO) via expresson of inducible NO synthase (iNOS), which has an inhibitory effect on parasite replication (Mordue and Sibley 2003, Borges and Johnson 1975). Monocytes also produce IL-1, which may act synergistically with IL-12 to induce IFNg production from both innate and

adaptive sources, and may mature into DCs and macrophages (Shibuya *et al.* 1998, Dupont *et al.* 2012). DCs are possibly the most important producers of IL-12, and are essential antigen-presenting cells (APCs), necessary for activating a number of adaptive immune processes (Liu *et al.* 2006). NK cells are of primary importance during the acute phase of infection and are essential producers of IFNg following stimulation with IL-12, as well as exhibiting cytotoxicity towards infected cells (Sher *et al.* 1993, Denkers *et al.* 1993).

The adaptive immune response, including both T and B cell mediated pathways, is also important in resistance to *T. gondii* for both humans and mice, particularly during the chronic phase of infection. CD4+ T cells are essential for mediation of B and CD8+ T cell responses, and also are primary producers of IFNg and CD40L during chronic infection (Lutgen *et al.* 2006, Reichmann *et al.* 2000). As mentioned, DCs are considered to be the most important group of APCs involved in CD4+ T cell activation in response to *T. gondii* infection, though B cells and macrophages are also capable of performing this function (Jenkins *et al.* 2001). CD8+ T cells are also critical for protection, through production of IFNg and CD40L like CD4+ T cells, and also via perforin-mediated lysis of infected cells (Denkers *et al.* 1997, Gazzinelli 1992). *T. gondii* antigens known to stimulate CD8+ T cell activity include Surface Antigen 1 (SAG1), dense granule proteins GRA4 and GRA6, and the rhoptry protein ROP7 (Dupont *et al.* 2012). Expression of the stimulatory GRA6 epitope is restricted to Type II strains (Blanchard *et al.* 2008). Humoral immunity has also been shown to be important for control in both humans and mice. In mice, antibodies are known to provide resistance through opsonization of parasites for phagocytosis, blockage of invasion, and activation of the classical complement pathway (Erbe *et al.* 1991, Vercammen *et al.* 1999, Schieber *et al.* 1980). As noted, CD4+ T cell activity is essential for effective B-cell stimulation and subsequent antibody production.

IFNg appears to be the primary mediator of control of acute infection (Dupont *et al.* 2014). The main function of this cytokine with regard to mouse resistance seems to be the upregulation of the p47 immunity-related GTPases (IRGs) and p65 guanylate binding proteins GBPs discussed above (Howard *et al.* 2011, Virreira et al 2011). Numerous IRGs and several GBPs have been implicated in resistance. IRGs may be grouped into effector (GKS) proteins, which bind sequentially to the PVM and effect mechanical disruption, and regulatory (GMS) proteins that bind to host intracellular membranes (golgi, ER etc.) and protect these from colonization and disruption by GKS proteins (Howard *et al.* 2011, Khaminets *et al.* 2010). GBPs also bind to the PVM, but appear to facilitate clearance through less direct mechanisms, including recruitment of IRGs and other immunity-related molecules (Yamamoto *et al.* 2012). Following disruption of the PV, exposed parasites may egress, be killed through permeabilization, or may be disposed of via the host's autophagic apparatus (Dupont *et al.* 2012). In addition to activating IRG-dependent parasite clearance, IFNg also performs several other known immunity-related functions. One of these is the promotion of tryptophan degradation in infected cells through the upregulation of the genes IDO1 and IDO2, which catalyze this process (Murray *et al.* 1989). Being auxotrophic for tryptophan, *T. gondii* cannot replicate if this amino acid is not provided by the host (Sibley *et al.* 1994). Additionally, expression of iNOS is increased in IFNg-stimulated macrophages, resulting in increased production of NO and inhibition of parasite replication in these and other cell types. However, upregulation of iNOS also requires additional signaling molecules, including TNFa and CD40L (Reichmann *et al.* 2000, Yap *et al.* 1998). These and other members of the TNF family of cytokines, including Lymphotoxin alpha (LTa), are required for protection during the

chronic stage of infection (Schluter *et al.* 2003). The primary contribution of TNFa to immunity appears to be through iNOS upregulation, but evidence suggests other functions may also be important (Yap et al 1998). CD40L acts in conjunction with IFNg in ways described above, but also has an IFNg-independent function relating to xenophagic parasite clearance in a manner dependent on the autophagy-associated protein Beclin-1 (Reichmann *et al.* 2000). LTa has also been shown to be essential for resistance in mice, though the precise role of this cytokine in immunity to *T. gondii* is not well understood (Schluter *et al.* 2003).

Population structure

Since the original genotyping work in which the three clonal lineages were identified in Europe and North America, subsequent genotyping studies using a variety of techniques including PCR-RFLP, microsatellite DNA analysis, intron sequencing and whole genome sequencing have revealed that the true global *T. gondii* population structure contains more complexity than described by the clonal model (Su *et al.* 2012). The European population appears to most closely resemble this model, being largely dominated by Type II and III strains, and with Type I strains making up a significant percentage as well (Howe and Sibley 1995). Even in this case, other so-called atypical strains also are quite prevalent, however (Herrmann *et al.* 2012). In North America, Type II and III strains are also most prominent, but several atypical strains closely related to the archetypal Type II lineage are also major components of the population (Dubey *et al.* 2011, Khan *et al.* 2011). In addition, many diverse atypical strain types have also been identified in small proportions. Furthermore, the Type I lineage makes up only a small percentage of the total population, with isolates of this genotype being outnumbered by numerous atypical genotypes (Darde *et al.* 2008, Dubey and Su 2009). In China and southeast Asia, the archetypal lineages are relatively small contributors to the regional population, which is instead dominated by a small set of atypical lineages (Dubey *et al.* 2007, Zhou *et al.* 2009, Qian *et al.* 2012) . It should be noted that while the populations of Europe, North America and southeast Asia are more complex than was originally thought and contain some highly dominant atypical lineages, they do still appear to embody largely clonal structures (Shwab *et al.* 2014). That is to say that a relatively small number of strain types compose a vast majority of the overall populations, suggesting that asexual or clonal reproduction has been the primary mode of expansion in these regions, with little sexual recombination between strains of different types. Genotyping studies of limited *T. gondii* isolates from South America, by contrast, have revealed completely different population structures. In these cases, a large diversity of atypical strains have been identified, most of these being common only in specific localities, with no single genotype approaching the dominance observed in the populations of the northern hemisphere (Pena *et al.* 2008, In South America, it thus appears that recombination among strains has been much more prominent, and a clonal model is therefore not appropriate for describing the population structures of this region (Dubey and Su 2009). The differences between the mechanisms of strain propagation in Northern continents and South America may reflect the endemic vs. epidemic nature of the parasite in these regions. The parasite is more abundant in South America than elsewhere on Earth, and thus coinfection of individual feline hosts with multiple strain types would be expected to be more common, increasing the likelihood of sexual recombination between strains.

Observations leading to current research

While numerous genotyping studies of *T. gondii* have been carried out, no formal attempt has previously been made to standardize the methodology to genotype *T. gondii*, so all available data reported from different researchers are fragmented and could not collectively establish a cohesive big picture of genetic diversity and population structure. This made it difficult to use available data in conjunction with knowledge of other aspects of parasite biology, specifically the host-specific molecular mechanisms of pathogenesis, to understand the principles that have shaped the present *T. gondii* population structure. Previous genotyping studies have for the most part been limited to small localities and particular host species. For example, a typical study might focus on isolates from chickens living on farms in a small region of Colombia. By itself, this information does not provide us with much new insight beyond the limited scope of the particular study. However, when hundreds of such studies are carried out, examining numerous host species in localities around the world, these can potentially contribute significantly to a larger understanding of *T. gondii* population genetics. For such comparisons between studies to be meaningful, however, it is necessary for the format of the data collected in each to be compatible. As such, we have focused first on developing and presenting a standardized method for *T. gondii* genotyping using PCR-RFLP targeting a specific set of ten genetic markers, presented in chapter two of this thesis. The standardized PCR-RFLP method has been applied to genotype over a thousand *T. gondii* samples worldwide, generated invaluable data within several years by many researchers. We have then proceeded to compile all of the data collected and published using this method through the end of the year 2012. This data is presented and analyzed as a whole in chapter three.

As noted, the diversity observed within the South American *T. gondii* population seems to correlate with more widespread and severe human disease in general, and the strain types associated with more serious symptoms of toxoplasmosis in humans also appear to have a greater tendency towards mouse virulence. Given these correlations, and the current rapid expansion of human populations in many South American countries, exacerbating the potential for exposure to new parasite strains and resultant disease outbreaks, it is important to understand the mechanisms governing the virulence of atypical *T. gondii* strains and to have a means of predicting the virulence phenotypes of uncharacterized isolates in order to assess their potential as threats to human health. Though a number of previously published genotyping studies have included mouse virulence assays of isolated strains, this is not the case for the majority of isolates and many strains therefore remain uncharacterized with regards to virulence. As noted above, molecular studies have identified the genes ROP5 and ROP18 as the key determinants of virulence differences among the archetypal *T. gondii* lineages. However, the extent to which these genes determine virulence in the large number of atypical strains identified has only been studied in a limited capacity. We were interested in determining the extent to which these two loci determine virulence in atypical strains, both because this knowledge would be useful in and of itself in helping to understand the mechanisms of *T. gondii* pathogenesis, and because genotyping at these loci could potentially provide a convenient method of predicting the mouse virulence of new isolates without requiring mouse mortality assays to be carried out. This would be particularly useful in cases where parasite DNA from infected animal tissue is obtainable but viable isolated parasites are not. To this end, we developed PCR-RFLP-based genotyping methods for ROP5 and ROP18, and proceeded to genotype a library of global *T. gondii* isolates

with both known and unknown mouse virulence phenotypes. In this way, we were able to both determine that, taken together, the ROP5 and ROP18 genotypes are highly predictive of mouse virulence, and to infer the probable mouse virulence phenotypes of a myriad of global isolates. These findings are presented in detail in chapter four of this dissertation. Additionally, we have genotyped this same set of isolates at ten additional loci for which we have developed novel PCR-RFLP genotyping protocols. When combined with the ten standardized markers discussed in chapter two, we were able to generate the most comprehensive picture of the global *T. gondii* population structure to date. This data is presented in Appendix A.

After obtaining as precise and complete an understanding of the global patterns of *T. gondii* diversity as possible, we sought to better determine the principles involved in shaping this population structure. It was clear from our own findings and those of other researchers that the archetypal Type II lineage and some closely related variants were consistently dominant in populations presenting clonal structures, such as those of Europe, North America and southeast Asia. We hypothesized, therefore, that such strains possessed some sort of competitive advantage over other strain types in these regions. As the primary phenotypic difference between *T. gondii* strains involves virulence to mice, we focused on this trait as the source of variable fitness between strain types. It has been well documented that Type II strains generally have mouse virulence levels intermediate to those of the highly virulent Type I and much less virulent Type III lineages. In this case, it is easy to understand why evolution towards lower virulence in this host species would be advantageous, as in the example of myxomatosis in Australian rabbits (Kerr 2012). In contrast to highly virulent strains which typically kill their hosts during the acute phase of infection, less virulent strains may progress to the chronic stage of infection, thus persisting within the host indefinitely and prolonging the time period in which passage to other hosts may occur. The answer to the question of why intermediately virulent Type II strains would have a fitness advantage over less virulent Type III strains seems less obvious. For a potential answer, we looked to studies involving direct competition between *T. gondii* strains for infection of murine hosts. A number of such studies have been published, typically involving the challenge of mice already chronically infected by one parasite strain with a second strain. A detailed summary of the findings of a number of relevant studies of this type is provided in chapter four of this work. A common finding of these studies is that, while prior chronic *T. gondii* infection in mice usually is protective against subsequent infections, strains with higher virulence are more likely to be able to overcome this protection and establish a secondary chronic infection. Therefore, in our current work we hypothesize that strains of intermediate virulence may have a competitive advantage over those of high and low virulence. Such strains may both be able to establish chronic infections in mice unlike highly virulent strains, and have a greater probability of reinfecting previously infected mice than strains with low virulence. In order to determine if such advantages could drive the expansion of intermediately virulent strains within populations over long periods of time, we employed an *in silico* modeling approach, the results of which are presented and discussed in chapter five. In short, the simulation results indicated that the two variables of mouse virulence and probability of successful sequential infection are both potentially influential to a strain's prevalence within a parasite population, and that strains which possess an optimal balance between these variables (most closely resembling the phenotypes of natural Type II strains) will be consistently dominant.

In addition to examining the relationship between mouse virulence and population genetics of *T. gondii*, we were interested in discovering more about the variations in host response to different parasite strains. While the differences between the three archetypal lineages and the ways in which these differences contribute to variable disease outcomes in mice have been studied extensively, the host side of these interactions is not as well understood. Previous studies have shown variations in macrophage cytokine production during in vitro infection with Type I and Type II parasite strains, and variations in macrophage activation pathways between Type II strains and Type I and III strains. Other cell types, including human foreskin fibroblasts, chicken embryonic fibroblasts, and human neuroepithelial cells also have been shown to produce different responses to infection with different strain types in vitro. Furthermore, overall transcript levels have been shown to be markedly different in mice during infection with Type I and II strains compared to Type III strains. These studies and others indicate the existence of strain-specific responses of mice to infection with different *T. gondii* strains, but little is currently known about the specific ways in which these responses differ. For this reason, we were interested in performing experiments focused on identifying strain-specific immune responses of mice. Specifically, we performed a time-course microarray study in which we analyzed total gene expression profiles of mice on a daily basis for seven days following infection with a Type I, II or III strain of *T. gondii*. In this way, we were able to identify unique host response profiles for each of the archetypal lineages. These results are discussed in chapter six of this dissertation.

Chapter II. Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*

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Introduction

Toxoplasma gondii is an obligate intracellular, apicomplexan parasite that infects all warm-blooded vertebrates, including mammals and birds. It is the only known species in the genus *Toxoplasma* and is considered to be one of themost successful eukaryotic pathogens in terms of the number of host species and percentage of animals infected worldwide. Up to one-third of the human population in the world is chronically infected (Dubey and Beattie, 1988; Tenter *et al.* 2000). Human infections are primarily caused by ingesting undercooked meat containing viable tissue cysts or by ingesting food or water contaminated with oocysts shed in the faeces from infected cats (Dubey, 2004). Primary infections in adults are mostly asymptomatic but lymphadenopathy or ocular toxoplasmosis can occur in some patients. Severe acute, disseminated toxoplasmosis can occur in immunocompetent human patients when infected with some isolates (Bossi and Bricaire, 2004). Infection acquired during pregnancy may spread and cause severe damage to the foetus. In immunocompromised patients, the reactivation of a latent infection can cause life-threatening encephalitis (Montoya and Liesenfeld, 2004). *T. gondii* has subpopulation structures in different geographical regions. It has what appears to be a clonal population structure in North America and Europe, with 3 predominant lineages (named types I, II and III) defined by multi-locus enzyme electrophoresis (MLEE), polymerase chain reactionrestriction fragment length polymorphism (PCRRFLP) or microsatellite analysis (Darde *et al.* 1992; Howe and Sibley, 1995; Ajzenberg *et al.* 2002a). A recent report suggests the same clonal structure in Africa (Velmurugan *et al.* 2008). However, *T. gondii* isolates from animals and human patients in South America are diverse and largely distinct from those in North America and Europe (Ajzenberg *et al.* 2004; Khan *et al.* 2006; Lehmann *et al.* 2006; Pena *et al.* 2008; Dubey *et al.* 2008d). Historically, these distinct and non-type I, II or III parasites were designated as the 'atypical ' or 'exotic' isolates. The low linkage disequilibrium among genetic loci of these isolates suggests that the parasites have undergone frequent sexual recombination (Ajzenberg *et al.* 2004; Lehmann *et al.* 2004; Su *et al.* 2006). The consequences of infection with *T. gondii* depend on the host species and parasite genotypes. In mice, type I lineages are uniformly lethal (LD_{100}=1); by contrast, the type II and III lineages are significantly less virulent (LD_{100}>=103) (Sibley and Boothroyd, 1992). In humans, disease manifestations vary widely, ranging from asymptomatic to severe acute toxoplasmosis (Bossi and Bricaire, 2004; Montoya

and Liesenfeld, 2004). Type II is the predominant lineage causing human toxoplasmosis. However, there are biases between disease presentations and parasite genotypes. For examples, type I or type I-like atypical isolates are more likely to be involved in severe toxoplasmic retinochoroiditis in human patients (Grigg *et al.* 2001) and the atypical isolates often cause severe acute, disseminated toxoplasmosis in immunocompetent patients (Bossi and Bricaire, 2004). Clinical symptoms of *T. gondii* infection are non-specific and unreliable for diagnosis. The conventional diagnosis of *T. gondii* infection usually employs serological tests, bioassays in cats and/or mice, or a combination of the 2 approaches (Dubey and Beattie, 1988). In the past 2 decades, the diagnosis of *T. gondii* infection by direct detection of parasite-specific DNA in biological samples using PCR-based molecular methods has gained popularity. The molecular diagnosis is more sensitive and cost-effective than the conventional methods (Schoondermark-Van De Ven *et al.* 1994; Bessie`res *et al.* 2009). There are hundreds of papers in current literature databases (such as PubMed) describing the use of these molecular methods. However, in this review article, rather than conduct an exhaustive review of all of the literature, we focus on key, representative articles to provide a perspective on establishing an integrated approach for the detection and characterization of *T. gondii*.

Molecular methods for detection and identification

Molecular methods rely on PCR for the specific detection or analysis of *T. gondii* DNA. These methods have proved to be simple, sensitive, reproducible and cost-effective, and have been applied to a variety of clinical samples from animals and humans (Bell and Ranford-Cartwright, 2002; Contini *et al.* 2005; Calderaro *et al.* 2006; Bastien *et al.* 2007). Molecular methods can be divided into 2 groups. The first group focuses on specific detection of *T. gondii* DNA in biological samples. The conventional PCR, nested PCR (n-PCR) and quantitative real-time PCR (qPCR) of repetitive DNA sequences belong to this group. The second group of molecular methods focuses on a high resolution identification of *T. gondii* isolates. The multilocus PCR-RFLP, microsatellite, and multilocus sequence typing (MLST) of singlecopy DNA sequences belong to this group. In the following sections, we discuss the above methods in detail. Molecular detection by conventional PCR, nested PCR (n-PCR) or quantitative real-time PCR (qPCR) of repetitive DNA sequences To achieve high sensitivity, PCR of a small, repetitive DNA sequence is preferred, because the efficiency of amplifying a small DNA fragment is higher than that of a large one. In addition, there are more template copies in a repetitive sequence per organism. Three repetitive DNA sequences are often used for the detection of *T. gondii* in biological samples, including the 35-copy B1 gene, the 300- copy 529 bp repeat element and the 110-copy internal transcribed spacer (ITS-1) or 18S rDNA gene sequences. A molecular detection method (conventional PCR) for *T. gondii*, targeting the B1 gene, was first developed by Burg *et al.* (1989). This method has since been modified and adapted in different laboratories (Khalifa *et al.* 1994; Liesenfeld *et al.* 1994; Bretagne *et al.* 1995; Pelloux *et al.* 1998; Contini *et al.* 2002, 2005; Reischl *et al.* 2003; Switaj *et al.* 2005; Bastien *et al.* 2007). The 529 bp repeat element was identified by Homan *et al.* (2000), and it was reported to be 10- to 100-times more sensitive than the B1 gene (Homan *et al.* 2000; Reischl *et al.* 2003; Calderaro *et al.* 2006). The 110-copy ITS-1 or 18S rDNA has been used as the target in a few studies (Hurtado *et al.* 2001; Jauregui *et al.* 2001; Calderaro *et al.* 2006) and showed similar sensitivity to the B1 gene. To achieve higher sensitivity, n-PCR of B1 gene and ITS-1 sequences has been

applied in some studies (Pelloux *et al.* 1998; Hurtado *et al.* 2001; Jauregui *et al.* 2001; Contini *et al.* 2002, 2005; Bastien *et al.* 2007), both markers showed high sensitivity, with the detection level being as low as 1 parasite (Hurtado *et al.* 2001; Jauregui *et al.* 2001; Calderaro *et al.* 2006). For a given repetitive sequence, n-PCR is more sensitive than conventional PCR. A case in point is that the n-PCR of the B1 gene is more sensitive than the conventional PCR for the detection of *T. gondii* in amniotic fluid samples from congenital toxoplasmosis of human (Okay *et al.* 2009). Recently, we employed a set of primers for n-PCR of *T. gondii* 18S rDNA, and found that it was more sensitive than the B1 gene (data not shown). The 18S rDNA marker is of particular interest, as it can distinguish several protozoan parasites which are closely related to *T. gondii*. There is further discussion of this marker in a later section (Toward an integrated approach for molecular detection and identification) of this article. In recent years, qPCR that targets the repetitive DNA sequences, has shown high sensitivity (detecting y1 parasite genome equivalent) (Jauregui *et al.* 2001; Reischl *et al.* 2003; Contini *et al.* 2005; Calderaro *et al.* 2006; Edvinsson *et al.* 2006). The qPCR approach has gained popularity for not only detecting but also quantifying *T. gondii* in biological samples (Bell and Ranford-Cartwright, 2002; Contini *et al.* 2005). Its sensitivity is superior to n-PCR assays (Contini *et al.* 2005) and can detect *T. gondii* over a range of 6–7 orders of magnitude (Lin *et al.* 2000; Jauregui *et al.* 2001; Contini *et al.* 2005). Quantification of *T. gondii* by qPCR has been applied to the detection of the parasite in animal tissues and whole blood or amniotic fluids of human clinical samples (Lin *et al.* 2000; Jauregui *et al.* 2001; Kupferschmidt *et al.* 2001; Contini *et al.* 2005). Since it can estimate the intensity of *T. gondii* infection in the patients following drug treatment, qPCR is extremely useful to monitor the progression of infections under treatment and to diagnose lowlevel infections or carrier states (Contini *et al.* 2005). The 529 bp element is a preferred target for qPCR, as it provides a sensitivity of 6 repeat equivalents, or 1/50 of a genome equivalent (Kasper *et al.* 2009), currently representing the most sensitive assay for the detection of *T. gondii*. Rapid and accurate detection of *T. gondii* infection is pivotal for prompt treatment of clinical toxoplasmosis. However, the above-mentioned detection methods (conventional PCR, n-PCR and qPCR of repetitive DNA sequences) provide no information beyond a positive diagnosis. To better understand the epidemiology of the parasite, there is an increasing interest and need in the genetic classification of *T. gondii*. In the following section, we discuss the methods used for the genetic identification and characterization of this parasite. Molecular characterization by multilocus PCR-RFLP, microsatellite or multilocus sequence typing (MLST) analysis For epidemiological studies, it is important to identify individual *T. gondii* isolates and to track the source of contamination. The commonly used methods for these purposes are multilocus PCR-RFLP, microsatellite or MLST typing. The PCR-RFLP is based on the ability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs), digest PCR products and subsequently display distinct DNA banding patterns on agarose gels by electrophoresis (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). Microsatellite analysis is based on the DNA sequence length polymorphisms of short nucleotide tandem repeats. The tandem repeats in *T. gondii* are often simple, consisting of as few as 2 nucleotides (di-nucleotide repeats), and occur 2–20 times (Blackston *et al.* 2001; Ajzenberg *et al.* 2002a, 2004). The MLST is based on DNA sequence polymorphisms including the SNPs, insertion and deletion of nucleotides in the sequence. SNPs and microsatellites are predicted to mutate at different evolutionary rates. For eukaryotes in general, the mutation rate for SNPs is estimated at 10x9–10x10 per nucleotide position per replication, whereas the rate for microsatellites is 10x2–10x5 per locus per replication (Goldstein and Schlotterer, 1999). Although the exact mutation rates of SNPs and microsatellites are not known in *T. gondii*, it is

clear that the latter have a higher mutation rate and can provide enhanced resolution in typing (Ajzenberg *et al.* 2002a). The microsatellites are useful in distinguishing genetically closely related *T. gondii* isolates. Since multilocus PCR-RFLP and microsatellite typing are simple and cost effective compared with MLST typing, they are preferred tools for characterization of *T. gondii* in epidemiological studies. A hallmark study of the epidemiology and population structure of *T. gondii* was conducted more than a decade ago on 106 isolates collected from humans and animals in North America and Europe (Howe and Sibley, 1995). In this study, 6 PCR-RFLP markers were used and 3 predominant lineages (types I, II and III) were identified. It was concluded that *T. gondii* had a clonal population structure (Howe and Sibley, 1995). Since then, different sets of multilocus PCR-RFLP or microsatellite markers have been applied to epidemiological and population studies (Blackston *et al.* 2001; Ajzenberg *et al.* 2002a, b, 2004, 2005; Lehmann *et al.* 2004, 2006; Khan *et al.* 2005a, b; Ferreira *et al.* 2006, 2008; Su *et al.* 2006). A key finding of these studies was that *T. gondii* isolates from South America were highly diverse and distinct from those of North America and Europe. It was concluded that, in South America, *T. gondii* has an epidemic population structure (Ajzenberg *et al.* 2004; Lehmann *et al.* 2004, 2006; Pena *et al.* 2008). However, the comparing of genotypes among different studies has been difficult or impossible, as different markers have been employed in different laboratories. The disadvantage of using a single-copy gene or genomic sequence is that the sensitivity is compromised compared with a highly repetitive sequence. A technical challenge to multilocus typing is that often samples are limited both in volume and in DNA amount, particularly from tissues from clinical cases or the mouse bioassay. To alleviate this problem, we have developed a multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) method employing 10 genetic markers, including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico (Dubey *et al.* 2007e). The sensitivity of this method is estimated at 10 or more *T. gondii* genome equivalents, based on a previous study with 4 (SAG2, SAG3, BTUB and GRA6) of these 10 markers (Khan *et al.* 2005a). In contrast, the sensitivity of conventional PCR-RFLP is estimated at 100 *T. gondii* genome equivalents (unpublished data). In Mn-PCR-RFLP typing, all markers are preamplified by multiplex PCR using external primers in a single reaction, and the pre-amplified PCR products are used as templates to amplify each individual marker by n-PCR (Figure II-1). The advantage of this approach is that only a limited amount of individual sample is needed, which is particularly useful when only small amounts of 'precious' samples are available. Mn-PCR-RFLP analysis has been applied to the genetic typing of a large number of animal isolates bioassayed in mice, generating a substantial amount of data regarding genetic diversity and population structure of the parasite (Dubey *et al.* 2007a-g, 2008a-e; Pena *et al.* 2008; Sundar *et al.* 2008; Velmurugan *et al.* 2008; Dubey and Su, 2009). This method has also been used for the genetic characterization of *T. gondii* DNA extracted from the brains of sea otters (Sundar *et al.* 2008) and arctic foxes (Prestrud *et al.* 2008). Application of this method to a broad range of samples will greatly facilitate our understanding of molecular epidemiology and population diversity of *T. gondii* in the near future. When the amount of DNA for *T. gondii* isolates is not limited, the MLST typing is the method of choice because it has the highest resolution among all typing methods. Recent studies of *T. gondii* using this approach have revealed alleles unique to parasite isolates in Brazil (Khan *et al.* 2006, 2007), emphasizing the importance of a sequencing approach for studying the population genetics and molecular phylogeny of *T. gondii*.

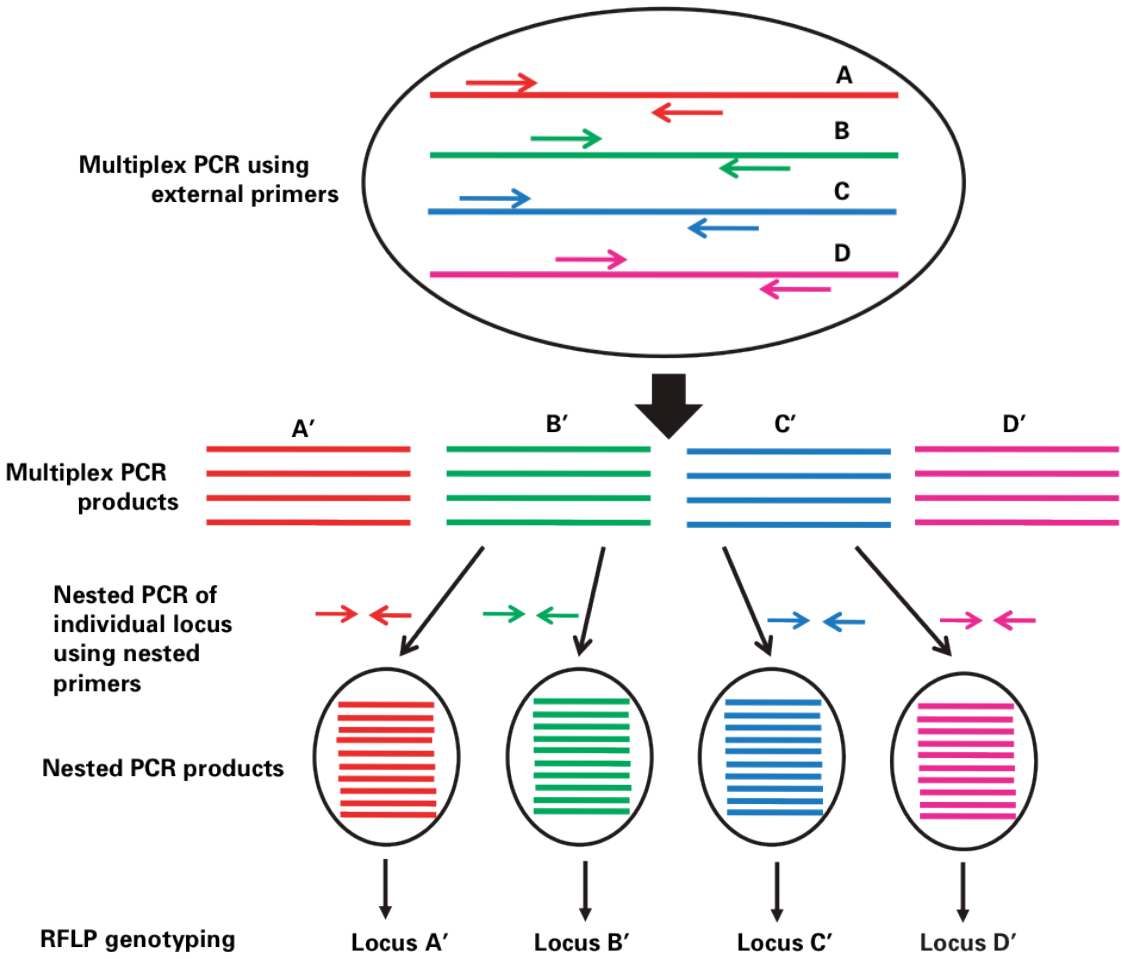


Figure II-1. Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) typing. Several genetic loci are pre-amplified by multiplex PCR with external primers. Then each individual locus is amplified by nested PCR using internal primers. The nested PCR products are treated with restriction enzymes and resolved in agarose gel to reveal strain-specific DNA banding patterns (RFLP typing). Horizontal lines represent DNA sequences and the horizontal arrows represent PCR primers.

Table II-1. Comparison of the methods for molecular detection and characterization of *T. gondii*

Method	Sensitivity (no. of parasites per PCR)	Advantages and Limitations	Suitable applications	References
qPCR of repetitive sequences	0.02 to 1 genome equivalents at 529-bp locus ~1 genome equivalent at B1 locus	Highly sensitive in detection. Isolation of parasite not needed. Not suitable for typing.	Diagnosis	(Edvinsson et al. 2006; Kasper et al. 2009; Reischl et al. 2003; Calderaro et al. 2006; Contini et al. 2002)
n-PCR of repetitive sequences	1 to 5 genome equivalents at B1 locus	Sensitive. Isolation of parasite not needed. Not suitable for typing.	Diagnosis	(Calderaro et al. 2006; Contini et al. 2002)
Mn-PCR-RFLP	~10 genome equivalents	Less sensitive. High resolution in typing. Isolation of parasite may not be needed.	Epidemiology and population genetics	(Khan et al. 2005a)
Mn-PCR-based sequencing	~10 genome equivalents	Less sensitive. High resolution in typing. Isolation of parasite may not be needed.	Epidemiology, population genetics and phylogenetics	This study
Conventional PCR-based sequencing	100 genome equivalents	Insensitive, high resolution in typing. Isolation of parasite needed.	Epidemiology, population genetics and phylogenetics	This study
Multiplex PCR of microsatellite markers	Not known	High resolution in typing	Epidemiology, population genetics	(Ajzenberg et al. 2005)

Table II-2. Summary of primers for multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP)

Markers	Multiplex PCR primers (external primers)*	Nested PCR primers (internal primers)	Nested PCR (bp)	Restriction enzymes, NEB buffers, incubation temperature and time	Reference
SAG1	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	390	Sau96I + HaeII (double digest). NEB4, BSA, 37C 1h, 2.5% gel.	(Grigg et al. 2001)
5'-SAG2	Not needed. The DNA fragment for 5'-SAG2 is covered by the external primers of alt. SAG2.	F: ATTCTCATGCCTCCGCTTC R: GCAAGAGCGAACTTGAACAC	242	MboI, NEB4, BSA, 37C 1h. 2.5% gel	(Howe et al. 1997; Su et al. 2006)
3'-SAG2	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	222	HhaI, NEB4, BSA, 37C 1h. 2.5% gel.	(Howe et al. 1997)
alt. SAG2	F: GGAACGCGAACAATGAGTTT R: GCACTGTTGTCCAGGGTTTT	F: ACCCATCTGCGAAGAAAACG R: ATTTCGACCAGCGGGAGCAC	546	HinfI + TaqI, NEB3, BSA, 37C 30 min, 65C 30 min. 2.5% gel	(Khan et al. 2005b; Su et al. 2006)

Table II-2 continued

Markers	Multiplex PCR primers (external primers)*	Nested PCR primers (internal primers)	Nested PCR (bp)	Restriction enzymes, NEB buffers, incubation temperature and time	Reference
BTUB	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	411	BsiEI + TaqI (double digest), NEB4, BSA, 60C 1h, 2.5% gel	(Khan et al. 2005b; Su et al. 2006)
GRA6	F: ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F: TTTCCGAGCAGGTGACCT R: TCGCCGAAGAGTTGACATAG	344	MseI, NEB2, BSA, 37C 1h, 2.5% gel.	(Khan et al. 2005b; Su et al. 2006)
C22-8	F: TGATGCATCCATGCGTTTAT R: CCTCCACTTCTTCGGTCTCA	F: TCTCTCTACGTGGACGCC R: AGGTGCTTGGATATTCGC	521	BsmAI + MboII (double digest), NEB2, BSA, 37C 30 min, 5C, 30 min, 2.5% gel	(Khan et al. 2005b; Su et al. 2006)

Table II-2 continued

Markers	Multiplex PCR primers (external primers)*	Nested PCR primers (internal primers)	Nested PCR (bp)	Restriction enzymes, NEB buffers, incubation temperature and time	Reference
C29-2	F: ACCCACTGAGCGAAAGAAA R: AGGGTCTCTTGCGCATACAT	F: AGTTCTGCAGAGTGTCGC R: TGTCTAGGAAAGAGGGCGC	446	HpyCH4IV + RsaI (double digest), NEB1, BSA, 37C 1h, 2.5% gel	(Khan et al. 2005b; Su et al. 2006)
L358	F: TCTCTCGACTTCGCCTCTTC R: GCAATTTCCTCGAAGACAGG	F: AGGAGGCGTAGCGCAAGT R: CCCTCTGGCTGCAGTGCT	418	HaeIII + NlaIII (double digest), NEB1, BSA, 37C 1h, 2.5% gel	(Khan et al. 2005b; Su et al. 2006)
PK1	F: GAAAGCTGTCCACCCTGAAA R: AGAAAGCTCCGTGCAGTGAT	F: CGCAAAGGGAGACAATCAGT R: TCATCGCTGAATCTCATTGC	903	AvaI + RsaI (double digest), NEB4, BSA, 37C 1h, 2.5% gel.	(Khan et al. 2005b; Su et al. 2006)
Apico	F: TGGTTTTAACCCTAGATTGTGG R: AAACGGAATTAATGAGATTTGAA	F: GCAAATTCTTGAATTCTCAGTT R: GGGATTCGAACCCTTGATA	640	AflII + DdeI (double digest), NEB4, BSA, 37C 1h, 3% gel	(Su et al. 2006)

*F, forward primer. R, reverse primer

Toward a molecular approach to identification and detection

Each of the detection and identification methods discussed above has its advantages and/or limitations, depending on application. A comparison of these methods is presented in Table II-1. At present, most clinical studies have focused on detecting the presence of *T. gondii* DNA in biological samples using the highly sensitive nested or qPCR of repetitive DNA sequences, but little has been done to further characterize the parasites. This is because each of these markers (B1 gene, 529 bp repetitive element, or ITS-1) has identical or near-identical repetitive sequences, therefore they are not adequate for genetic typing. The conventional multilocus PCR-RFLP analysis method relies on single-copy polymorphic DNA sequences, and usually a relatively large amount of starting templates from the parasite is required. Therefore, it has been difficult to genetically identify *T. gondii* in clinical samples, due to the extremely small amounts of parasite DNA available. The recent development of the Mn-PCR-RFLP method makes it possible to genetically characterize or classify *T. gondii* from clinical samples with high resolution. Here, we propose an integrated approach for the detection and genetic characterization of *T. gondii*. This approach should include detection of *T. gondii* infection in biological samples by n-PCR or qPCR of repetitive DNA sequences (employing the B1 gene or the 529-bp repeat element), followed by Mn-PCR-RFLP for the subsequent identification of *T. gondii*. There are a number of protocols developed in several laboratories for the detection of *T. gondii* by qPCR or n-PCR (Costa *et al.* 2000; Reischl *et al.* 2003; Edvinsson *et al.* 2006; Calderaro *et al.* 2006; Kasper *et al.* 2009). Although the sensitivity of these protocols varies among different laboratories, all studies demonstrate that the 529 bp repeat element is the most sensitive target, with a detection limit of 1/50 of a genome equivalent (Kasper *et al.* 2009).

Regardless of the protocol employed, all positive-testing samples should be further characterized genetically by the Mn-PCR-RFLP typing method. The PCR primer sequences and experimental conditions for this typing method are listed in Table II-2. This integrated approach will provide maximum information for epidemiological studies in order to address questions regarding the association of disease manifestations with parasite genotypes and virulence. For phylogenetic studies, MLST is preferred for its high resolution. Conventional PCR based MLST requires the samples to have a relatively high concentration of genomic DNA (100 genome equivalents per PCR). Taking the Mn-PCR approach, we have been able to generate quality DNA sequence data from samples containing as low as 10 genome equivalent (data not published). The major precaution for this approach is that, if errors occur in the early cycles of PCR, erroneous sequences may be generated. This problem can be solved by sequencing at least 2 independent PCR products from the same sample.To standardize studies and facilitate comparison among different laboratories, it is strongly recommended that all sequencing efforts include a few commonly used introns such as UPRT-1, UPRT-7, EF1 and HP (Khan *et al.* 2006, 2007) and the GRA6 gene (Fazaeli *et al.* 2000). For genomic DNA samples extracted directly from animal tissues, we suggest screening all samples using n-PCR targeting the 18S rDNA sequence prior to Mn-PCR-RFLP typing for *T. gondii*. By sequence analysis of the 18S rDNA for a number of closely related apicomplexan parasites, we identified SNPs that can distinguish *T. gondii*, Hammondia hammondi, Neospora caninum and Sarcocystis neurona by RFLP analysis (Fig. II-2). For n-PCR of 18S rDNA, the external primers are: Tg18s48F, CCA TGCATGTCTAAGTATAAGC and Tg18s359R, GTTACCCGTCACTGCCAC, which amplify a 312bp DNA fragment from *T. gondii*. The nested PCR primers are: Tg18s58F,

CTAAGTATAAGC TTTTATACGGC and Tg18s348R, TGCCACG GTAGTCCAATAC, which amplify a 291bp DNA fragment from *T. gondii*. These primers were designed based on sequences conserved for the above 4 parasites. The GenBank Accession numbers of these sequences are M97703 (*T. gondii*), AF096498 (H. hammondi), U17346 (N. caninum) and U07812 (S. neurona). Using this marker, we recently detected a mixed infection of Sarcocystis tenella and *T. gondii* from sheep brain tissues (data not published). The 18S rDNA marker provides an excellent tool for detecting apicomplexans that are closely related to *T. gondii* in animal samples, which will add additional value to the studies on *T. gondii* infection in animal populations. To monitor contamination of PCR amplification in Mn-PCR-RFLP genotyping, negative controls without DNA template should be included in each batch of experiments. In addition, a set of reference *T. gondii* isolates should be included to monitor the efficiency of PCR amplification and restriction enzyme digestion. This is very important, because for each batch of experiments there can be variation in efficiency of restriction digestion and sometimes incomplete digestion, and the concentration of electrophoretic gel can also vary slightly among experiments. Including *T. gondii* reference isolates in every experiment and on every electrophoretic gel will ensure the accuracy of results. The suggested references include RH88, GT1, PTG, CTG, TgCgCa1 (a.k.a. Cougar, COUG),MAS, TgCatBr5, TgCatBr64 and TgRsCr1 (Su *et al.* 2006; Dubey *et al.* 2009). The genetic data for these reference strains are summarized in Table II-3. A set of gel images is presented in Figure II-3.

Currently, there is a lack of standard nomenclature for *T. gondii* genetic typing. Historically, genetic markers were selected based on polymorphisms in DNA or protein sequences among the clonal type I, II, and III lineages, and the corresponding alleles were designated either as I, II, III, or 1, 2, 3, respectively (Darde *et al.* 1992; Sibley and Boothroyd, 1992; Ajzenberg *et al.* 2004; Ferreira *et al.* 2006; Khan *et al.* 2006; Su *et al.* 2006; Pena *et al.* 2008). Alleles which are distinct from those of types I, II or III were designated as either unique-1 (u-1), unique-2 (u-2), unique-3 (u-3) etc., or 4, 5, 6, etc. In Table II-3 and Figure II-3, we use the former scheme to describe new alleles. In summary, the application of an integrated approach, combining molecular detection and high resolution genetic characterization, should assist in enhancing our understanding of the molecular epidemiology, population genetics and phylogeny of *T. gondii*, which will be beneficial to the control of *T. gondii* transmission and a reduction of toxoplasmosis in humans and animals.

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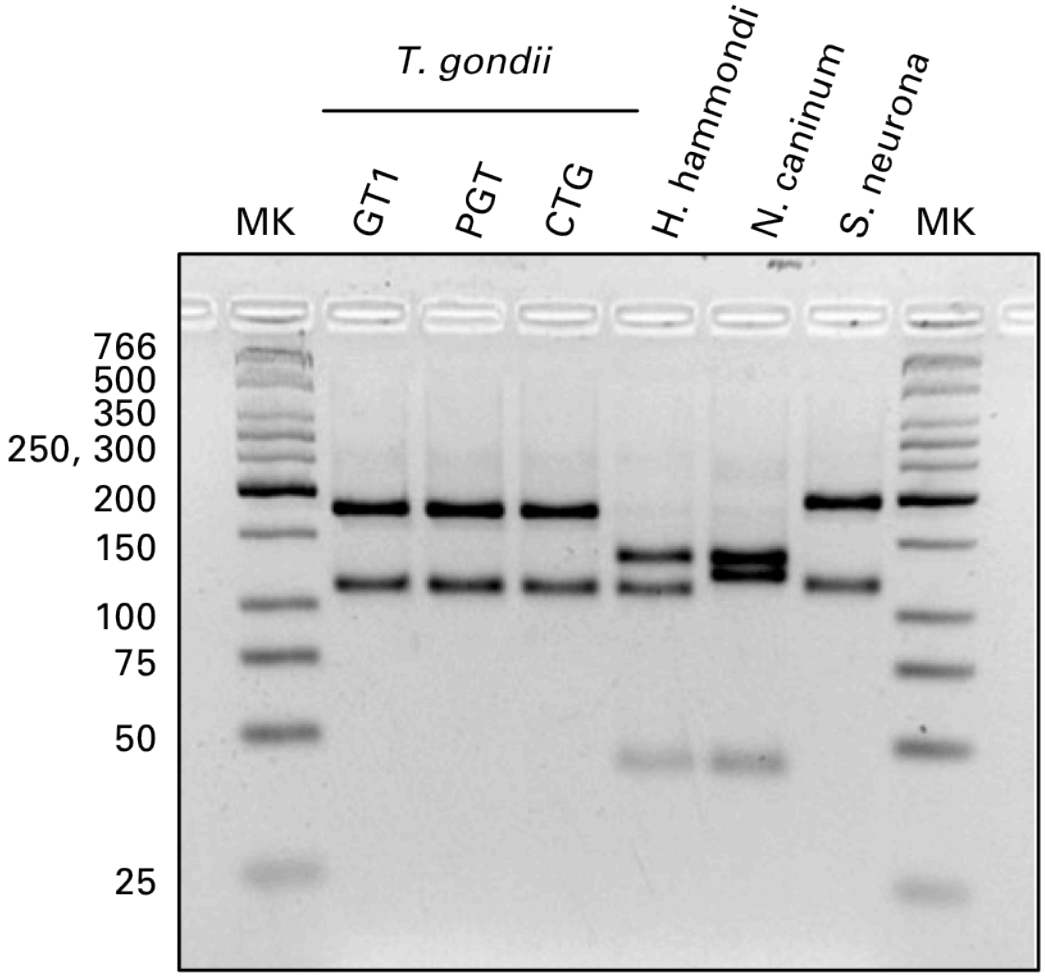


Figure II-2. PCR-RFLP gel image for *T. gondii*, *Hammondia hammondi*, *Neospora caninum* and *Sarcocystis neurona* based on the 18S rDNA marker. PCR products from each parasite species were separated in 3% agarose gels. Samples GT1, PTG, and CTG represent *T. gondii*. All 4 apicomplexan species have unique banding patterns and can be clearly distinguished (mouse and human DNA were used as negative controls and no PCR products were amplified, not shown). MK: Low molecular weight DNA ladder (New England Biolabs Inc.).

Table II-3 Summary of multilocus PCR-RFLP typing for *T. gondii* reference isolates

	Genetic markers										
T. gondii reference isolates	SAG1[1]	(5'+3') SAG2[2]	alt. SAG2[3]	SAG3	BTUB	GRA6	C22-8	C29-2	L358	PK1	Apico
GT1, RH88 (Type I)	I	I	I	I	I	I	I	I	I	I	I
PTG (Type II)	II/III	II	II	II	II	II	II	II	II	II	II
CTG (Type III)	II/III	III	III	III	III	III	III	III	III	III	III
TgCgCa1 (cougar, COUG)	I	II	II	III	II	II	u-1	u-1	I	u-2	I
MAS	u-1	II	III	III	III	III	I	I	I	III	I
TgCatBr5	I	III	III	III	III	III	I	I	I	u-1	I
TgCatBr64	I	u-1	III	III	III	III	I	I	III	III	I
TgRsCr1	u-1	II	I	III	I	III	I	I	I	III	I

[1] At SAG1 locus, Type II and III are indistinguishable.
[2] SAG2 marker based on 5' and 3' ends of the gene sequence (Howe et al. 1997).
[3] A SAG2 marker based on the 5' end of the gene sequence but different from 5' SAG2 (Su et al. 2006).
u-1 and u-2 are new alleles that are different from the clonal Type I, II and III alleles.

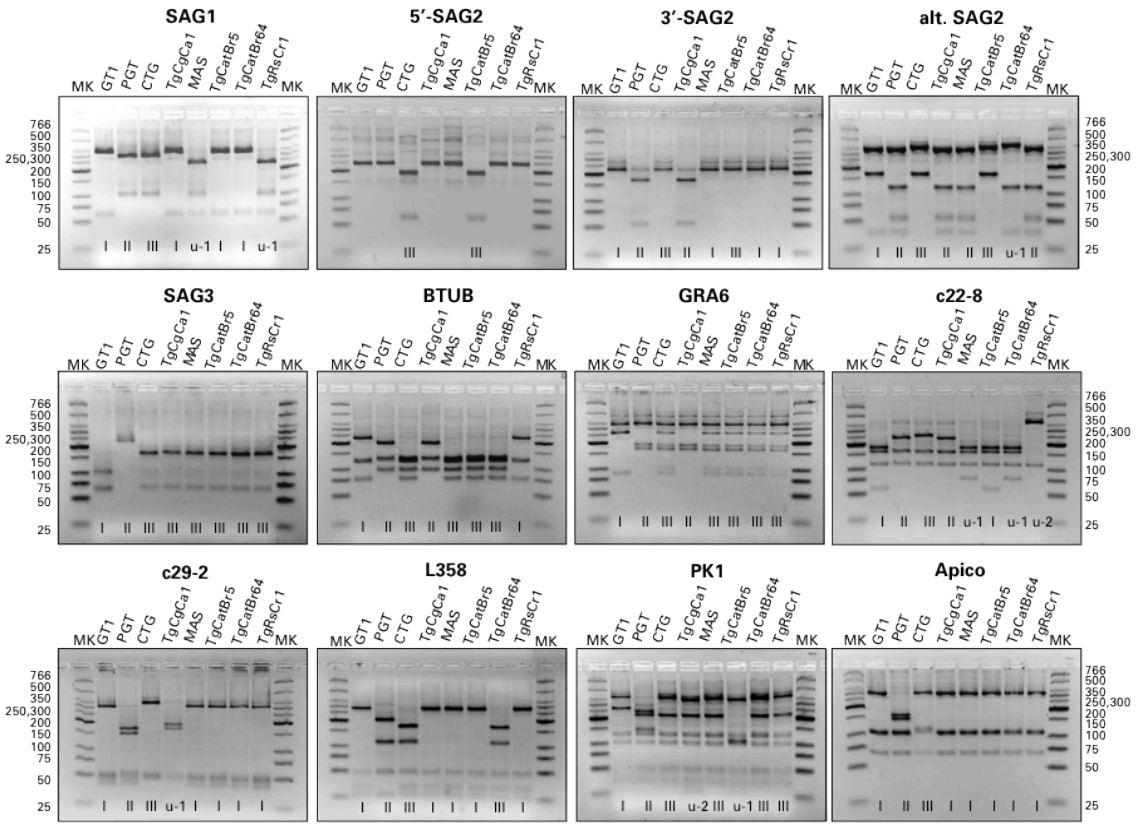


Figure II-3. Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) analysis of *T. gondii* samples using 10 different genetic markers. *T. gondii* reference strains are GT1 (or RH88), PTG, CTG, TgCgCa1 (a.k.a. Cougar, COUG), MAS, TgCatBr5, TgCatBr64 and TgRsCr1. Nested PCR products from each marker were digested with selected restriction enzymes (Table II-2), and DNA fragments were separated in agarose. All markers were resolved in 2.5% gels, except that Apico was resovled in 3% gels. MK, low molecular weight DNA ladder (New England Biolabs Inc.)

Chapter III. Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping

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Introduction

Toxoplasma gondii is a parasitic protozoan parasite belonging to the phylum Apicomplexa. It parasitizes mammals and birds, and one third of the world's human population is infected (Dubey, 2010). *T. gondii* generally forms chronic infections in its hosts, embedding in muscle and brain tissue (Tenter *et al.* 2000). In immunocompromised hosts such as AIDS patients, infection with *T. gondii* may result in encephalitis, and in developing fetuses acute toxoplasmosis may result in blindness, deformation, mental retardation, or even death (Montoya and Liesenfeld, 2004). In veterinary medicine, toxoplasmosis is a major contributor to abortion in a number of economically important livestock animals such as sheep and goats (Buxton *et al.* 2007). Recently, chronic toxoplasmosis has been shown to cause behavioral changes in mice, making them attracted rather than repelled by the scent of cat urine, attributable to inhibition of neuronal function and alteration of neurotransmitter levels (Haroon *et al.* 2012; Gatkowska *et al.* 2012). In humans, the condition has also been shown to correlate with schizophrenia (Yolken *et al.* 2001; Torrey *et al.* 2007) and bipolar disorder (Pearce *et al.* 2012; Hamdani *et al.* 2013).

Unlike many other parasites in the apicomplexan phylum, which exhibit stronger host specificity, *T. gondii* possesses an extremely broad host range, infecting both mammals and birds (Dubey, 2010). The parasite has been isolated from a wide range of hosts, including both terrestrial and marine animals. In addition to having this expansive host range, *T. gondii* also seems to be nearly ubiquitous geographically, having been isolated from a variety of climatic regions on every continent surveyed (Dubey, 2010). It seems that this expansion of the parasite occurred relatively recently in its evolutionary history (Khan *et al.* 2007). Because of the remarkable evolutionary success of *T. gondii* that has resulted in such broad expansion, it is important to understand transmission patterns and the factors that influence such patterns. In 1995, Howe and Sibley published a landmark article showing evidence for a clonal population structure in Europe and North America, in which the vast majority of *T. gondii* strains could be grouped into three lineages, namely Types I, II and III (Howe and Sibley, 1995). There appeared to be very limited sexual recombination between strains belonging to each of the lineages. While this model of the

T. gondii population structure is still upheld to a large extent, it later became clear that the actual structure was more complex than was originally thought. This was found to be particularly true in Central and South America, where an abundance of atypical (non-clonal) strain types have been found (Lehmann *et al.* 2006; Ferreira *et al.* 2006; Khan *et al.* 2007; Pena *et al.* 2008; Su *et al.* 2012). Moreover, improvements in genotyping techniques have revealed greater complexity not only in Central and South America, but also in North America (Rajendran *et al.* 2012; Su *et al.* 2012).

Several molecular techniques including polymerase chain reaction - restriction fragment length polymorphism analysis (PCR-RFLP) (Howe and Sibley, 1995; Su *et al.* 2006, 2010), microsatellite DNA analysis (Ajzenberg *et al.* 2002, 2010) and multilocus DNA sequence typing of introns (Khan *et al.* 2011; Khan *et al.* 2007) have been used to study the genetic makeup of *T. gondii* strains. A comprehensive review of these methods and their contribution to the state of molecular epidemiology and population genetics of *T. gondii* can be found in a recent publication (Dardé *et al.* 2014). Among these methods, PCR-RFLP analysis of ten markers (Su *et al.* 2006, 2010) was applied to more than a thousand *T. gondii* isolates worldwide, generating a significant amount of data and revealing the genetic diversity of the parasite. Here, we summarize the data generated to the end of 2012 using this approach. This data comes from numerous publications originating from studies performed on every continent except Antarctica, and provides information for 1457 isolates representing 189 different genotypes. A number of *T. gondii* isolates previously typed by other methods were genotyped by the ten PCR-RFLP markers in our laboratory and the results were integrated into this review article. Together, this information makes up the most comprehensive and cohesive picture of the global *T. gondii* population structure yet compiled.

Results

Geographical patterns of genetic diversity

At present, there is no standard nomenclature in designating genotypes for *T. gondii*. Conventional designation defined the clonal Type I, II and III lineages, and lumped up all others as atypical or exotic genotypes (Howe and Sibley, 1995; Grigg *et al.* 2001; Su *et al.* 2003). The subsequently identified major genotypes were added to the list including Type BrI, BrII, BrIII and BrIV, Type 12, Africa 1, and Chinese 1 (Chen *et al.* 2011; Khan *et al.* 2011; Mercier *et al.* 2010; Pena *et al.* 2008). However, this scheme of genotype designation is too cumbersome to define hundreds of genotypes identified by the multilocus PCR-RFLP method. To overcome this problem, a scheme has been adopted in which each genotype is designated as a "ToxoDB PCR-RFLP genotype" followed by a specific numeral. A comparison of conventional nomenclature and ToxoDB PCR-RFLP designations for major genotypes is provided in Table III-1. To find a more comprehensive comparison of genotype designation schemes, see the recent publication by Dardé *et al.* (2014).

A total of 189 ToxoDB PCR-RFLP genotypes identified from 1457 samples are summarized in Table E-1 (Appendix E) . The compiled data were based on published results reported up to the end of 2012. The vast majority of *T. gondii* isolates were obtained from domestic and wild

animals with chronic infection. For these samples, infection of *T. gondii* was first determined by the presence of anti-*Toxoplasma* antibodies in their sera, followed by bioassay of seropositive animal tissues (brain and/or muscle) in mice or cats. *T. gondii* isolates obtained from experimentally infected mice or cats were further processed for genotyping. The global distribution of the 189 genotypes is illustrated in Figure III-1. The ten most frequently identified genotypes are genotype #2, #3, #1, #5, #4, #9, #6, #7, #8 and #10, accounted for 13.8, 12.6, 12.2, 5.0, 4.5 3.8, 3.3, 2.6, 2.3 and 2.1% of the samples, respectively. Genotypes #1 and #3, which differ only at the Apico locus, together compose the conventional Type II lineage, accounted for 24.8% (362/1457) of the population. Genotype #1 is also referred to as Type II clonal, whereas #3 as Type II variant (Table III-1). As noted, genotype #2, also known as Type III, accounted for 13.8% (201/1457) of the samples. Genotypes #4 and #5, which differ only at the SAG1 locus and are collectively known as Type 12, accounted for 9.5% (139/1457) of the population. The results showed that genotypes #1, #2 and #3 (Type II clonal, Type III and Type II variant) are identified worldwide. These three genotypes are highly prevalent in Europe. Genotypes #1, #2, #3, #4 and #5 dominate in North America. Genotypes #2 and #3 (Types III and II variant) dominate in Africa, and genotypes #9 and #10 (Chinese 1 and Type I) are prevalent in east Asia. In the paragraphs below, we have summarized the genotyping results by major geographical regions. Due to small sample size (only 3 isolates), Australia is not discussed further.

North America

In North America, including the USA and Canada, a total of 501 isolates (including 2 samples from Hawaii) have been typed to 40 genotypes. The majority of the samples (86.4%, 433/501) fall into genotypes #1, #2, #3, #4 and #5 (Figure. III-1). Genotypes #1 (29.7%, 149/501) and #3 (14.2%, 71/501), which are the conventional Type II lineage, accounted for 43.9% (220/501) of the population. Genotype #2 (Type III), accounted for 18.2% (91/501) of the samples. Genotypes #4 (10.0%, 50/501) and #5 (14.4%, 72/501) (together as Type 12) accounted for 24.4% (122/501) of the population. Type 12 is now recognized as the fourth clonal lineage in North America (Khan *et al.* 2011). It was shown that Type 12 is a dominant lineage in wildlife in North America (Dubey *et al.* 2011). *Toxoplasma gondii* strains belonging to this lineage, also designated as Type X, were associated with high mortality in sea otters along the coast of California (Miller *et al.* 2008). Genotype #10 (Type I), previously considered as one of several dominant types in North America, accounted only for 0.8% (4/501) of the population.

Europe

Of the 64 samples obtained from European countries, 9 genotypes were identified (Figure III-1). Nearly three quarters (64.1%, 41/64) belong to the genotypes #1 (25.0%, 16/64) and #3 (39.1%, 25/64), which are recognized as the Type II lineage. Genotype #2 (Type III) makes up 12.5%, (8/64) of the samples. Genotype #10 (Type I) and genotype #6 (a.k.a Type BrI, *Africa 1*), both have relatively high frequencies, accounted for 9.4% (6/64) and 4.7% (3/64), respectively. This genetic uniformity gives Europe the closest resemblance to the three-lineage model proposed by Howe and Sibley (Howe and Sibley, 1995). The prevalence of genotypes #4 and #5 (also collectively designated as Type 12) in North America provides contrast with Europe,where genotype #4 has not been found and genotype #5 makes up only a minor part of the population. More sampling from a greater diversity of hosts is needed from European countries for better understanding of the region's *T. gondii* population structure.

Table III-1. *T. gondii* genotype designations for common lineages

Conventional genotype designations	ToxoDB PCR-RFLP Genotypes	Representative isolates	References
Type I, type 1	#10	GT1	Su et al. (2012)
Type II, type 2 (type 2 clonal)	#1	PTG	Su et al. (2012)
Type II, type 2 (type 2 variant)	#3	PRU	Su et al. (2012)
Type III, type 3	#2	VEG	Su et al. (2012)
Type 12, atypical, exotic	#4	B41	Khan et al. (2011); Su et al. (2012)
Type 12, atypical, exotic, includes Type X and Type A	#5	ARI	Khan et al. (2011); Su et al. (2012)
Type BrI, atypical, exotic, Africa 1	#6	FOU, TgCatBr2	Pena et al. (2008); Mercier et al. (2010); Su et al. (2012)
Type BrII, atypical, exotic	#11	TgCatBr1	Pena et al. (2008); Su et al. (2012)
Type BrIII, atypical, exotic	#8	P89 (TgPgUs15), TgCatBr3	Pena et al. (2008); Su et al. (2012)
Type BrIV, atypical, exotic	#17	MAS, TgCkBr147	Pena et al. (2008); Su et al. (2012)
Chinese 1, atypical, exotic	#9	TgCtPRC4	Dubey et al. (2007b); Chen et al. (2011); Su et al. (2012)

Figure III-1. Geographical distribution of *T. gondii* genotypes. Black dots indicate locations from which *T. gondii* isolates were obtained and genotyped using the PCR-RFLP method. The numbers around pie chart edges indicate ToxoDB PCR-RFLP genotypes. In some cases, alternative strain type designations are overlaid on pie charts in bold lettering (also see Table III-1 for correlations between nomenclatures). Sizes of pie charts correlate with total number of isolates (n), and colors indicate different genotypes. Two strains (GANGI and WIK) reported from Africa without specific countries of origin were listed in Supplemental Table S1, but not included on this map.

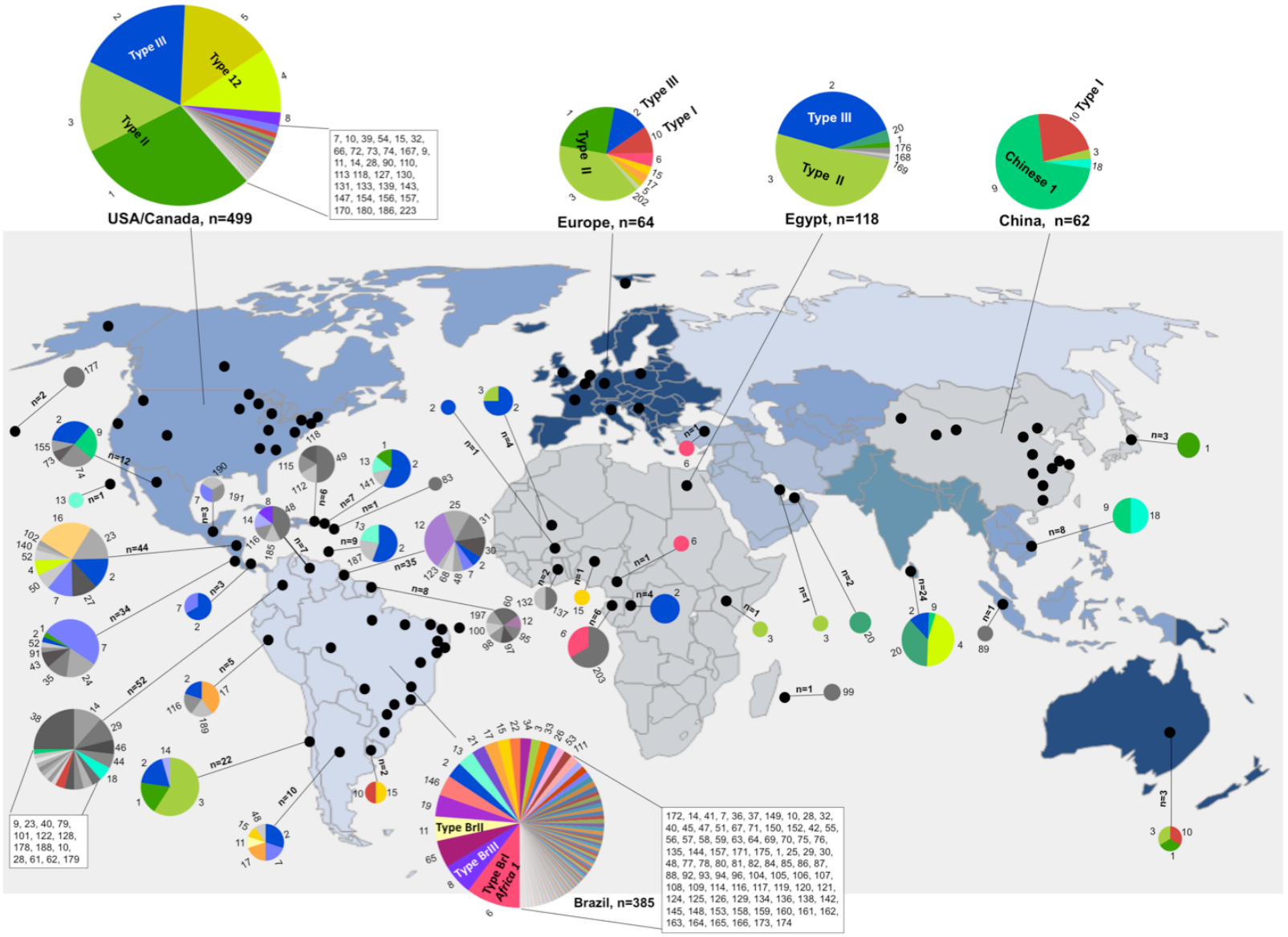


Figure III-1 continued

Asia

In Asia, there also appears to be a high degree of genetic uniformity. From 102 samples, 10 genotypes were identified. Genotype #9 (*Chinese 1*) is by far the most commonly found, accounting for 48.0% (49/102) of samples. It is present in China, Vietnam and Sri Lanka, indicating a widespread distribution in Eastern Asia. Genotype #10 is also common in China, unlike in most other countries (13.7%, 14/102 in Asia). Genotypes #4, #18 and #20 have relatively high frequencies among the samples in Asia. Genotype #20 has been identified in a wide range of areas, from Sri Lanka in south Asia to Egypt in north Africa. Thus far, most of the sampling from Asia has been from the more populous eastern regions. More sampling is thus needed from the western areas where the climate and geography, as well as the distribution of human populations are markedly different.

Africa

A total of 141 samples were typed and 13 genotypes identified in Africa. Most samples (118/141) in Africa were from Egypt. Overall, genotype #3 (45.4%, 64/141) and #2 (39.0%, 55/141) are the two dominant types, accounting for 84.4% of the samples. Genotype #6 was identified for several samples. The limited data that are available from sampling in Western Africa seem to suggest a high level of diversity, with a relatively low frequency of common genotypes. However, more sampling is required in Africa before strong conclusions can be drawn regarding the makeup of the *T. gondii* population of that continent. It would be of special interest to have more information regarding *T. gondii* in the tropical regions of Africa.

Central and South America

A total of 646 samples were typed and 156 genotypes identified in Central and South America. Unlike in the northern hemispheric countries discussed above, *T. gondii* in this region displays a highly diverse population structure, in which no genotype appears to be clearly dominant. The top ten genotypes are #2, #6, #7, #8, #11, #3, #65, #13, #19 and #146, with frequencies of 6.8, 6.2, 5.0, 3.7, 3.3, 3.1, 3.1, 2.5, 2.5 and 2.3%, respectively. The genotypes #1, #2, #3 and #10 are at low frequencies and have not taken over the genetic landscape as in the north. Of these genotypes, the #2 (Type III) is the most frequently occurring (6.8%, 44/646) and is found in many of the countries in this region, even those for which the sample size is low, such as Panama, Peru and Grenada. Even in these regions however, genotype #2 is often less frequent than many other genotypes. An exception to this trend is found in Chile, where we see a genetic makeup more similar to that found in North America and Europe, in which genotypes #1, #2 and #3 predominate. Chile is located on the west side of the Andes Mountains which separate it from most of the countries in South America. On its west side is the Pacific Ocean, which provides ports for maritime transportation to other continents. Trading with countries on different continents might bring genotypes #1, #2 and #3 to Chile, eventually allowing them to become dominant in this region (Rajendran *et al.* 2012).

Quantitative comparison of genetic diversity

To quantify differences of genetic diversity among *T. gondii* populations from major geographical areas, we analyzed the typing data based on different continents. Within- and between-population diversity and shared genotypes were analyzed by Arlequin v3.5 as described in previous reports (Rajendran *et al.* 2012; Excoffier and Lischer, 2010). The results are summarized in Table III-2, Figure III-2 and Table III-3. Among the five major regions including Africa, Asia, Europe, North America, and Central/South America, the genotype diversity (gene diversity) indices are 0.6433, 0.7280, 0.7679, 0.8285 and 0.9792, respectively, indicating high diversity in Central/South America, followed by North America (Table III-2). Pairwise comparison revealed that there are significant differences among populations in Africa, Asia, Europe, North America, and Central/South America, with only the populations of Europe and North America exhibiting similar genetic composition to each other (Figure III-2). Furthermore, the difference between Central/South America and other areas is more prominent (Figure III-2). Analysis of shared genotypes revealed the distribution of common genotypes (Table III-3). Genotypes #1, #2 and #3 exist in all five continents. Genotype #6 (BrI, Africa 1) has been identified in all but North America, genotype #10 (Type I) has been identified in all but Africa, and genotype #15 identified in all but Asia. The North and Central/South Americas shared 17 genotypes, the highest among all populations compared.

Discussion

The compiled PCR-RFLP typing results of 1457 *T. gondii* samples revealed 189 genotypes. Overall, geographical differences are readily recognized. Only a few genotypes dominate in the northern hemisphere, whereas hundreds of genotypes coexist with a few having a relatively higher frequency in the southern hemisphere. These results suggest a clonal population structure in the north and an epidemic population structure in the south. This finding is in strong agreement with the conclusion from a smaller sample size that was analyzed by combined typing methods (Dardé *et al.* 2014). Though the frequencies of genotypes may change with more intensive sampling, we expect that the compiled results of 1457 samples have captured the big picture of global *T. gondii* diversity.

Understanding *T. gondii* population structure is of great interest, as it may provide us with essential information regarding the transmission and evolution of this widespread zoonotic parasite. Several major factors may have been involved in shaping the modern-day *T. gondii* population structure. First, the rise of human agriculture about 11,000 years ago in Mesopotamia and its expansion to the New World in the last few hundred years may have significantly transformed our biological environment and reduced genetic diversity of animal parasites (Rosenthal, 2009). In North America, Europe and east Asia, large-scale farming and the erection of cities have largely transformed the landscape throughout much of these regions, and likewise many of the ancestral wild animal species have been replaced with genetically uniform domestic animals. Such transformation of geography and reduced animal diversity may have led to expansion of a few *T. gondii* genotypes that adapted to the new environment. The limited data lend support to the idea that the clonal *T. gondii* lineages may have become dominant in a manner that was concomitant with the beginnings of human agriculture in the region of

Mesopotamia, whereupon these lineages then spread outward alongside the flow of human migration. However, more data is certainly required from the Middle East and other regions before more definitive conclusions can be drawn regarding this matter.

Second, the low genetic diversity of *T. gondii* observed in the northern hemisphere may be a result of the founder effect. The high genetic diversity observed in South and Central America suggests that *T. gondii* originated in this region (Lehmann et al., 2006). It is possible that a small number of founding strains were introduced from South America to other continents by maritime trading of goods, transportation of pet animals such as cats, or accidental transport of infected rodents within the last five centuries (Lehmann et al., 2006). These founder populations could then have expanded throughout their respective geographic regions with little competition or recombination between strains, leading to the observed clonal population structures.

Another possible explanation for the high genetic diversity found in South and Central America, in contrast to the low diversity of the northern hemisphere, is that the tropical and subtropical zones in the former region support a greater diversity and number of animal hosts, each of which might favor the selection of different *T. gondii* genotypes, enabling a wider variety of strains to proliferate. Additionally, the warmer climate may allow a greater number of oocysts to survive in nature for a longer period of time, resulting in more infection of hosts overall as well as decreased selective pressure compared to the northern hemisphere. Thus far, sampling in tropical regions other than those of South and Central America has been sparse and so understanding of the *T. gondii* population structures in these areas remains limited. If additional sampling were to reveal that regions with similar climate and host species diversity such as those found in Africa and southeast Asia also supported highly diverse *T. gondii* populations, such a finding would lend credence to this alternative hypothesis.

Future perspective

The existing genotyping studies have revealed essential information regarding the global diversity of *T. gondii* and its population structure, providing us preliminary data for further study on the origin, transmission, ecology and evolution of this highly successful protozoan parasite. To start tackling these topics, more in-depth sampling of *T. gondii* is necessary for several aspects. First, sampling of *T. gondii* in wildlife from remote areas that are not disturbed by human civilization is needed to better assess the effect of human settlement on the diversity of *T. gondii*. A recent study in French Guiana showed that a human environment may favor clonal expansion and reduce *T. gondii* diversity (Mercier *et al.* 2011). Given that human agriculture arose in the Fertile Crescent about 11,000 years ago in Mesopotamia (Diamond and Bellwood, 2003), it might have led to expansion of genotypes #1, #2 and #3 and significantly reduced *T. gondii* diversity in Europe and Southwest Asia. However, we would expect that high genetic diversity may still be maintained in wildlife from remote geographical regions that have not been disturbed by human settlement in Europe and Southwest Asia. Similar studies are also necessary in tropical Africa, the Amazon rainforest, South Asia and Australia to better understand the effect of human societies on *T. gondii* genetic diversity. Availability of these data will allow us to understand the global transmission patterns of *T. gondii.*

Second, potential long distance transmission of *T. gondii* by migratory marine mammals and birds is poorly understood. It was shown that striped and bottlenosed dolphins, as well as a variety of Antarctic pinnipeds were seropositive for *T. gondii* infection (Dubey *et al.* 2007a, 2008; Rengifo-Herrera *et al.* 2012). Dolphins may migrate over relatively large distances and thus may be important vectors for the long distance spread of *T. gondii*. Several studies have focused on the prevalence of *T. gondii* in marine mammals such as sea otters along the coastlines of California, Washington and Alaska. A genotype designated as 'Type X' (genotype #5) was dominant in these sea otters (Conrad *et al.* 2005; Sundar *et al.* 2008). The same genotype seemed to be the major cause of high mortality in otters in California (Conrad *et al.* 2005). Though sea otters are not migratory themselves, they may be sentinels of a route by which *T. gondii* might be introduced into the marine ecosystem (or at least an indicator of the types of strains present in such ecosystems), through which it seems likely that intercontinental transmission may occur. Migratory birds may also be an important contributor to the transmission of *T. gondii* between continents. However, few studies have been performed targeting such species. A recent study reported genotyping of a single isolate from a wild pigeon in Mexico (Alvarado-Esquivel *et al.* 2011). Other studies have found sporadic cases of *T. gondii* infection in wild bird species (Dubey *et al.* 2011a,b). Given the potential role of migrating marine mammals and birds in long distance transmission of *T. gondii*, more studies on these animals are needed to better understand their contribution to the evolution of *T. gondii* on a global scale.

Third, most *T. gondii* samples studied so far have been from nonhuman animal origin. Only a limited number of samples have been isolated from human infections. A total of only 84 human samples have been analyzed by the 10 PCR-RFLP markers that are the focus of this review article. These samples have revealed a variety of genotypes with no clear pattern emerging. Atypical genotype #65 was found to occur in human patients in Sao Paulo, Brazil in a single study (Ferreira *et al.* 2011). Another feature of note seems to be that genotype #10 (Type I), the highly mouse-virulent lineage, appears to be more prevalent in human infections than is seen in other hosts. A larger sample size is necessary to determine the significance of this feature, and human toxoplasmosis patients need to be sampled from a greater variety of geographic regions in order to determine whether this is a global phenomenon. It would also be of interest to determine the mouse-virulence of the atypical strains found in human hosts in order to investigate the possibility that such murine virulence also leads to an increased ability to cause disease in *Homo sapiens*.

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Table III-2. Basic statistics of *T. gondii* populations from different geographical regions

	Africa	Asia	Europe	North America	Central/South America
Number of isolates	141	102	64	501	646
Number of genotypes	13	10	9	40	156
Gene diversity	0.6433+/-0.0256	0.7280+/-0.0391	0.7679+/-0.0351	0.8285+/-0.0086	0.9792+/-0.0017
Mean no. of pairwise differences over loci	5.5687+/-2.6895	4.9293+/-2.4182	5.0968+/-2.6495	5.0968+/-2.4762	6.1121+/-2.9118

Due to the small sample size of only 3 isolates, Australia was not included for analysis in this table

Table III-3. Shared genotypes among geographical regions

	Africa	Asia	Europe	North America
Africa				
Asia	1,2,3,6,20			
Europe	1,2,3,6,15	1,2,3,6,10		
North America	1,2,3,15	1,2,3,4,9,10	1,2,3,5,10,15	
Central/South America	1,2,3,6,15	1,2,3,4,6,9,10,18	1,2,3,6,10,17	1,2,3,4,7,8,9,10,11,14,15,28,32,73,74,118,157

Due to the small sample size of only 3 isolates, Australia was not included for analysis in this table

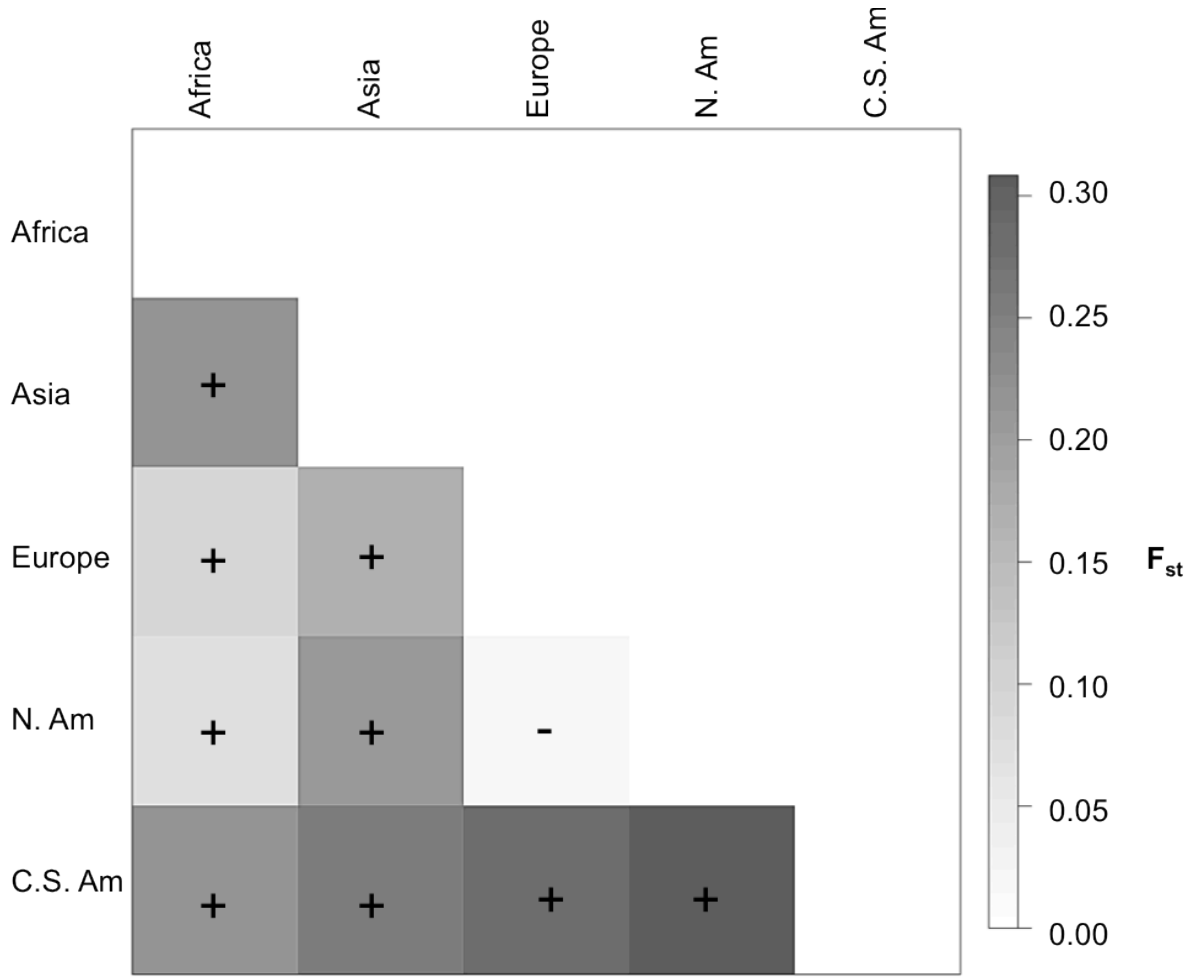


Figure III-2. Pairwise Fst of five *T. gondii* populations from different geographical regions. Comparison of populations were conducted using Arlequin ver 3.5. Statistical significance is determined at P=0.001. The "+" sign indicates significant difference between two populations, whereas "-" indicates insignificance. N. am = North America, C.S. Am = Central and South America. The heat map indicates the Fst value.

Chapter IV. The ROP5 and ROP18 genotypes are highly predictive of virulence across globally distributed strains of *Toxoplasma gondii*

Introduction

Toxoplasma gondii is among the most widespread eukaryotic parasites known. It is thought to chronically infect approximately one third of the world's human population and has been isolated from an extremely diverse array of mammals and birds from every continent besides Antarctica. Toxoplasmosis is a leading cause of death among AIDS patients and can also cause birth defects and mortality in infants when transmitted vertically during pregnancy (Dubey, 2010). In immunocompetent adults, asymptomatic chronic infections generally develop following a brief and mild symptomatic period, though more severe symptoms may also occur. Ocular infections are associated with approximately 2 percent of *T. gondii* infections, and these may result in visual impairment or blindness if improperly treated. Additionally, in some cases severe and potentially deadly systemic toxoplasmosis, sometimes referred to as Amazonian toxoplasmosis, may occur in otherwise healthy adults. This form of the disease often requires intensive medical care and has resulted in deaths due to multiple organ failure (Demar *et al.* 2007, 2012).

Though a link between parasite genotype and the severity of human disease has not been definitively established, an increasing abundance of circumstantial evidence lends support to this probability. We have recently compiled all of the PCR-RFLP genotyping data that had been published as of the end of 2012 using a widely-employed set of ten genetic markers, which included a total of 1,475 isolates comprising 189 distinct genotypes (Shwab *et al.* 2014). These isolates were collected from all continents with the exception of Antarctica, and from a variety of mammalian and avian species, both wild and domesticated. Our analysis revealed distinct population structures for each major geographic region examined, with striking contrast between the largely clonal populations found in North America, Europe and Asia, wherein only a few dominant genotypes account for the overwhelming majority of isolates and highly diverse, endemic structure of the South/Central American region wherein many genotypes coexist with none being notably dominant. In addition to being more genetically diverse, South American strains are also more highly mouse-virulent on average than strains identified on other continents. In mouse bioassays described in the studies referenced in Shwab *et al.* (2014), 61 percent of South and Central American strains caused mortality following ingestion of infected tissue by mice, compared to only 7 percent of North American strains. North American and European populations are dominated by the archetypal Type II and III clonal types (toxoDB genotypes #1, 2 and 3) which exhibit low to intermediate mouse virulence, while the Asian population is dominated by the Type II-related genotype #9.

Evidence indicates a correlation between the extreme diversity of atypical parasite genotypes found in South America and the manifestation of more dangerous forms of human disease. In Brazil for example, severe symptoms were found to be associated with congenital ocular toxoplasmosis at a rate five times higher than that recorded in Europe (Gilbert *et al.* 2008), and when 20 tissue samples from a diverse assortment of toxoplasmosis patients from multiple hospitals and clinics in Sao Paulo state were genotyped, 18 were determined to be infected with

the atypical mouse-virulent ToxoDB genotype #65, which makes up only a small fraction of Brazilian *T. gondii* isolates, suggesting a possible association of this genotype with human disease (Ferriera *et al.* 2011). In French Guiana, more than 50 cases of Amazonian toxoplasmosis have been recorded since 1998. This form of the disease appears to be associated with a sylvatic cycle of parasite transmission, through wild felids and intermediate hosts as opposed to domesticated animals, as patients generally reported recent rainforest-related activities such as consumption of undercooked wild game or surface water. In cases where parasites have been isolated from patient tissues, isolates were found to possess atypical genotypes that were highly virulent in mice (Darde *et al.* 1998, Demar *et al.* 2007, Carme *et al.* 2002, 2009). Though not yet identified outside of French Guiana, it is expected that the strain types responsible may be present in adjacent areas inhabited by wild felids (Carme *et al.* 2009). Even among cases of toxoplasmosis in regions with predominantly clonal population structures, bias appears to exist towards infection by mouse virulent or atypical genotypes. In a study performed in the United States, two thirds of genotyped isolates from patients with ocular toxoplasmosis were found to belong to the virulent archetypal Type I or Type I-related atypical genotypes despite the overwhelming dominance of less virulent Type II and III strains in North America (Grigg *et al.* 2001). Disproportionate association of atypical genotypes with severe clinical symptoms has also been observed in Europe (Bossi and Bricaire 2004, Delhaes 2010).

If mouse virulence of *T. gondii* strains correlates with human disease, as suggested by the aforementioned observations, it is of great interest to have a means of predicting the potential virulence of newly identified genotypes, as well as to understand the mechanisms governing pathogenesis in these strains. The rhoptry-secreted proteins ROP18 and ROP5, have been identified as the key determinants of virulence in murine hosts among the archetypal parasite lineages (Taylor *et al.* 2006, Saeij et el. 2006, Reese *et al.* 2011). A third polymorphic rhoptry protein, ROP16 has also been shown to be important in modulating the host response to parasite invasion in mice (Saeij *et al.* 2006, Yamamoto *et al.* 2009, Ong *et al.* 2010). It appears to be variations in the assortment of alleles of these genes that constitute the functional differences between the three archetypal lineages with regard to pathogenesis in mice. Type I strains possess functional ROP5 and ROP18 alleles, which work in tandem to inactivate host immunity-related GTPases that would otherwise target and disrupt the parasitophorous vacuole membrane, thus allowing these strains to propagate in a rapid and unchecked manner, resulting in a high degree of mortality (Refer to chapter I of this work and Niedelman *et al.* 2012, Etheridge *et al.* 2014, Reese *et al.* 2014). Type III strains, by contrast, do not express ROP18 and thus are easily overcome by the host's defenses, making these strains largely avirulent to mice (Boyle *et al.* 2008, Niedelman *et al.* 2012). Type II strains are the most distantly related of the three lineages, and appear to employ a somewhat different pathogenic strategy in mice, determined by their particular set of virulence gene alleles. They express a functional version of ROP18, but a deficient version of ROP5, making them vulnerable to host defenses (Saeij *et al.* 2006, Behnke *et al.* 2011). However, they also express a different ROP16 allele from those of Type I and III strains, resulting in altered host gene expression and a more severe inflammatory response compared to that associated with Type III invasion, which is sufficient to cause mortality in some cases (Saeij *et al.* 2006, Yamamoto *et al.* 2009, Ong *et al.* 2010). The key role played by configurations of these polymorphic virulence genes in the murine virulence of *T. gondii* has been demonstrated conclusively in representatives of the three archetypal lineages, using both forward and reverse genetic approaches, and for a subset of atypical strains as well (Taylor *et al.*

2006, Saeij *et al.* 2006, Reese *et al.* 2011, Etheridge *et al.* 2014, Niedelman *et al.* 2012). These data suggest that this may be a general phenomenon across all strains of *T. gondii*. If this is the case, then identification of strain genotypes at these loci would presumably be an excellent indicator of strain virulence, and an important means of classifying strains based on biologically relevant genetic variation.

Though ROP5, ROP18, and to a lesser extent ROP16, have been shown in molecular studies to play key roles in determining the virulence of laboratory *T. gondii* strains, the distribution of their alleles in natural populations and the broad influence of these allele combinations on *T. gondii* virulence have not been examined in depth. Dubey *et al.* (2014) identified a correlation between ROP18 and ROP5 genotypes for a set of 23 strains, comprising isolates from North American wildlife along with several reference strains. Here, we have sought to expand upon these findings by genotyping a broader assortment of strain types, focusing on diverse atypical South American strains as well as Asian isolates. We have employed a PCR-RFLP-based genotyping strategy for these three polymorphic, pathogenesis-associated loci similar to that of Dubey *et al.* (2104), though with a higher resolution protocol for the ROP5 locus. In this manner, we demonstrate through correlation of identified PCR-RFLP genotypes of 308 *T. gondii* strains with published mouse virulence data that the combination of ROP5 and ROP18 (but not ROP16) alleles is highly predictive of *T. gondii* virulence across a broad range of global *T. gondii* isolates.

Materials and methods

PCR-RFLP Protocols

PCR-RFLP protocols for ROP16 and ROP18 were identical to those employed by Dubey *et al.* 2014, except that restriction enzyme digestion was deemed unnecessary in the case of ROP18 as different alelles produced nested PCR products of distinguishably different lengths. In the case of ROP5, a protocol was developed based on the ROP5 sequences identified by Niedelman *et al.* (2012) in order to differentiate the major identified ROP5 allele complements. For each locus, PCR was performed on parasite genomic DNA using external primers, followed by a second round of PCR with nested primers using external primer products as template, based on the strategy described by Su *et al.* (2010). Restriction enzymes targeting single nucleotide polymorphisms identified between strain types were then used to differentially digest PCR amplified DNA. For ROP18, two sets of internal primers were used, one to amplify the upstream promoter insertion sequence (UPS) of the archetypal Type III allele, and one to amplify a repetitive sequence (DEL) in the promoters of the archetypal Type I and II alleles. No restriction enzyme digestion was required to distinguish ROP18 genotypes as different genotypes produce nested PCR products of different lengths, as noted. For ROP16, nested PCR products were digested with NlaIII and TaqaI enzymes. For ROP5, nested PCR products were digested with FspBI and HaeII enzymes. Samples with ROP5 genotype 5 RFLP pattern were further digested with BstUI to distinguish genotypes 5 and 8. Primers and restriction enzymes for each marker are shown in Table IV-1. All DNA samples from *T. gondii* isolates were amplified in 96-well PCR plates via multiplex PCR using all external primers simultaneously according to Su *et al.* (2010). Amplification for multiplex PCR used FastStart DNA Polymerase (Roche). The thermocyler

program was as follows: 4 minutes at 95C, 30 cycles of 30 seconds at 95C, 1 minute at 55C, and 2 minutes at 74C. This was followed by nested PCR amplification using PAQ5000 DNA polymerase (Stratagene) with individual marker primers and a thermocycler program of 4 minutes at 95C, 35 cycles of 30 seconds at 95C, 1 minute at 58C, and two minutes at 72C. Restriction enzyme digestions were carried out using PCR amplified DNA in 96-well plates. Digested products were separated via electrophoresis in 2.5% agarose gels stained with ethidium bromide and visualized under UV light. Each gel contained a set of standard *T. gondii* reference strains including GT1, PTG, CTG. MAS, COUG, TgCtBr5, TgCtBr64 and TOUCAN which aided in genotype identification. Each plate also contained multiple no-template negative controls.

T. gondii isolate DNA

Genomic DNA samples from *T. gondii* strains were provided by numerous laboratories from around the world. Specific information regarding isolate origins is provided in Shwab *et al.* (2014). Genomic DNA stocks were stored at -20C prior to analysis.

Strain virulence assessement

Strain virulence information was based on published mouse bioassay data. References for each strain are provided in Shwab *et al.* (2014). Bioassays typically consisted of oral inoculation of mice with tissue from chronically infected animals. Each strain was categorized as lethal or nonlethal based on virulence in lab mice. Strains were categorized as lethal if death was caused in all infected mice within 30 days post infection, while strains were cateforized as nonlethal if all or some infected (seropositive) mice survived for at least 30 days and produced tissue cysts. Statistical analysis of the correlation between genotype (ROP5, ROP16 and ROP18) and mouse mortality was performed using the non-parametric Kruskal-Wallis (group analysis) and Mann Whitney U (individual comparisons) tests.

Network Analysis

Allelic data from multilocus analysis of PCR-RFLP markers were used to generate a network of *T. gondii* strain polymorphisms from 308 samples. Multilocus PCR-RFLP typing data were coded for ROP18 and ROP5 loci. For a given locus, presence or absence of DNA restriction fragments was coded as either 1 or 0, respectively. SplitsTree v4.4 was used to generate an unrooted network using the neighbor-net method with 1,000 bootstrap replicates.

Results

Identification of PCR-RFLP genotypes at ROP18, ROP5 and ROP16 loci

PCR-RFLP protocols were developed in order to identify SNPs present at the ROP18, ROP5 and ROP16 loci. Figure IV-1 depicts the RFLP patterns at each of these loci for a set of eight diverse reference strains representing a number of major distinct phylogenetic clusters of *T. gondii*

Table IV-1. Primer sequences and restriction enzymes for PCR-RFLP genotyping of ROP16, ROP18 and ROP5 loci.

Marker	External Primers (5' to 3')	Internal Primers (5' to 3')	Restriction Enzymes
ROP16	ROP16F-ext: ATCTGCTTATCCGGCGACTA ROP16R-ext: TCCGTTGGCATTTATCATCA	ROP16F-int: TACCAAACCCAGCTTTCACC ROP16R-int: TCGTCAACAGCTGACTCCAC	NlaIII+TaqaI
ROP18	ROP18-DelFext: CTCGTCGACCACACAGCTAA ROP18-UPSRext: GAGTGCTTTCTGTCGCTCCT	ROP18-DelFint: AGTTCCCTTCCCTGGTGTCT ROP18-DelRint: ACAAACTGGACTGGGGTGAG Rop18-UPSFint: CACAGCATGAGCTTAAGAGTTG Rop18-UPSRint: CACCGCAAGACAGGCTGTCTTC	None required
ROP5	ROP5F-ext: GGACAGACGCAGGCTTTTAC ROP5R-ext: TCAAACGTCCTGACACTTCG	ROP5F-int: TGTGGCAGTTCAGTCTCAGC ROP5R-int: TCGAAGTTGAGGAACCGTCT	FspBI+HaeII BstUI

isolates identified previously (Su *et al.* 2010). These reference strains include GT1 (archetypal Type I), PTG (archetypal Type II), CTG (archetypal Type III), MAS, COUG (TgCgCa1), TgCatBr5, TgCatBr64 and TOUC (TgRsCr1). These reference strains capture the majority of the genetic diversity identified at each locus, however in some instances additional alleles were identified that were not present in any of these reference strains. Three alleles were identified for ROP16 markers, four alleles were identified for ROP18 and eight separate alleles were identified for ROP5. In some cases, alleles were very rare and were limited to a few or even only a single strain out of the 308 analyzed.

Genotyping of 308 T. gondii isolates at ROP18, ROP5 and ROP16 loci

As the ROP18 and ROP5 (and to a lesser extent ROP16) genes have been shown to heavily influence mouse virulence phenotype in archetypal *T. gondii* lineages, PCR-RFLP markers were developed for these loci and the genotypes of 308 isolates, representing 143 ToxoDB genotypes, were determined. Table E-2 (Appendix E) shows the complete genotypic data at all 3 loci for each isolate. A total of 28 combinations of these genotypes were identified. Also indicated are the mouse mortality data from bioassays of each strain, where available (for strain references see Shwab *et al.* 2014, except where noted in Table E-2). The ROP16, ROP18 and ROP5 genotypes and the combined ROP18/5 haplotype were plotted against this published mouse mortality data for 239 strains with virulence data (representing 121 ToxoDB genotypes) in order to determine whether a correlation exists between mouse virulence and genotype at these loci. Of these, strains belonging to ToxoDB genotypes #6, 10, 11, 14, 15, 17, 21, 28, 34, 46, and 70 demonstrated both lethal and nonlethal phenotypes. In the case of genotype #6, strain BOF possesses an alternate ROP5 genotype (6 as opposed to 3 for other #6 strains) which may account for the nonlethal phenotype of this strain. TgCatBr54 also displays a nonlethal phenotype despite having ROP5 genotype 3 (though mortality was fairly high at 75 percent). For genotype #17, the three nonlethal strains were found to possess ROP18 genotype 3, while the five lethal strains were found to possess ROP18 genotype 4. This was also the case for genotype #28, wherein the one nonlethal strain showed ROP18 genotype 3 while the two lethal strains showed ROP18 genotype 4. In ToxoDB genotypes #10, 11, 14, 15, 21, 34, 46 and 70, differences in lethality were not associated with variations in ROP18, 5 or 16 genotypes. Though all strains of the ToxoDB genotype #9 (Chinese I) displayed nonlethal phenotypes, 5 of these 21 strains were found to possess ROP18 genotype 3 (generally associated with avirulence) while the others were found to possess ROP18 genotype 2 (generally associated with virulence).

ROP16

As noted, three ROP16 alleles were identified. ROP16 genotype 1 identifies the similar archetypal Type I and Type III alleles, while genotype 2 identifies the archetypal Type II allele. ROP16 genotype 3 represents an atypical allele. ROP16 genotype 1 was identified in 168 of the strains examined, while genotype 2 was identified in 76 strains, and genotype 3 in only 4 strains. When the average mouse mortality during bioassay associated with each ROP16 genotype was determined (Figure IV-2A), strains with genotype I were found to have produced an average of about 63 percent mortality, genotype 2 about 33 percent average mortality and the four strains with genotype 3 each produced 100 percent mortality. The standard deviations of virulence for

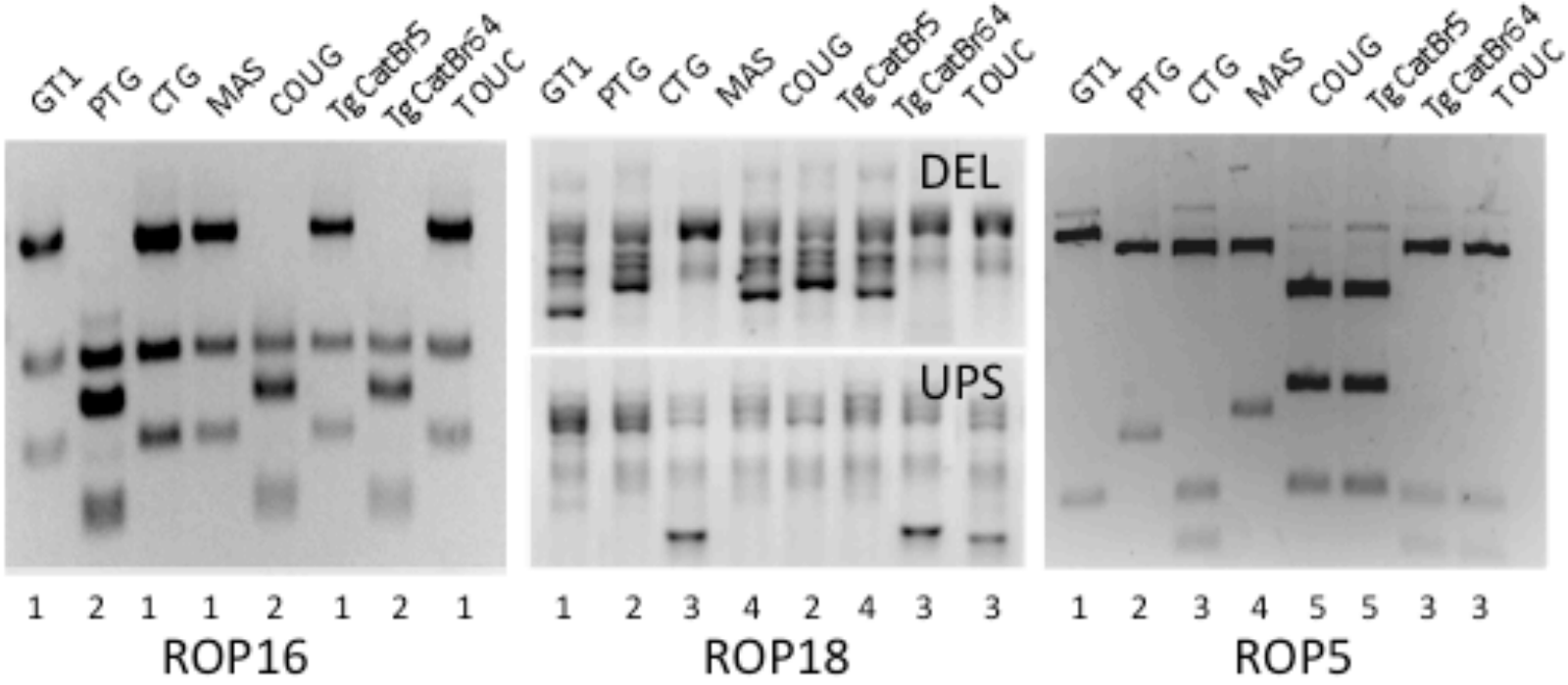


Figure IV-1. Reference strain RFLP patterns for 3 PCR-RFLP markers. Restriction fragments of reference strains GT1, PTG, CTG, MAS, COUG, TgCatBr5, TgCatBr64 and TOUCAN for the 3 described PCR-RFLP markers using primers and restriction enzymes as given Materials and Methods. Strain IDs are presented above each column of gels and identified genotypes are indicated below each gel lane. Fragments were visualized under UV light following electrophoresis in 2.5% agarose gels containing ethidium bromide.

genotype 1 and 2 strains were relatively high, and no statistically significant difference was observed between the mortality caused by each of these two groups. Genotype 3 correlated with high virulence and was significantly different from genotype 2, but due to the small sample size of this group the implications of this correlation are limited.

ROP18

Four ROP18 genotypes were identified, with genotypes 1, 2 and 3 indicating similiarity to the archetypal Type I , II and III alleles respectively, and genotype 4 identifying alleles with similarity to the ToxoDB #17 reference strain MAS. 47 isolates with mouse virulence data were found to be of ROP18 genotype 1, 29 of genotype 2, 68 of genotype 3, and 93 of genotype 4. Association of each of the four ROP18 genotypes with mouse virulence are shown in Figure IV-2B. Strains with genotypes 1 and 4 produced averages of 85 and 89 percent mortality, respectively, and were highly significantly different in this regard from strains with genotypes 2 and 3, which produced averages of approximately 4 and 15 percent mortality, respectively. Thus, ROP18 genotypes 1 and 4 appear to be heavily associated with a highly mouse virulent phenotype within this sample set, whereas genotypes 2 and 3 are associated with low mouse virulence. Genotype 3 includes the archetypal Type III allele of the gene as mentioned, found to associate with low virulence in QTL mapping studies, while the archetypal Type II allele (represented by ROP18 genotype 2) has been found to associate with a highly virulent phenotype (Taylor *et al.* 2006). It therefore seems likely that the correlation between ROP18 genotype and avirulence observed here may be the indirect result of co-segregation of this allele with avirulent Type II-like forms of the ROP5 locus (Behnke *et al.* 2011).

ROP5

Eight distinct ROP5 genotypes were identified among the 330 isolates examined. Genotype 3 (associated with the archetypal Type III allele) was the most commonly identified with 138 occurrences out of 248 strains with mouse virulence data. This was followed by genotype 4 (associated with reference strain MAS) with 31 strains, genotype 5 (associated with reference strain COUG) with 30 strains, genotype 1 (associated with archetypal Type I) with 25 strains, genotype II (associated with archetypal Type II) with 16 strains, genotype 8 (associated with VAND and related virulent Guyana strains) with 6 strains, and genotypes 6 and 7 with only one occurrence each (BOF and TgCkGh1, respectively). Figure IV-2C shows the mouse mortality associated with the six genotypes with more than one occurrence. The majority of these groups could be distinguished from one another statistically though standard deviations were high for genotypes 1, 3, 4 and 5, presumably due mainly to the presence of different ROP18 alleles within these groups. Genotypes 2 and 6 are more uniform and were found to be tightly associated with avirulence and high virulence, respectively.

ROP18/5 haplotype

Because ROP18 and ROP5 were found to be by far the strongest indicators of virulence among the archetypal *T. gondii* strains, analysis of the correlation between mouse virulence and the combined ROP18/5 haplotype for the 308 strains examined was performed. 17 different haplotypes were identified, 4 of which had only one occurrence. The mouse mortalities

Figure IV-2. Mouse virulence of strains categorized by ROP16, ROP18 and ROP5 genotypes. Average mouse mortality for 239 *T. gondii* strains with published bioassay results were categorized by ROP16 (A), ROP18 (B) and ROP5 (C) genotypes and ROP18/5 haplotypes (D). Average percent mouse mortality is plotted against genotype and error bars indicate plus or minus one standard deviation. Number of strains representing each genotype are given in parentheses below genotype IDs. Different letters above data points indicate statistical differences as determined by Mann-Whitney U test. Data are significant at maximum P-values of 0.05, 0.01, $5x10^{-5}$ and 0.001 for A, B, C and D, respectively.

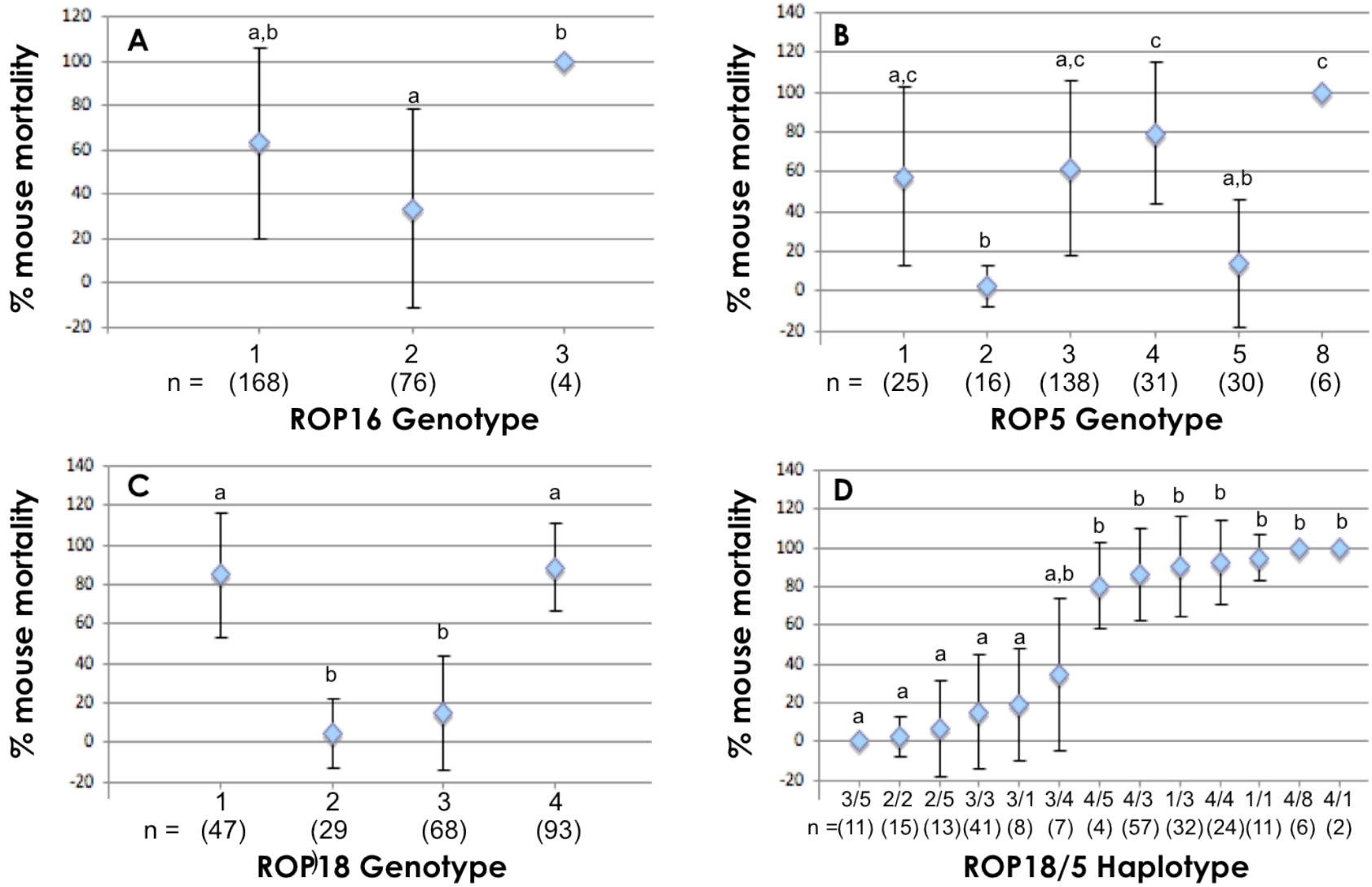


Figure IV-2 continued

associated with the other 13 haplotypes are presented in Figure IV-2D. clear and statistically significant segregation with either high or low mouse virulence was observed for each haplotype with the exception of haplotype 3/4, which includes several strains with intermediate to high virulence, despite possession of the avirulence-associated Type III ROP18 allele, including the virulent CASTELLS strain.

Network analysis of ROP18/5 haplotypes was also performed in order to visualize the relationships between groups. Qualitative mouse virulence of each network node/haplotype, grouped into lethal (100 percent mortality) and nonlethal (no or partial mortality) categories is presented in Figure IV-3. The majority of haplotypes were observed to associate mainly with high or low virulence in most cases, though a range of phenotypes, including both lethal and nonlethal strains, was observed for all but a few. This would seem to indicate that the ROP18 and ROP5 genotypes are strong predictors of strain virulence but that other factors also contribute to this phenotype. Some of the observed variation is likely due to the fact that the data presented were collected from many different sources with inevitable differences in inoculation technique, parasite dosage and other factors, but it may also be the case that some strains utilize alternative pathogenic mechanisms that allow them to be highly mouse virulent despite possession of canonically avirulent ROP18 and/or ROP5 alleles (as in the case of CASTELLS) and vice versa.

Discussion

In the current study, we have shown that, as in the archetypal lineages which have been intensively studied on a molecular level, the ROP5 and ROP18 genes are strong determinants of virulence even among highly diverse atypical *T. gondii* strains. This was evidenced by the strong overall correlation of the ROP18/5 haplotype with mouse virulence. Using our PCR-RFLP approach, we were able to distinguish 4 ROP18 genotypes. In a previous study (Khan *et al.* 2009), ROP18 loci from a number of strains were sequenced, leading to the identification of three major alleles, termed I*, II* and III*, and the I* allele was further subdivided into alleles I*a, I*b and I*c.. Based on the strains sequenced, our ROP18 genotypes 2 and 3 appear to correlate with the II* and III* alleles from this previous study, while genotype 4 seems to correlate with the I*c allele, and genotype 1 appears to combine both alleles I*a and I*b. As alleles I*a, b and c were all found to confer virulence in transgenic strains, this grouping is consistent with the lethality found herein to be associated with ROP18 genotypes 1 and 4. For ROP5, 8 distinct RFLP genotypes were identified. As strains generally possess multiple copies of ROP5, alleles of this locus have been more difficult to characterize than for ROP18 (Niedelman *et al.* 2012). ROP5 genotypes 1, 2 and 3 appear to correlate with the archetypeal Type I, II and III ROP5 allele complements, based on their association with reference strains GT1 (Type I), PTG (Type II) and CTG(Type III), respectively. The Type I and III allele complements have been shown to be very similar in sequence and to confer virulence in transgenic strains, while the Type II complement is more divergent and was associated with avirulence in transgenic strains (Benkhe *et al.* 2011, Fleckenstein *et al.* 2011). ROP18 genotype 4 was associated with the highly virulent reference strain MAS in this study, suggesting that this genotype represents another virulent ROP5 allele complement. Niedelman *et al.* (2012) found ROP5 alleles to be highly expressed in the MAS strain, and for this strain to be resistant to IFNγ-

Figure IV-3. Network analysis of ROP18/5 haplotypes and associated lethality in mice . A neighbor-joining network was generated for 330 strains using Splitstree v.2.2.based on the ROP18/5 haplotypes. Haplotype IDs are indicated at each node in bold lettering, as well as a pie chart indicating mouse mortality for haplotypes with such data available. Number of strains representing each haplotype are given in parentheses beside corresponding pie charts. Strains in each haplotype are divided into lethal (complete mortality during mouse bioassay) and nonlethal (no or partial mortality during mouse bioassay) categories and these categories are color-coded as red and blue, respectively. Sizes of pie charts are proportional to the number of strains belonging to each haplotype.

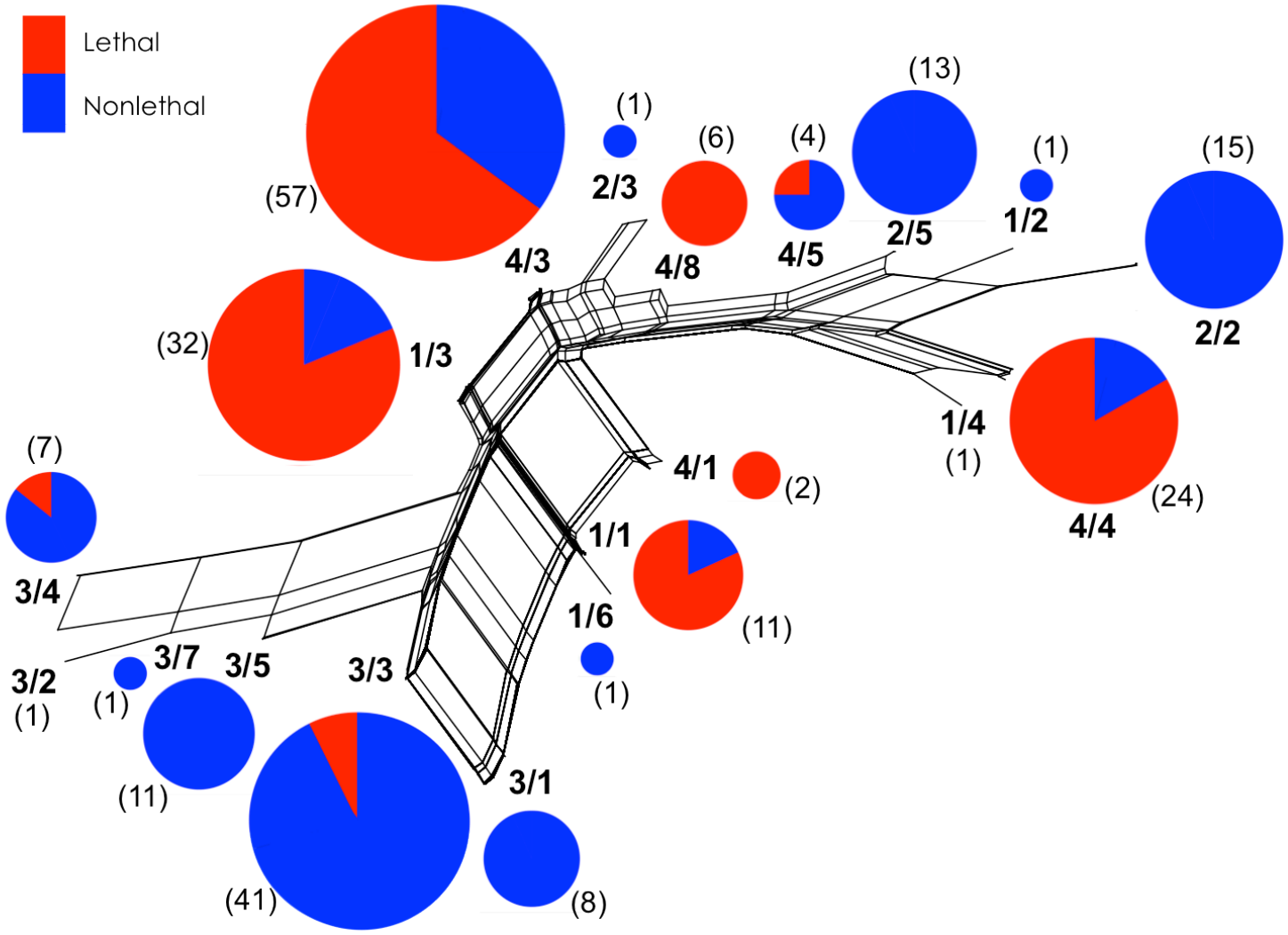


Figure IV-3 continued

dependent host immune responses in cell culture experiments. ROP5 genotype 5 was associated with the nonlethal COUGAR strain. Since this strain has been found to highly express ROP18 (Niedelman et al 2012), it may be that this ROP5 genotype is the cause of avirulence in this case, though this would need to be tested experimentally. If this is the case, it would potentially explain the nonlethal phenotypes observed for the ToxoDB genotype #9 (Chinese I) strains analyzed in this study, all of which possessed the virulence-associated ROP18 genotype 2 in combination with ROP5 genotype 5. However, another Chinese isolate with this same ROP5 genotype, TgCatPR5 (ToxoDB genotype #49), was found to have a lethal phenotype. This latter result may be a reflection of higher intermediate level of virulence, however, as a closely related strain (TgCatPR6, genotype #112) with the same ROP18/5 complement produced 92 percent mortality in mouse bioassay. ROP5 genotype 6 was only identified in the nonlethal strain BOF, which has been previously shown to have very low ROP5 expression, and thus is likely to represent an avirulent form of this locus (Niedelman *et al.* 2012). ROP5 genotype 7 was only identified in strain TgCkGh1, which has not been characterized with regard to virulence phenotype. ROP5 genotype 8 was associated with a clade of highly virulent strains, members of which were isolated from human patients with severe acquired toxoplasmosis in French Guiana (Carme *et al.* 2002), suggesting association of this allele with a virulent phenotype.

Statistically, the ROP18 genotype alone was found here to be the strongest predictor of mouse virulence, moreso than the combined ROP18/5 haplotype; however, this is likely due in part to the fact that fewer ROP18 genotypes were identified compared to ROP18/5 haplotypes, meaning that each ROP18 group comprised a higher number of strains, contributing to statistical significance, and also due to the apparent relative lack of recombination between archetypal Type II-related strains, associated with the avirulent Type II form of the ROP5 locus, and other strains with virulent forms of ROP5. Thus, although the Type II ROP18 allele has been linked to mouse virulence in molecular studies, in nature this allele is generally associated with an avirulent form of ROP5, resulting in an indirect correlation with avirulence in strains possessing the Type II ROP18 allele. For this reason, to use the ROP18 genotype alone as a predictor of mouse virulence is to run the risk of overlooking potentially virulent strains possessing a Type II-related ROP18 allele in combination with a virulent form of the ROP5 locus. In the current study, no strains were identified which possessed ROP18 genotype 2 in combination with either of the known virulent forms of the ROP5 locus (represented by ROP5 genotypes 1 and 3), but association of ROP18 genotype 2 with ROP5 genotype 5 was observed in the ToxoDB #9 strains discussed above. While this haplotype was associated with low virulence, this same ROP5 genotype was associated with high virulence in combination with ROP18 genotype 4 (TgCatPr5). Thus, it is not entirely clear to what extent the ROP5 genotype may be contributing to mouse virulence phenotype in these cases. Regardless of the overall correlation with virulence, within the majority of ROP18/5 haplotypes, both lethal and nonlethal strains were identified. In some cases this is likely due to variations in dosage administered, as bioassays were primarily done through the feeding of infected tissue to mice, without precise measurement of parasite load in these tissues. However, in other cases counterpredicted phenotypes may be indicative of alternative pathogenic mechanisms in these strains. That appears to be the case for the strain CASTELLS, which has previously been demonstrated to have a highly virulent phenotype despite possessing the unexpressed Type III ROP18 allele (Khan *et al.* 2009). Further analysis of strains with unexpected virulence phenotypes identified here using more standardized virulence assays may thus aid in the discovery of alternative mechanisms leading to parasite

virulence. Of particular interest are strains of the predominantly avirulent 3/3 ROP18/5 haplotype that produced high mouse mortality, including TgDgCo8, TgCkNi4, and the reference strain TgCatBr64. Overall, the ROP18/5 haplotype provides a strong prediction of strain virulence that should be useful for future characterization of new *T. gondii* strains, particularly in cases where parasite genomic DNA is accessible but viable parasites cannot be successfully isolated for determination of parasite virulence via bioassay.

Interestingly, the dominant Asian strain type, ToxoDB genotype #9 (Chinese I) was in this study separated into two subtypes, differing only at the ROP18 locus (Table E-2). The first subtype (genotype combination 10) possesses ROP18 genotype 2, associated with the virulent Type II allele, while the second subtype (genotype combination 16) possesses ROP18 genotype 3, associated with the avirulent Type III allele. It has been previously noted that genotype #9 strains include both virulent and avirulent forms (Li *et al.* 2014), and the identification here of two subgroups differing at the ROP18 locus seems likely to be relevant to this observation. While the genotype #9 strains used in this study for which virulence data was available all displayed nonlethal phenotypes in mice, it seems plausible that virulent genotype #9 strains may also possess ROP18 genotype 2, possibly in combination with an alternate ROP5 genotype. It is necessary to perform ROP18 and ROP5 genotyping on virulent genotype #9 isolates in order to elucidate the genetic basis for the observed variation of genotype #9 strains with regard to virulence.

The work described herein indicates a high degree of universality in the principles governing virulence of *T. gondii* strains. The high proportion of mouse-virulent strain types observed in South America compared to elsewhere in the world appears to be largely a result of virulent combinations of ROP18 and ROP5 alleles in these strains. Though it is clear that the South American *T. gondii* population differs dramatically with regard to strain virulence as compared to elsewhere in the world, the evolutionary reasons for this discrepancy are not well understood. It may be considered that the high degree of strain variability found in South America provides evidence that the parasite originated in this region, and thus has resided there longer than in other regions of the world and has therefore had more time to evolve a diversity of forms. By contrast, *T. gondii* populations found on other continents would likely then be the result of more recent founding events, seeded by limited numbers of strains carried either by wild migratory animals, or by intercontinental transport of domesticated host animals by humans. This scenario would thus explain the reduced diversity observed outside of South and Central America. Another, not-mutually-exclusive possibility is that the inordinately high host species diversity found in the tropical regions of South and Central America has also promoted the diversification of parasite strains exposed to varying selective pressures within different host organisms. While the domestic cat is by far the most abundant definitive host for *T. gondii* worldwide, making it and its primary prey species the house mouse essential for parasite transmission and thus key sources of selective pressure, in many regions of South America wild felid hosts are still prevalent, as are a diverse assortment of wild prey species. Thus, parasite transmission via the sylvatic cycle is likely prominent, allowing the propagation and persistence of highly mouse virulent strains which would be poorly adapted to domestic cycle transmission (Carme *et al.* 2009). Genotyping information from other geographic regions with high species diversity, such as the tropical rainforests of Africa and southeast Asia, could provide valuable insight as to the influence of

host species variability on the *T. gondii* population structure and the distribution of virulent strain types. However, such data is unfortunately quite scarce.

Given the correlation that has been observed between the severity of human disease and mouse virulence of infecting *T. gondii* strains, the results of this study may also help researchers to better evaluate the medical risks posed to humans by parasite strains identified in a given geographic region. Our results lend additional interest to the findings of Sanchez *et al.* (2013) in which the researchers used a PCR-based genotyping approach to determine that a higher proportion of strains isolated from human patients in Colombia with symptomatic ocular toxoplasmosis were negative for an upstream promoter sequence (UPS) responsible for the suppression of the Type III ROP18 allele (and thus presumably expressing functional ROP18) than those isolated from asymptomatic patients. The findings of our study support the notion that these UPS-negative strains are likely to be mouse virulent, providing further evidence for a correlation between mouse virulence and human disease. As many South American countries (Brazil in particular) are among the fastest developing in the world today, the geographic expansion of these societies as sparsely inhabited areas become more populous may lead to human contact with previously unidentified strains of *T. gondii*, generating a possible risk of exposure to more dangerous genotypes such as those responsible for the numerous cases of Amazonian toxoplasmosis recorded in French Guiana (Carme *et al.* 2009). This work should provide researchers with an enhanced set of tools for future studies, and a means of quickly identifying potentially virulent strain types without the requirement of parasite isolation, thus facilitating a higher degree of preparedness for disease outbreaks.

Chapter V. The impact of agrarian environment on evolution of population structure and virulence in *Toxoplasma gondii*

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Introduction

Among eukaryotic pathogens, the protozoan parasite *Toxoplasma gondii* is perhaps the most ubiquitous, having been identified in the tissues of myriad animal hosts, including both mammalian and avian species. Moreover, *T. gondii* is estimated to chronically infect approximately one third of the world's human population, causing ocular toxoplasmosis in immunocompetent individuals and often-fatal encephalitis in the immunocompromised, as well as birth defects and mortality following vertical transmission to developing fetuses (Dubey, 2010; Montoya and Liesenfeld, 2004). In a recent study, genotypic data from more than 1,400 globally distributed *T. gondii* samples were compiled, providing the most comprehensive picture of the global *T. gondii* population structure yet assembled (Shwab *et al.* 2014). This analysis revealed distinct population structures for each major geographic region examined, with striking contrast between the highly diverse, endemic structure of the Central/South American region and the more clonal populations found in all other areas, wherein only a few genotypes account for the overwhelming majority of isolates. In particular, PCR-RFLP genotypes #1 and #3 (collectively known as Type II), #2 (a.k.a. Type III), #4 and #5 (collectively known as Type 12) dominate in North America, genotypes #1, #2 and #3 dominate in Europe and northern Africa, and genotype #9 (Chinese 1) dominates in east Asia (Shwab *et al.* 2014).

The prevalence of a few dominant *T. gondii* genotypes in much of the world could be the result of the founder effect. Alternatively, it could be the consequence of natural selection. The most notable phenotype among *T. gondii* strains that might potentially be under natural selection pertains to murine virulence. Due to polymorphisms in several key effector proteins secreted into host cells during infection, different genotypes of *T. gondii* produce markedly different disease outcomes in the house mouse *Mus musculus* (Taylor *et al.* 2006; Saeij *et al.* 2006; Reese *et al.* 2011). This variability provides the primary systematic contrast between the three archetypal lineages including the PCR-RFLP genotypes #1, #2, #3 and #10. The PCR-RFLP genotype #10 (Type I) strains are known to be lethal to house mice, causing rapid death within two to three

weeks after infection with only 1 viable parasite, whereas genotypes #1 and #3 (Type II) and genotype #2 (Type III) infections are non-lethal to mice and can generally establish an asymptomatic chronic infection (Sibley and Boothroyd, 1992). However, while genotype #2 strains are essentially non-virulent to mice except at very high dosages ($LD_{50} > 10^5$ parasites) , genotype #1 and #3 strains display a wider and intermediate range of virulence , causing mortality in infected mice at lower dosages than #2 strains (Khan *et al.* 2009).

Prior studies have shown that more virulent parasite strains have an advantage in the context of reinfection of mice already chronically infected with a less virulent *T. gondii* strain (Krahenbuhl *et al.* 1971; Reikvam *et al.* 1976; Araujo *et al.* 1997; Dao *et al.* 2001; Brandao *et al.* 2009; Silva *et al.* 2012). While a previous *T. gondii* infection is often protective against subsequent infections, it appears that strains with higher virulence are frequently able to overcome this protective immunity and establish a secondary infection. Under these circumstances, a greater number of murine hosts should be susceptible to infection by more virulent strains as compared to strains with low virulence, giving the former group a distinct advantage. For highly virulent strains, this advantage would presumably be offset by the high mortality rate (often 100%) associated with infection of naïve mice, which reduces the likelihood of transmission to new hosts. This dichotomy provides selective pressure which may favor or against *T. gondii* virulence depends on host sensibility to infection in the transmission cycle of the parasite.

We hypothesize that agricultural development and urbanization in the past 11,000 years has enhanced *T. gondii* transmission through the domestic life cycle, within which the virulence of *T. gondii* in house mice has a significant impact on parasite fitness and the global *T. gondii* population structure. To test this hypothesis, we used an *in silico* modeling approach that simulates the life cycle of the parasite, monitoring transmission of lethal (highly virulent) and non-lethal (intermediately virulent and non-virulent) strains of *T. gondii* between intermingled populations of domestic cats and mice. Under a variety of simulation conditions, we found that the advantage conferred to strains of intermediate virulence during sequential infection was sufficient to allow this strain type to dominate in populations, at the expense of the lethal and non-virulent strains, generating population structures that closely resemble those observed in nature.

Materials and Methods

Collection and analysis of mouse virulence data from South, Central and North America, and determination of virulence of dominant PCR-RFLP genotype #9 from East Asia

Data regarding mouse virulence of South, Central and North American *T. gondii* isolates were obtained from references in a recent review paper (Shwab et al., 2014). Specific information regarding methods for virulence determination may be found in the original publications. The majority of data were obtained via bioassay, in which mice were orally inoculated with tissue from infected host animals and observed for disease presentation and mortality. Some studies also included inoculation via IP injection of tachyzoites from cell cultured strains, or via oral inoculation with oocysts.

Collection of previously published data on sequential infection of mice with different T. gondii strains

Available data on competition of different *T. gondii* strains in sequential infection of mice were collect by reviewing published research papers. Details regarding methodology for previously published studies may be found in the original articles.

Simulation of sequential infection of T. gondii strains with different virulence phenotypes in house mice.

To assess the potential impact an advantage for more virulent strains during reinfection of previously infected mice could have on the evolution of the *T. gondii* population structure, a model was developed to simulate transmission dynamics of *T. gondii* in domestic and sylvatic life cycles (Figure V-1). The simulation was run using the software NetLogo version 10.1.0 (https://ccl.northwestern.edu/netlogo/). Because many of the parameters involved in this model are not clearly defined by experimental data, we carried out simulations under a variety of conditions relating to mouse mortality rate and probability of reinfection for each parasite virulence type, as well as host and parasite starting population sizes and duration of the simulation. Simulated environments consisted of variables including cats, mice, the environment and the parasites. The parasites comprise three categories: non-virulent (NV), intermediately virulent (IV) and highly virulent (HV). The cats and mice were programmed to move randomly within the area of a spatial grid, interacting when a cat and a mouse occupied the same point on the grid. Such an interaction resulted in the cat consuming the mouse. If the mouse were a carrier of *T. gondii*, the cat would then become infected. If the consumed mouse were infected with two different parasite strains, the cat would randomly become infected by one or the other strain. Following infection, cats shed oocysts into the environment, which persisted for a specified period of time. Encounter of oocysts by mice or other cats resulted in those animals becoming infected. If mice were to become infected by *T. gondii*, they would have a specified percentage chance of dying between 7 and 14 days later that was specifically indicated for each parasite virulence category. Mice that did not die would remain chronic carriers of the parasite for the remainders of their life spans. If a chronically infected mouse were to encounter an oocyst of a different virulence category, the strain would have a certain percentage chance of reinfecting that mouse based on a probability value specified for each virulence category. Birth and natural death rates of cats and mice were also separately specified. Simulations were run for specified numbers of days, with one event occurring for each organism per day. That is, each organism would move to one new location each day and process any developments that the new location and/or progression of time might dictate, including birth, death, carnivory, infection or oocyst shedding.

Results

Virulence of T. gondii in house mice

For the analysis of mouse virulence, we obtained data from previously published papers on *T. gondii* population genetic studies (Shwab *et al.* 2014) *Toxoplasma gondii* isolates that caused mortality in mice within a month post infection, with any surviving mice being seronegative or

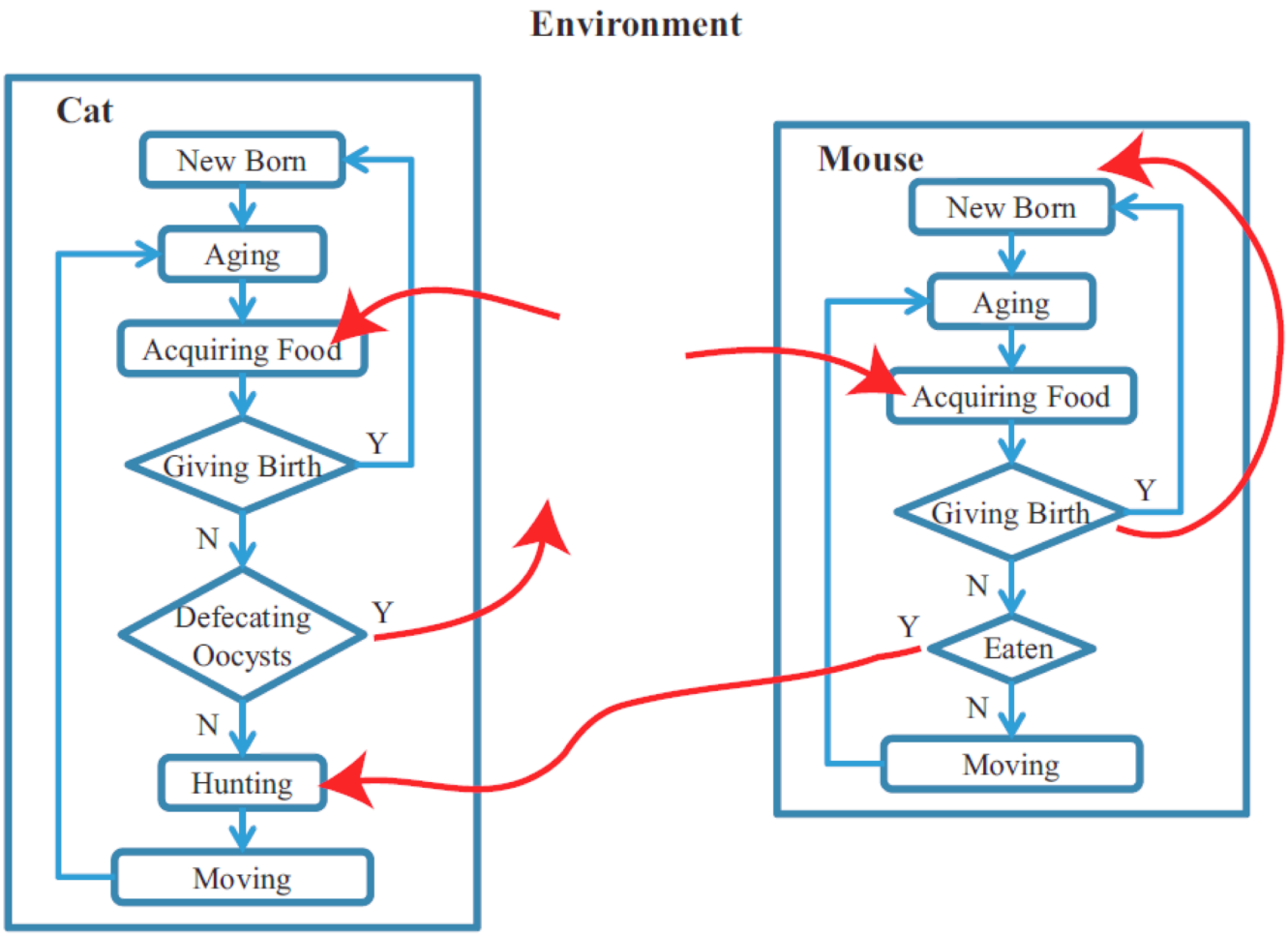


Figure V-1. Flow chart of possible cat and mouse actions within the simulated environment

free of tissue cysts, were considered lethal (highly virulent, HV), while those that could establish chronic infection and produced less than 100% mortality in mice were considered non-lethal (intermediately virulent, IV or non-virulent, NV). The results showed that overall 61% of 427 isolates characterized from South/Central America have been determined to be lethal to mice, compared to only 7% of 193 isolates from North America. This difference is quite striking and underscores the evolutionary divergence of *T. gondii* in Central/South America compared to North America.

For the dominant *T. gondii* genotypes (genotypes #1, #2, #3, #4, #5, #9) in the north hemisphere, and the genotype #6, which has a relatively higher frequency in South America and Africa, we examined parasite virulence in greater detail by compiling cumulative mortality rates published in literature (Table V-1). Reliable data for genotype #9 was not available at the time of this study, so we tested 3 isolates for this genotype. At the time of writing this paper, a new study on mouse virulence of genotype #9 became available (Li et al., 2014) and we compiled those results in Table V-1 as well. For this comparison, isolates were roughly grouped into three categories based on the cumulative percentage of mortality caused in mice. The first group comprised strains that produced less than 20% mouse mortality (non- virulent), the second included strains producing between 20 and 95% mouse mortality (intermediately virulent), and the third group consisted of strains producing 95 to 100% mortality (highly virulent, lethal). We found that the dominant strain types including genotypes #1, #4, #5 and #9 are intermediately virulent, whereas genotypes #2 and #3 were non-virulent to mice. However, genotype #6 was highly virulent (Table V-1). It is worth noticing that, though mouse mortality may vary among isolates belonging to the same genotypes and the grouping of mouse virulence is subjective, it does reflect the overall levels of virulence among these genotype groups. Together, these data demonstrate that, in addition to markedly different patterns of parasite diversity, the northern hemisphere and South America also differ greatly with regard to the mouse virulence of evolutionarily successful strains from each region.

Strains of higher virulence demonstrate a greater frequency of successful reinfection of chronically infected mice than strains of lower virulence

To determine how the virulence level of a *T. gondii* strain affects the strain's ability to super infect mice already infected with a non-lethal strain of the parasite, we identified a number of published studies in which sequential *T. gondii* infections had been performed (Table V-2). Araujo *et al.* (1997) orally challenged Swiss-Webster mice chronically infected with ME49 with tissue cysts of the relatively virulent strain C56 and its highly virulent variant R-C56, and identified successful infection of mouse brain tissue with both challenge strains. Dao *et al.* (2001) orally challenged OF1 mice chronically infected by the low virulence strain 76K with another strain of low virulence, PRU, and mice chronically infected by PRU with the more highly virulent NED strain. In this study, no reinfection was detected in the brains of mice challenged by PRU, while 44% of the NED-challenged mice did show successful reinfection. Brandao *et al.* (2009) challenged BALB/c mice chronically infected by the low virulence strain D8 with either the intermediately virulent strain CH3 or the highly virulent strain EGS. These researchers identified a reinfection rate of 38% for CH3 and 88% for EGS. Silva *et al.* (2012) challenged BALB/c mice chronically infected by either ME49 or D8 with either CH3 or EGS, and found that CH3 had 20% and 44% success rates for reinfecting mice chronically infected by

Table V-1. Mouse virulence (based on accumulative mortality) of dominant *T. gondii* strains from different geographic regions

ToxoDB PCR-RFLP genotype	Representative strains	Region	Avg. mouse mortality %	Mortality range %	Virulence category	References
#1 (Type II)	ME49, PIH, PE	N. America, Europe	24	0-40	Intermediate	Khan *et al.* 2009
#2 (Type III)	CTG, VEG, STRL, C56	N. America, Europe	3	0-13	Low	Khan *et al.* 2009
#3 (Type II)	DEG, PRU	N. America, Europe	3	0-6	Low	Khan *et al.* 2009; Li *et al.* 2014
#4 (Type 12)	B41	N. America	71	71	Intermediate	Khan *et al.* 2011
#5 (Type 12)	ARI, T61, WTD3	N. America	37	10-80	Intermediate	Khan *et al.* 2009; Khan *et al.* 2011
#6 (BrI)	FOU, TgCatBr2, TgCatBr9	S. America	100	100	High	Khan *et al.* 2009
#9 (Chinese I)	TgCtgy1, TgCtwh3, TgCtwh6	E. Asia	66	24-94	Intermediate	Li *et al.* 2014

Table V-2. Summary of previous studies relating to *T. gondii* sequential infection of mice

Mouse line	Inoculation method	Primary infecting strain	Inoculum (cysts)	% Mortality (naive mice)	Secondary infecting strain	Inoculum (cysts)	% Mortality (naive mice)	% Re-infection	Reference
Swiss-Webster	IP	C37	10^5 Tachy-zoites	0	RH	10^6 Tachy-zoites	ND	≥33	Krahenbuhl *et al.* 1971
NMRI/ Bom	Subcuta-neous	BEV	Cysts (indetermi nate)	20	RH	Cysts (indeterm inate)	ND	≥53	Reikvam *et al.* 1976
Swiss-Webster	Oral	ME49	≤500	0	C56	5	100	100	Araujo *et al.* 1997
Swiss-Webster	Oral	ME49	≤500	0	RC56	5	100	100	Araujo *et al.* 1997
OF1	Oral	76K	10	0	PRU	10	0	0	Dao *et al.* 2001
OF1	Oral	PRU	10	0	NED	10	ND	44	Dao *et al.* 2001
BALB/c	Oral	D8	20	0	CH3	20	100	38	Brandao *et al.* 2009
BALB/c	Oral	D8	20	0	EGS	20	100	88	Brandao *et al.* 2009
BALB/c	Oral	D8	20	0	CH3	20	100	20	Silva *et al.* 2012
BALB/c	Oral	D8	20	0	EGS	20	100	71	Silva *et al.* 2012
BALB/c	Oral	ME49	20	0	CH3	20	100	44	Silva *et al.* 2012
BALB/c	Oral	ME49	20	0	EGS	20	100	100	Silva *et al.* 2012

D8 and ME49, respectively, while EGS had success rates of 71% and 100% in reinfecting mice chronically infected by these same strains. The results of these studies indicate a positive correlation between the relative virulence of the primary and secondary infecting strains and the frequency of successful reinfection of chronically infected mice. Krahenbuhl *et al.* (1971) and Reikvam *et al.* (1976) reported that, when mice chronically infected with the low virulence C37 and BEV strains were challenged with the highly virulent RH strain, partial mortality in mice was observed. The full extent of reinfection was not determined in these studies, and was only inferred through mortality. However, it is likely that mice that did not succumb to secondary infection may still have been reinfected. In all cases where the challenging strain showed higher virulence than the chronically infecting strain, a reinfection was found to develop in at least some percentage of the mice. Moreover, in the studies by Brandao *et al.* (2009) and Silva *et al.* (2012), the highly virulent EGS strain showed a higher rate of reinfection in mice chronically infected with D8 and ME49 than did the intermediately virulent challenge strain CH3, suggesting that higher virulence may correlate with a higher rate of reinfection. Only in the case of challenge of 76K-infected mice with PRU, a strain of approximately equal virulence, was no reinfection observed. Though no data on the virulence of the RH and NED strains were provided in the studies cited, these strains have previously been established as having high and intermediate virulence, respectively (Khan *et al.* 2009, Li *et al.* 2014; Sahm *et al.* 1998).

Simulation of parasite transmission in silico.

Transmission of *T. gondii* was simulated using the program NetLogo version 10.1.0. There are three major components in the transmission cycle: the definitive host, the intermediate host and the environment. Simulations were carried out in two scenarios including the domestic and sylvatic cycles. For the domestic cycle, the three components are the domestic cat definitive host, the house mouse intermediate host, and the environment. Given that most mice may become infected by ingesting a small amount of *T. gondii* oocysts in the environment, the mortality rates in mice infected with non-lethal types are expected to be low. We set the mortality rate to be at 0.1% for the non-virulent type (NV), 1% for the intermediately virulent type (IV), and 90% for the highly virulent type (HV). For reinfection, the HV parasites were able to infect NV infected mice at a rate of 70%, and the IV parasites could infect NV infected mice at a rate of 25%. The simulations were run for 100 years with and without allowing reinfection of the second *T. gondii* strains. Figure V-2 shows the results of representative simulations. When reinfection of chronically infected mice with a more virulent genotype was enabled, the outcome of transmission changed (Figure V-2A-C,). Under this condition, the HV genotype circulated in the cats, mice and the patches a little longer than without reinfection, though it still quickly disappeared. The NV genotype circulated in the animal population and the environment for several decades, but was subsequently cleared. However, the IV genotype was able to maintain transmission for 100 years. Running the simulation for 500 years did not change the outcome (data not shown). When no reinfection was allowed, the HV *T. gondii* type rapidly disappeared from cats, mice and the patches in the environment (Figure V-2D-F). Though the IV genotype could circulate in the cats, mice and the patches for some time (about a decade or two), its transmission was not sustainable and disappeared eventually. However, the NV genotype was maintained in the population. The results did not change even when the simulation was run for 500 years (data not shown).

For the sylvatic cycle, the three components are the definitive host (wild cats), the intermediate host (wildlife that are resistant to *T. gondii*), and the environment. Given that most wildlife are resistant to *T. gondii*, and the infection is generally chronic, we assumed that there is no significant difference and set the mortality rates at 0.1% for all three groups (HV, IV and NV) of *T. gondii*. For reinfection rate, the HV type was programmed to infect NV-infected wildlife at a rate of 10%, and the IV type was programmed to infect NV infected wildlife at a rate of 5%. The simulations were run for 100 years with and without allowing reinfection of the second *T. gondii* strains. Figure V-3 shows the results of representative simulations. When reinfection of chronically infected wildlife with a more mouse-virulent genotype was activated, the outcome of transmission changed dramatically (Figure V-3A-C). Under this condition, the HV quickly dominated over both the IV and NV genotypes and became fixed within a period of 25 years, whereas the IV and NV genotypes became extinct. When no reinfection was allowed, all categories of *T. gondii* types (HV, IV and NV) randomly circulated in the population, each with the same probability of becoming fixed or distinct (Figure V-3D-F).

The effects of increasing virulence of the IV genotype on transmission dynamics in the domestic cycle were evaluated and are shown in Figure V-4. Here, the NV and HV types were set at mortality rates of 0.1 and 90 percent, respectively. Probability of sequential infection was set at zero percent for the NV type and 100 percent for the IV and HV types. As the virulence of the IV type was increased from 0.1 to 2 percent, the dominance of this type in the population was found to decrease correspondingly with regard to infected cats, mice and patches with shed oocysts (Figure V-4A). Prevalence in mouse hosts decreased more precipitously than prevalence in cats or shed oocysts. At 2 percent mortality, the prevalence of the IV type in mice fell below 50 percent, though it remained over 50 percent in cats and oocysts. Figures V-4B, C and D show representative graphs of population structure evolution at different IV mouse mortality rates. Figure S1E illustrates the effect of increasing probability of sequential infection for the IV strain type. Here, mouse mortality rates caused by the NV, IV and HV types were set at 0.1, 1 and 90 percent, respectively, and probabilities of sequential infection for the NV and HV types were set at 0 and 100 percent. As the probability of sequential infection for the IV type was increased from 0 to 100 percent, it was observed that the prevalence of this type in cats, mice and oocysts all increased as well. Increases between reinfection probabilities of zero and 20 percent were the most dramatic, though notable differences were observed between probabilities of 25 and 50 percent as well as 75 and 100 percent. Below a reinfection probability of 10 percent, the IV type became dominated by the NV type, and at 0 probability, the IV type was found to be consistently eliminated from all three populations within about 40 years. Figures V-4F, G and H provide representative examples of population structure fluctuation at different IV probabilities of sequential infection. If the possibility of co-infection was removed from the simulation, all dominance of IV strains was eliminated, and NV parasites consistently became the most prevalent type, with the IV lineage decreasing to low levels and eventually disappearing from the population when mortality rates were set to 1% and 0.1% for IV and NV (Figure V-4F). In all the aforementioned cases, the mouse mortality rate of the highly virulent (HV) type was set to 90%. Under this condition, HV rapidly declined and was consistently eliminated from the simulated environment, as the hosts carrying it were killed off (Figure V-4C,D,G,H). In cases where the NV and IV strain types were assigned equal mortality rates (Figure V-4B), eliminating any advantage for the NV strain type, in addition to the IV type becoming dominant in the population the HV type also persisted in higher proportion than the

Figure V-2. Transmission dynamics in susceptible intermediate hosts. The lines show frequency of NV (blue), IV (green) and HV (red) *T. gondii* genotypes in the form of oocysts contaminating patches (C,F) of environment and bradyzoites in infected cats (A,D) and mice (B,E) over the indicated time courses. (A, B, C) With reinfection of mice enabled or (D, E, F) without reinfection of mice enabled. Mouse mortality rates of NV, IV and HV types are set to 0.1, 1 and 90%, respectively.

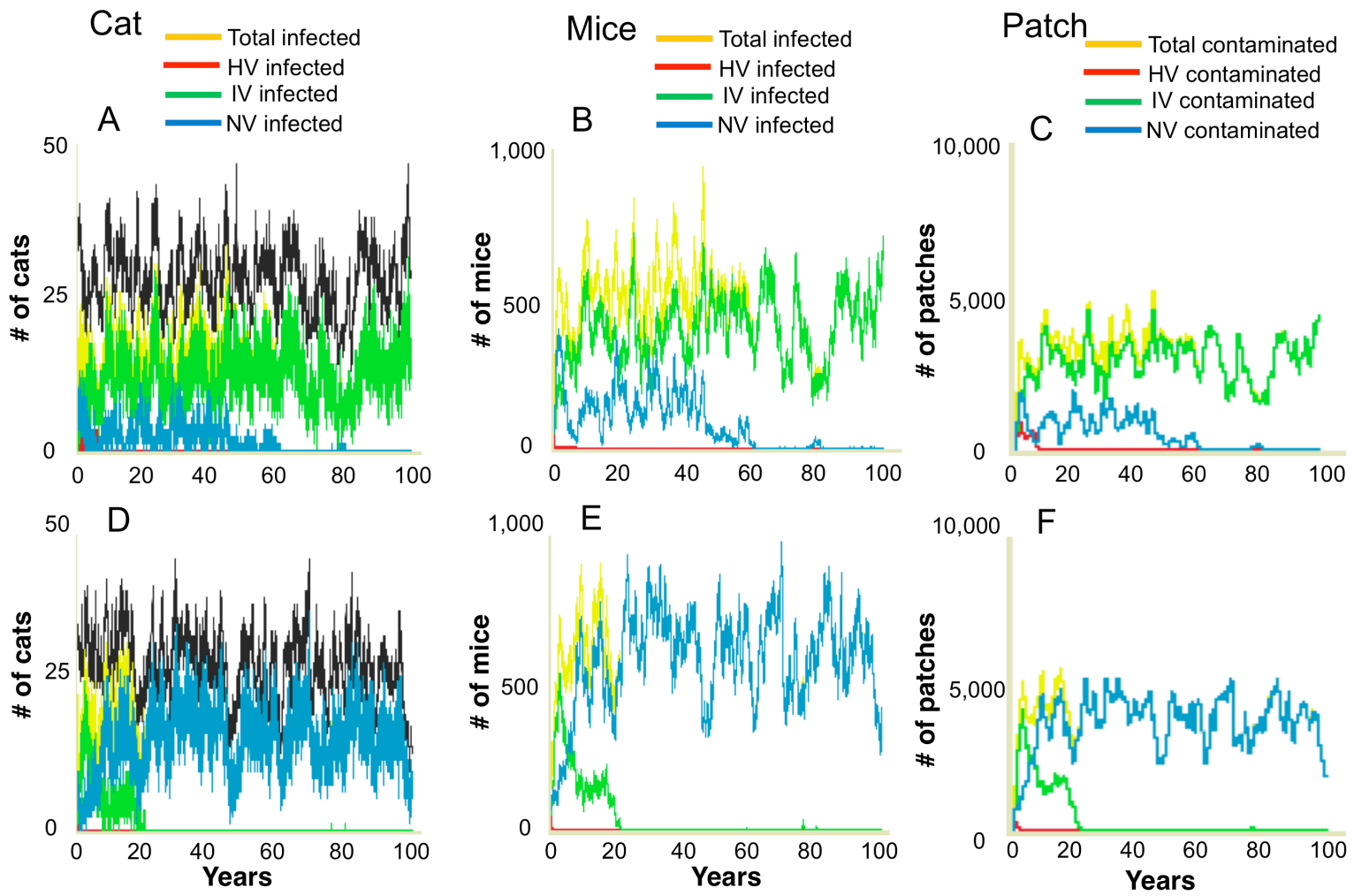


Figure V-2 continued

Figure V-3. Transmission dynamics in resistant intermediate hosts. The lines show fluctuations in frequency of NV (blue), IV (green) and HV (red) *T. gondii* lineages in the form of oocysts contaminating patches (C,F) of environment and bradyzoites in infected cats (A,D) and mice (B,E) over the indicated time courses. (A, B, C) With reinfection of mice enabled or (D, E, F) without reinfection enabled.

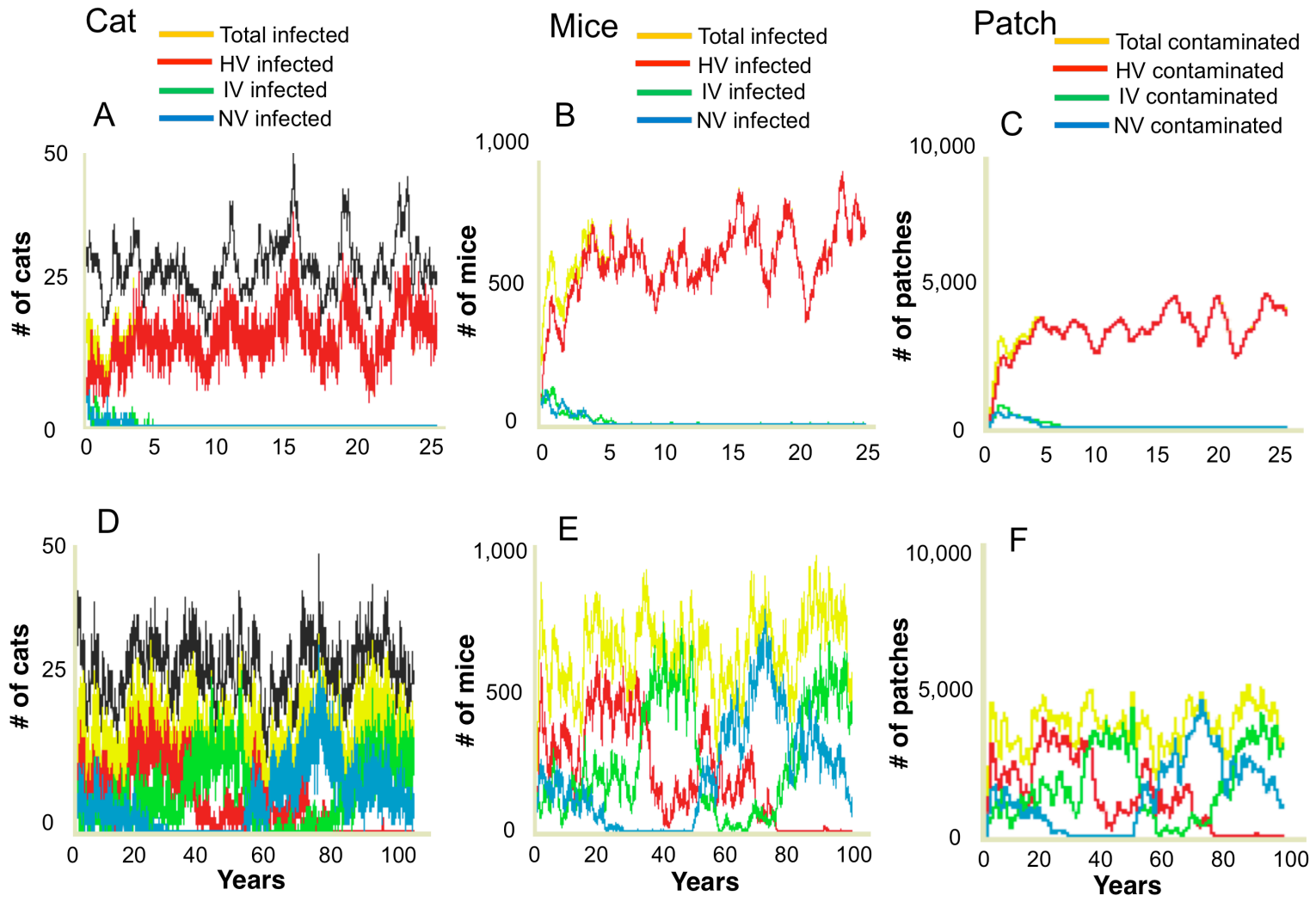


Figure V-3 continued

Figure V-4. Dominance of intermediately virulent *T. gondii* lineage is dependent on mouse mortality and sequential infection rates. (A and E) Graph showing average proportion of IV strain type in simulated populations over 100,000 days (~275 years) at various levels of mouse virulence (A) and probability of sequential infection (E). For (A), NV and HV mouse mortality set at 0.1 and 90%, respectively, and sequential infection probabilities set at 0,100 and 100% for NV, IV and HV strains, respectively. For (E), NV, IV and HV mouse mortality set at 0.1, 1 and 90%, respectively, and sequential infection probabilities set at 0, and 100% for NV and HV strains, respectively. (B,C,D,F,G,H) Histograms showing fluctuations in frequency of NV (blue), IV (green) and HV (red) *T. gondii* lineages in the form of oocysts contaminating patches of environment and tachyzoites and bradyzoites in infected cats and mice over the indicated time courses. (B,C,D) NV and HV mouse mortality set at 0.1 and 90%, respectively, and sequential infection probabilities set at 0,100 and 100% for NV, IV and HV strains, respectively. IV mouse mortality indicated to the right of each set of graphs. (F,G,H) NV, IV and HV mouse mortality set at 0.1, 1 and 90%, respectively, and sequential infection probabilities set at 0, and 100% for NV and HV strains, respectively. IV sequential infection probability is indicated to the right of each set of graphs.

A

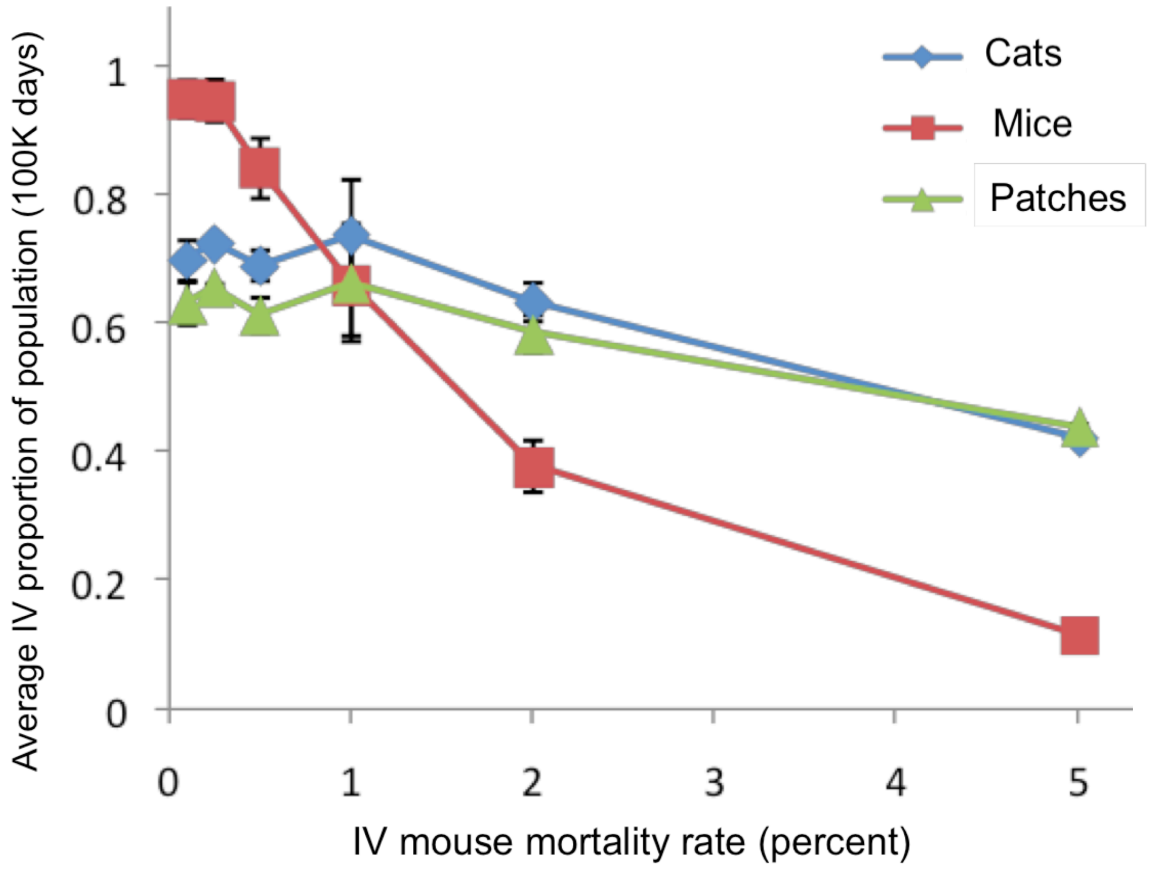


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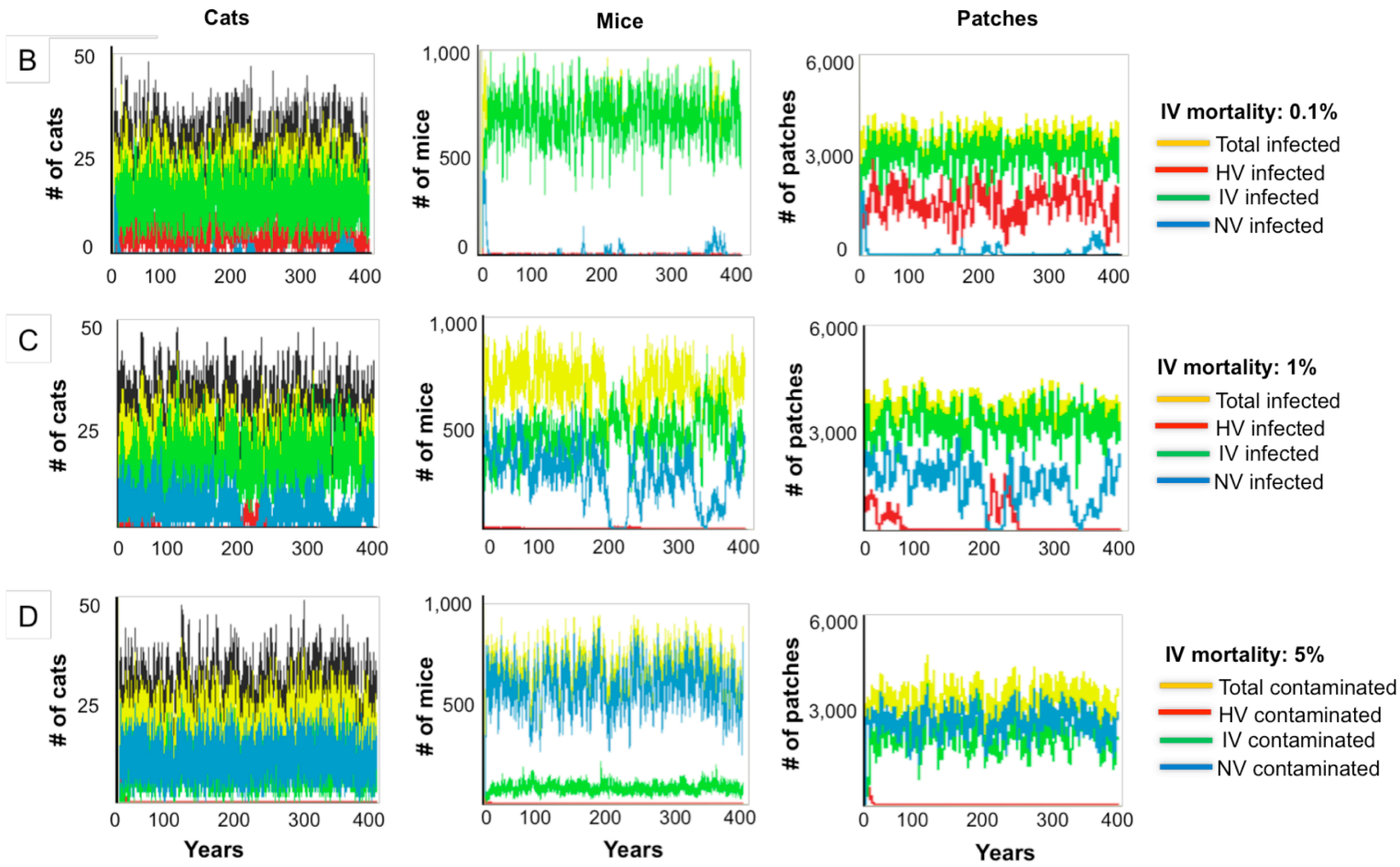


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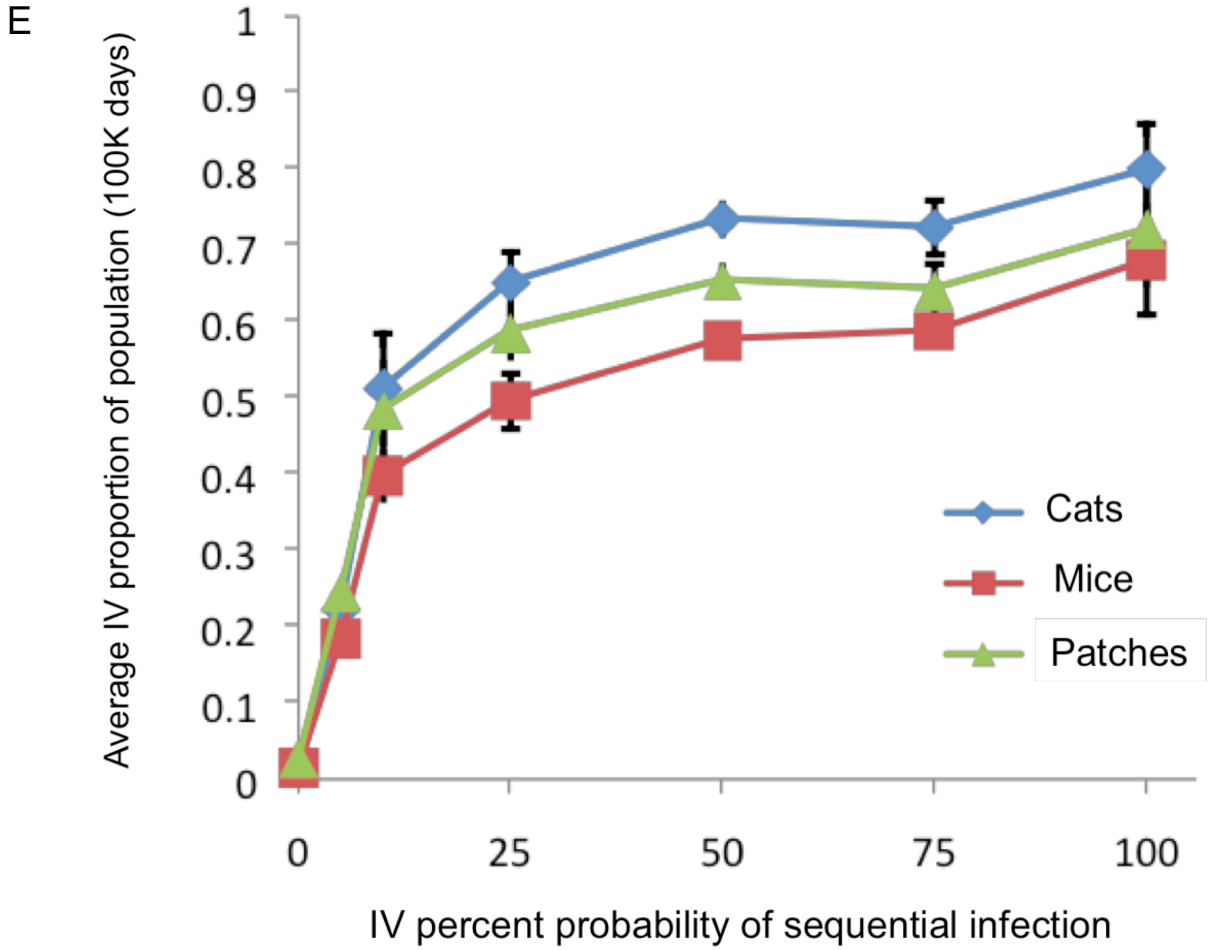


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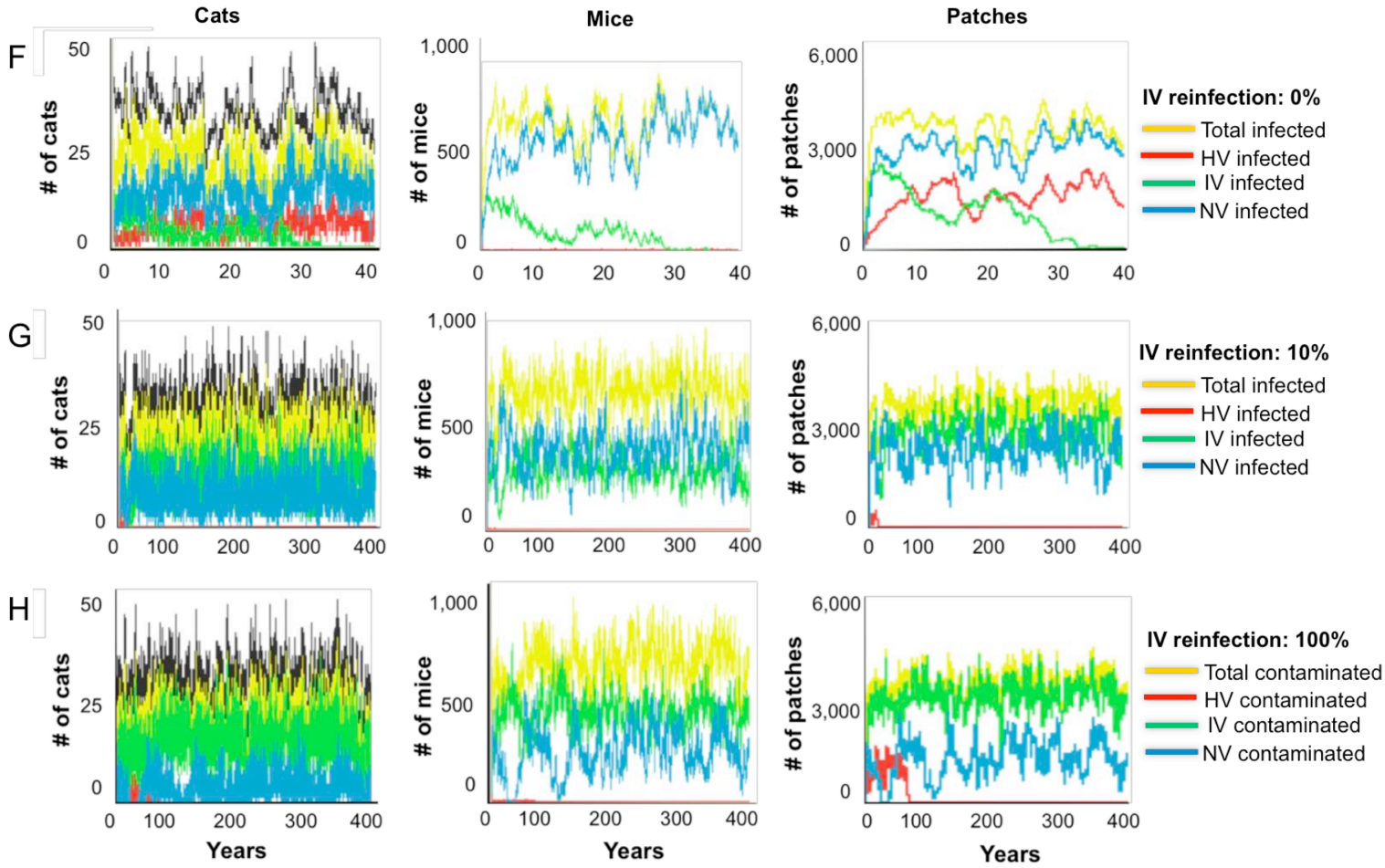


Figure V-4 continued

NV type in cat infection and patch contamination, though not in mice infection. Likewise, if the probability of reinfection was reduced to zero for the IV type, eliminating any advantage over NV for this type, the HV type became dominant over IV in cat and patch populations though again not in mice (Figure V-4F).

Discussion

The strikingly different population structures and distribution of mouse-virulence for *T. gondii* strains observed in Europe, Africa, Asia and North America versus in South America is of great interest for understanding the transmission and evolution of this ubiquitous protozoan parasite. Of the most common lineages, PCR-RFLP genotypes #1, #2 and #3 dominate in Europe and northeast Africa, genotypes #1, #2, #3, #4 and #5 dominate in North America, and genotype #9 dominates in eastern Asia. However, a highly diverse population is observed in South America with genotype #6 having the highest relative frequency (Shwab et al., 2014). Phenotypically, all dominant genotypes in Europe, northeast Africa, Asia and North America are non-lethal to house mice whereas the most common South American genotype, #6, is virulent (Table V-1). In addition, comparing the populations of North and South America (with larger sample sizes), it is obvious that more *T. gondii* genotypes in South America are virulent than in North America. There are two likely explanations for this geographical partition of genotypes and mouse virulence, including: 1) the founder effect of a few *T. gondii* genotypes which originally existed in the northern hemisphere; and 2) the consequence of natural selection of strains with discrete virulence phenotypes. Here, we have investigated the life cycle of *T. gondii* and integrated information regarding population structure, mouse-virulence, sequential infection of mice with different genotypes and variations in environmental conditions to gain a better understanding of the factors that may have shaped the modern day population structure and virulence distribution patterns of *T. gondii*.

T. gondii has a complex life cycle in which felines serve as the definitive hosts, while intermediate hosts include a wide variety of mammals and birds

Figure V-5 illustrates the sykvatic and domestic life cycles of the parasite. Sexual reproduction of the parasite occurs only within the small intestines of feline hosts, following ingestion of infected intermediate hosts, which results in the production of large numbers of oocysts which are shed into the environment. Intermediate hosts become infected by ingesting oocysts from contaminated food or water, or tissue cysts in infected prey animals. The life cycle is completed by predation of infected intermediate hosts by felids (Dubey, 2010). It is notable that whereas house mice are highly sensitive and may succumb to infection depending on the infecting parasite genotype, most other mammals and birds are resistant and infection generally results in the establishment of asymptomatic chronic infections regardless of strain genotype (Dubey, 2010). The high degree of variability in mouse virulence observed in *T. gondii* underscores the evolutionary significance of house mice (*M. musculus*) as an intermediate host for the parasite. Prior to the rise of human agrarian societies approximately 11,000 years ago, transmission of *T. gondii* would have presumably occurred through a variety of wild felines and intermediate host species, in what is termed the sylvatic life cycle. However, the more simplified domestic cycle,

involving only transmission between domestic cats and house mice, may be more important in association with human settlements (Figure V-5).

Cats play a central role in T. gondii transmission

Modern felid species originated in Asia about 10 million years ago and subsequently radiated to all continents during the next 3 million years (Johnson et al., 2006). Genetic assessment of domestic cats and their wild progenitors has revealed that cats were domesticated in the Near East, coincident with agricultural village development in the Fertile Crescent, where they probably began their association with humans as a commensal, feeding on the rodent pests such as house mice that infested the grain stores of the first farmers (Driscoll *et al.* 2007). The earliest evidence of feline domestication by humans dates back to approximately 9500 years ago in Cyprus (Vigne *et al.* 2004).

The house mouse, *Mus musculus*, is considered an important host within the domestic cycle of *T. gondii*. It has an exceptionally widespread geographical range and is the most successful and ubiquitous invasive mammal other than humans (Cucchi *et al.* 2012). *Mus musculus* arose and diverged in the vicinity of the Indo-Pak subcontinent between 1.7 and 0.7 million years ago, and its three major subspecies *M. m. musculus*, *M. m. domesticus*, and *M. m. castaneus* became adapted to life in woodland, shrub land, and grassland steppe, and radiated to different regions of the Eurasian continent and north Africa within the past 100,000 years (Figure V-6A) (Suzuki and Aplin, 2012). The house mouse is remarkable in its propensity for commensalism with humans in the context of a settled agricultural environment. Though the house mouse species arose an order of magnitude earlier than any form of settled human existence, it began developing its commensal relationship with humans starting with the very beginnings of agriculture and the creation of the first grain stores in the Fertile Crescent about 11,000 years ago (Bonhomme and Searle, 2012). The association of house mice with human settlements led to a period of rapid geographical expansion and allowed them to expand their range well beyond their natural limits of tolerance. Therefore, human agriculture is considered to be the key factor in the evolution of house mice (Cucchi *et al.* 2012). Today, the three major subspecies of *M. musculus* have different geographical distributions: *M. m. castaneus* has colonized southeastern Asia, *M. m. musculus* has colonized eastern and central Asia and northern Europe, whereas *M. m. domesticus* has colonized southwestern Asia, the Mediterranean, western Europe and northern Africa. Furthermore, *M. m. domesticus* eventually spread to regions other than Eurasia and northern Africa through long-distance colonization by the European expansion to the New World and Australia during the past 500 years (Figure V-6A)(Bonhomme and Searle, 2012).

Human agricultural societies independently arose from several locations worldwide between about 11,000 and 4,000 years ago. Among these locations, southwestern Asia, central China, Mexico and western Africa are the cradles for the domestication and cultivation of grain crops. Wheat was first cultivated about 11,000 years ago in the Fertile Crescent in southwest Asia, the rice 9,000 years ago in central China, corn 5,000 years ago in Mexico, and legumes 5,000 years ago possibly in western Africa (Figure V-6B) (Bellwood, 2005). Agriculture in the Fertile Crescent and central China originated much earlier than in Mexico and western Africa, and development occurred on a much larger scale and had a broader impact on human societies in these regions. Within a few millennia, the farming system developed in Fertile Crescent had

Figure V-5. The life cycle of *T. gondii*. Parasites are shed by definitive feline hosts in the form of oocysts, which may then be ingested by intermediate hosts. Transmission back to definitive hosts may then occur by ingestion of infected intermediate host tissue containing bradyzoites in tissue cysts. The sylvatic cycle (left) includes many definitive wild feline host species and varied mammalian and avian intermediate host species, whereas the domestic cycle (right) includes only the domestic cat as definitive host and the house mouse as intermediate host. Prehistorically, the sylvatic cycle was presumably the major means of *T. gondii* transmission, but as the world's human population increased due to the expansion of agricultural communities (bottom), domestic cats came to far outdistance other definitive host species, with the house mouse serving as their major prey species. Thus the domestic life cycle became of primary evolutionary importance for transmission of the parasite.

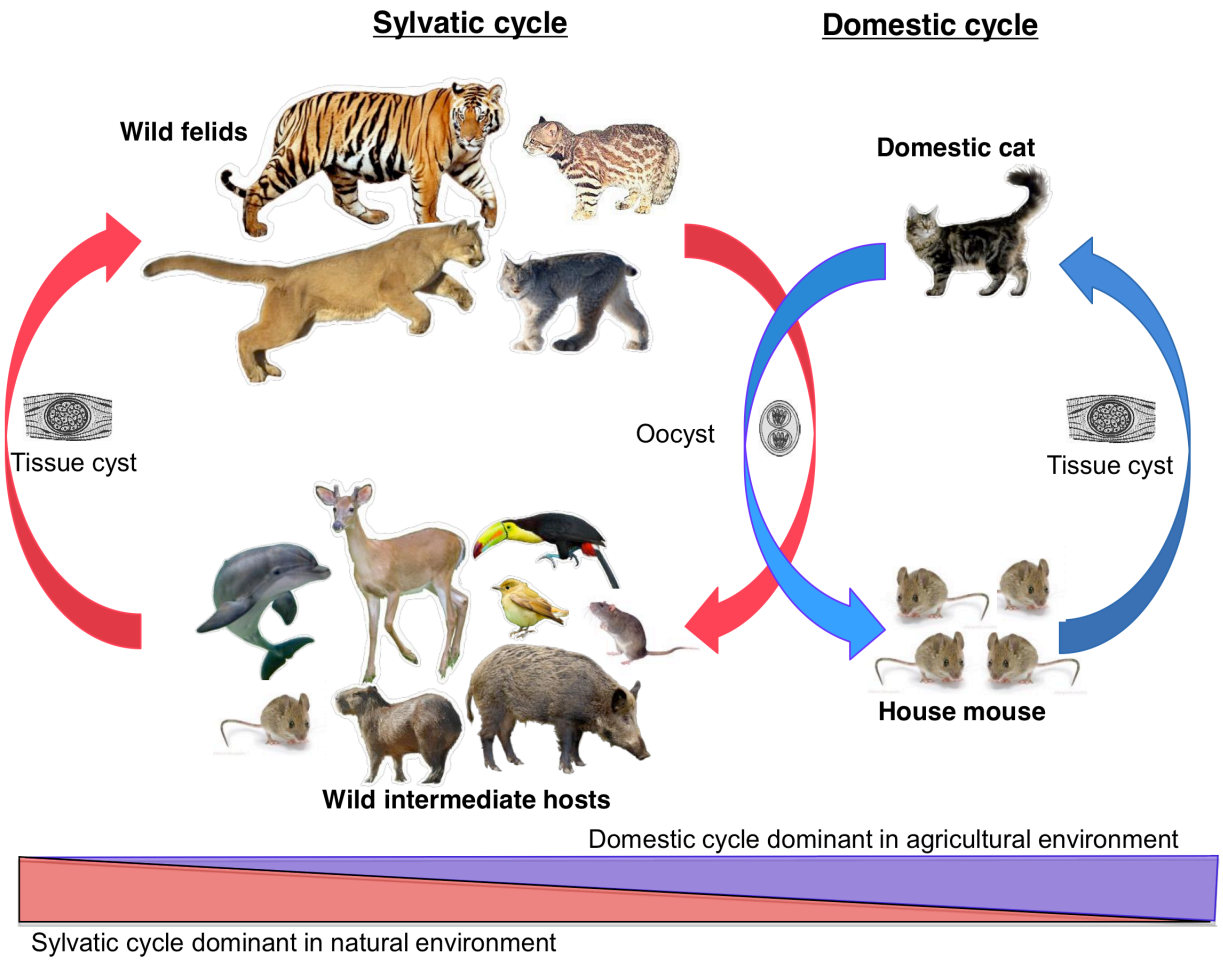


Figure V-5 continued

Figure V-6. Relationships between global expansion and distribution of house mice, human agriculture and *T. gondii*. (A) Expansion patterns and current global distribution of the three major house mouse subspecies, *Mus musculus domesticus* (red), *M. m. musculus* (green), and *M. m. castaneus* (yellow) (Bonhomme and Searle 2012). Colored arrows indicate directions of mouse subspecies expansion over the indicated time frames. (B) Origins of agriculture and association with domestic cats and house mice (Bellwood 2005). Indicated are cultivation of wheat in the Middle East, rice in southeast Asia (both associated with domestic cats and house mice), corn in South and Central America and sorghum in Africa. Arrows indicate directions of outward agricultural expansion, including migration of European settlers to North America and concomitant exportation of domestic cats and house mice. (C) Distribution of *T. gondii* lineages by continent (Shwab et al. 2014). Pie charts indicate proportions of color-coded *T. gondii* PCR-RFLP genotypes present on corresponding continents. Populations in the northern hemisphere are largely clonal in structure, with small numbers of highly dominant lineages, whereas the South American population is much more diverse, without notably dominant individual genotypes.

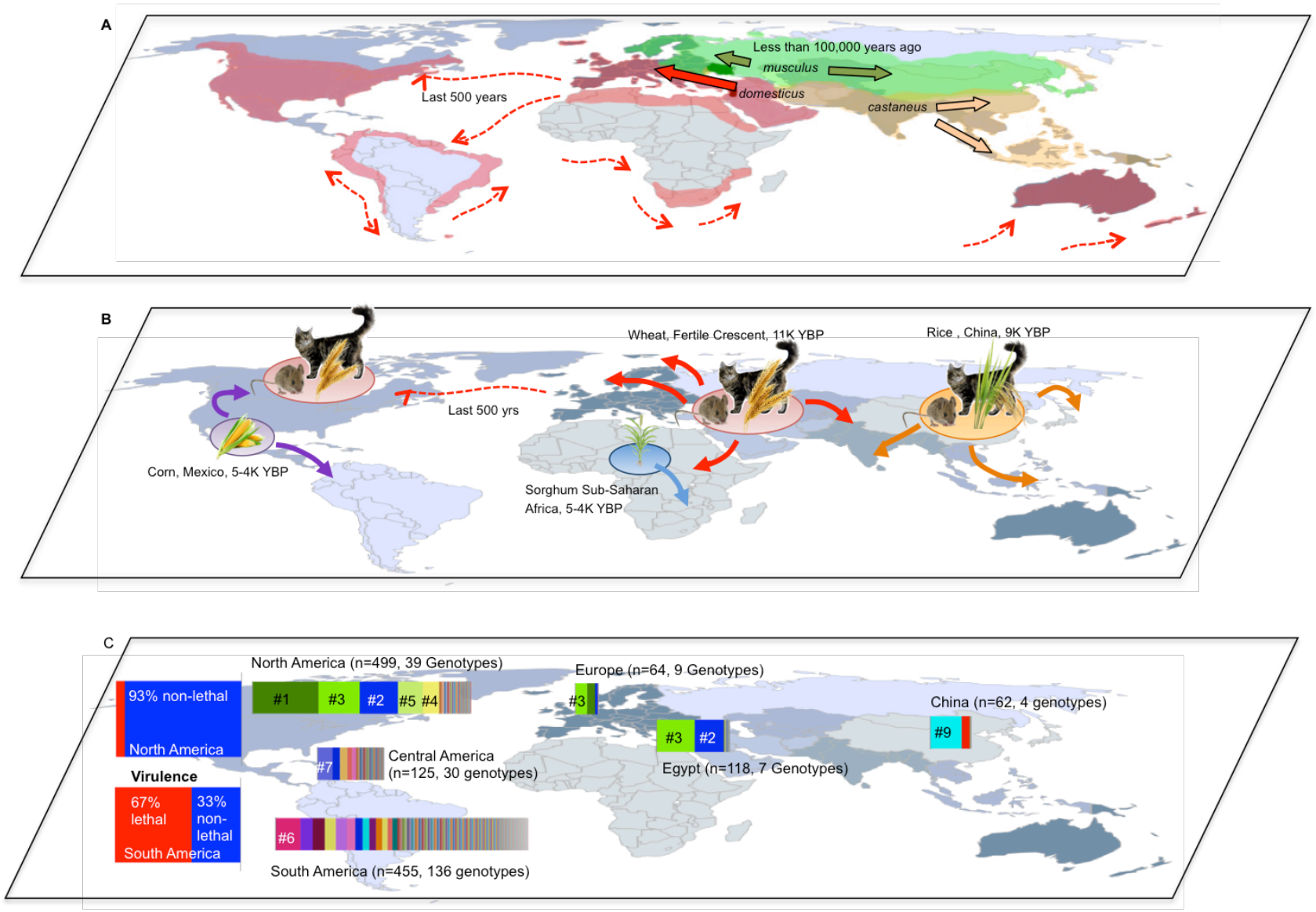


Figure V-6 continued

spread over vast areas of the Old World: to Britain in the west, to central Asia in the east, to Egypt and north Africa in the south, and to Pakistan and India in the southeast. Similarly, agriculture developed in central China also dispersed to a wide area, reaching southeastern Asia and eastern India rapidly. Following the initial domestication of plants, sedentary agricultural societies began to develop, enabling the production of reliable and more abundant food sources, and subsequently leading to rapid expansion of the human population (Bellwood, 2005). Such changes inevitably impacted the regional ecosystems in numerous ways, including facilitation of the expansion and dispersal of house mice. To this end, abundant data are available from the Fertile Crescent, the earliest origin of human agriculture. Zooarchaeological studies of Neolithic sites of Syria and Turkey revealed that the occurrence of house mice among small mammals was less than 5% in the vicinities of hunter-gatherer communities of 12,000 years ago, but this proportion jumped to an overwhelming 80% in association with the earliest human agricultural settlements of 11,000 years ago (Cucchi *et al.* 2012). House mice would likely have been a major prey animal for domestic cats throughout their history, and in fact control of such rodent species was likely a major incentive for the initial domestication of felines (Driscoll *et al.* 2007). Prior to the rise of human agrarian societies, transmission of *T. gondii* would have presumably occurred through a variety of wild definitive felid hosts and intermediate hosts within the sylvatic cycle (Figure V-5). However, the human agricultural environment has unintentionally created a unique niche to transmit *T. gondii* among domestic cats and house mice by way of the domestic cycle (Figure V-5). The impact of this contribution has been twofold: 1) cats and house mice have been brought together into a close proximity, and 2) higher densities of cats and mice have been sustained in the same environment. Because of its high abundance worldwide and sympatry with the abundant domestic cats, *M. musculus* is probably the most evolutionarily important intermediate host for *T. gondii* (Lilue *et al.* 2013). The shift in transmission from sylvatic life cycle to domestic life cycle in settled human agricultural environments would be expected to have altered the selective pressures on this parasite, requiring its adaptation for survival in and transmission by the highly susceptible house mouse host. Unlike most other mammals, house mice, particularly the variety of lab mice which are derived from *M. m. domesticus*, are sensitive to *T. gondii* infection. Their response to different lineages of *T. gondii* can be markedly different. The commonly used lab mice are highly sensitive to genotype #10 (Type I) *T. gondii* strains, and can quickly succumb to the parasites within 2 to 3 weeks post infection. However, they are resistant to genotypes #1 and #3 (Type II) and genotype #2 (Type III) and most infections become chronic if inoculated at low doses (Sibley and Boothroyd 1992; Howe and Sibley 1995). The difference in sensitivity of mice to different *T. gondii* genotypes may have affected the long-term fitness of these parasites. Simultaneously, the virulence of certain genotypes of *T. gondii* may have placed a strong selective pressure on the evolution of defense mechanisms in the house mice. One example is the family of IFNγ-inducible, 47 kDa immunity-related GTPases (IRGs). House mice (both laboratory and wild lineages) have two adjacent clusters of IRGs on chromosome 11 and one cluster on chromosome 18. These IRGs are highly polymorphic, particularly among wild mice (Lilue *et al.* 2013). The IRG proteins are essential for mouse immunity to *T. gondii*, defending mice against the non-lethal *T. gondii* strains (genotype #1 - Type II and genotype #2 - Type III) but not the lethal strain (genotype #10 - Type I). This is due to expression by genotype #10 *T. gondii* of polymorphic rhoptry proteins 5 (ROP5) and 18 (ROP18), which together confer resistance to the destruction by host IRG proteins of the parasite parasitophorous vacuole, the membrane protecting the parasite within host cells (Howard at al. 2011). The existence of polymorphisms both in host IRG proteins and the parasite ROP proteins

suggest that the house mice and *T. gondii* have had a long-term association, and that house mice are an evolutionarily important intermediate host for the parasite.

Recent studies in lab mice have provided evidence that though the highly virulent parasite strains cause high mortality in naïve mice, they have an advantage in the context of reinfection of mice already chronically infected with a less virulent *T. gondii* strain (Table V-2) (Krahenbuhl *et al.* 1971; Reikvam *et al.* 1976; Araujo *et al.* 1997; Dao *et al.* 2001; Brandao *et al.* 2009; Silva *et al.* 2012). The preexisting chronic infection of a low virulence *T. gondii* strain likely provides sufficient immune protection for the mice to survive what would normally be a fatal infection by a highly virulent strain. This would lend an advantage to transmission of virulent parasites. However, such an advantage would presumably be offset by the high mortality rate associated with infection of naïve mice, which would reduce the likelihood of transmission to new hosts.

To assess the consequences of virulence phenotype on transmission of *T. gondii* strains within an agricultural environment, we performed analyses using a simulation of domestic cycle parasite transmission. The results showed that, when reinfection of chronically infected mice with more virulent genotypes is enabled, the IV genotype is able to maintain transmission and becomes clearly and consistently dominant over the NV genotype (Figure V-1A-C). This is likely due to a favorable balance between strain lethality and the ability to reinfect intermediate hosts by the intermediately virulent *T. gondii* genotype, which invades more aggressively but still does not generally cause significant mortality. The ability to utilize previously infected mice as viable hosts may provide a significant competitive advantage. The consequence of such evolutionary selection would be expected to be reduced genetic diversity and dominance of a small number of clonal *T, gondii* genotypes. This outcome is in agreement with the fact that only a few intermediately virulent *T. gondii* genotypes have become dominant in Europe (#1, #2, and #3), northern Africa (#1, #2, and #3), North America (#1, #2, #3, #4 and #5), and China (#9) (Figure V-6C). Such a population structure superimposes nicely with human agriculture and domestic cat/house mice geographic distributions (Figure V-6A-B). These dominant strains have all been shown to display intermediate virulence in mice, with the exception of genotype #3 which seems to have relatively lower virulence (Table 1). Within the simulated populations, when assuming a complete immunity upon initial chronic infection that prevents sequential reinfection of mice with a second *T. gondii* strain, only the non-virulent genotype was found to maintain infection in the population (Figure V-1D-F). The virulent *T. gondii* strains quickly disappeared, followed by the intermediately virulent genotype. The rate of disappearance was correlated with the level of virulence (mortality): the higher the virulence, the faster the parasite disappeared (Figure V-1D-F). This result suggests that, under the situation of a clonal population structure, evolutionary selection reduces virulence in *T . gondii*. This principle may help explain the dominance of the less-virulent genotype #3 in Europe (Table V-1).

To simulate the sylvatic cycle of *T. gondii* transmission, we set the mortality rate for all three strain types at a very low value (0.1%), equivalent to the mouse non-virulent genotype. This is based on the fact that most wildlife are resistant to *T. gondii*, and infections are generally chronic. When reinfection of chronically infected wildlife with more mouse-virulent genotypes (HV and IV) was enabled, HV quickly became dominant, but the mouse IV and NV populations diminished to extinction within a few decades (Figure V-3A-C). When we examined the murine virulence data for globally isolated *T. gondii* strains, a far greater proportion of highly mouse-

virulent strains were identified in South/Central America compared to elsewhere in the world. The outcome of the simulation is thus more or less in line with the modern-day population structure in Central/South America, suggesting mouse virulent *T. gondii* genotypes are evolutionarily favored in this tropical environment. In contrast to the northern hemisphere, the highly diverse felids and intermediate hosts of Central/South America may differentially select heterologous *T. gondii* genotypes with varying virulence phenotypes, thereby maintaining a genetically diverse parasite population. When reinfection was disabled in simulated environments with sylvatic transmission, each of the three categories of *T. gondii* genotypes was observed to randomly circulate within the population with equal probability of becoming fixed or extinct (Figure V-3D-F). As this is a deviation from the observed populations of Central/South America wherein more virulent phenotypes appear to have an evolutionary advantage, this seems unlikely to be an important selective mechanism for the population.

Because the exact parameters regarding mortality rates and sequential infection probabilities during mouse infections in nature are difficult to ascertain, we also examined the dynamics of simulated populations over a range of values for both of these variables (Figure v-4). We found that dominance of the IV strain type decreased as its virulence was increased and the rate of decreasing is higher in mice than in cats and in contaminated patches (Figure v-4A-D). At 5% mortality, dominance in all three categories was lost (Figure v-4A,D). In nature, we generally expect mortality rates of mice infected with intermediately virulent strains to be minimal, as only small doses of parasite are likely to be encountered and ingested. In bioassay studies such as those cited in Shwab *et al.* (2014), wherein potentially infected tissue is fed to laboratory mice for the purposes of detecting and isolating *T. gondii*, nonvirulent and intermediately virulent strains are rarely distinguishable from one another, in that the dosages are only sufficient to cause mortality from highly virulent strains, and higher concentrations of parasite are required to identify strains of intermediate and low virulence. In the case of reinfection probability, we found that dominance of IV strains increased rapidly up to about a 10% reinfection rate, and then more gradually above this threshold (Figure v-4E-H). Taken together, virulence in terms of mouse mortality and the capability of invading existing chronic infection (reinfection) are two important aspects that likely under evolutionary selection in *T. gondii*.

Aside from house mice, any intermediate hosts sensitive to *T. gondii* infection and which have differential susceptibility to distinct parasite genotypes may exert selective pressure on the parasite. There are a few candidate intermediate hosts of possible of importance in this regard. Wild rabbits (lagomorphs) have been reported to be the major diet for domestic cats and bobcats (Liberg *et al.* 1984; Delibes *et al.* 1997). Experimental challenges of laboratory rabbits showed that they are highly sensitive to *T. gondii* infection (Dubey, 2010). Given the worldwide distribution and abundance of lagonorphs, more studies are needed to determine their role in *T. gondii* transmission. Another potentially important intermediate host of *T. gondii* is the deer mouse (*Peromyscus maniculatus*). Among the small rodents in North America, the deer mouse is the most abundant species with a wide distribution from the Yukon Territory in Canada in the north to the Mexican Plateau in the south (Witmer and Moulton, 2012). It was found to be the most common rodent in a variety of crop fields (Kaufman and Kaufman, 1990). Experimentation has shown that deer mice can be easily infected with*T. gondii* and that infections may be transmitted vertically to offspring as in house mice (Rejmanek *et al.* 2010). Therefore, deer mice

may play an important role in *T. gondii* transmission in North America where the genotypes #4 and #5 dominate in wildlife (Khan et al., 2011, Dubey et al, 2011).

Our study has shown that the domestic and sylvatic transmission cycles of *T. gondii* are under different selective pressures, with the key factor under selection being parasite virulence. The domestic life cycle associated with human agricultural settlements is expected to promote an evolutionary trajectory favoring the development of the more clonal population structure dominated by less virulent *T. gondii* genotypes. While the virulent strains pay a high fitness costs during infection of naïve mice, they benefit from the ability to overcome host immunity and reinfect mice chronically infected with less virulent *T. gondii* genotypes. In this situation, strains of intermediate virulence may strike a balance which provides them with an overall selective advantage. In contrast, within natural environments such as the tropical Amazon rainforest, the sylvatic cycle favors development of a diverse population supporting highly virulent *T. gondii* genotypes. This is due to the high diversity of host species that are resistant to *T. gondii*, alleviating the selective pressure associated with strain virulence. Thus, it appears that the South American population structure likely resembles the primordial state of the parasite, while the genetic uniformity observed elsewhere represents a relatively recent, human influenced state. This makes sense as much of South America, particularly the Amazon rainforest region, remains undeveloped compared to countries in the northern hemisphere. In addition, a greater variety of animal species are present in this region than in any other part of the world, which would presumably contribute to a corresponding diversification of parasite strains (Dirzo and Raven, 2003). More extensive sampling in other less developed regions of the world with a high diversity of wildlife, such as the tropical areas of Africa and southeast Asia, is necessary to further examine the extent to which these variables influence the population structure of T. gondii.

Our findings suggest that, as human influence continues to expand and more of Earth's ecosystems are altered, the global *T. gondii* population structure may continue to evolve towards uniformity and reduced mouse virulence. South American countries such as Brazil are currently among the most rapidly developing in the world. As the human populations of these countries grow and more land is transformed by agriculture and urbanization, we may see a decline in the prevalence of more highly mouse-virulent South American strains. But in the meantime, encroachment into unexplored natural environments such as the tropical Amazon will potentially expose us to more virulent *T. gondii* strains in the wild, increasing the risk of contracting more severe forms of toxoplasmosis. While humans are more resistant to *T. gondii* infection than mice in general, some evidence suggests an increased association of highly mouse virulent strains with acute toxoplasmosis in immunocompetent humans (Ferreira *et al.* 2011; Dubey *et al.* 2012). Though the long term effect of human agricultural expansion has been to reduce the genetic diversity and virulence of *T. gondii* throughout much of the world, human-associated changes to ecosystems such dramatic increases in densities of house mouse and domestic cat populations (including feral cats) may lead to higher infection rates of *T. gondii* in these animals, resulting in heavier environmental contamination and an eventual increase in infection rates within human populations.

Chapter VI. Dynamics of gene expression in mice infected with different genotypes of *T. gondii*

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Introduction

Toxoplasma gondii is one of the most successful protozoan parasites infecting mammals and birds (Dubey, 2010). It is estimated that up to one-third of the world's human population is chronically infected with this parasite (Dubey 2010, Tenter *et al.* 2000). Genetic analyses showed that, in Europe and North Africa, two *T. gondii* lineages, namely type II (ToxoDB PCR-RFLP genotype #1 and #3) and type III (genotype #2) predominate. In North America type II, type III and type 12 (genotype #4 and #5) are the major lineages. A previously defined type I (genotype #10), was widely spread globally, but it was isolated with a low frequency. Though genetic variation between these major lineages is about 1-2 % (Khan *et al.* 2009), the type I, II and III strains differ widely in virulence, persistence, migratory capacity, attraction of different cell types, and induction of cytokine expression (Saeij *et al.* 2005). Type I strains are highly virulent in mice with an LD_{100} as low as a single parasite, while strains of type II and III are less virulent (LD_{100}>1000), and as such are able to establish chronic infections in mice readily (Sibley and Boothroyd 1992, Sibley *et al.* 2009). The variability of disease outcome in human infections may also be linked to the type of strains that cause the infection. Type I strains are often found in severe acquired toxoplasmosis and congenital infection (Xiao *et al.* 2009, Melo *et al.* 2011, Khan *et al.* 2005). Type II strains cause the majority (70 to 80%) of human cases of toxoplasmosis in North America and Europe (Howe and Sibley 1995, Ajzenberg *et al.* 2009).

To understand how these distinct *T. gondii* lineages lead to different outcomes in infected hosts, strain-specific host responses are of interest for investigation. A previous study of macrophage cytokine production after *in vitro* infection with parasites of virulent type I and intermediate type II genotypes showed that a type II strain induced nearly 200-fold higher level of IL-12 than a type I strain after 24-h of infection (Robben *et al.* 2004). Macrophages were activated through the classical (Th1 response-associated) pathway by type II infection, but the alternative (Th2 response-associated) pathway by type I and III infections (Jensen *et al.* 2011). The *Toxoplasma* rhoptry kinase ROP16 (type I and III alleles) was reported to be responsible for alternative

activation through activating STAT6 (Butcher *et al.* 2011). The type II dense granule protein GRA15 is able to promote classical activation of macrophages by activating the NF-kB pathway (Jensen *et al.* 2011). Strain-dependent host response was also found in human foreskin fibroblasts (Saeij *et al.* 2007, Rosowski *et al.* 2011), chicken embryonic fibroblasts (Ong *et al.* 2011), and human neuroepithelial cells (Xiao *et al.* 2011). Our previous study also showed that overall transcription levels in mice were similar in type I and type II infections, and both had greater changes than the infection with type III on day 5 post-infection (Hill *et al.* 2012).

Based on these studies, it is clear that there are significant differences in the host response to different *T. gondii* genotypes. However, the knowledge of host response to these different genotypes is confined to *in vitro* model and/or limited time points sampled. To provide a broad spectrum of host response to different *T. gondii* strains, we therefore conducted a time course study in mice to reveal dynamics of gene transcription, and expression networks in mice infected with different lineages of *T. gondii.* Through gene ontology and network analysis, major cause-effect relationship among a number of transcripts of immune and inflammatory responses are proposed and discussed.

Materials and Methods

Parasite strains and infection in mice

T. gondii Type I strain GT1 (high virulence), Type II strain PTG (intermediate virulence), and Type III strain CTG (non-virulence) were propagated in confluent human foreskin fibroblast (HFF) monolayers as described previously (Hill *et al.* 2012). The tachyzoites were harvested by filtering through 3-μm-pore-size polycarbonate filters (catalog # 420400; Fisher Scientific, Hanover Park, IL) and counted with a hemocytometer. Five hundred tachyzoites were inoculated into 6-8-week old female outbred CD-1 mice (ICR-Harlan Sprague) by intraperitoneal injection. Four mice were euthanized on day 1, 2, 3, 4, 5 and 7 post infection, respectively. To collect peritoneal cells, 5 ml of ice-cold phosphate-buffered saline (PBS) was injected into the peritoneal cavity, and then peritoneal lavage was collected. The cells were collected by centrifugation at 1,000×g for 10 min at 4 °C, resuspended in 1 ml of RNAlater solution (Ambion, Austin, TX), incubated in 4°C for 1 h, and then stored at -70°C before RNA extraction. The mouse spleens were collected, weighed, homogenized, and homogenized in PBS to 0.1 g/ml for DNA extraction and subsequent determination of parasite burden. Four uninfected mice were used as negative controls. The use of mice for this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Knoxville.

Microarray analysis

Microarray analysis was performed by the method described previously (Hill *et al.* 2012). In brief, total RNA from peritoneal cells was extracted using Qiagen RNeasy Plus Mini Kit (catalog #74131; Qiagen,Valencia, CA) following the manufacturer's instructions. The quality of RNA was tested by agarose gel electrophoresis, and the concentration of RNA was determined by reading the absorbance at 260/280 nm. Affymetrix Mouse Exon 1.0 ST array containing 35,556 transcripts was used. RNA samples were processed according to the Affymetrix protocol for one-

cycle DNA synthesis using a Message Amp II-Biotin Enhanced Kit (P/N AM1791; Ambion, Austin, TX). Five micrograms of fragmented cRNA was hybridized to the Affymetrix GeneChip. Arrays were processed using the Affymetrix Hyb/Stain kit PN900720. Arrays were immediately scanned using the Affymetrix 7G scanner. Array images were visually inspected for anomalies. The individual chip scans were quality checked for the presence of control genes and background signal values. All microarray procedures were done at the University of Tennessee Affymetrix Core Facility.

Analysis of differential gene expression

Gene expression analysis was performed using Affymetrix® Expression Console and Transcriptome Analysis Console (TAC) 2.0 Software (www.affymetrix.com). The raw data in the form of Affymetrix CEL files from the arrays were normalized with the robust multichip algorithm (RMA-Sketch). The analysis was performed to detect genes with statistically significant expression levels between *T. gondii*-infected mice and the uninfected control mice. Gene transcripts were considered to be differentially expressed when there was at least a 2-fold change (up or down) from the uninfected controls with a false discovery rate (FDR) p value of ≤ 0.05. The expression analysis was performed at gene level (not exon level).

Analysis of biological pathways enriched during T. gondii infections.

In order to identify enriched biological functions, the Database for Annotation, Visualization and Integrated Discovery (DAVID) v.6.7 bioinformatics software was employed (Huang et al, Nature Protocols, 2009). This online software allows for analysis of large gene lists based on functional annotations from multiple databases. In our analysis, lists of Affymetrix exon gene IDs for significantly up- or downregulated genes ($q \leq 0.05$ based on FDR analysis) from each of the three infection types across all time points were uploaded into the DAVID functional annotation tool. The output of Functional Annotation Chart was obtained based on GO term - biologic process (GOTERM_BP). Functional Annotation Clustering analysis identified groups of functionally related genes that were enriched among the listed transcripts, meaning that genes belonging to these function were represented significantly more frequently than would be expected in a random set of *M. musculus* transcripts. An enrichment score was generated for each cluster, calculated by averaging the negative logarithms of the modified Fisher Exact P-values (EASE scores) of the individual pathways in the cluster. For clustering analysis, the stringency level was set as “high”.

Microarray data validation by quantitative real-time RT-PCR (qRT-PCR)

To validate the microarray data, a few up- and down-regulated transcripts at different time points were selected for qRT-PCR analyses using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (catalog #4374966; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. One microliter of RNA was transcribed into cDNA, and Quantitative PCR was performed using TaqMan Gene Expression Assays and Mouse ACTB Endogenous Control (Assay ID 4352341E; Applied Biosystems, Foster City, CA) in the iCycler thermal cycler (iQ5, Bio-Rad Laboratories, Inc. Hercules, CA). The TaqMan Gene Expression Assays consist of pre-formulated PCR primers and a FAM dye-labelled TaqMan probe for selected

target transcripts. The Mouse ACTB Endogenous Control contained pre-formulated PCR primers and a VIC dye-labelled TaqMan probe of β-actin. Multiplex PCR reaction mix had a volume of 25 µl containing 9 µl of H_2O, 12.5 µl of 2×TaqMan Fast Advanced Master Mix (catalog #4444964; Applied Biosystems, Foster City, CA), 1.25 µl of 20X TaqMan® Gene Expression Assay buffer, 1.25 µl of ACTB Endogenous Control, and 1 µl of cDNA. The reaction was carried out using the following cycling conditions: 50°C for 2 min, 94°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec. The comparative CT method (△△CT) was chosen for the relative quantification of gene expression (Schmittgen and Livak 2008).

Parasite load determination by quantitative PCR (qPCR)

Parasite load in mouse spleen tissue was determined as described previously (Hill *et al.* 2012) with modifications. Briefly, 100 µl of homogenized spleen tissue at a concentration of 0.1 g/ml in PBS was diluted with 100 µl of PBS, then 50 µl of 10 mg/ml proteinase K was added, and the mixture was incubated at 55°C for 2 h. DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (catalog number 69504; Qiagen, Valencia, CA). For qPCR standard controls, the spleen of an uninfected mouse was homogenized and diluted in PBS, and *T. gondii* tachyzoites were spiked into the homogenized spleen to make a series of concentrations of 1×10^7 to 1×10^3 tachyzoites/ml. All homogenized spleen samples were adjusted to a volume of 0.1 g/ml in PBS, and genomic DNA was purified using the same procedure as detailed above. The concentration of parasites in mouse spleen was estimated by qPCR using a TaqMan probe targeting the ITS1 sequence (GenBank accession number AY143141). The primers for PCR amplification were ITS1-Fx (GAAGGGGCTCAATTTCTGG) and ITS1-Rx (TGTTCCTCAGATTTGTTGTTTGA), which amplify a 117-bp sequence. The ITS1 probe was 5'-5-FAM-CGTGTCTCTGTTGGGATACTGATTTCCAGG- BHQ-1-3', with the 5' end labeled with 6-carboxyfluorescein (FAM) and the 3' end labeled with Black Hole Quencher-1(BHQ-1) (Integrated DNA Technologies, Inc., Coralville, IA). The qPCR mixture had a total volume of 25 µl containing 10.9 µl of H_2O, 12.5 µl of 2×TaqMan Fast Advanced Master Mix(catalog number 4444964; Applied Biosystems, Foster City, CA), 0.15 µl of 50 µM ITS1-Fx and ITS1-Rx primers, 0.3 µl of 50 µM ITS1 probe, and 1 µl of purified DNA. The reaction was carried out using the iCycler thermal cycler (iQ5, Bio-Rad Laboratories, Inc. Hercules, CA) under the following conditions: 94°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec. The CT value of each sample was compared to values of the standard controls to estimate the concentration of parasite per gram of spleen.

Results

Microarray analysis

Two to four high-quality RNA samples from mice infected with each of the three *T. gondii* strains at each of the six time points were used for microarray experiments. A total of 45 mouse peritoneal cell samples were processed and analyzed including two samples for each strain at each time point with exception of three samples for Type III (CTG) on day 5 and day 7, three for Type I (GT1) on day 7, four samples for Type I on day 5 and four for uninfected mice. Differentially expressed genes (DEGs) were identified based on signal intensity values and fold

changes compared to values from the uninfected mice. For Type I infected mice, 9, 75, 1830, and 1307 genes were significantly changed on days 3, 4, 5, and 7, respectively. For Type II infected mice, 58, 71, 124 and 1029 genes were significantly changed on days 2, 3, 4 and 5, respectively. For Type III infected mice, 112, 52, 111, 64 and 158 genes were significantly changed on days 2, 3, 4, 5 and 7, respectively. No significant differences in gene expression were observed for other days. Changes in DEG number for each strain type over the course of the experiment are shown in Figure VI-1A. In both Type II and Type III infected mice, the number of DEGs increased markedly on day 2. However, in the case of Type III, the number of DEGs then remained relatively constant through day 7, while in the case of Type II, DEGs increased again nearly ten-fold on day 5, then had dropped completely by day 7. The pattern of gene expression observed for Type I infection was quite distinct, as no significant change was observed until day 3, followed by roughly ten-fold increases in DEGs on days 4 and 5. This peak number of DEGs was then approximately maintained through day 7. Thus, each of the three strain types elicited a unique response in terms of the temporal patterns of gene expression, with Type III and Type II eliciting an earlier response than Type I, which then remained constant for Type III while spiking on day 5 and then dropping on day 7 for Type II. Type I, on the other hand, elicited a delayed response which increased steadily to reach a peak level similar to that of Type II on day 5, but which unlike Type II was maintained through day 7. The change of up-regulated genes in all three infections followed the same trend of total number of DEGs (Figure VI-1B). On day 2 and day 3, clearly there are higher numbers of genes up-regulated in Type II and Type III infection than in that of Type I. Downregulated genes were considerably lower in all strains, but increased in Type I and to a lesser extent Type II on day 5, followed by decreases on day 7 (Figure VI-1C).

Functional analysis of DEGs in mice infected with the three distinct T. gondii strains.

Functional analysis of DEGs was performed by DAVID ver 6.7. GO TERM biological processes (GO TERM_BP) were evaluated by Functional Analysis Charting, and the enrichment of related biological function was evaluated by Functional Analysis Clustering. Statistical significance was set at FDR q<=0.5, the stringency for the latter analysis was set at "high". For all *T. gondii* infections, immune response was the top affected function (Table VI-1). The number of DEGs for this response is summarized in Figure VI-2. The trend of change among the three *T. gondii* strains follow the total number of DEGs and the up-regulated DEGs nicely. Overall, Type III infection caused least number of DEGs, whereas Type I caused the highest. For Type III infected mice, innate defense response is the main scheme of reaction, dictated by the up-regulation of GBPs. Inflammatory response is quite weak. For Type I infected mice, inflammatory responses dominate, particularly on days 4, 5 and 7, whereas Type II infected mice had intermediate levels of inflammatory response, particularly on day 5.

Time course of DEGs

Genes that had at least 2-fold change in expression, which were also statistically significant (FDR q<=0.05) for at least 4 time points among infection of the three genotypes of parasites, were selected for time course analysis. The results are summarized in Figure VI-3. These genes include several immunity-related GTPases (IRGs), guanylate-binding proteins (GBPs), INF-γ, IL1b, TNF-α, CXCL9, CXCL10/IP-10, CCL12, chitinase 3-like 3 (Chi3l3/ECF-L/Ym1), serine

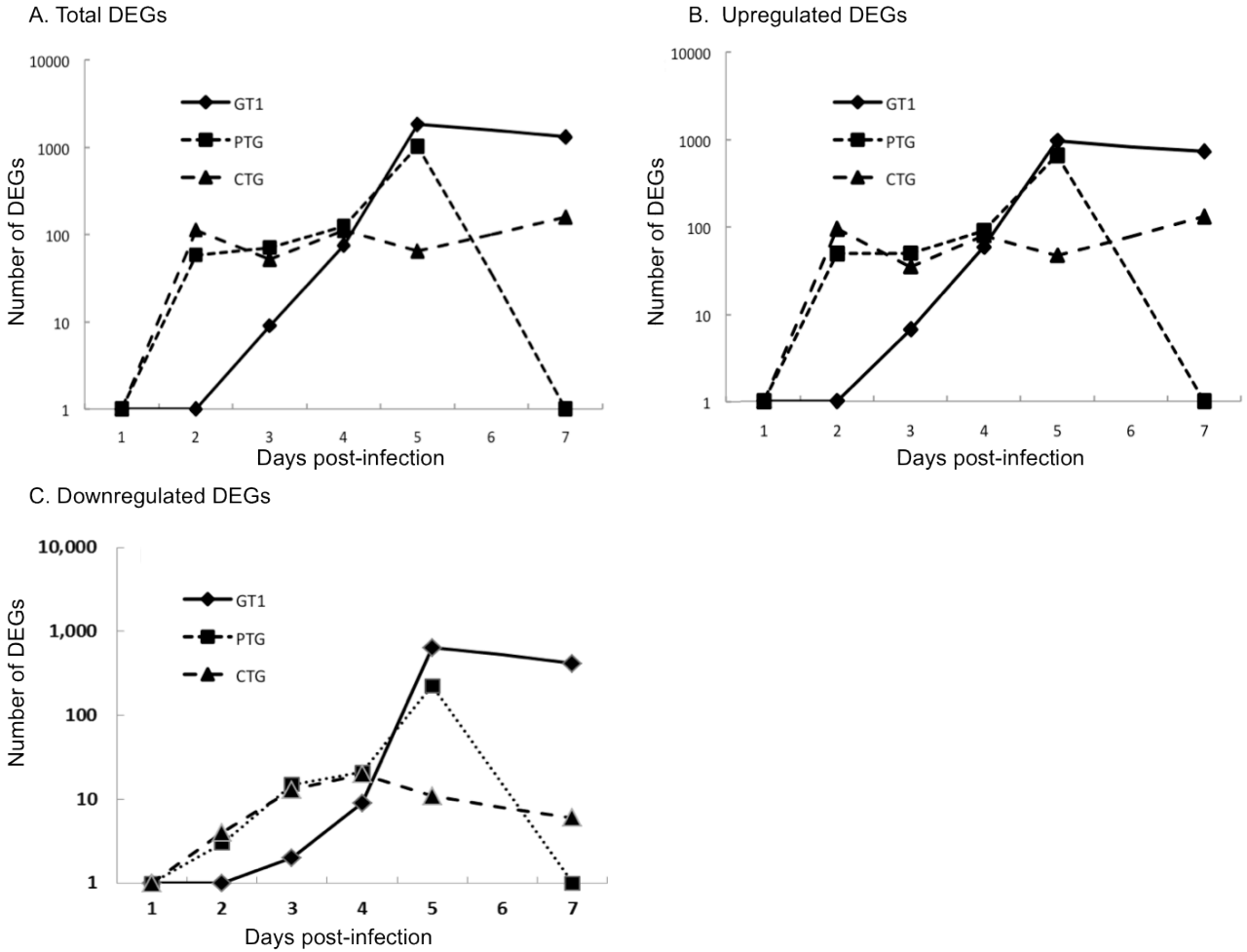


Figure VI-1. Temporal patterns of differentially expressed genes (DEGs). (A) Total DEGs over seven days post-infection for GT1 (Type I, diamonds), PTG (Type II, squares) and CTG (Type III, triangles). (B) Upregulated transcripts. (C) Downregulated transcripts.

Table VI-1. Immune response in mice infected with GT1, PTG and CTG strains.

Type I	day2	day3 (8, 8 w/ID)	day4 (77, 64 w/IDs)	day5 (1554, 1402 w/ IDs)	day7 (1293, 1182 w/ IDs)
	No significant changes	Immune Response (6), genes include CCL12, CXCL13, complement factor properdin, (CFP), complement component 1, s subcomponent (C1s), and T-cell spedific GTPase (Tgtp1/Irgb6), GBP1. Most enriched cluster (enrichment score 3.35) - Inflammatory response/response to wounding/defense response.	Immune Response (16), Regulation of T-cell Proliferation (5), Defense Response (9). Genes for Immune Response include XCL1, CCL4, CXCL9, CXCL10, CXCL13, INF-g, IL12b, interleukin 18 receptor accessory protein (IL18RAP), TNF-a, TNFRSF14, radical S-adenosyl methionine domain containing 2 (RSAD2), Tgtp1/Irgb6, Irgm1, GBP1, GBP4, GBP8/GBP10. Most enriched cluster (score: 4.87) -- cytokine-cytokine receptors.	Immune Response (134), Defense response (89), Response to wounding (74), Inflammatory response (57), Positive regulation of immune system process (50), negative regulation of immune system process (19). Most enriched cluster (score: 9.32): regulation of: cell activation/leukocyte activationn/lymphocyte activation.	Immune Response (129), Positive regulation of immune system process (62), Cell cycle (99), Defense response (81), Cell cycle process (70), Response to wounding (64), Inflammatory response (55). Negative regulation of immune system process (25). Most enriched cluster (score: 21.23) -- regulation of: cell activation/leukocyte activationn/lymphocyte activation.

Table VI-1 continued

Type II	day2 (59, 50 w/ ID)	day3 (72, 63 w/ID)	day4 (130, 108 wi/IDs)	day5 (775, 662 w/ IDs)	day7
	Immune Response (11), including Fc receptor, IgG, high affinity I (Fcgr1), IL18R, Iigp1/Irga6, Irgm1, Tgtp1/Irgb6, GBP1, GBP4, GBP8/GBP10, GBP9. Most enriched cluster (score: 5.92) -- GBP proteins.	Immune Response (13), Inflammatory response (7), Positive regulation of response to stimulus (6). Genes for Immune response include Fcgr1, CFP, CXCL9, CXCL10, IL1b, TNF-a (inflammatory response), IL1f9, macrophage activation 2 like (Mpa2L), IDO1, Tgtp1/Irgb6, GBP1, GBP4, GBP8/GBP10. Most enriched cluster (score: 4.64) -- GBP proteins.	Immune Response (14), genes include Fcgr1, CXCL9, CXCL10, CXCL13, IL12b, myosin 1f, collectin sub-family member 12, Iigp1/Irga6, Irgm1, Tgtp1/Irgb6, GBP1, GBP4, GBP8/GBP10, GBP9. Most enriched cluster (score: 4.76) -- GBP proteins.	Immune Response (72), Defense response (50), Inflammatory response (32), Response to wounding (36), Positive regulation of immune system process (25), negative regulation of immune system process (13). Most enriched cluster (score: 8.01) -- lectin/sugar binding/carbohydrate binding. At the medium level of stringency by DAVID clustering analysis, the most enriched cluster (score: 10.07) is defense response/inflammatory response/response to wounding.	No significant changes

Table VI-1 continued

Type III	day2 (111, 94 w/ ID)	day3 (51, 46 w/IDs)	day4 (126, 104 w/IDs)	day5 (68, 56 w/ID)	day7 (147, 131 w/IDs)
	Immune Response (21). Cell division (11), Mitosis (9). Genes for immune response include Fcgr1, C1s, XCL1, CXCL9, CXCL10, INF-g (inflammatory response), interferon induced with helicase C domain 1 (IFIH1), macrophage activation 2 like (Mpa2L), myxovirus (influenza virus) resistance 1 (mx1), serine (or cysteine) peptidase inhibitor, clade G, member 1 (SERPING1), toll-like receptor 2 (TLR2), tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10), TNFSF14, IigP1/Irga6, Tgtp1/Irgb6, Irgb10, Irgm1, GBP1, GBP4, GBP8/10, GBP9. Most enriched cluster (enrichment score: 6.29) -- GBP proteins.	Immune Response (9), Inflammatory Response (5), Positive Regulation of Apoptosis (5). Genes for Immune Response include Fcgr1, CXCL10, CXCL13, , Tgtp1/Irgb6, TNF-a, Iigp1/Irga6, GBP1, GBP4, GBP8/10. Most enriched cluster (enrichment score: 2.95) -- GBP proteins.	Immune Response (23), Defense Response (13). Inflammaroty Response (9), Positive Regulation of Response to Stimulus (8). Genes for Immune Response include Fcgr1, XCL1, CXCL9, CXCL10, CXCL13, CCL12, IL1b, IL1f9, CFP, IL18RAP, Mpa2L, TNF-a, TNFSF14, radical S-adenosyl methionine domain containing 2 (RSAD2), similar to T cell signal transduction molecule1 SAP; SH2 domain protein 1A (Sh2d1a), Iigp1/Irga6, Tgtp1/Irgb6, Irgb10, Irgm1, GBP1, GBP4, GBP8/10, GBP9. Most enriched cluster (score: 6.08) -- GBP proteins.	Immune Response (17). Genes include 2'-5' oligoadenylate synthetase 3 (Oas3), IL18RAP, IFIH1, interferon regulatory factor 7 (IRF7), Mpa2L, SERPING1, XCL1, tumor necrosis factor (ligand) superfamily, member 13b (TNFSF13b), Tgtp1/Irgb6, Ligp1/Irga6, Irgb10, Irgm1, GBP1, GBP3, GBP4, GBP8/10, GBP9. Most enriched cluster (score: 9.58) -- GBP proteins.	Immune Response (25), Regulation of Immue Effector Process (8). Genes for Immune Response include Fcgr1, XCL, INF-g, Oasl2, growth arrest and DNA-damage-inducible 45 gamma (Gadd45g), histocompatibility 2, Q region locus 1 (H2-Q1), histocompatibility 2, T region locus 9 (H2-T9), interferon regulatory factor 7 (TRF7), IL18BP, Mpa2L, SERPING1, CCL12, CXCL10, RSAD2, interferon gamma inducible protein 47 (Ifi47), Iigp1/Irga6, Irgb10, Irgm1, Tgtp1/Irgb6, GBP1, GBP2, GBP3, GBP4, GBP5, GBP8/10, GBP9. Most enriched cluster (score: 12.56) -- GBP proteins.

(or cysteine) peptidase inhibitor, clade G, member 1 (SerpinG1), serine (or cysteine) peptidase inhibitor, clade B, member 6b (SerpinB6b), Z-DNA binding protein 1 (Zbp1), Fc receptor, IgG, high affinity I (Fcgr1), Fc receptor, IgG, low affinity IV (Fcgr4), lymphocyte antigen 6 complex, locus C1 (Ly6c1), lymphocyte antigen 6 complex, locus I (Ly6i), V-set and immunoglobulin domain containing 4 (Vsig4), CXCL13, arachidonate 15-lipoxygenase (Alox15), and apolipoprotein E (Apoe).

Immunity-related GTPase (IRG) gene family upregulation

Among the highly up-regulated genes, a number of IRG proteins were identified, including Irga6, Irgb6, Irgm1 and Irgm3. Irga6 was shown to be highly upregulated during Type III infection from day 2 through the end of the study, with a dramatic peak on day 2 followed by more moderate upregulation during subsequent time points. In the case of Type II infection, upregulation was maintained from days 2-4, followed by a peak on day 5 and a marked decline on day 7. For Type I infection, upregulation declined during days 2-4, then increased on days 5 and 7. In the case of Irgb6, all three strains also caused early upregulation starting on day 2, though this was more marked in Type II and Type III infections than in Type I infection. Upregulation was maintained steadily during Type III infection, increased over time for Type I infection, and dropped to zero by day 7 for Type III infection. The regulatory IRGs Irgm1 and Irgm3 also displayed significant levels of upregulation. For both genes, this upregulation persisted from days 2 to 7 during Type III infection, though declining overall with time. Type II infection resulted in strong upregulation from day 2 to 5, followed by a drop to zero on day 7. Type I caused a relatively constant level of upregulation from days 2 through 7.

In addition to the IRGs, numerous genes encoding members of the p65 guanylate-binding protein (GBP) family were found to be upregulated during infection with all three strain types including the chromosome 3 GBPs Gbp1, Gbp3, Gbp5 and Gbp7, and the chromosome 5 GBPs Gbp4, Gbp6, Gbp8, Gbp9 and Gbp11. The chromosome 3 BGP genes all followed similar patterns of upregulation, showing strong upregulation on day two for Type III and Type II infections with comparatively lower upregulation for Type I infection. Upregulation associated with Type II and Type III infections dropped on day 3 and then rose through day 5, while levels associated with Type I infection remained relatively constant during this period. Upregulation remained relatively high on day 7 of Type III and Type I infections, but dropped to zero for Type II infection. For chromosome 5, Gbp4, Gbp6, Gbp8, Gbp9 and Gbp11 also all showed similar temporal patterns of expression, with upregulation observable for all strains on day 2, highest during Type III infection and lowest during Type I infection. Upregulation then declined through day 4 for Type III infection, then increased gradually through day 7. In the case of Type II infection, upregulation was maintained relatively constantly until dropping completely or to a low level on day 7. Type I infection, by contrast, resulted in a gradual and somewhat steady increase in upregulation over the course of the experiment, ultimately reaching levels comparable to those generated by Type III infection.

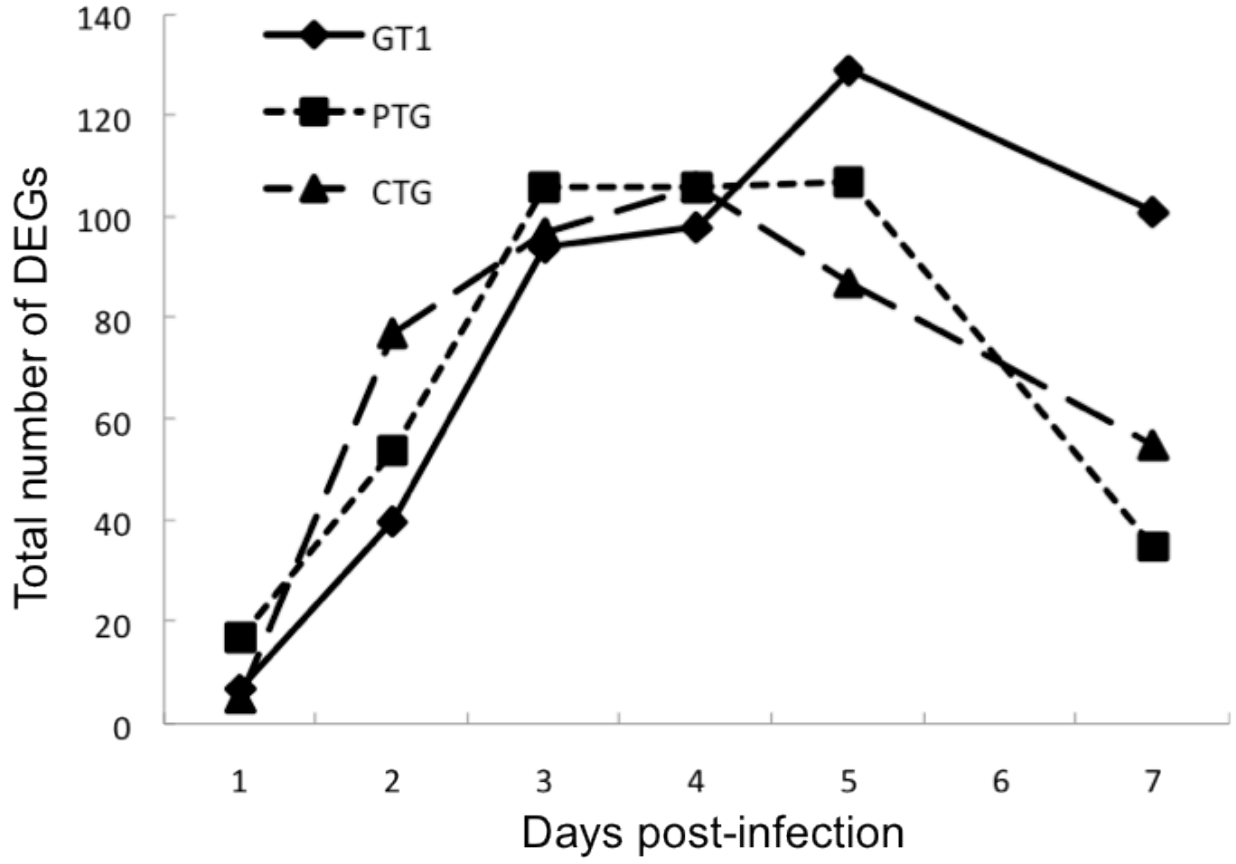


Figure VI-2. Temporal expression over seven days of mouse genes involved in immune response.

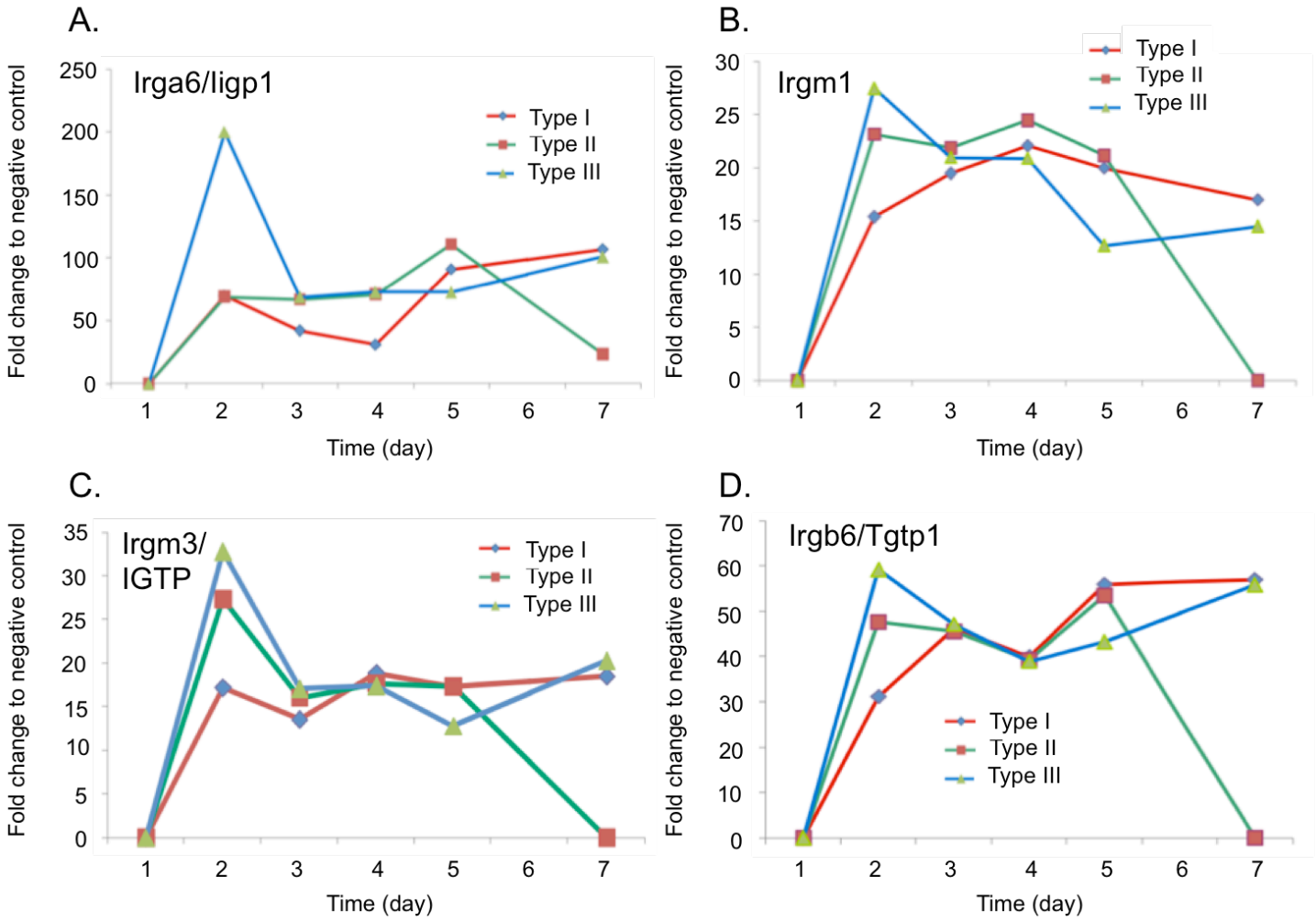


Figure VI-3. Expression of specific immunity related genes. Expression of genes over seven days follwing infection with Type I (GT1, red line), Type II (PTG, green line) or Type III (CTG, blue line).

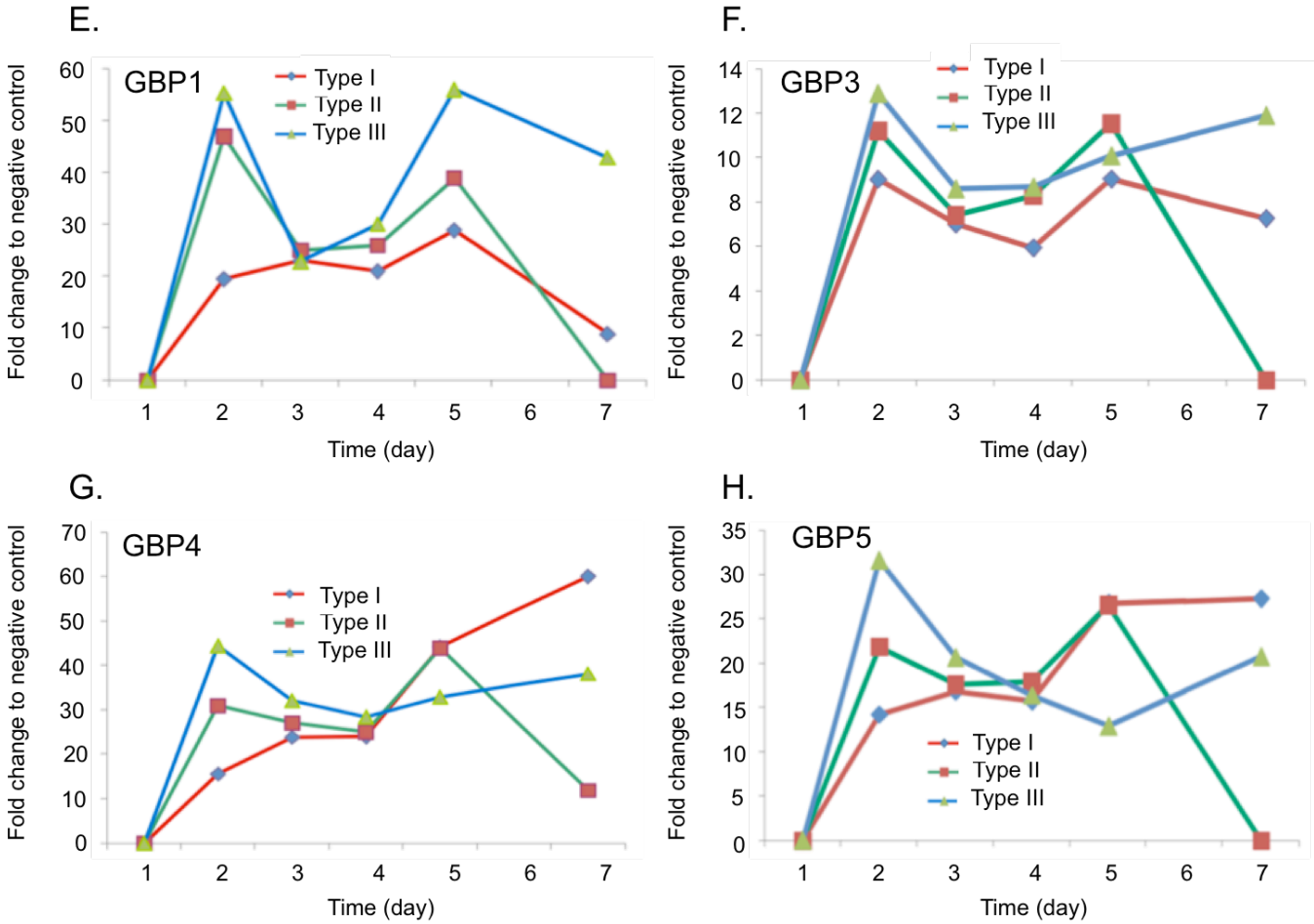


Figure VI-3. Expression of specific immunity related genes (continued).

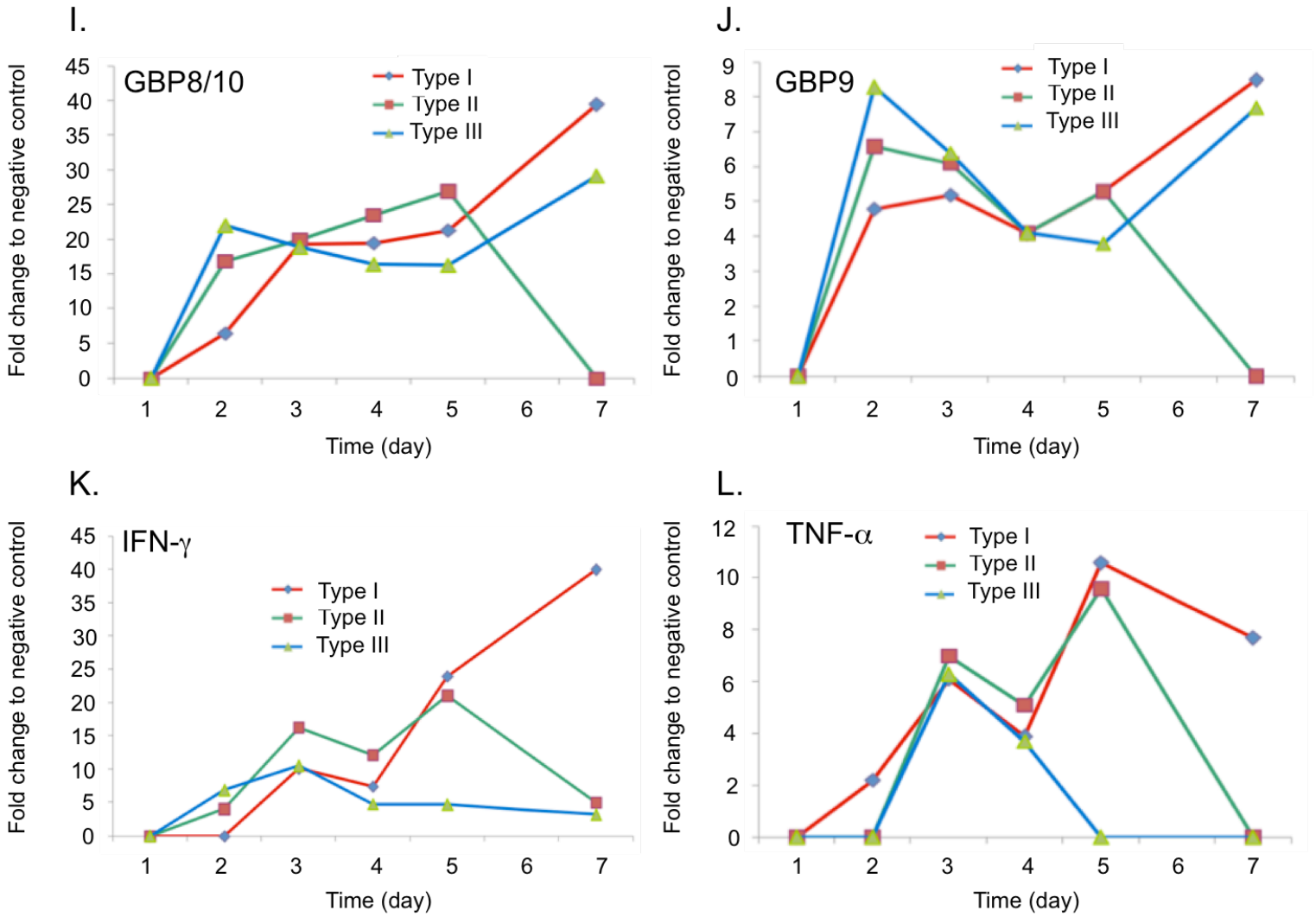


Figure VI-3. Expression of specific immunity related genes (continued).

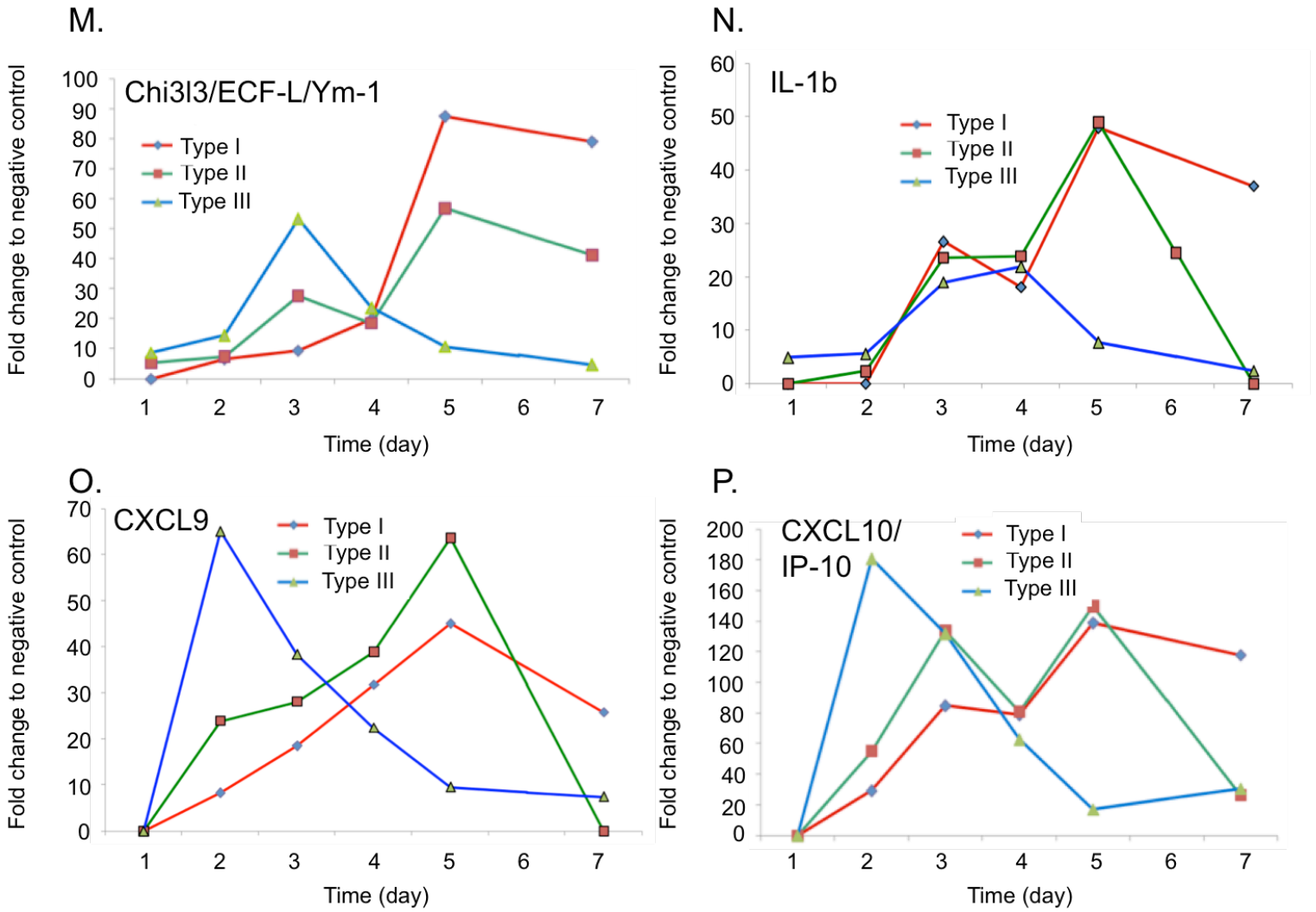


Figure VI -3. Expression of specific immunity related genes (continued).

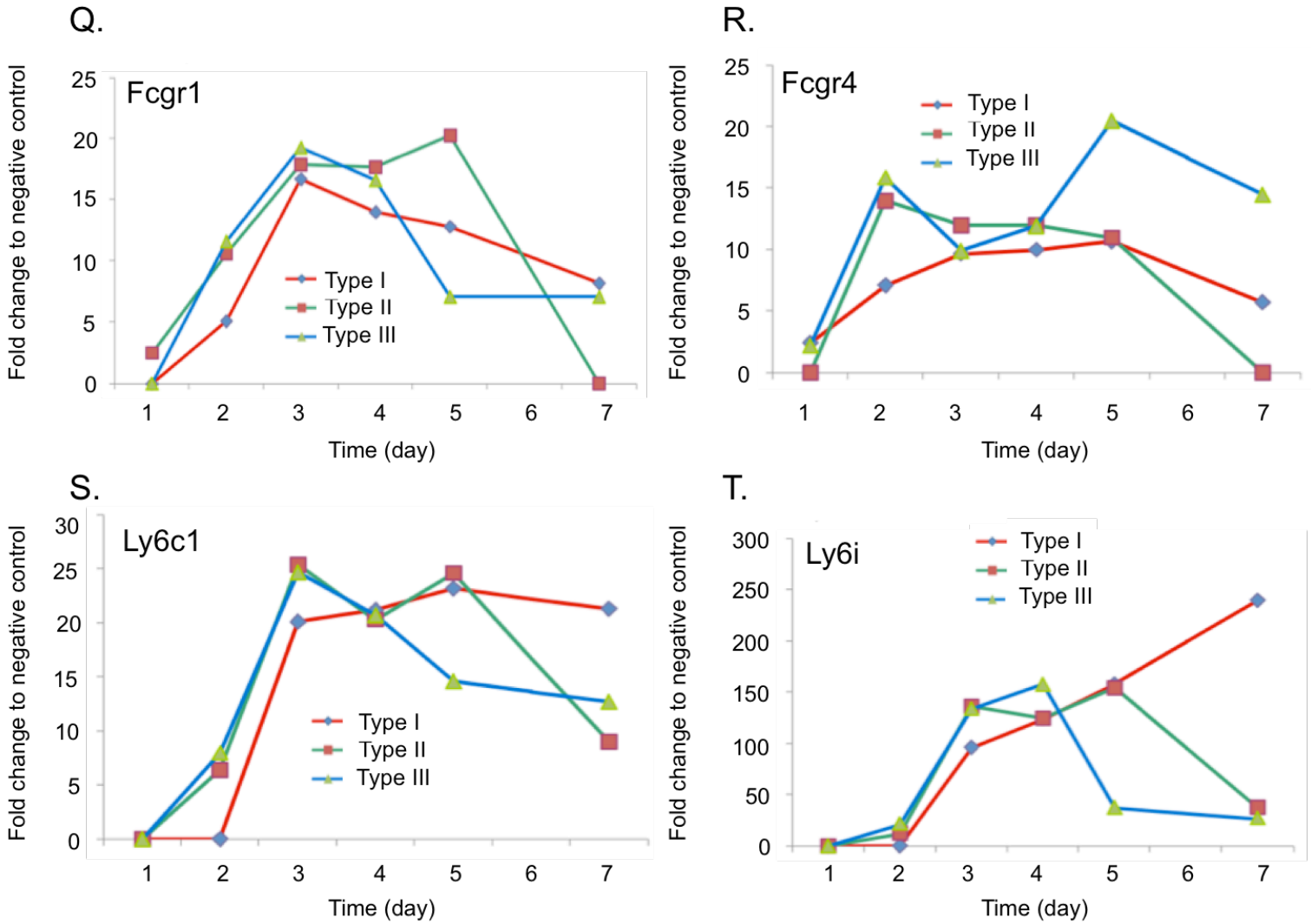


Figure VI-3. Expression of specific immunity related genes (continued).

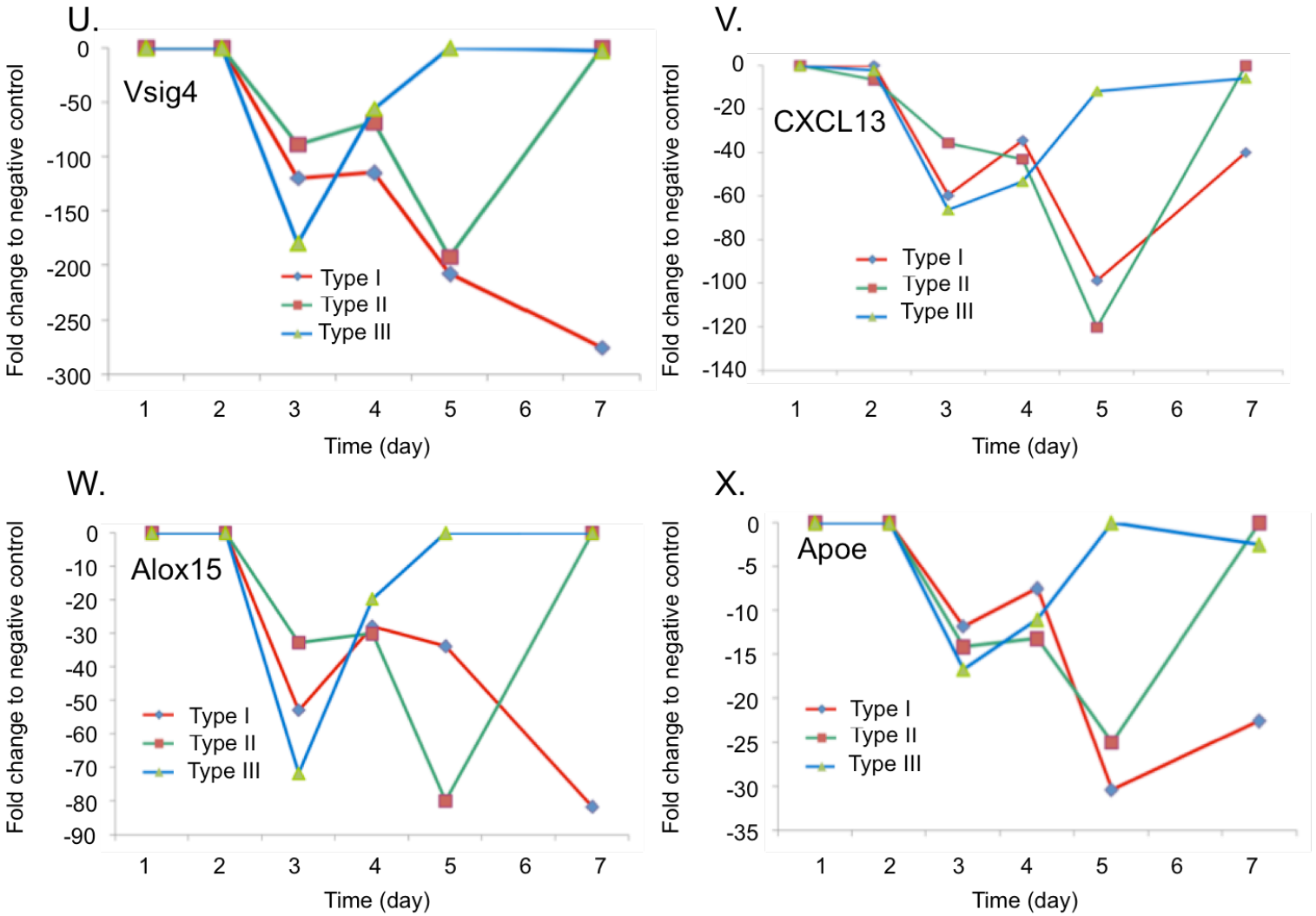


Figure VI-3. Expression of specific immunity related genes (continued)

Chemokine gene upregulation

In addition to the IRG and GBP genes, other immunity-related genes also showed significant upregulation. The chemokine gene CXCL10 was strongly upregulated on day 2 of Type III infection, then declined steady through day 7. Type II and Type I resulted in lower level upregulation on day 2, fluctuating and dropping to a minimal level on day 7 for Type II, and increasing steadily during the course of the experiment for Type I. The CXCL11 gene showed a sharp rise in upregulation during Type II infecton between days 1 and 3, followed by an equally sharp decline to zero between days 5 and 7. For Type III infection, only a single sharp spike on day 3 was observed, followed by a decline to zero upregulation by day 5. Type I showed no upregulation until day 3, followed by maintenance of fairly constant upregulation for the duration of the experiment. CXCL9 showed an early spike in upregulation on day 2 of Type III infection, followed by a steady decline to a minimal level by day 5. Upregulation of this gene increased steadily from days 2 through 5 for both Type II and Type I infections, then dropped for both strain types on day 7, completely so for Type II and only moderately for Type I. CCL2, CCL3, CCL4 CCL7, CCL8, CCL12 and CCL22 all showed similar patterns of expression, with a sharp peak in expression on day 3 of Type III infection, followed by a decrease to near zero by day 7 (data not shown). Type II infection resulted in fairly strong upregulation between days 3 and 5, followed by a sharp decline on day 7. Type I infection resulted in a sharp increase in upregulation from day 3 to 5 (with a moderate dip on day 4 in most cases), followed by a more gradual drop on day 7. In the case of CCL22, the increase from day 4 to 5 during Type I infection was especially dramatic relative to the expression levels associated with infection by other strain types (data not shown).

Upregulation of other immunity-related signaling molecule genes and related effectors

The IFNg displayed weak upregulation during Type III infection peaking at day 3, somewhat stronger upregulation during Type II infection peaking at day 5, and weak upregulation during Type I infection starting on either day 3 or 4, followed by a steady increase to high levels by day 7. TNF-a also was moderately upregulated, with all three strains producing a similar increase in expression on day 3, which then dropped to zero by day 5 for Type III infection and fluctuated before dropping to zero by day 7 for Type II infection. Type I infection produced a similar pattern to Type II infection through day 5, but dropped only slightly on day 7, so that unlike in the cases of Type II and Type III infection, upregulation was still high at the end of the experiment during Type I infection.

Other upregulated genes

Other genes were also upregulated, including the chitinase-like gene Ym1, which spiked on day three during Type III infection, then declined rapidly, while spiking and gradually declining on day 5 for Type II and Type I infections, with a stronger spike in the latter than in the former case. The high-affinity IgG receptor gene Fcgr1 showed similar patterns of upregulation for all three strains in the earlier stages of infection, increasing on days 2 and 3, followed by a plateau or small decline on day 4. After this point, upregulation showed a consistent but slow decline for Type I infection, a sharp drop followed by a plateau for Type III infection, so that both Type I and Type III infections produced similar expression levels on day 7, and a continued increase

followed by a sharp decline to zero on day 7 for Type II infection. The IgG/IgE receptor gene Fcgr4 showed stronger upregulation on day 2 for Type III and Type II infections than for Type I infection, followed by declines associated with the former strains and an increase associated with the latter, so that all three strains produced similar expression on days 3 and 4. However, while Type I and Type II infections both showed declines from days 5 to 7, upregulation associated with Type III infection increased considerably on day 5, followed by a moderate decline that still resulted in relatively strong upregulation on day 7.
Ly6c1 showed a dramatic increase in upregulation on day three during infections with all three strains types, followed by declines associated with Type II and Type III infections, while upregulation during Type I infection remained relatively constant. Ly6i showed a similar pattern of upregulation, except that upregulation was much higher overall, and during Type I infection it increased consistently from day 3 to day 7, rather than plateauing as in the case of Ly6c1 expression. The peptidase inhibitor Serpinb6b displayed a similar pattern of upregulation to Ly6i, though like Ly6c1 this was also on a smaller scale. Upregulation of the IFNg-induced Z-DNA-binding protein gene Zbp1 followed a pattern similar to that of Ly6c1, and on a similar scale, though upregulation dropped to zero during day 7 of Type II infection.

Downregulated genes

In addition to upregulation, many genes were also found to be downregulated during *T. gondii* infection, examples are Vsig4, CXCL13, Alox15 andApoE. Expression of these genes showed a general trend of strong downregulation on day 3 of infection with each strain type, followed by a return to control or near-control levels of expression on day 5 for Type III infections and day 7 for Type II infections. During Type I infections, downregulation of these genes generally fluctuated between days 4 and 7, though always ending up with significant downregulation on day 7.

Annotated pathway enrichment analysis

Bioinformatic analysis using the DAVID software revealed that sets of up- and downregulated genes associated with infection by each of the three *T. gondii* strains were enriched for genes associated with markedly different biological pathways, as shown in Tables VI-2-4. Type III infection was associated with enrichment of the smallest number of pathways annotated in the KEGG PATHWAY and BioCarta databases (Table VI-2). These comprised clusters of genes involved in cell-mediated immunity and inflammation (Including MHC Class I genes, perforin 1, IFNg, TNFa and IL-1) and cell cycle regulation (cyclins and cyclin kinase inhibitors).

For Type II infection, 7 enriched clusters were identified (Table VI-3). All of these comprised cell-mediated immunity-related pathways such as those relating to CD4+ T Cell activation and Th1 differentiation (IL-12, IL-12 receptor, CD3, IFNg, IFNg receptor, TNFa), proinflammatory cytokine production and cytotoxicity (IFNg, TNFa, IL-10, CCL3, CCL4, IL-1, granzyme B, Nos2), T-Cell and other leukocyte signaling and surface molecules (selectins, ICAM-1, Lck, Zap70) and apoptosis (Fas, FasL, Caspase 7, IkBa).

Type I infection was associated with enrichment of the largest number of identified clusters, 15 in all (Table VI-4). These included many immunity-related gene clusters similar to those

enriched during Type II and Type III infection, but in general the number of DEGs per cluster was higher, and comprised a greater diversity of pathways. These included immune pathways relating to CD4+ T-Cell differentiation and proliferation (CD28, CD4, CD3, IL-2, IL-12, IL-12 receptor, IL-18 receptor, IL-4 receptor, CXCR3, colony-stimulating factor 2), inflammation (CCL3, CCL4, CCR2, CCR5, IFNg, IFNg receptor, iNOS2), apoptotic pathways (fas, fasL, fas death domain associated protein (DAXX), MAPKAPK2, caspase 3, 7, cytochrome C, TNFa, TNFa receptor, mapk13, IkBa, PKC), the complement pathway (complement component 1, 6, C1, C3 prepropeptide, complement component factor H, factor B, complement receptor 2) and T Cell signaling (CD3, CD4, CD8, CD28, IL-6, fas, fasL, fyn proto oncogene, lymphocyte protein tyrosine kinase, zap kinase, ctl associated protein4, inducible T-cell co-stimulator). Pathway clusters not directly related to the immune response were also enriched, including those associated with DNA replication and damage response (dna primase p49 subunit, DNA replication helicase 2 homologue (yeast), ATP-dependent DNA ligase I, DNA polymerase subunits, replication factors, exonuclease I, checkpoint kinase 1 homologue (S. pombe), polo-like kinase, Brca1), carbohydrate metabolism (galactose, starch, sucrose, amino sugar and nucleotide sugar metabolic enzymes), muscle functioning and Calcium channel regulation (voltage dependent calcium channel subunits, insulin-like growth factor 1, integrins, AAPK gamma2 subunit, cardiac ryanodine receptor, solute carrier family proteins, TGFb, actinin A1, gap junction protein alpha 1, junction plakoglobin, cardiac RyR2), cell-cycle regulation (cell division cycle protein homologues (S. pombe), cyclins, CDPK6, P21 CDPK inhibitor, dihyrofolate reductase, IkBa), transcriptional regulation and cellular signaling (calmodulin, jun oncogene, phospholipase C, PKC, vav1 oncogene, endothelin1, Fyn protooncogene, STAT1, Igf1, inositol monophosphatase , phosphoinositide-3-kinase, neuregulin 4, jun oncogene, MyD88). This latter category also included genes related to B-cell response and humoral immunity (B-Cell Linker protein, CD79B, high affinity Ige Fc receptor (alpha polypeptide), membrane spanning 4 domains subfamily A2).

Microarray data validation by quantitative real-time RT-PCR (qRT-PCR)

To validate the microarray data, four up-regulated transcripts (IFN-γ, iNOS, IL-12β and TNF) and one down-regulated gene (CXCL13) at five time points (days 1, 2, 3, 4, and 5) were used for qRT-PCR analysis. RNA samples from day 7 were accidently lost during storage and could not be included in analysis. TaqMan Gene Expression Assays (Applied Biosystems) were applied for IFN-γ (assay ID: Mm01168134_m1), iNOS (Mm00440502_m1), IL-12β (Mm00434174_m1), TNF (Mm00443260_g1), and CXCL13 (Mm04214185_s1). As shown in Figure VI-4, the qRT-PCR expression data of five transcripts at five time points revealed overall agreement with the microarray data (R^2=0.7964). There is some degree of variation between the microarray and qRT-PCR data, but magnitudes of expression changes measured by each method were similar.

Determination of parasite load by qPCR

Parasite loads in the spleens of mice infected with 500 tachyzoites on day 1, 2, 3, 4 and 5 post-infection were quantified. Since the parasite was not detectable in some samples on day 1, the data generated from day 1 were excluded in the following analysis. Parasites load on day 2 through day 5 in three infection groups are displayed in Figure VI-5. No significant differences in parasite loads were observed from day 2 to day 4 among the three groups; however, the

Table VI-2. Enrichment of functional gene clusters during CTG infection

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
1	1.52	Cell-mediated immunity (Th1 response)	KEGG Pathway	Type I diabetes mellitus	6	1.40E-03	7.10E+00
			KEGG Pathway	Allograft rejection	5	7.00E-03	6.40E+00
			KEGG Pathway	Autoimmune thyroid disease	3	2.50E-01	3.10E+00
			KEGG Pathway	Viral myocarditis	3	3.50E-01	2.40E+00
2	1.16	Cell Cycle Regulation	KEGG Pathway	Cell cycle	6	2.70E-02	3.50E+00
			KEGG Pathway	p53 signaling pathway	4	6.30E-02	4.30E+00
			KEGG Pathway	Oocyte meiosis	4	1.90E-01	2.60E+00

Table VI-3. Enrichment of functional gene clusters during PTG infection

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
1	3.33	Cell-mediated immunity/ Th1 response/ inflam-mation	KEGG Pathway	Graft-versus-host disease	13	3.10E-06	5.40E+00
			KEGG Pathway	Allograft rejection	10	5.40E-04	4.20E+00
			KEGG Pathway	Type I diabetes mellitus	10	1.00E-03	3.80E+00
			KEGG Pathway	Autoimmune thyroid disease	8	2.80E-02	2.70E+00
2	2.27	T-Cell surface proteins/ migration	BIOCARTA	CTL mediated immune response against target cells	7	3.20E-05	9.10E+00
			BIOCARTA	T Cytotoxic Cell Surface Molecules	5	6.90E-03	6.00E+00
			BIOCARTA	T Helper Cell Surface Molecules	5	6.90E-03	6.00E+00
			KEGG Pathway	Leukocyte transendothelial migration	6	5.50E-01	1.20E+00

Table VI-3. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
3	1.93	CD4 Th1 Differen-tiation/ NK cyto-toxicity	BIOCARTA	Selective expression of chemokine receptors during T-cell polarization	7	6.80E-03	3.80E+00
			BIOCARTA	NO2-dependent IL 12 Pathway in NK cells	5	9.40E-03	5.50E+00
			BIOCARTA	IL12 and Stat4 Dependent Signaling Pathway in Th1 Development	6	9.50E-03	4.30E+00
			BIOCARTA	Th1/Th2 Differentiation	5	3.10E-02	4.00E+00
4	1.64	Cytokines and Inflammatory Response	KEGG Pathway	Allograft rejection	10	5.40E-04	4.20E+00
			BIOCARTA	Cytokine Network	4	1.10E-01	3.40E+00
			BIOCARTA	Cytokines and Inflammatory Response	4	2.10E-01	2.50E+00

Table VI-3. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
5	1.14	Apoptosis/ NF-kB	KEGG Pathway	Apoptosis	9	2.60E-02	2.50E+00
			BIOCARTA	NFkB activation by Nontypeable Hemophilus influenzae	5	6.90E-02	3.10E+00
			BIOCARTA	Signal transduction through IL1R	5	8.80E-02	2.90E+00
			BIOCARTA	NF-kB Signaling Pathway	4	1.70E-01	2.70E+00
6	1.12	Leukocyte Surface Molecules	BIOCARTA	Monocyte and its Surface Molecules	4	3.50E-02	5.20E+00
			BIOCARTA	Neutrophil and Its Surface Molecules	3	1.00E-01	5.40E+00
			BIOCARTA	Adhesion Molecules on Lymphocyte	3	1.20E-01	4.80E+00

Table VI-3. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
7	1.19	T Cell Signaling/ activation	KEGG Pathway	T cell receptor signaling pathway	12	9.20E-03	2.50E+00
			BIOCARTA	Lck and Fyn tyrosine kinases in initiation of TCR Activation	4	2.00E-02	6.30E+00
			BIOCARTA	The Co-Stimulatory Signal During T-cell Activation	4	9.30E-02	3.60E+00
			BIOCARTA	T Cell Receptor Signaling Pathway	6	9.70E-02	2.40E+00
			BIOCARTA	Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor	4	1.10E-01	3.40E+00
			KEGG Pathway	Primary immunodeficiency	3	4.40E-01	2.00E+00

Table VI-4. Enrichment of functional gene clusters during GT1 infection

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
1	2.7	T-Cell Differentiation/ inflam-mation	BIOCARTA	Selective expression of chemokine receptors during T-cell polarization	15	1.70E-04	2.80E+00
			BIOCARTA	NO2-dependent IL 12 Pathway in NK cells	9	1.50E-03	3.30E+00
			BIOCARTA	IL12 and Stat4 Dependent Signaling Pathway in Th1 Development	11	3.20E-03	2.60E+00
			BIOCARTA	Th1/Th2 Differentiation	9	1.90E-02	2.40E+00
2	2.67	DNA replication/ repair	KEGG Pathway	DNA replication	17	6.20E-07	4.10E+00
			KEGG Pathway	Mismatch repair	7	3.90E-02	2.70E+00
			KEGG Pathway	Nucleotide excision repair	7	4.10E-01	1.40E+00

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
3	1.78	T Cell Signaling	BIOCARTA	HIV Induced T Cell Apoptosis	8	3.80E-04	4.30E+00
			BIOCARTA	CTL mediated immune response against target cells	8	2.30E-03	3.50E+00
			BIOCARTA	Lck and Fyn tyrosine kinases in initiation of TCR Activation	7	3.60E-03	3.70E+00
			BIOCARTA	T Helper Cell Surface Molecules	8	4.60E-03	3.20E+00
			BIOCARTA	T Cytotoxic Cell Surface Molecules	8	4.60E-03	3.20E+00
			BIOCARTA	The Co-Stimulatory Signal During T-cell Activation	8	3.10E-02	2.40E+00
			BIOCARTA	IL 17 Signaling Pathway	6	1.10E-01	2.20E+00
			BIOCARTA	Role of Tob in T-cell activation	6	1.50E-01	2.10E+00

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
3 (cont.)	1.8	T Cell Signaling	BIOCARTA	T Cell Receptor and CD3 Complex	3	1.90E-01	3.60E+00
			BIOCARTA	Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor	6	2.70E-01	1.70E+00
4	1.76	Fas Mediated Apoptosis	BIOCARTA	HIV Induced T Cell Apoptosis	8	3.80E-04	4.30E+00
			BIOCARTA	Bystander B Cell Activation	4	1.10E-01	3.20E+00
			BIOCARTA	Antigen Dependent B Cell Activation	5	1.30E-01	2.40E+00

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
5	1.27	Cell-mediated immunity	KEGG Pathway	Graft-versus-host disease	15	6.80E-03	2.20E+00
			KEGG Pathway	Type I diabetes mellitus	14	3.20E-02	1.90E+00
			KEGG Pathway	Allograft rejection	12	7.70E-02	1.70E+00
			KEGG Pathway	Autoimmune thyroid disease	10	4.90E-01	1.20E+00
6	1.03	Comple-ment	KEGG Pathway	Complement and coagulation cascades	18	6.00E-03	2.00E+00
			BIOCARTA	Classical Complement Pathway	6	1.10E-01	2.20E+00
			BIOCARTA	Complement Pathway	7	1.50E-01	1.90E+00
			BIOCARTA	Lectin Induced Complement Pathway	3	7.90E-01	1.10E+00

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
7	0.62	Fas Signaling/ Apoptosis	BIOCARTA	Stress Induction of HSP Regulation	7	2.30E-02	2.80E+00
			BIOCARTA	Regulation of transcriptional activity by PML	5	3.80E-01	1.60E+00
			BIOCARTA	FAS signaling pathway (CD95)	8	4.00E-01	1.30E+00
			BIOCARTA	Keratinocyte Differentiation	10	4.20E-01	1.20E+00
			BIOCARTA	HIV-I Nef	12	5.30E-01	1.10E+00
8	0.61	Carbo-hydrate Metabolism	KEGG Pathway	Galactose metabolism	7	9.30E-02	2.20E+00
			KEGG Pathway	Starch and sucrose metabolism	7	2.50E-01	1.60E+00
			KEGG Pathway	Amino sugar and nucleotide sugar metabolism	6	6.10E-01	1.10E+00

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
9	0.35	Heart Disease, muscle function, Ca channel regulation	KEGG Pathway	Hypertrophic cardiomyopathy (HCM)	13	2.90E-01	1.30E+00
			KEGG Pathway	Dilated cardiomyopathy	12	5.40E-01	1.10E+00
			KEGG Pathway	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	10	5.40E-01	1.10E+00
10	0.31	Caspase mediated apoptosis	BIOCARTA	Apoptotic Signaling in Response to DNA Damage	7	2.20E-01	1.70E+00
			BIOCARTA	Induction of apoptosis through DR3 and DR4/5 Death Receptors	7	5.80E-01	1.20E+00
			BIOCARTA	Caspase Cascade in Apoptosis	5	6.60E-01	1.10E+00
			BIOCARTA	Role of Mitochondria in Apoptotic Signaling	5	6.60E-01	1.10E+00

Table VI-4. Continued

Cluster	Enrich-ment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
11	0.3	Leukocyte surface molecules	BIOCARTA	Monocyte and its Surface Molecules	4	4.10E-01	1.70E+00
			BIOCARTA	Neutrophil and Its Surface Molecules	3	5.20E-01	1.80E+00
			BIOCARTA	Adhesion Molecules on Lymphocyte	3	5.90E-01	1.60E+00
12	0.27	Cell Cycle Regulation	BIOCARTA	Cyclins and Cell Cycle Regulation	7	3.40E-01	1.50E+00
			BIOCARTA	Cell Cycle	6	6.20E-01	1.20E+00
			BIOCARTA	Influence of Ras and Rho proteins on G1 to S Transition	5	7.70E-01	1.00E+00
13	0.26	DNA Damage Response	BIOCARTA	cdc25 and chk1 Regulatory Pathway in response to DNA damage	3	4.40E-01	2.10E+00
			BIOCARTA	Cell Cycle	6	5.40E-01	1.30E+00
			BIOCARTA	RB Tumor Suppress/Checkpoint	3	7.00E-01	1.30E+00

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
14	0.21	Transcriptional regulation/ signaling pathways	BIOCARTA	Pertussis toxin-insensitive CCR5 Signaling in Macrophage	8	3.10E-02	2.40E+00
			BIOCARTA	Effects of calcineurin in Keratinocyte Differentiation	5	2.20E-01	2.00E+00
			BIOCARTA	Phospholipase C Signaling Pathway	3	4.40E-01	2.10E+00
			BIOCARTA	Role of EGF Receptor Transactivation by GPCRs in Cardiac Hypertrophy	5	4.80E-01	1.40E+00
			BIOCARTA	BCR Signaling Pathway	7	5.80E-01	1.20E+00
			BIOCARTA	Fc Epsilon Receptor I Signaling in Mast Cells	7	6.80E-01	1.00E+00
			BIOCARTA	Signaling Pathway from G-Protein Families	5	8.00E-01	9.60E-01

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
14 (cont.)	0.2	Transcript-ional regulation/ signaling pathways	BIOCARTA	Bioactive Peptide Induced Signaling Pathway	6	8.30E-01	9.00E-01
			BIOCARTA	Links between Pyk2 and Map Kinases	5	8.40E-01	8.90E-01
			BIOCARTA	Angiotensin II mediated activation of JNK Pathway via Pyk2 dependent signaling	5	8.60E-01	8.60E-01
			BIOCARTA	TPO Signaling Pathway	4	8.90E-01	8.30E-01
			KEGG Pathway	Glioma	6	8.90E-01	7.90E-01
			KEGG Pathway	Phosphatidylinositol signaling system	7	9.00E-01	7.80E-01
			BIOCARTA	Signaling of Hepatocyte Growth Factor Receptor	5	9.30E-01	7.50E-01
			BIOCARTA	EGF Signaling Pathway	4	9.50E-01	6.90E-01
			BIOCARTA	PDGF Signaling	4	9.50E-01	6.90E-01

Table VI-4. Continued

Cluster	Enrichment Score	Function	Database	Pathway	DEG Count	P-Value	Pathway Enrichment
14 (cont.)	0.2	Transcriptional regulation/ signaling pathways	KEGG Pathway	ErbB signaling pathway	7	9.60E-01	6.70E-01
			BIOCARTA	Agrin in Postsynaptic Differentiation	3	9.80E-01	5.50E-01
15	0.21	NF-kB/AP-1 stress response regulation	BIOCARTA	Signal transduction through IL1R	7	4.20E-01	1.30E+00
			BIOCARTA	Cadmium induces DNA synthesis and proliferation in macrophages	4	5.80E-01	1.40E+00
			BIOCARTA	TNF/Stress Related Signaling	3	9.80E-01	5.80E-01

parasite load on day 5 in the GT1 group was significantly higher than that of the PTG and CTG groups. The mean value of the parasite loads in GT1, PTG and CTG were 2.05×10^7, 4.89×10^6, and 3.63×10^6 parasites per gram of spleen, respectively.

Discussion

The purpose of this study was to reveal how the host responds to infection by distinct *T. gondii* strains using the murine model. Our results showed that the host responses were dynamic and different among the three *T. gondii* strains tested. At the early stage of acute infection (i.e., from day 1 through day 3), the number of DEGs in the type I strain GT1 infection was lower than those of the type II PTG and type III CTG infections. However, by day 4, all three strain types showed a comparable number of DEGs. While DEGs associated with CTG infection then reached a plateau, the numbers associated with GT1 and PTG continued to increase on day 5 to an approximately equal level, which is maintained by GT1 infection through day 7, while dropping to zero for PTG infection at this point. The dynamic changes are more prominent in the up-regulated genes (Fig. VI-1B). Due to the lack of data in day 6, it is not clear the number of DEGs associated with PTG infection started to decline on day 5 or day 6, but likely peaked between day 5 and day 6. For this strain type, it seems that the period of day 5 to day 7 is an important window when the host response underwent drastic changes to *T. gondii* infection. These changes may be the consequence of negative feedback of host responses to dampen detrimental responses of *T. gondii* infection. The degree of expression and composition of genes affected may play a key role in determining different outcomes of infection with three distinct strains.

Though a wide range of functional groups were found to be significantly changed by *T. gondii* infection, immune response is the major groups affected (Figure VI-2). Regarding inflammatory and immune response, the number of genes affected follow the same trend for all genes counted (Figure VI-1A and Figure VI-2A). However, the majority of genes for inflammatory and immune response are up-regulated from day 1 to day 7. GT1 strain infection induced a remarkable increase in expression of the inflammation and immune response associated genes compared with PTG and CTG strains infection on day 7 (Figure VI-2). Clinically, the mice infected with the GT1 strain had a significantly higher parasite load than that of PTG and CTG strains on day 5 (Figure VI-6), and the animals started to show severe symptoms of infection on day 7, while those infected by the type II or III did not have obvious symptoms of disease. In accordance with our previous data (Xiao *et al.* 2011), many genes such as CCL2, CCL3, CCL4, CCL7, CCL8, CCL12, CCL22, CXCL10, CXCL11, Myd88 and TNF, were highly expressed in GT1 infection at later stage of acute infection, which may contribute to a lethal inflammatory response to this highly virulent strain. Many of the genes of inflammatory and immune response, such as Iigp1/Irga6, Irgm1, CXCL10/IP-10, INF-γ, iNOS, CXCL13, CCL2, CCL3, CCL7, CCL8, CCL12, CCL22, CXCL11, IL-1b, Myd88, and TNF, showed bimodal responses with the first peak expression at day 3 (except Iigp1/Irga6 at day 2), and then a second peak expression mostly at day 5. These responses may reflect oscillations which are common in the immune system (Stark *et al.* 2007). In addition to bimodal responses, expression levels of these genes vary widely among the infections of the three different *T. gondii* strains (Figure VI-3), which may contribute to different outcome in infected hosts. Of these genes, Iigp1/Irga6, CXCL10/IP-10, INF-γ and iNOS are known host factors that are important to control *T. gondii* infection.

One of the genes found to be most highly upregulated during infections with all three strain types was Iigp1/Irga6, a member of the interferon-inducible immunity-related GTPases (IRG proteins/p47 GTPases), which are key factors in murine defense against *T. gondii* infection (Dupont *et al.* 2012). In INF-γ-stimulated cells, the IRGs have been found to accumulate on *T. gondii* parasitophorous vacuole membranes (PVM) in a stepwise manner, resulting in constriction and ultimate disruption of the PVM, followed by parasite clearance. *Mus musculus* expresses at least 19 of these genes, which are clustered on chromosomes 11 and 18. Several other members of this class were also found to be highly upregulated in this study, including Irgb6, Irgm1 and Igrm3. Irgb6 and Irga6 have been identified as two of the earliest IRGs recruited to the PVM, and their presence is also necessary for localization of subsequent IRGs, making them highly important to this defense mechanism. Irgm1 and Irgm3 also play a key role by associating with host intracellular membranes, specifically those of the lysosomes and the Golgi apparatus in the case of Irgm1 and the endoplasmic reticulum in the case of Irgm3, protecting them from disruption by the effector IRGs like Irga6 and Irgb6 and thus preventing deleterious autoimmune effects. Deletion of either Irgm1 or Irgm3 has previously been shown to result in strongly increased susceptibility of mice to *T. gondii* infection. Parasite defense against the IRG immune response involves the virulence factors ROP18 and ROP5, which in Type I strains inactivate IRG proteins in a collaborative manner. During infection by virulent *T. gondii* strains including Type I strains such as GT1, the rhoptry kinase ROP18 is secreted into the host cytoplasm where, with catalytic activity enabled by interaction with the rhoptry pseudokinase ROP5, it phosphorylates IRGs at specific conserved threonine residues, thereby preventing their colonization of the PVM. Type III strains such as CTG do not express ROP18 and thus are unable to prevent parasite clearance in this manner, while Type II strains such as PTG lack the proper allele or alleles of ROP5 needed to enhance the kinase activity of ROP18, and thus are also deficient in protecting against clearance (Fleckenstein *et al.* 2012). In the current experiment, both effector and regulatory IRGs showed similar patterns of expression, with upregulation occurring early (day 2) in the course of PTG and CTG expression and later (days 3 through 5) during GT1 infection. Then upregulation was found to diminish to insignificant levels between days 5 and 7 in PTG infection while being maintained through day 7 for CTG and GT1 infections.

In addition to the IRG proteins, *Mus musculus* also expresses a second family of IFNg-activated GTPases known as the p65 guanylate-binding proteins (GBPs), the 11 active genes encoding which are clustered in tandem on chromosomes 3 and 5. Loss of the chromosome 3 GBPs has been shown to dramatically increase susceptibility of mice to *T. gondii*. Like the IRGs, GBPs also accumulate on the PVM shortly after invasion by *T. gondii*, an action which is inhibited by virulent strains of the parasite. Unlike the IRGs, which cause direct disruption of the PVM, GBPs act by recruiting protein complexes to the membrane, such as the inflammosome, autophagy apparatus, and NOX2 NADPH oxidase, which have antimicrobial functions. GBPs are also blocked from binding to "self" intracellular membranes by IRGM proteins in a similar manner to IRGs. Furthermore, ROP18 has recently been found to interact with both murine and human GBPs in vitro (Sibley, unpublished). In the current study, the majority of known GBPs from both the chromosome 3 and 5 clusters were found to be upregulated during infection with all three strain types. It is noteworthy that all of the GBPs clustered on chromosome 5 followed essentially the same pattern of expression as was observed for the IRGs, whereas the chromosome 3 GBPs were only found to be upregulated starting at day 5 for all strain types, with

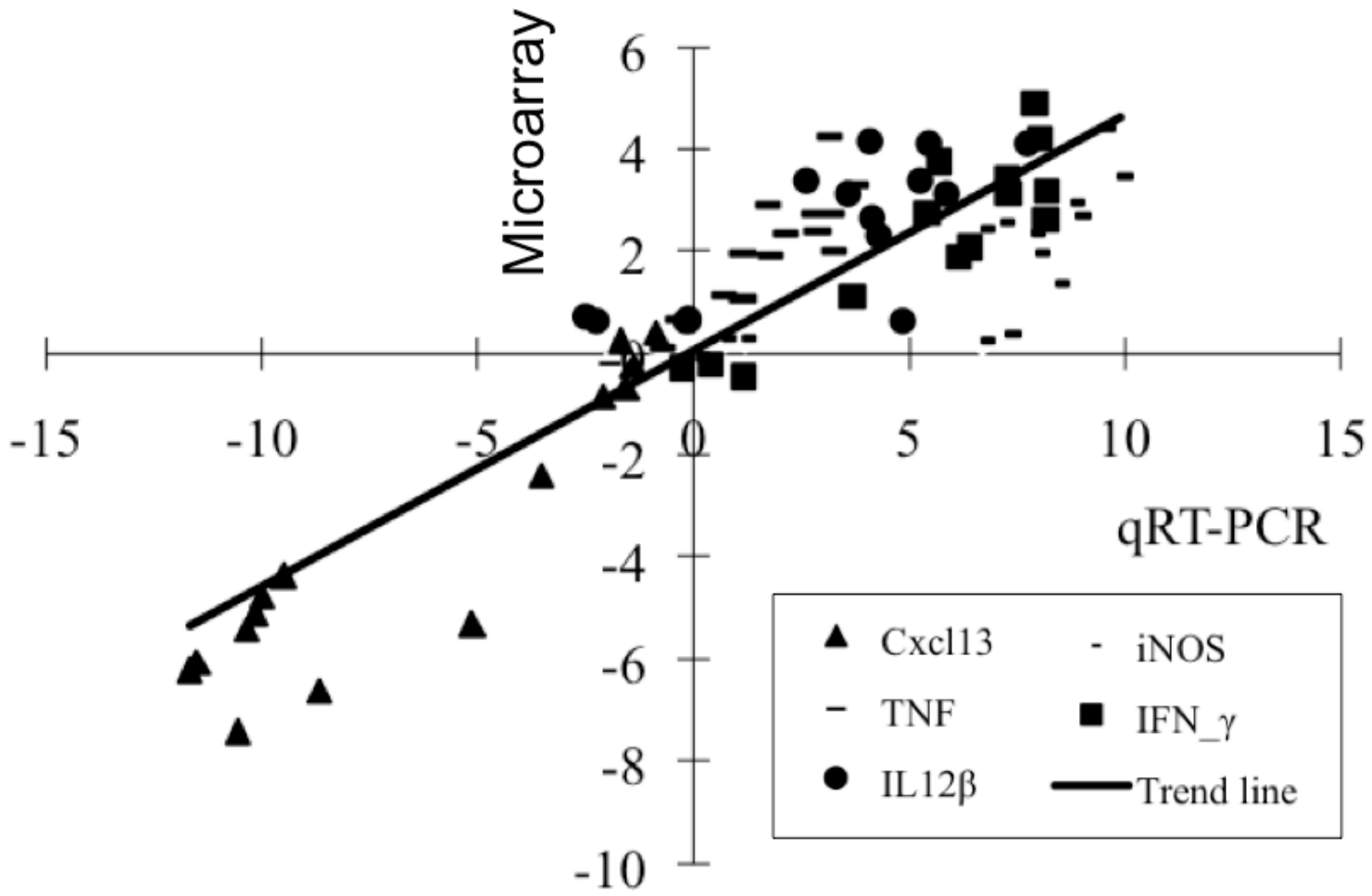


Figure VI-4. Comparison of microarray and qRT-PCR expression data. Expression levels of five genes as determined by qRT-PCR (X-axis) plotted against expression levels determined via microarray (Y-axis). Genes include Cxcl13 (triangles), TNF (dashes), IL-12B (circles), iNOS (small dashes) and IFNg (squares). Trendline for data is shown (R^2=0.7964).

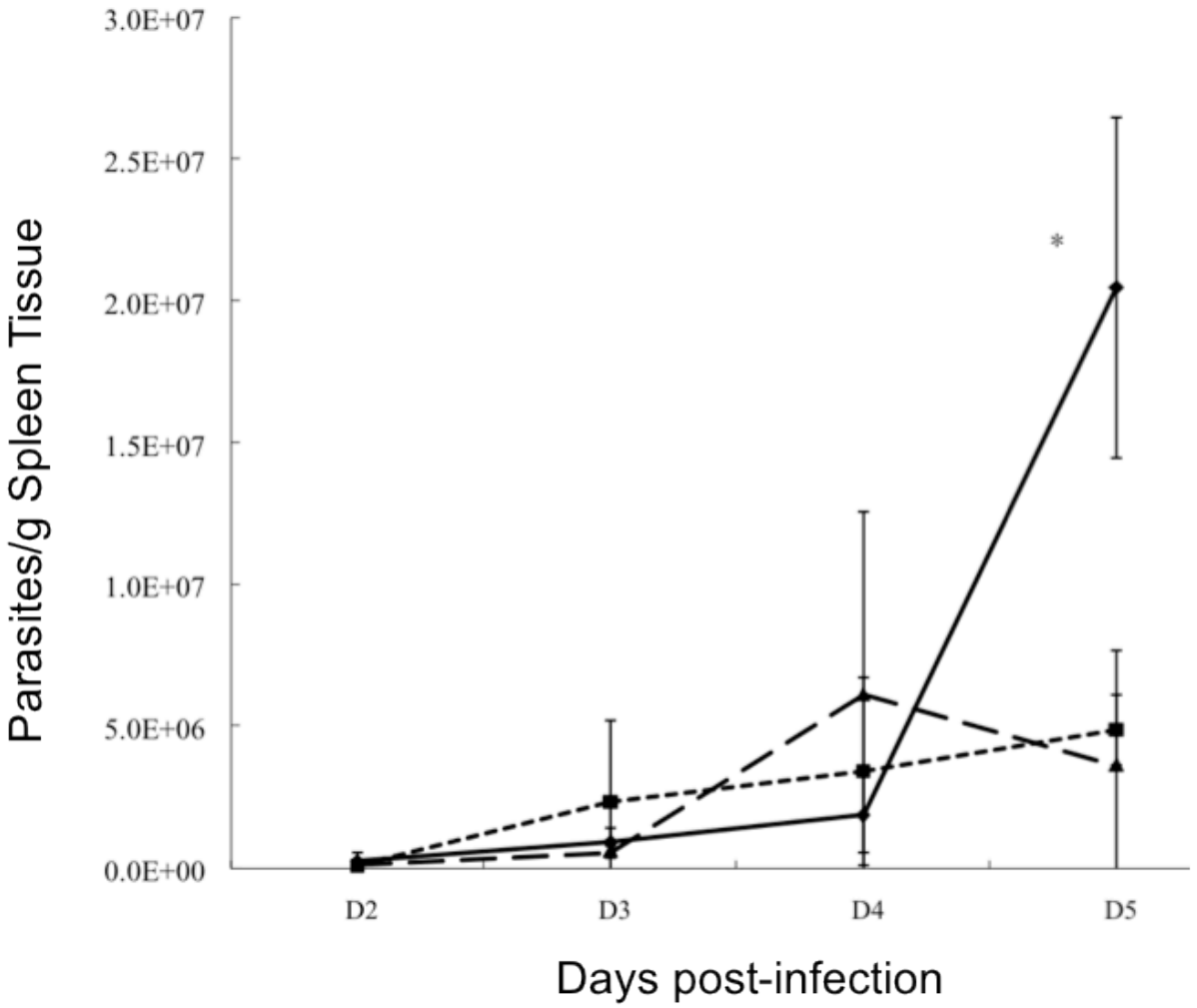


Figure VI-5. Parasite load in spleen tissue. Parasites per gram of spleen tissue from days 2-5 post infection of mice with GT1 (diamonds), PTG (squares) or CTG (triangles) strains, as determined by qPCR

the exception of Gbp1, a chromosome 3 GBP that followed the chromosome 5 expression pattern closely. Thus far, only the contribution of chromosome 3 GBPs to defense against *T. gondii* infection has been assessed experimentally, so that the importance of the chromosome 5 GBPs in this regard is not certain. The data presented here would appear to suggest a more central role for the chromosome 5-encoded GBPs in the immune response to *T. gondii* than their chromosome 3 counterparts, underscoring the necessity of more thorough investigation into this potentially important group of proteins.

It is likely that these differential patterns of IRG and GBP expression reflect the varied pathogenic strategies of the Type I, II and III lineages. By avoiding the IRG defense system in early-infected host cells, Type I strains may be able to replicate more freely within PVs, without the strong activation of the inflammatory immune response resulting from the PVM disruption, parasite clearance and programmed host cell death associated with the earlier stages of Type II and III infections. Thus, it is not until the later stages of acute infection, when host cells are lysed by replicating tachyzoites and the parasite burden begins to expand exponentially as subsequent rounds of infection occur, that strong activation of the host immune response occurs. IFNg itself was not found to be strongly upregulated during the early stages of any infection type, but the upregulation of many IFNg-responsive genes suggests functionally significant increases in the activity of this cytokine. The reduced upregulation of IRGs and GBPs observed towards the end of the experimental period for PTG infections in contrast to CTG and GT1 infections may be related to unique mechanisms of host gene regulation employed by Type II strains. For example, Type II strains are known to express different alleles of at least two important regulators of host transcription, ROP16 and GRA15, which act on the JAK-STAT3/6 and NF-kB pathways, respectively, and have been shown to alter the expression of a number of host cytokines during infection (Saeij *et al.* 2006, Rosowski *et al.* 2011). More investigation into these possible mechanisms is of interest.

In summary, using a mouse model and cDNA microarray technology, we found that mice infected with type I, II and III strains of *T. gondii* displayed distinct gene expression profiles. In the late stage of acute infection, the differentially expressed genes and affected functional groups had a remarkable difference between the type I infection and the type II / III infection, which may partly explain the different outcomes after infection with three distinct lineages of *T. gondii*. We also identified several potential target genes that may be critical in regulating the inflammatory and immune response to type I and type II infections. These results present an overall picture of host responses to three distinct lineages of *T. gondii*. Future experiments, such as using gene-knockout mice, are needed to validate the roles of the candidate regulatory genes in the pathology of toxoplasmosis caused by distinct *T. gondii* strains.

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Chapter VII. Future directions

In this work, we have begun to examine the population genetics of *T. gondii* with emphasis on understanding the role played by mice in shaping the structures of *T. gondii* populations. Our findings should provide a basis for the considerable research that remains to be done in this area, and also suggest novel directions for future work in other related areas. The following are a number of specific ideas for research projects with potential importance for understanding the evolution, pathogenesis and epidemiology of *T. gondii*.

Determine the extent of correlation between *T. gondii* type and severity of symptoms in human infection.

Data regarding *T. gondii* genotypes responsible for human infections are very limited at present. However, the information that is available shows a disproportionate abundance of atypical strains associated with more severe clinical cases. While many atypical strains, particularly from South and Central America, have been bioassayed and found to be highly mouse virulent, these represent only a small fraction of all isolates. Thus, the mouse virulence of the majority of atypical isolates remains unknown. The work described in chapter five of this dissertation provides evidence that the ROP5 and ROP18 genotypes can provide a reasonable indication of the mouse virulence phenotypes of *T. gondii* strains, and this data indicates that most of the atypical strains associated with human infections are most likely mouse virulent. However, we also found that a significant number of strains have virulence phenotypes that are not predicted by the ROP5/ROP18 genotype. Thus, in order to more accurately determine the extent to which mouse virulence correlates with human disease, it is necessary to perform mouse virulence assays on any available human isolates or isolates that share these genotypes. Understanding this relationship is of importance in that it would facilitate prediction of the likelihood and potential severity of toxoplasmosis outbreaks in human populations, based on *T. gondii* genotypes identified in non-human hosts from the same vicinities.

Development of serological test to distinguish between major *T. gondii* lineages in human infections

The work presented in chapter six of this thesis builds on previous findings that infection with the three archetypal lineages of *T. gondii* results in markedly different host responses in mice. It is also well established that different strains of *T. gondii* express different proteins that are secreted into host cells, or varied forms of homologous proteins. Thus, it is likely that infection by specific strains of *T. gondii* would result in the development of particular serum protein profiles in infected hosts, and that certain proteins might be useable as targets for distinguishing strain types associated with infections using antibody-based serological testing. Indeed, preliminary evidence for this has been obtained using 2-D Western blotting of proteins extracted from mice infected with each of the archetypal *T. gondii* lineages. This data indicated the presence of several serum proteins unique to specific *T. gondii* strains. Future work in this area should involve expansion of this preliminary work to produce a more robust and reliable set of potential targets for serological testing. Of particular interest will be the ROP18 and ROP5 proteins, as these may also provide information about the potential virulence of the infecting

strain. Protein identification should be followed by purification and identification of candidate proteins, followed by the production of antibodies specific to them. These antibodies could then be tested for efficacy in detection of infection by specific *T. gondii* strains in mice. Finally, these same tests could be applied to serum samples from human patients infected with known strains of *T. gondii*. If this mouse-developed testing procedure did not prove effective in humans, human serum samples could be used as a basis for the development of human-specific tests. If successful, the development of such assays would allow for a better understanding of the relationship between strain type and human disease symptoms, and would thus also enable the early diagnosis of potentially more severe cases of toxoplasmosis, allowing for appropriate measures to be taken regarding clinical treatment.

Determine pathogenic mechanisms of *T. gondii* strains with unexpected mouse virulence phenotypes.

As noted in chapter five of this dissertation, while the ROP5/ROP18 genotype is largely predictive of mouse virulence, this is not always the case. A number of strains, such as CASTELLS, show highly virulent phenotypes despite possessing alleles of ROP5 and/or ROP18 that are associated with avirulence according to the classical model of *T. gondii* pathogenesis in mice. Conversely, other strains possessed of classically virulent ROP5 and ROP18 alleles display avirulent phenotypes, based on previous findings. It is of interest first to determine whether or not the published mouse virulence data is accurate, and if so, to identify the unique molecular mechanisms that govern virulence in these strains. This could potentially be achieved using a forward genetics approach similar to that used to identify the major virulence determinants of the archetypal lineages. In this case, an atypical virulent strain could be crossed with a control archetypal strain in a feline host. Offspring strains could then be assayed for mouse virulence and virulence-associated loci could be identified via QTL-mapping. The control strain used for the cross would depend on the particular genotype of the atypical strain. For example, if the strain possessed an avirulent ROP18 allele, crossing with a Type III strain would be appropriate, as no virulent ROP18 alleles would be present in the cross, allowing for identification of other virulence-associated loci. Once such loci were identified, the specific gene/s involved and the associated mechanisms of action could be investigated using standard molecular reverse genetics approaches. Understanding these atypical mechanisms of pathogenesis could aid in the future identification of potentially virulent strains, help to develop new drug treatments, and provide new insight into selective pressures that have contributed to the evolution of the parasite.

Determine precise mechanisms governing success of reinfection of mice.

The work presented in chapter four of this dissertation indicates a correlation between mouse virulence of a *T. gondii* strain and the strain's likelihood of being able to successfully reinfect an already chronically infected mouse. This could provide an advantage to more virulent strains sufficient to promote their expansion within populations in which the domestic life cycle is prominent. As this phenomenon may have been and likely continues to be an important factor in governing the evolution of *T. gondii*, it is of interest to understand the mechanism or mechanisms by which more mouse virulent strains are able to overcome the protective immunity normally conferred by a previous establishment of chronic infection. In order to accomplish this, a detailed analysis of reinfection by virulent and avirulent strains needs to be undertaken. This would

involve quantification of parasite load, cytokine and antibody production, as well as microarray analysis of gene expression by both parasite and host during the initial stage of infection. This approach should allow for the identification of specific differences between sequential infection by virulent and avirulent strains. This work should also be aimed at identifying the stage of infection at which the challenging strain is inhibited, such as host cell invasion, replication, egress, cyst formation etc. This could be accomplished using microscopic analysis of tissues extracted from naïve or previously infected mice challenged with fluorogenic virulent and avirulent *T. gondii* strains and counting numbers of infected cells, number of parasites per host cell and number of cysts at various time points. A better understanding of the mechanisms of protective immunity and how it may be overcome by certain parasite strains would enhance our knowledge of specific principles underlying parasite fitness and therefore driving parasite evolution, and could also potentially allow for the development of vaccines aimed at enhancing protective immunity in humans.

Analyze expansion patterns of Type II strains using microsatellite analysis.

Through the extensive genotyping efforts presented in this dissertation, we have a fairly detailed picture of the global *T. gondii* population structure (though of course gaps still exist). However, this data provides only limited information regarding the directions in which strain expansion/migration has occurred. Elucidation of evolutionary relationships between different strain types is difficult due to continued recombination, but it should be more feasible to conduct experiments aimed at inferring relationships between different populations of the same strain type. As noted, the Type II archetypal lineage is the most abundant strain type worldwide, and is the most frequently identified genotype in Europe, the Middle East, and North America. While PCR-RFLP analysis is only capable of distinguishing between two variants of the Type II lineage, a higher resolution technique could allow subsets of this line to be further differentiated. Microsatellite analysis seems to be the technique best suited for this particular task, as microsatellite DNA repeats are highly variable and thus provides high resolution analysis of genetic differences between strains. Furthermore, the technique is relatively simple and rapid, and therefore suitable for high throughput analysis of genomic DNA samples from large numbers of isolates at low cost, comparable to PCR-RFLP. This is in contrast to other potential high resolution genotyping techniques such as intron sequencing, which would be quite costly when large sample numbers are involved. By analyzing multiple microsatellite loci for length polymorphisms from Type II (as determined by PCR-RFLP analysis) strains isolated from different geographic regions, it should be possible to ascertain the relative ages of these populations. For example, it seems likely that the Type II population of North America is a result of European migration to the Americas. Thus, we would hypothesize that the European population of Type II strains would be older and therefore more polymorphic with regard to microsatellite repeat number. This technique could be applied to Type III and other prominent strains as well, and to other geographic regions with somewhat clonal population structures. It would presumably be less effective with regard to strains from South and Central American populations, where it appears that much more sexual recombination has occurred, thus blurring evolutionary relationships between genotypes and separate populations of single genotypes. This work would be valuable in understanding the historical pathways of *T. gondii* expansion, giving us insight into the evolution of the parasite and the impact of human global migration on *T. gondii* and microbial communities in general.

Literature cited

Ajzenberg D, Banuls, AL, Su C, Dumetre A, Demar M, Carme B, Darde ML. (2004). Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *International Journal for Parasitology* **34**, 1185-1196.

Ajzenberg D, Banuls AL, Tibayrenc M, Darde ML. (2002a). Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *International Journal for Parasitology* **32**, 27–38.

Ajzenberg D, Cogne N, Paris L, Bessieres MH, Thulliez P, Filisetti D, Pelloux H, Marty P, Darde ML. (2002b). Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. *Journal of Infectious Diseases* **186**, 684–689.

Ajzenberg D, Collinet F, Mercier A, Vignoles P, Dardé ML. (2010). Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. *Journal of Clinical Microbiology* **48**, 4641-4645.

Ajzenberg, D., Dumetre, A. and Darde, M.-L. (2005). Multiplex PCR for typing strains of *Toxoplasma gondii*. *Journal of Clinical Microbiology* **43**, 1940–1943.

Ajzenberg D, Yera H, Marty P, Paris L, Dalle F, Menotti J, Aubert D, Franck J, Bessières MH, Quinio D, Pelloux H, Delhaes L, Desbois N, Thulliez P, Robert-Gangneux F, Kauffmann-Lacroix C, Pujol S, Rabodonirina M, Bougnoux ME, Cuisenier B, Duhamel C, Duong TH, Filisetti D, Flori P, Gay-Andrieu F, Pratlong F, Nevez G, Totet A, Carme B, Bonnabau H, Dardé ML, Villena I. (2009) Genotype of 88 *Toxoplasma gondii* isolates associated with toxoplasmosis in immunocompromised patients and correlation with clinical findings. *Journal of Infectious Disease*. **199**(8), 1155-67.

Al-Kappany, Y. M., Rajendran, C., Abu-Elwafa, S. A., Hilali, M., Su, C., and Dubey. J. P. (2010). Genetic diversity of *Toxoplasma gondii* isolates in Egyptian feral cats reveals new genotypes. *Journal of Parasitology*. **96**, 1112-1114.

Alvarado-Esquivel, C., Rajendran, C., Ferreira, L.R., Kwok, O.C., Choudhary, S., Alvarado-Esquivel, D., Rodríguez-Peña, S., Villena, I. and Dubey, J.P. (2011). Prevalence of *Toxoplasma gondii* infection in wild birds in Durango, Mexico. *Journal of Parasitology* **97**, 809-812.

Al-Qassab, S., Reichel, M.P., Su, C., Jenkins, D., Hall, C., Windsor, P.A., Dubey, J.P. and Ellis, J. (2009). Isolation of *Toxoplasma gondii* from the brain of a dog in Australia and its biological and molecular characterization. *Veterinary Parasitology* **164**, 335-339.

Araújo, J.B., da Silva, A.V., Rosa, R.C., Mattei, R.J., da Silva, R.C., Richini-Pereira, V.B. and Langoni, H. (2010). Isolation and multilocus genotyping of *Toxoplasma gondii* in seronegative rodents in Brazil. *Veterinary Parasitology* **174**, 328-331.

Araujo F, Slifer T, Kim S. (1997). Chronic infection with *Toxoplasma gondii* does not prevent acute disease or colonization of the brain with tissue cysts following reinfection with different strains of the parasite. *Journal of Parasitology*. **83**(3), 521-2

Barbour KW, Wei F, Brannan C, Flotte TR, Baumann H, Berger FG. (2002) The murine alpha(1)-proteinase inhibitor gene family: polymorphism, chromosomal location, and structure. *Genomics*. **80**(5), 515-22.

Bastien, P., Jumas-Bilak, E., Varlet-Marie, E. , Marty, P. (2007). Three years of multi-laboratory external quality control for the molecular detection of *Toxoplasma gondii* in amniotic fluid in France. *Clinical Microbiology and Infection* **13**, 430–433.

Belaaouaj A, Kim KS, Shapiro SD. (2000) Degradation of outer membrane protein A in Escherichia coli killing by neutrophil elastase. *Science*. **289**(5482), 1185-8.

Bell, A. and Ranford-Cartwright, L. (2002). Real-time quantitative PCR in parasitology. *Trends in Parasitology* **18**, 337–342.

Benarafa C. (2011) The SerpinB1 knockout mouse a model for studying neutrophil protease regulation in homeostasis and inflammation. *Methods in Enzymology*. **499**, 135-48.

Bessieres, M. H., Berrebi, A., Cassaing, S., Fillaux, J., Cambus, J. P., Berry, A., Assouline, C., Ayoubi, J. M. and Magnaval, J. F. (2009). Diagnosis of congenital toxoplasmosis: prenatal and neonatal evaluation of methods used in Toulouse University Hospital and incidence of congenital toxoplasmosis. *Memorias do Instituto Oswaldo Cruz* **104**, 389–392.

Blackston, C. R., Dubey, J. P., Dotson, E., Su, C., Thulliez, P., Sibley, D. Lehmann, T. (2001). High-resolution typing of *Toxoplasma gondii* using microsatellite loci. *Journal of Parasitology* **87**, 1472–1475.

Boehm U, Guethlein L, Klamp T, Ozbek K, Schaub A, Futterer A, Pfeffer K, Howard JC. (1998). Two families of GTPases dominate the complex cellular response to IFN-gamma. *Journal of Immunology*. **161,**6715-6723.

Borriello F, Krauter KS. (1991) Multiple murine alpha 1-protease inhibitor genes show unusual evolutionary divergence. *Proceedings of the National Academy of Sciences of the U S A*. **88**(21), 9417-21.

Bossi, P. and Bricaire, F. (2004). Severe acute disseminated toxoplasmosis. *The Lancet* 364, 579.

Bourguin I, Chardès T, Bout D. (1993). Oral immunization with *Toxoplasma gondii* antigens in association with cholera toxin induces enhanced protective and cell-mediated immunity in C57BL/6 mice. *Infection and Immunity*. **61**(5),2082-8.

Boursot P, Aujfrai J-C, Britton-Davidian J, and Bonhomme F (1993). The evolution of house mice. Annual Review of Ecological Systems. **24**,119-52

Boyle JP, Saeij JP, Hrada SY, Ajioka JW, Boothroyd JC (2008) Expression QTL mapping of *Toxoplasma* genes reveals multiple mechanisms for strain-specific differences in gene expression. *Eukaryotic Cell* **7**, 1403–1414.

Brandão GP, Melo MN, Gazzinelli RT, Caetano BC, Ferreira AM, Silva LA, Vitor RW. (2009). Experimental reinfection of BALB/c mice with different recombinant type I/III strains of *Toxoplasma gondii*: involvement of IFN-gamma and IL-10. Mem Inst Oswaldo Cruz. 104(2),241-5

Brandão GP, Melo MN, Caetano BC, Carneiro CM, Silva LA, Vitor RW. (2011). Susceptibility to re-infection in C57BL/6 mice with recombinant strains of *Toxoplasma gondii*. *Experimental Parasitology*. **128**(4), 433-7

Bretagne, S., Costa, J. M., Fleury-Feith, J., Poron, F., Dubreuil-Lemaire, M. L. and Vidaud, M. (1995). Quantitative competitive PCR with bronchoalveolar lavage fluid for diagnosis of toxoplasmosis in AIDS patients. *Journal of Clinical Microbiology* **33**, 1662–1664.

Burg, J. L., Grover, C. M., Pouletty, P. and Boothroyd, J. C. (1989). Direct and sensitive detection of a pathogenic protozoan *Toxoplasma gondii*, by polymerase chain reaction. *Journal of Clinical Microbiology* **27**, 1787–1792.

Butcher BA, Fox BA, Rommereim LM, Kim SG, Maurer KJ, Yarovinsky F, Herbert DR, Bzik DJ, Denkers EY. 2011. *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathogens*. **7:**e1002236.

Calderaro, A., Piccolo, G., Gorrini, C., Peruzzi, S., Zerbini, L., Bommezzadri, S., Dettori, G. and Chezzi, C. (2006). Comparison between two real-time PCR assays and a nested-PCR for the detection of *Toxoplasma gondii*. *Acta Bio Medica* **77**, 75–80.

Driscoll CA, Menotti-Raymond M, Roca AL, Hupe K, Johnson WE, Geffen E, Harley EH, Delibes M, Pontier D, Kitchener AC, Yamaguchi N, O'Brien SJ, and Macdonald DW. (2007).The Near Eastern Origin of Cat Domestication**.** *Science*; **317**, 519-523

Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, De Mol M, Mulligan RC, Collen D. (1993) Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *Journal of Clinical Investigation* **92**(6), 2756-60.

Carme B, Bissuel F, Ajzenberg D, Bouyne R, Aznar C, Demar M, Bichat S, Louvel D, Bourbigot AM, Peneau C, Neron P, Dardé ML. (2002). Severe acquired toxoplasmosis in immunocompetent adult patients in French Guiana. *Journal of Clinical Microbiology*. **40**(11):4037-44.

Carme B, Demar M, Ajzenberg D, Dardé ML. (2009). Severe acquired toxoplasmosis caused by wild cycle of *Toxoplasma gondii*, *French Guiana. Emerging Infectious Diseases*. **15**(4), 656-8.

Cavailles P, Flori P, Papapietro O, Bisanz C, Lagrange D, Pilloux L, Massera C, Cristinelli S, Jublot D, Bastien O, Loeuillet C, Aldebert D, Touquet B, Fournié GJ, Cesbron-Delauw MF. (2014). A highly conserved Toxo1 haplotype directs resistance to toxoplasmosis and its associated caspase-1 dependent killing of parasite and host macrophage. *PLoS Pathogens*. **10**(4):e1004005.

Chen, Z.W., Gao, J.M., Huo, X.X., Wang, L., Yu, L., Halm-Lai, F., Xu, Y.H., Song, W.J., Hide, G., Shen, J.L. and Lun, Z.R. (2011). Genotyping of *Toxoplasma gondii* isolates from cats in different geographic regions of China. *Veterinary Parasitology* **183**, 166-170.

Cho JH, Kim HO, Webster K, Palendira M, Hahm B, Kim KS, King C, Tangye SG, Sprent J. (2011). Calcineurin-dependent negative regulation of CD94/NKG2A expression on naive CD8+ T cells. *Blood*. **118**(1), 116-28.

Conrad, P.A., Miller, M.A., Kreuder, C., James, E.R., Mazet, J., Dabritz, H., Jessup, D.A., Gulland, F. and Grigg, M.E. (2005). Transmission of *Toxoplasma*: clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment. *International Journal for Parasitology* 35, 1155-68.

Contini, C., Cultrera, R., Seraceni, S., Segala, D., Romani, R., Fainardi, E., Cinque, P., Lazzarin, A. and Delia, S. (2002). The role of stage-specific oligonucleotide primers in providing effective laboratory support for the molecular diagnosis of reactivated *Toxoplasma gondii* encephalitis in patients with AIDS. *Journal of Medical Microbiology 51*, 879–890.

Contini, C., Seraceni, S., Cultrera, R., Incorvaia, C., Sebastiani, A. and Picot, S. (2005). Evaluation of a Real-time PCR-based assay using the lightcycler system for detection of *Toxoplasma gondii* bradyzoite genes in blood specimens from patients with toxoplasmic retinochoroiditis. *International Journal for Parasitology* **35**, 275–283.

Costa, J. M., Pautas, C., Ernault, P., Foulet, F., Cordonnier, C. and Bretagne, S. (2000). Real-time PCR for diagnosis and follow-up of *Toxoplasma* reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. *Journal of Clinical Microbiology* **38**, 2929–2932.

Cruz Mde A, Ullmann LS, Montaño PY, Hoffmann JL, Langoni H, Biondo AW. (2011) Seroprevalence of *Toxoplasma gondii* infection in cats from Curitiba, Paraná, *Brazil. Rev Bras Parasitol Vet*, **20**(3),256-158.

Dao A, Fortier B, Soete M, Plenat F, Dubremetz JF. (2001). Successful reinfection of chronically infected mice by a different *Toxoplasma gondii* genotype. *International Journal for Parasitology*. **31**(1), 63-5.

Dardé ML. (2008). *Toxoplasma gondii*, "new" genotypes and virulence. *Parasite*. **15**(3):366-71.

Dardé, M.L., Ajzenberg, D. and Su, C. (2014). Molecular epidemiology and population structure of *Toxoplasma gondii*. In *Toxoplasma gondii* - The Model Apicomplexan: Perspectives and Methods, 2nd edition (Weiss, L. M. and Kim, K.), pp. 61-97. Elsevier Ltd., San Diego, CA, USA.

Darde, M. L., Bouteille, B. and Pestre-Alexandre, M. (1992). Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. *Journal of Parasitology* **78**, 786–794.

Dardé ML, Villena I, Pinon JM, Beguinot I. (1998). Severe toxoplasmosis caused by a *Toxoplasma gondii* strain with a new isoenzyme type acquired in French Guyana. Journal of Clinical Microbiology. **36**(1), 324.

da Silva, D.S., Bahia-Oliveira, L.M.G., Shen, S.K., Kwok, O.C.H., Lehman, T. and Dubey, J.P. (2003). Prevalence of *Toxoplasma gondii* in chickens from an area in southern Brazil highly endemic to humans. *Journal of Parasitology* **89**, 394–396.

da Silva, R.C., Langoni, H., Su, C. and da Silva, A.V. (2011). Genotypic characterization of *Toxoplasma gondii* in sheep from Brazilian slaughterhouses: new atypical genotypes and the clonal Type II strain identified. *Veterinary Parasitology* **175**, 173-177.

de-la-Torre A, Sauer A, Pfaff AW, Bourcier T, Brunet J, Speeg-Schatz C, Ballonzoli L, Villard O, Ajzenberg D, Sundar N, Grigg ME, Gomez-Marin JE, 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 Neglected Tropical Diseases*. **7**(11), e2541.

Delhaes L, Ajzenberg D, Sicot B, Bourgeot P, Dardé ML, Dei-Cas E, Houfflin-Debarge V. (2010). Severe congenital toxoplasmosis due to a *Toxoplasma gondii* strain with an atypical genotype: case report and review. *Prenatal Diagnostics*. **30**(9), 902-905.

de Oliveira LN, Costa Junior LM, de Melo CF, Ramos Silva JC, Bevilaqua CM, Azevedo SS, Muradian V, Araújo DA, Dubey JP, Gennari SM. (2009). *Toxoplasma gondii* isolates from free-range chickens from the northeast region of Brazil. Journal of Parasitology. **95**(1), 235-7.

Demar M, Ajzenberg D, Maubon D, Djossou F, Panchoe D, Punwasi W, Valery N, Peneau C, Daigre JL, Aznar C, Cottrelle B, Terzan L, Dardé ML, Carme B. (2007). Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical, and parasitological aspects. *Clinical Infection and Disease*. **45**(7), e88-95.

Demar M, Hommel D, Djossou F, Peneau C, Boukhari R, Louvel D, Bourbigot AM, Nasser V, Ajzenberg D, Darde ML, Carme B. (2012). Acute toxoplasmoses in immunocompetent patients hospitalized in an intensive care unit in French Guiana. Clinical Microbiological Infection. **18**(7), E221-31.

Diamond, J. and Bellwood, P. (2003). Farmers and their languages: the first expansions. *Science*. **300**, 597-603.

Dirzo R, Raven PH (2003) Global state of biodiversity and loss. *Annual Review of Environmental Resources*. **28**,137–167.

Dubey JP. (2009). History of the discovery of the life cycle of *Toxoplasma gondii*. *International Journal of Parasitology*. **39**(8), 877-82.

Dubey, JP (2010). Toxoplasmosis of animals and humans, second edition. CRC Press.

Dubey, JP (2004). Toxoplasmosis – a waterborne zoonosis. *Veterinary Parasitology*. **126**, 57–72.

Dubey, J.P., Applewhaite, L., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C., Hill, R. and Su, C. (2007a). Molecular and biological characterization of *Toxoplasma gondii* isolates from free-range chickens from Guyana, South America, identified several unique and common parasite genotypes. *Parasitology* **134**, 1559-1565.

Dubey, J. P. and Beattie, C. P. (1988). Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, FL, USA.

Dubey, J. P., Cortés-Vecino, J.A., Vargas-Duarte, J.J, Sundar, N., Velmurugan, G.V., Bandini, L.M., Polo, L.J., Zambrano, L., Mora, L.E., Kwok, O.C.H., Smith, T. and Su, C. (2007b). Prevalence of *Toxoplasma gondii* in dogs from Colombia, South America and genetic characterization of *T. gondii* isolates. *Veterinary Parasitology* **145**, 45-50.

Dubey, J.P., Fair, P.A., Sundar, N., Velmurugan, G., Kwok, O.C.H., McFee W.E., Majumdar, D. and Su, C. (2008). Isolation of *Toxoplasma gondii* from bottlenose dolphins (*Tursiops truncatus*). *Journal of Parasitology* **94**, 821-823.

Dubey, J.P., Gamble H.R., Hill, D.E., Sreekumar, C., Romand, S. and Thulliez, P. (2002a). High prevalence of viable *Toxoplasma gondii* infection in market weight pigs from a farm in Massachusetts. *Journal of Parasitology* **88**, 1234-1238

Dubey, J.P., Gennari, S.M., Labruna, M.B., Camargo, L.M., Vianna, M.C., Marcet, P.L. and Lehmann, T. (2006a). Characterization of *Toxoplasma gondii* isolates in free-range chickens from Amazon, Brazil. *Journal of Parasitology* **92**, 36-40.

Dubey, J.P., Gennari, S.M., Sundar, N. Vianna., M.C., Bandini, L.M., Yai, L.E., Kwok, C.H. and Su, C. (2007c). Diverse and atypical genotypes identified in *Toxoplasma gondii* from dogs in Sao Paulo, Brazil. *Journal of Parasitology* **93**, 60-64.

Dubey, J.P., Graham, D.H., Blackston, C.R., Lehmann, T., Gennari, S.M., Ragozo, A.M.A., Nishi, S.M., Shen, S.K., Kwok, O.C.H., Hill, D.E. and Thulliez, P. (2002b). Biological and genetic characterisation of *Toxoplasma gondii* isolates from chickens (*Gallus domesticus*) from Sao Paulo, Brazil: unexpected findings. *International Journal for Parasitology* **32**, 99-105.

Dubey, J.P., Graham D.H., da Silva D.S., Lehmann, T. and Bahia-Oliveira, L.M. (2003a). *Toxoplasma gondii* isolates of free-ranging chickens from Rio De Janeiro, Brazil: mouse mortality, genotype, and oocyst shedding by cats. *Journal of Parasitology* **89**, 851-853.

Dubey, J,P., Graham, D.H., De Young, R.W., Dahl, E., Eberhard, M.L., Nace, E.K., Won, K., Bishop, H., Punkosdy, G., Sreekumar, C., Vianna, M.C., Shen, S.K., Kwok, O.C., Sumners, J.A., Demarais, S., Humphreys, J.G. and Lehmann, T. (2004a). Molecular and Biologic Characteristics of *Toxoplasma gondii* isolates from wildlife in the United States. *Journal of Parasitology* **90**, 67-71.

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. and 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. *Journal of Parasitology* **91**, 1082-1093.

Dubey, J.P., Hill, D.E., Rozeboom, D.W., Rajendran, C., Choudhary, S., Ferreira, L.R., Kwok, O.C.H. and Su, C. (2012). High prevalence and genotypes of *Toxoplasma gondii* isolated from organic pigs in northern USA. *Veterinary Parasitology* **188**, 14-18.

Dubey, J.P., Hill, D.E., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C., Pierce, V., Kelly, K., Dulin, M., Thulliez, P., Iwueke, C. and Su, C. (2008b). Endemic toxoplasmosis in pigs on a farm in Maryland: isolation and genetic characterization of *Toxoplasma gondii*. *Journal of Parasitology* **94**, 36-41.

Dubey, J.P., Huong, L.T.T., Sundar, N. and Su, C. (2007d). Genetic characterization of *Toxoplasma gondii* isolates in dogs from Vietnam suggests their South American origin. *Veterinary Parasitology* **146**, 347-351.

Dubey, J.P., Huong, L.T., Lawson, B.W., Subekti, D.T., Tassi, P., Cabaj, W., Sundar, N., Velmurugan, G.V., Kwok, O.C. and Su, C. (2008c). Seroprevalence and isolation of *Toxoplasma gondii* from free-range chickens in Ghana, Indonesia, Italy, Poland, and Vietnam. *Journal of Parasitology* **94**, 68-71.

Dubey JP, Lago EG, Gennari SM, Su C, Jones JL. (2012). Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. *Parasitology*. **139**(11), 1375-424.

Dubey, J.P., Lopez-Torres, H.Y., Sundar, N., Velmurugan, G.V., Ajzenberg, D., Kwok, O.C.H., Hill, R., Darde, M.L. and Su, C. (2007e). Mouse-virulent *Toxoplasma gondii* isolated from feral cats on Mona Island, Puerto Rico. *Journal of Parasitology* **93**, 1365-1369.

Dubey, J. P., Mergel, J., Gehring, E., Sundar, N., Velmurugan, G., Kwok, O.C.H., Grigg, M.E., Martineau, D. and Su, C. (2009a). Toxoplasmosis in captive dolphins (*Tursiops truncatus*) and walrus (*Odobenus rosmrus*). *Journal of Parasitology* **95**, 82-85.

Dubey, J.P., Morales, J.A., Sundar, N., Velmurugan, G.V., González-Barrientos, C.R., Hernández-Mora, G. and Su, C. (2007a). Isolation and genetic characterization of *Toxoplasma gondii* from striped dolphin (*Stenella coeruleoalba*) from Costa Rica. *Journal of Parasitology* **93**, 710-711.

Dubey, J.P., Moura, L., Majumdar, D., Sundar, N., Velmurugan, G.V., Kwok, O.C.H., Kelly, P., Krecek, R.C., and Su, C. (2009b). Isolation and characterization of viable *Toxoplasma gondii* isolates revealed possible high frequency of mixed infection in feral cats (*Felis domesticus*) from St. Kitts, West Indies. *Parasitology* **136**, 589-594.

Dubey, J.P., Navarro, I.T. Graham, D.H., Dahl, E., Freire, R.L., Prudencio, L.B., Sreekumar, C., Vianna, M.C. and Lehmann, T. (2003b). Characterization of *Toxoplasma gondii* isolates from free range chickens from Paraná, Brazil. *Veterinary Parasitology* **117**, 229-234.

Dubey, J.P., Navarro, I.T., Sreekumar, C., Dahl, E., Freire, R.L., Kawabata, H.H., Vianna, M.C., Kwok, O.C., Shen, S.K., Thulliez, P. and Lehmann, T. (2004b). *Toxoplasma gondii* infections in cats from Parana, Brazil: seroprevalence, tissue distribution, and biologic and genetic characterization of isolates. *Journal of Parasitology* **90**, 721-726.

Dubey, J.P., Pas, A., Rajendran, C., Kwok, O.C.H., Ferreira, L.R., Martins, J., Hebel, C., Hammer, S. and Su, C. (2010a). Toxoplasmosis in sand cats (*Felis margarita*) and other animals in the Breeding Centre for Endangered Arabian Wildlife in the United Arab Emirates and Al Wabra Wildlife Preservation, the state of Qatar. *Veterinary Parasitology* **172**, 195-203.

Dubey, J.P., Passos, L.M.F., Rajendran, C., Ferreira, L.R., Gennari, S.M. and Su, C. (2011a). Isolation of viable *Toxoplasma gondii* from feral guinea fowl (*Numida meleagris*) and domestic rabbits (*Oryctolagus cuniculus*) from Brazil. *Journal of Parasitology* **97**, 842-845.

Dubey, J.P., Patitucci, A.N., Su, C., Sundar, N., Kwok, O.C.H. and Shen, S. K. (2006b). Characterization of *Toxoplasma gondii* isolates in free-range chickens from Chile, South America. *Veterinary Parasitology* **140**, 76-82.

Dubey, J.P., Passos, L.M.F., Rajendran, C., Ferreira, L.R., Gennari, S.M. and Su, C. (2011a). Isolation of viable *Toxoplasma gondii* from feral guinea fowl (*Numida meleagris*) and domestic rabbits (*Oryctolagus cuniculus*) from Brazil. *Journal of Parasitology* **97**, 842-845.

Dubey, J.P., Quirk, T., Pitt, J.A., Sundar, N., Velmurugan, G.V., Kwok O.C., Hill, R. and Su, C. (2008d). 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. *Journal of Parasitology* **94**, 42-45.

Dubey, J.P., Rajapakse, R.P.V.J., Wijesundera, R.R.M.K.K., Sundar, N., Velmurugan, G.V., Kwok, O.C.H. and Su, C. (2007g). Prevalence of *Toxoplasma gondii* in dogs from Sri Lanka and genetic characterization of the parasite isolates. *Veterinary Parasitology* **146**, 341-346.

Dubey, J.P., Rajendran, C., Costa D.G.C., Ferreira, L.R., Kwok, O.C.H., Qu, D., Su, C., Marvulo, M.F.V., Alves, L.C., Mota, R.A. and Silva, J.C.R. (2010b). New *Toxoplasma gondii* genotypes isolated from free-range chickens from the Fernando De Noronha, Brazil: unexpected findings. *Journal of Parasitology* **96**, 709-712.

Dubey, J.P., Rajendran, C., Ferreira, L.R., Kwok, O.C.H., Sinnett, D., Majumdar, D. and Su, C. (2010c). A new atypical highly mouse virulent *Toxoplasma gondii* genotype isolated from a wild black bear in Alaska. *Journal of Parasitology* **96**, 713-716.

Dubey, J.P., Rajendran, C., Ferreira, L.R., Martins, J., Kwok, O.C.H., Hill, D.E., Villena, I., Zhou, H., Su, C. and Jones, J.L. (2011b). High prevalence and genotypes of *Toxoplasma gondii* isolated from goats, from a retail meat store, destined for human consumption in the USA. *International Journal for Parasitology* **41**, 827-833.

Dubey, J. P. and Su, C. (2009). Population biology of *Toxoplasma gondii*: what's out and where did they come from. *Memorias do Instituto Oswaldo Cruz.* **104**, 190–195.

Dubey, J.P., Su, C., Cortes, J.A., Sundar, N., Gomez-Marin, J.E., Polo, L.J., Zambrano, L., Mora, L.E., Lora, F., Jimenez, J., Kwok, O.C.H., Shen, S.K., Zhang, X., Nieto, A. and Thulliez, P. (2006c). Prevalence of *Toxoplasma gondii* in cats from Colombia, South America and genetic characterization of *T. gondii* isolates. *Veterinary Parasitology* **141**, 42-47.

Dubey, J.P., Su, C., Oliveira, J., Morales, J.A., Bolanos, R.V., Sundar, N., Kwok, O.C.H., and Shen, S.K. (2006d). Biologic and genetic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Costa Rica, Central America. *Veterinary Parasitology* **139,** 29-36.

Dubey, J.P., Sundar, N., Gennari, S.M., Minervino, A.H.H., Farias, N.A.d.R., Ruas, J.L., dos Santos, T.R.B., Cavalcante, G.T., Kwok, O.C.H., and Su, C. (2007h). Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from the northern Para state and the southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations. *Veterinary Parasitology* **143**, 182-188.

Dubey, J.P., Sundar, N., Hill, D., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Majumdar, D., and Su, C. (2008e). High prevalence and abundant atypical genotypes of *Toxoplasma gondii* isolated from lambs destined for human consumption in the USA. *International Journal for Parasitology* **38**, 999-1006.

Dubey, J.P., Sundar, N., Nolden, C.A., Samuel, M.D., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., Bodenstein, B., and Su, C. (2007i). Characterization of *Toxoplasma gondii* from raccoons (*Procyon lotor*), coyotes (*Canis latrans*), and striped skunks (*Mephitis mephitis*) in Wisconsin identified several atypical genotypes. *Journal of Parasitology*. **93,** 1524-1527.

Dubey, J.P., Sundar, N., Pineda, N., Kyvsgaard, N.C., Luna, L.A., Rimbaud, E., Oliveira, J.B., Kwok, O.C., Qi, Y., and Su, C. (2006e). Biologic and genetic characteristics of *Toxoplasma gondii* isolates in free-range chickens from Nicaragua, Central America. Veterinary Parasitology. **142**, 47-53.

Dubey, J.P., Thulliez, P., and Powell, E.C. (1995). *Toxoplasma gondii* in Iowa sows: comparison of antibody titers to isolation of *T. gondii* by bioassays in mice and cats. *Journal of Parasitology* **81**, 48-53.

Dubey, J.P., Velmurugan, G.V., Alvarado-Esquivel, C., Alvarado-Esquivel, D., Rodríguez-Peña, S., Martínez-García, S., González-Herrera, A., Ferreira, L.R., Kwok. O.C. and Su, C. (2009c). Isolation of *Toxoplasma gondii* from animals in Durango, Mexico. *Journal of Parasitology* ***95***, 319-322.

Dubey, J.P., Velmurugan, G.V., Chockalingam, A., Pena, H.F.J., de Oliveira, L.N., Leifer, C.A., Gennari, S.M., Bahia Oliveira, L.M.G., and Su, C. (2008f). Genetic diversity of *Toxoplasma gondii* isolates from chickens from Brazil. *Veterinary Parasitology* **157,** 299-305.

Dubey, J.P., Velmurugan, G.V., Morales, J.A., Arguedas, R. and Su, C. (2009d). Isolation of *Toxoplasma gondii* from the keel-billed toucan (*Ramphastos sulfuratus*) from Costa Rica. *Journal of Parasitology* **95**, 467-468.

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, E., Shearn-Bochsler, V., Kwok, O.C.H., Ferreira, L.R., Choudhary, S., Faria, E.B., Zhou, H., Felix, T.A. and Su, C. (2011b). Genetic characterisation of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. *International Journal for Parasitology* **41**, 1139-1147.

Dubey, J.P., Velmurugan, G.V., Ulrich, V., Gill, J., Carstensen, M., Sundar, N., Kwok, O.C.H., Thulliez, P., Majumdar, D., and Su, C. (2008g). Transplacental toxoplasmosis in naturally-infected white-tailed deer: Isolation and genetic characterisation of *Toxoplasma gondii* from foetuses of different gestational ages. *International Journal for Parasitology* **38**, 1057-1063.

Dubey, J.P., Webb, D.M., Sundar, N., Velmurugan, G.V., Bandini, L.A., Kwok, O.C.H., and Su, C. (2007j). 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*). *Veterinary Parasitology* **148**, 207-212.

Dubey, J.P., Zhu, X.Q., Sundar, N., Zhang, H., Kwok, O.C.H., and Su, C. (2007b). Genetic and biologic characterization of *Toxoplasma gondii* isolates of cats from China. *Veterinary Parasitology* **145**, 352-356.

Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. 2002. IFN gamma-Inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *Journal of Immunology*. **168:**3195-3204.

Edvinsson, B., Lappalainen, M. and Evengard, B. (2006). Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Clinical Microbiology and Infection* **12**, 131–136.

Egan CE, Cohen SB, Denkers EY. (2012). Insights into inflammatory bowel disease using *Toxoplasma gondii* as an infectious trigger. *Immunology and Cell Biology*. **90**(7):668-75.

Egan CE, Sukhumavasi W, Butcher BA, Denkers EY. (2009). Functional aspects of Toll-like receptor/MyD88 signalling during protozoan infection: focus on *Toxoplasma gondi*i. *Clinical and Experimental Immunology*. **156,** 17-24.

Elzey BD, Tian J, Jensen RJ, Swanson KA, Lees JR, Lentz SR, Stein CS, Nieswandt B, Wang YQ, Davidson BL, Ratliff TL. (2003). Platelet-mediated modulation of adaptive immunity: A communication link between innate and adaptive immune compartments. *Immunity* **19,** 9-19.

Etheridge RD, Alaganan A, Tang K, Lou HJ, Turk BE, Sibley LD. (2014). The *Toxoplasma* pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. *Cell Host Microbe*. **15**(5), 537-50.

Eurich K, Segawa M, Toei-Shimizu S, Mizoguchi E. (2009). Potential role of chitinase 3-like-1 in inflammation-associated carcinogenic changes of epithelial cells. *World Journal of Gastroenterology*. **15,** 5249-5259.

Excoffier, L. and Lischer, H.E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**, 564-567.

Fazaeli, A., Carter, P. E., Darde, M. L. and Pennington, T. H. (2000). Molecular typing of *Toxoplasma gondii* strains by GRA6 gene sequence analysis. *International Journal for Parasitology* **30**, 637–642.

Fekkar A, Ajzenberg D, Bodaghi B, Touafek F, Le Hoang P, Delmas J, Robert PY, Dardé ML, Mazier D, Paris L. (2011). Direct genotyping of *Toxoplasma gondii* in ocular fluid samples from 20 patients with ocular toxoplasmosis: predominance of type II in France. *Journal of Clinical Microbiology*. **49**(4), 1513-1517.

Fentress SJ, Behnke MS, Dunay IR, Mashayekhi M, Rommereim LM, Fox BA, Bzik DJ, Taylor GA, Turk BE, Lichti CF, Townsend RR, Qiu W, Hui R, Beatty WL, Sibley LD. (2010). Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii*-secreted kinase promotes macrophage survival and virulence. *Cell Host Microbe*. **8**(6), 484-95.

Ferreira, I.M.R., Vidal, J.E., de Mattos, C.d.C.B., de Mattos, L.C., Qu, D., Su, C. and Pereira-Chioccola, V.L. (2011). *Toxoplasma gondii* isolates: Multilocus RFLP-PCR genotyping from human patients in Sao Paulo State, Brazil identified distinct genotypes. *Experimental Parasitology* **129**, 190-195.

Ferreira, I. M., Vidal, J. E., Costa-Silva, T. A., Meira, C. S., Hiramoto, R. M., Penalva De Oliveira, A. C. and Pereira-Chioccola, V. L. (2008). *Toxoplasma gondii*: genotyping of strains from Brazilian AIDS patients with cerebral toxoplasmosis by multilocus PCR-RFLP markers. *Experimental Parasitology* **118**, 221–227.

Ferreira AdM, Vitor RWA, Carneiro ACAV, Brandao GP, Melo MN. (2004). Genetic variability of Brazilian *Toxoplasma gondii* strains detected by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and simple sequence repeat anchored-PCR (SSR-PCR). *Infection, Genetics and Evolution*. **4,** 131-142.

Ferreira, A. D. M., Vitor, R. W., Gazzinelli, R. T. and Melo, M. N. (2006). Genetic analysis of natural recombinant Brazilian *Toxoplasma gondii* strains by multilocus PCR-RFLP. *Infection, Genetics and Evolution* **6**, 22–31.

Fleckenstein MC, Reese ML, Konen-Waisman S, Boothroyd JC, Howard JC, Steinfeldt T. (2012). A *Toxoplasma gondii* pseudokinase inhibits host IRG resistance proteins. *PLoS Biology*. **10,** e1001358.

Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, Akira S, Aderem A. (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* **432:**917-921.

Freyre A, Falcón J, Mendez J, Correa O, Morgades D, Rodríguez A. (2004). An investigation of sterile immunity against toxoplasmosis in rats. *Experimental Parasitology*. **107**(1-2), 14-9

Fux, B., Rodrigues, C.V., Portela, R.W., Silva, N.M., Su, C., Sibley, D., Vitor, R.W., Gazzinelli, R.T. (2003) Role of cytokines and major histocompatibility complex restriction in mouse resistance to infection with a natural recombinant strain (type I-III) of *Toxoplasma gondii*. *Infection and Immunity*. **71**, 6392-6401.

Gamble, H.R., Dubey, J.P. and 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.

Gatkowska, J., Wieczorek, M., Dziadek, B., Dzitko, K. and Dlugonska, H. (2012). Sex-dependent neurotransmitter level changes in brains of *Toxoplasma gondii* infected mice. *Experimental Parasitology* **133**, 1-7.

Gazzinelli RT, Wysocka M, Hieny S, Scharton-Kersten T, Cheever A, Kühn R, Müller W, Trinchieri G, Sher A. (1996). In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *Journal of Immunology*. **157**(2), 798-805.

Gilbert RE, Freeman K, Lago EG, Bahia-Oliveira LM, Tan HK, Wallon M, Buffolano W, Stanford MR, Petersen E (2008). European Multicentre Study on Congenital Toxoplasmosis (EMSCOT). Ocular sequelae of congenital toxoplasmosis in Brazil compared with Europe. *PLoS Negled Tropical Diseases*. **2**(8), e277.

Goldstein, D. B. and Schlotterer, C. (1999). Microsatellites : Evolution and Applications. Oxford University Press, Oxford, UK.

Grigg, M.E., Ganatra, J., Boothroyd, J.C. and Margolis, T.P. (2001). Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *Journal of Infectious Diseases* **184**, 633-639.

Hamdani, N., Daban-Huard, C., Lajnef, M., Richard, J.R., Delavest, M., Godin, O., Le Guen, E., Vederine, F.E., Lépine, J.P., Jamain, S., Houenou, J., Le Corvoisier, P., Aoki, M., Moins-Teisserenc, H., Charron, D., Krishnamoorthy, R., Yolken, R., Dickerson, F., Tamouza, R., Leboyer, M. (2013). Relationship between *Toxoplasma gondii* infection and bipolar disorder in a French sample. *Journal of Affective Disorders* **148,** 444-448.

Haroon, F., Handel, U., Angenstein, F., Goldschmidt, J., Kreutzmann, P., Lison, H., Fischer, K.D., Scheich, H., Wetzel, W., Schlüter, D. and Budinger, E. (2012). *Toxoplasma gondii* actively inhibits neuronal function in chronically infected mice. *PLoS One*. **7**, e35516.

Hayashi H, Matsubara H, Yokota T, Kuwabara I, Kanno M, Koseki H, Isono K, Asano T, Taniguchi M. (1992). Molecular cloning and characterization of the gene encoding mouse melanoma antigen by cDNA library transfection. *Journal of Immunology*. **149**(4), 1223-9.

Herrmann DC, Pantchev N, Vrhovec MG, Barutzki D, Wilking H, Fröhlich A, Lüder CG, Conraths FJ, Schares G. (2010). Atypical *Toxoplasma gondii* genotypes identified in oocysts shed by cats in Germany. *International Journal for Parasitology*. **40**(3), 285-92.

Hill RD, Gouffon JS, Saxton AM, Su C. (2012). Differential gene expression in mice infected with distinct *Toxoplasma* strains. *Infection and Immunity*. **80**(3), 968-74.

Holland GN. (2003). Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease. *American Journal of Ophthalmology*. **136**(6):973-88.

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

Howard JC, Hunn JP, Steinfeldt T. (2011). The IRG protein-based resistance mechanism in mice and its relation to virulence in *Toxoplasma gondii*. *Current Opinions in Microbiology*. **14**(4), 414-21.

Howe, D. K., Honore, S., Derouin, F. and Sibley, L. D. (1997). Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. *Journal of Clinical Microbiology* **35**, 1411–1414.

Howe, D.K. and Sibley, L.D. (1995). *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *Journal of Infectious Diseases* **172**, 1561-1566.

Huang, S.Y., Cong, W., Zhou, P., Zhou, D.H., Wu, S.M., Xu, M.J., Zou, F.C., Song, H.Q. and Zhu, X.Q. (2012). First report of genotyping of *Toxoplasma gondii* isolates from wild birds in China. *Journal of Parasitology* **98,** 681-682.

Hurtado, A., Aduriz, G., Moreno, B., Barandika, J. and Garcia-Perez, A. L. (2001). Single tube nested PCR for the detection of *Toxoplasma gondii* in fetal tissues from naturally aborted ewes. *Veterinary Parasitology* **102**, 17–27.

Jacobs L, JS Remington and ML Melton. *1960*. A survey of meat samples from swine cattle, and sheep fort he presence of encysted *Toxoplasma*. *Journal of Parasitology*. **46**: 23-28.
Jauregui, L. H., Higgins, J., Zarlenga, D., Dubey, J. P. and Lunney, J. K. (2001). Development of a real-time PCR assay for detection of *Toxoplasma gondii* in pig and mouse tissues. *Journal of Clinical Microbiology* **39**, 2065–2071.

Jensen KD, Wang Y, Wojno ED, Shastri AJ, Hu K, Cornel L, Boedec E, Ong YC, Chien YH, Hunter CA, Boothroyd JC, Saeij JP. 2011. *Toxoplasma* polymorphic effectors determine macrophage polarization and intestinal inflammation. *Cell Host Microbe* **9**, 472-483.

Warren E. Johnson, Eduardo Eizirik, Jill Pecon-Slattery, William J. Murphy, Agostinho Antunes, Emma Teeling, and Stephen J. O'Brien. (2006). The Late Miocene Radiation of Modern Felidae: A Genetic Assessment. *Science.* **311**, 73-77.

Jones PP, Murphy DB, McDevitt HO. (1981). Variable synthesis and expression of E alpha and Ae (E beta) Ia polypeptide chains in mice of different H-2haplotypes. *Immunogenetics*. **12**(3-4). 321-37.

Kasper, D. C., Sadeghi, K., Prusa, A.-R., Reischer, G. H., Kratochwill, K., FoÅNrster-Waldl, E., Gerstl, N., Hayde, M., Pollak, A. and Herkner, K. R. (2009). Quantitative real-time polymerase chain reaction for the accurate detection of *Toxoplasma gondii* in amniotic fluid. *Diagnostic Microbiology and Infectious Disease* **63**, 10–15.

Kerr, PJ. 2012. Myxomatosis in Australia and Europe: a model for emerging infectious diseases. *Antiviral Research*. **93**(3):387-415.

Khalifa, K. E. S., Roth, A., Roth, B., Arasteh, K. N. and Janitschke, K. (1994). Value of PCR for evaluating occurrence of parasitemia in immunocompromised patients with cerebral and extracerebral toxoplasmosis. *Journal of Clinical Microbiology* **32**, 2813–2819.

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. *International Journal for Parasitology* **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. *Proceedings of the National Academy of Sciences of the USA* **104**, 14872-14877.

Khan, A., Jordan, C., Muccioli, C., Vallochi, A. L., Rizzo, L. V., Belfort Jr., R., Vitor, R. W. A., Silveira, C. and Sibley, L. D. (2006). Genetic divergence of *Toxoplasma gondii* strains associated with ocular Toxoplasmosis. *Brazil. Emerging and Infectious Diseases* **12**, 942–949.

Khan IA, MacLean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, Luster AD. (2000). IP-10 is critical for effector T cell trafficking and host survival in *Toxoplasma gondii* infection. Immunity **12,** 483-494.

Khan, A., Su, C., German, M., Storch, G. A., Clifford, D. B. and Sibley, L. D. (2005a). Genotyping of *Toxoplasma gondii* Strains from immunocompromised patients reveals high prevalence of type I strains. *Journal of Clinical Microbiology* **43**, 5881–5887.

Khan A, Taylor S, Ajioka JW, Rosenthal BM, Sibley LD. (2009). Selection at a single locus leads to widespread expansion of *Toxoplasma gondii* lineages that are virulent in mice. *PLoS Genetics*. **5,** e1000404.

Khan, A., Taylor, S., Su, C., Mackey, A. J., Boyle, J., Cole, R., Glover, D., Tang, K., Paulsen, I. T., Berriman, M., Boothroyd, J. C., Pfefferkorn, E. R., Dubey, J. P., Ajioka, J. W., Roos, D. S., Wootton, J. C. and Sibley, L. D. (2005b). Composite genome map and recombination parameters derived from three archetypal lineages of *Toxoplasma gondii*. *Nucleic Acids Research* **33**, 2980–2992.

Kim K, Weiss LM. (2008). *Toxoplasma*: the next 100 years. *Microbes and Infection*. **10**(9), 978-84.

Krahenbuhl JL, Blazkovec AA, Lysenko MG. (1971) The use of tissue culture-grown trophozoites of an avirulent strain of *Toxoplasma gondii* for the immunization of mice and guinea pigs. *Journal of Parasitology*. **57**(2), 386-90.

Krücken J, Dkhil MA, Braun JV, Schroetel RM, El-Khadragy M, Carmeliet P, Mossmann H, Wunderlich F. (2005) Testosterone suppresses protective responses of the liver to blood-stage malaria. *Infection and Immunity*. **73**(1), 436-443.

Kupferschmidt, O., Kruger, D., Held, T. K., Ellerbrok, H., Siegert, W. and Janitschke, K. (2001). Quantitative detection of *Toxoplasma gondii* DNA in human body fluids by TaqMan polymerase chain reaction. *Clinical Microbiology and Infection* **7**, 120–124.

Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang MJ, He CH, Takyar S, Elias JA. (2011). Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annual Review of Physiology*. **73:**479-501.

Lehmann, T., Graham, D. H., Dahl, E. R., Bahia- Oliveira, L. M., Gennari, S. M. and Dubey, J. P. (2004). Variation in the structure of *Toxoplasma gondii* and the roles of selfing, drift, and epistatic selection in maintaining linkage disequilibria. *Infection, Genetics and Evolution* **4**, 107–114.

Lehmann, T., Marcet, P.L., Graham, D.H., Dahl, E.R. and Dubey, J.P. (2006). Globalization and the population structure of *Toxoplasma gondii*. *Proceedings of the National Academy of Sciences of the USA* **103**, 11423-11428.

Li M, Mo XW, Wang L, Chen H, Luo QL, Wen HQ, Wei W, Zhang AM, Du J, Lu FL, LunZR, Shen JL. (2014). Phylogeny and virulence divergency analyses of *Toxoplasma gondii* isolates from China. *Parasites and Vectors*. **7**, 133.

Liesenfeld O. (2002). Oral infection of C57BL/6 mice with *Toxoplasma gondii*: a new model of inflammatory bowel disease? *Journal of Infectious Disease*. **185**, S96-S101.

Liesenfeld O, Kosek J, Remington JS, Suzuki Y. Association of CD4+ T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with *Toxoplasma gondii*. J Exp Med. 1996 Aug 1;184(2):597-607. PubMed PMID: 8760813; PubMed Central PMCID: PMC2192709.

Liesenfeld O, Parvanova I, Zerrahn J, Han SJ, Heinrich F, Munoz M, Kaiser F, Aebischer T, Buch T, Waisman A, Reichmann G, Utermohlen O, von Stebut E, von Loewenich FD, Bogdan C, Specht S, Saeftel M, Hoerauf A, Mota MM, Konen-Waisman S, Kaufmann SH, Howard JC. (2011). The IFN-gamma-inducible GTPase, Irga6, protects mice against *Toxoplasma gondii* but not against *Plasmodium berghei* and some other intracellular pathogens. *PLoS One* **6:**e20568.

Liesenfeld, O., Roth, A., Weinke, T., Foss, H. D. and Hahn, H. (1994). A case of disseminated toxoplasmosis-value of PCR for the diagnosis. *Journal of Infection* **29**, 133–138.

Lin, M. H., Chen, T. C., Kuo, T., Tseng, C. C. and Tseng, C. P. (2000). Real-time PCR for quantitative detection of *Toxoplasma gondii*. *Journal of Clinical Microbiology* **38**, 4121–4125.

Martens S, Parvanova I, Zerrahn J, Griffiths G, Schell G, Reichmann G, Howard JC. (2005). Disruption of *Toxoplasma gondii* parasitophorous vacuoles by the mouse p47-resistance GTPases. *Plos Pathogens*. **1,** 187-201.

McLeod, R., Eisenhauer, P., Mack, D., Brown, C., Filice, G. and Spitalny, G. (1989) Immune responses associated with early survival after peroral infection with *Toxoplasma gondii*. *Journal of Immunology.* **142**, 3247-3255.

Melo MB, Jensen KD, Saeij JP. (2011). *Toxoplasma gondii* effectors are master regulators of the inflammatory response. *Trends in Parasitology*. **27,** 487-495.

Melo MB, Kasperkovitz P, Cerny A, Konen-Waisman S, Kurt-Jones EA, Lien E, Beutler B, Howard JC, Golenbock DT, Gazzine lli RT. (2010). UNC93B1 mediates host resistance to infection with *Toxoplasma gondii*. *PLoS Pathogens*. **6,** e1001071.

Mercier, A., Ajzenberg, D., Devillard, S., Demar, M.P., de Thoisy, B., Bonnabau, H., Collinet, F., Boukhari, R., Blanchet, D., Simon, S., Carme, B. and Darde, M.L. (2011). Human impact on genetic diversity of *Toxoplasma gondii*: Example of the anthropized environment from French Guiana. *Infection, Genetics and Evolution.* **11**, 1378-1387.

Mercier, A., Devillard, S., Ngoubangoye, B., Bonnabau, H., Bañuls, A.-L., Durand, P., Salle, B., Ajzenberg, D. and Dardé, M.-L. (2010). Additional haplogroups of *Toxoplasma gondii* out of Africa: population structure and mouse-virulence of strains from Gabon. *PLoS Neglected Tropical Diseases* **4**, e876.

Miller, M.A., Miller, W.A., Conrad, P.A., James, E.R., Melli, A.C., Leutenegger, C.M., Dabritz, H.A., Packham, A.E., Paradies, D., Harris, M., Ames, J., Jessup, D.A., Worcester, K. and Grigg, M.E. (2008). Type X *Toxoplasma gondii* in a wild mussel and terrestrial carnivores from coastal California: new linkages between terrestrial mammals, runoff and toxoplasmosis of sea otters. *International Journal for Parasitology.* **38**, 1319-1328.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. J.Immunol. **164**, 6166-6173.

Montoya, J.G. and Liesenfeld, O. (2004). Toxoplasmosis. *Lancet* **363**, 1965-1976.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. (2001). Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology*., **19**, 683-765.

Morrison DA. (2009). Evolution of the Apicomplexa: where are we now? *Trends in Parasitology*. **25**(8), 375-82.

Muradian, V., Ferreira, L.R., Lopes, E.G., Esmerini, P.d.O., Pena, H.F., Soares, R.M. and Gennari, S.M. (2012). A survey of *Neospora caninum* and *Toxoplasma gondii* infection in urban rodents from Brazil. *Journal of Parasitology* **98**, 128-134.

Norose K, Kikumura A, Luster AD, Hunter CA, Harris TH. (2011). CXCL10 Is Required to Maintain T-Cell Populations and to Control Parasite Replication during Chronic Ocular Toxoplasmosis. *Investations in Ophthalmology and Vision Science.* **52,** 389-398.

Okay, T. S., Yamamoto, L., Oliveira, L. C., Manuli, E. R., Andrade Junior, H. F. D. and Del Negro, G. M. B. (2009). Significant performance variation among PCR systems in diagnosing congenital toxoplasmosis in Sao Paulo, Brazil: analysis of 467amniotic fluid samples. *Clinics*. **64**, 171–176.

Ong YC, Boyle JP, Boothroyd JC. (2011). Strain-dependent host transcriptional responses to *Toxoplasma* infection are largely conserved in mammalian and avian hosts. *PLoS One.* **6,** e26369.

Ong YC, Reese ML, Boothroyd JC. (2010). *Toxoplasma* rhoptry protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6. *Journal of Biology and Chemistry*. **285**(37), 28731-40.

Opgen-Rhein R, Strimmer K. (2007). From correlation to causation networks: a simple approximate learning algorithm and its application to high-dimensional plant gene expression data. *BMC Systems Biology*. **1,** 37-46.

Opgen-Rhein R, Strimmer K. (2006). Using regularized dynamic correlation to infer gene dependency networks from time-series microarray data, p73-76. *Proceeding of the 4th International Workshop on Computational Systems Biology*. Tampere University of Technology, Tampere, Finland.

Parameswaran, N., Thompson, R.C., Sundar, N., Pan, S., Johnson, M., Smith, N.C. and Grigg, M.E. (2010). Non-archetypal Type II-like and atypical strains of *Toxoplasma gondii* infecting marsupials of Australia. *International Journal for Parasitology* **40**, 635-640.

Pawlowski N, Khaminets A, Hunn JP, Papic N, Schmidt A, Uthaiah RC, Lange R, Vopper G, Martens S, Wolf E, Howard JC. 2011. The activation mechanism of Irga6,
an interferon-inducible GTPase contributing to mouse resistance against *Toxoplasma gondii*. BMC Biol. **9,** 7-21.

Pearce, B.D., Kruszon-Moran, D., Jones, J.L. (2012). The relationship between *Toxoplasma gondii* infection and mood disorders in the third National Health and Nutrition Survey. *Biological Psychiatry* **72**, 290-295.

Pelloux, H., Guy, D., Angelici, M. C., Aspock, H., BessieÅL res, M., Blatz, R., Del Pezzo, M., Girault, V., Gratzl, R., Holberg-Petersen, M., Johnson, J., Kruger, D., Lappalainen, M., Naessens, A. and Olsson, M. (1998). A second European collaborative study on polymerase chain reaction for *Toxoplasma gondii*, involving 15 teams. *FEMS Microbiology Letters.* **165**, 231–237.

Pena, H.F.J., Gennari, S.M., Dubey, J.P. and Su, C. (2008). Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil. *International Journal for Parasitology.* **38**, 561-569.

Pena, H.F.J., Marvulo, M.F.V., Horta, M.C., Silva, M.A., Silva, J.C.R., Siqueira, D.B., Lima, P.A.C.P., Vitaliano, S.N. and Gennari, S.M. (2011). Isolation and genetic characterisation of *Toxoplasma gondii* from a red-handed howler monkey (*Alouatta belzebul*), a jaguarundi (*Puma yagouaroundi*), and a black-eared opossum (*Didelphis aurita*) from Brazil. *Veterinary Parasitology.* **175**, 377-381.

Pena, H.F.J., Soares, R.M., Amaku, M., Dubey, J.P. and Gennari, S.M. (2006). *Toxoplasma gondii* infection in cats from Sao Paulo state, Brazil: Seroprevalence, oocyst shedding, isolation in mice, and biologic and molecular characterization. *Research in Veterinary Science.* **81**, 58-67.

Pena HF, Marvulo MF, Horta MC, Silva MA, Silva JC, Siqueira DB, Lima PA, Vitaliano SN, Gennari SM (2011). Isolation and genetic characterisation of *Toxoplasma gondii* from a red handed howler monkey (*Alouatta belzebul*), a jaguarundi (*Puma yagouaroundi*), and a black-eared opossum (*Didelphis aurita*) from Brazil. Veterinary Parasitology. **175**(3-4), 377-81.

Perkins FO, Barta JR, Clopton RE, Peirce MA, Upton SJ (2000). "Phylum Apicomplexa". In Lee JJ, Leedale GF, Bradbury P. *An Illustrated guide to the Protozoa : organisms traditionally referred to as protozoa, or newly discovered groups* **1** (2nd ed.). Society of Protozoologists. pp. 190–369

Pollard AM, Knoll LJ, Mordue DG. 2009. The role of specific *Toxoplasma gondii* molecules in manipulation of innate immunity. *Trends in Parasitology*. **25:**491-494.

Prestrud, K.W., Åsbakk, K., Mørk, T., Fuglei, E., Tryland, M. and Su, C. (2008a). Direct high-resolution genotyping of *Toxoplasma gondii* in arctic foxes (*Vulpes lagopus*) in the remote arctic Svalbard archipelago reveals widespread clonal Type II lineage. *Veterinary Parasitology* **158**, 121-128.

Prestrud, K.W., Dubey, J.P., Asbakk, K., Fuglei, E. and Su, C. (2008b). First isolate of *Toxoplasma gondii* from arctic fox (*Vulpes lagopus*) from Svalbard. *Veterinary Parasitology* **151**, 110-114.

Pusch L, Romeike B, Deckert M, Mawrin C. (2009). Persistent *Toxoplasma* bradyzoite cysts in the brain: incidental finding in an immunocompetent patient without evidence of a toxoplasmosis. *Clinical Neuropathology*. **28**(3), 210-2.

Qian, W., Wang, H., Su, C., Shan, D., Cui, X., Yang, N., Lv, C. and Liu, Q. (2012). Isolation and characterization of *Toxoplasma gondii* strains from stray cats revealed a single genotype in Beijing, China. *Veterinary Parasitology* **187**, 408-413.

Ragozo, A.M.A., Pena, H.F.J., Yai, L.E.O., Su, C. and Gennari, S.M. (2010). Genetic diversity among *Toxoplasma gondii* isolates of small ruminants from Brazil: Novel genotypes revealed. *Veterinary Parasitology* **170**, 307-312.

Rajendran, C., Su, C. and Dubey, J.P. (2012). Molecular genotyping of *Toxoplasma gondii* from Central and South America revealed high diversity within and between populations. *Infection, Genetics and Evolution* **12**, 359-368.

Reese ML, Shah N, Boothroyd JC. (2014). The *Toxoplasma* Pseudokinase ROP5 Is an Allosteric Inhibitor of the Immunity-related GTPases. Journal of Biology and Chemistry. **289**(40), 27849-58.

Reese ML, Zeiner GM, Saeij JP, Boothroyd JC, Boyle JP. (2011). Polymorphic family of injected pseudokinases is paramount in *Toxoplasma* virulence. Proceedings of the National Academy of Sciences of the U S A. **108**(23), 9625-30.

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

Reikvam A, Lorentzen-Styr AM. (1976). Virulence of different strains of *Toxoplasma gondii* and host response in mice. *Nature*. **261**(5560):508-9.

Remington JS, Cavanaugh EN. (1965). Isolation of the encysted form of *Toxoplasma gondii* from human skeletal muscle and brain. New England Journal of Medicine. **273**(24), 1308-10.

Rengifo-Herrera, C., Ortega-Mora, L.M., Alvarez-García, G., Gómez-Bautista, M., García-Párraga, D., García-Peña, F.J. and Pedraza-Díaz, S. (2012). Detection of *Toxoplasma gondii* antibodies in Antarctic pinnipeds. *Veterinary Parasitology* **190,** 259-262.

Robben PM, Mordue DG, Truscott SM, Takeda K, Akira S, Sibley LD. (2004). Production of IL-12 by macrophages infected with *Toxoplasma gondii* depends on the parasite genotype. *Journal of Immunology*. **172:**3686-3694.

Rosenthal, B.M. (2009). How has agriculture influenced the geography and genetics of animal parasites? *Trends in Parasitology* **25,** 67-70.

Rosowski EE, Lu DN, Julien L, Rodda L, Gaiser RA, Jensen KDC, Saeij JPJ. 2011. Strain-specific activation of the NF-kappa B pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. J. Exp. Med. **208:**195-212.

Sahm M, Gross U, Reiter-Owona I, Seitz HM. (1998). Infection and stage conversion during murine pulmonary toxoplasmosis: a study with three different strains of *Toxoplasma gondii*. *Journal of Parasitology*. **84**(4), 723-9.

Saeij JP, Boyle JP, Boothroyd JC. (2005). Differences among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host. Trends Parasitol. **21:**476-481.

Saeij JP, Boyle JP, Coller S, Taylor S, Sibley LD, Brooke-Powell ET, Ajioka JW, Boothroyd JC. (2006). Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science*. **314**(5806):1780-3.

Saeij JP, Coller S, Boyle JP, Jerome ME, White MW, Boothroyd JC. (2007). *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature*. **445,** 324-327.

Schäfer J, Opgen-Rhein R, Strimmer K. (2006). Reverse Engineering Genetic Networks using the GeneNet Package. *R News*. **6**, 50-53.

Schafer J, Strimmer K. (2005). An empirical Bayes approach to inferring large-scale gene association networks. *Bioinformatics*. **21,** 754-764.

Schmidt-Ott KM, Mori K, Li JY, Kalandadze A, Cohen DJ, Devarajan P, Barasch J. (2007). Dual action of neutrophil gelatinase-associated lipocalin. Journal of the American Society for Nephrology. **18,** 407-413.

Schmittgen TD, Livak KJ. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*. **3**, 1101-1108.

Schoondermark-Van De Ven, E., Melchers, W., Camps, W., Eskes, T., Meuwissen, J. and Galama, J. (1994). Effectiveness of spiramycin for treatment of congenital *Toxoplasma gondii* infection in Rhesus monkeys. *Antimicrobial Agents and Chemotherapy* **38**, 1930–1936.

Shapiro SD, Goldstein NM, Houghton AM, Kobayashi, DK, Kelley DG, Belaaouaj A. (2003). Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. American Journal of Pathology. **163**, 2329–2335.

Shwab EK, Zhu XQ, Majumdar D, Pena HF, Gennari SM, Dubey JP, Su C. (2014). Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. *Parasitology*. **5**, 1-9

Sibley, L. D. and Boothroyd, J. C. (1992). Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature*, **359**, 82–85.

Sibley LD, Khan A, Ajioka JW, Rosenthal BM. 2009. Genetic diversity of *Toxoplasma gondii* in animals and humans. *Philos. Trans. R. Soc. Lond., B, Bio. Sci*. **364,** 2749-2761.

Silva LA, Brandão GP, Pinheiro BV, Vitor RW. (2012). Immunosuppression with cyclophosphamide favors reinfection with recombinant *Toxoplasma*gondii strains.. *Parasite*. **19**(3), 249-57.

Soares, R.M., Silveira, L.H., da Silva, A.V., Ragozo, A., Galli, S., Lopes, E.G., Gennari, S.M. and de Jesus Pena, H.F. (2011). Genotyping of *Toxoplasma gondii* isolates from free range chickens in the Pantanal area of Brazil. *Veterinary Parasitology* **178**, 29-34.

Stark J, Chan C, George AJ. 2007. Oscillations in the immune system. Immunology Reviews. **216,** 213-231.

Steinfeldt T, Könen-Waisman S, Tong L, Pawlowski N, Lamkemeyer T, Sibley LD, Hunn JP, Howard JC. (2010). Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii*. *PLoS Biology*. **8**(12), e1000576.

Stumhofer JS, Laurence A, Wilson EH, Huang E, Tato CM, Johnson LM, Villarino AV, Huang Q, Yoshimura A, Sehy D, Saris CJ, O'Shea JJ, Hennighausen L, Ernst M, Hunter CA. (2006). Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nature Immunology*. **7**(9):937-45.

Su, C. and Dubey, J. P. (2009). *Toxoplasma*. In Molecular Detection of Foodborne Pathogens (ed. Liu, D.), pp. 741–753. CRC Press, Boca Raton, FL, USA.

Su, C., Evans, D., Cole, R.H., Kissinger, J.C., Ajioka, J.W. and Sibley, L.D. (2003). Recent expansion of *Toxoplasma* through enhanced oral transmission. *Science* **299,** 414-416.

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. and Sibley, L.D. (2012). Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. *Proceedings of the National Academy of Sciences of the USA* **109**, 5844-5849.

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.

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. *International Journal for Parasitology* **36**, 841-848.

Sukhumavasi W, Egan CE, Warren AL, Taylor GA, Fox BA, Bzik DJ, Denkers EY. 2008. TLR adaptor MyD88 is essential for pathogen control during oral *Toxoplasma gondii* infection but not adaptive immunity induced by a vaccine strain of the parasite. *Journal of Immunology*. **181**, 3464-3473.

Sundar, N., Cole, R.A., Thomas, N.J., Majumdar, D., Dubey, J.P. and Su, C. (2008). Genetic diversity among sea otter isolates of *Toxoplasma gondii. Veterinary Parasitology.* **151**, 125-132.

Suzuki Y, Conley FK, Remington JS. (1989). Differences in virulence and development of encephalitis during chronic infection vary with the strain of *Toxoplasma gondii*. *Journal of Infection and Disease*. **159**(4):790-4

Suzuki Y, Yang Q, Remington JS. (1995). Genetic resistance against acute toxoplasmosis depends on the strain of *Toxoplasma gondii*. Journal of Parasitology. **81**(6), 1032-4.

Switaj, K., Master, A., Skrzypczak, M. and Zaborowski, P. (2005). Recent trends in molecular diagnostics for *Toxoplasma gondii* infections. *Clinical Microbiology and Infection*. **11**, 170–176.

Suzuki Y, Orellana MA, Schreiber RD, Remington JS. (1988). Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science*. **240**, 516-518.

Takacs AC, Swierzy IJ, Luder CGK. 2012. Interferon-gamma restricts *Toxoplasma gondii* development in murine skeletal muscle cells via nitric oxide production and immunity-related GTPases. *PLoS One.* **7**, e45440.

Takeda K, Akira S. (2004). TLR signaling pathways. Seminars in Immunology. **16,** 3-9.

Taylor S, Barragan A, Su C, Fux B, Fentress SJ, Tang K, Beatty WL, Hajj HE, Jerome M, Behnke MS, White M, Wootton JC, Sibley LD. (2006). A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. S*cience*. **314**(5806):1776-80.

Tenter, A.M., Heckeroth, A.R. and Weiss, L.M. (2000). *Toxoplasma gondii*: from animals to humans. *International Journal for Parasitology* **30**, 1217-1258.

Torrey, E.F., Bartko, J.J., Lun, Z.R. (2007). Antibodies to *Toxoplasma gondii* in patients with schizophrenia: a meta-analysis. *Schizophrenia Bulletin* **33**, 729–736.

Velmurugan, G.V., Dubey, J.P. and Su, C. (2008). Genotyping studies of *Toxoplasma gondii* isolates from Africa revealed that the archetypal clonal lineages predominate as in North America and Europe. *Veterinary Parasitology* **155**, 314-318.

Velmurugan, G.V., Su, C. and Dubey, J.P. (2009). Isolate designation and characterization of *Toxoplasma gondii* isolates from pigs in the United States. *Journal of Parasitology* **95**, 95-99.

Virreira Winter S, Niedelman W, Jensen KD, Rosowski EE, Julien L, Spooner E, Caradonna K, Burleigh BA, Saeij JP, Ploegh HL, Frickel EM. (2011). Determinants of GBP recruitment to *Toxoplasma gondii* vacuoles and the parasitic factors that control it. *PLoS One*. **6**(9), e24434.

Wang D, Wang W, Dawkins P, Paterson T, Kalsheker N, Sallenave JM, Houghton AM. (2011). Deletion of Serpina1a, a murine α1-antitrypsin ortholog, results in embryonic lethality. *Experimental Lung Research*. **37**(5), 291-300.

Wen XS, Kudo T, Payne L, Wang XS, Rodgers L, Suzuki Y. (2010). Predominant interferon-gamma-mediated expression of CXCL9, CXCL10, and CCL5 proteins in the brain during chronic infection with *Toxoplasma gondii* in BALB/c mice resistant to development of toxoplasmic encephalitis. *Journal of Interferon Cytokine Research*. **30,** 653-660.

Xiao J, Buka SL, Cannon TD, Suzuki Y, Viscidi RP, Torrey EF, Yolken RH. (2009). Serological pattern consistent with infection with type I *Toxoplasma gondii* in mothers and risk of psychosis among adult offspring. *Microbes and Infection*. **11,** 1011-1018.

Xiao J, Jones-Brando L, Talbot CC, Jr., Yolken RH. (2011). Differential effects of three canonical *Toxoplasma* strains on gene expression in human neuroepithelial cells. Infection and Immunity. **79,** 1363-1373.

Yai, L.E.O., Ragozo, A.M.A., Soares, R.M., Pena, H.F.J., Su, C. and Gennari, S.M. (2009). Genetic diversity among capybara (*Hydrochaeris hydrochaeris*) isolates of *Toxoplasma gondii* from Brazil. *Veterinary Parasitology* **162**, 332-337.

Yan HK, Yuan ZG, Song HQ, Petersen E, Zhou Y, Ren D, Zhou DH, Li HX, Lin RQ, Yang GL, Zhu XQ. (2012). Vaccination with a DNA vaccine coding for perforin-like protein 1 and MIC6 induces significant protective immunity against *Toxoplasma gondii*. *Clinical Vaccine Immunology*. **19**(5), 684-9

Yamamoto M, Standley DM, Takashima S, Saiga H, Okuyama M, Kayama H, Kubo E, Ito H, Takaura M, Matsuda T, Soldati-Favre D, Takeda K. (2009). A single polymorphic amino acid on *Toxoplasma gondii* kinase ROP16 determines the direct and strain-specific activation of Stat3. *Journal of Experimental Medicine*. **206**(12), 2747-60.

Yamamoto M, Okuyama M, Ma JS, Kimura T, Kamiyama N, Saiga H, Ohshima J, Sasai M, Kayama H, Okamoto T, Huang DC, Soldati-Favre D, Horie K, Takeda J, Takeda K. (2012). A cluster of interferon-γ-inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. *Immunity*. **37**(2), 302-13.

Yolken, R.H., Bachmann, S., Ruslanova, I., Lillehoj, E., Ford, G., Torrey, E.F., Schroeder, J. (2001). Antibodies to *Toxoplasma gondii* in individuals with first-episode schizophrenia. *Clinical Infectious Diseases.* **32**, 842-844.

Yu J, Wei M, Mao H, Zhang J, Hughes T, Mitsui T, Park IK, Hwang C, Liu S, Marcucci G, Trotta R, Benson DM Jr, Caligiuri MA. (2009). CD94 defines phenotypically and functionally distinct mouse NK cell subsets. *Journal of Immunology*. **183**(8), 4968-74.

Zaki MM. 2011. Parasite platelet interactions. *Parasitologists United Journal.* **4:**127-136.

Zenner L, Darcy F, Capron A, Cesbron-Delauw MF. (1998). *Toxoplasma gondii*: kinetics of the dissemination in the host tissues during the acute phase of infection of mice and rats. *Experimental Parasitology*. **90**(1), 86-94.

Zhou, P., Nie, H., Zhang, L.X., Wang, H.Y., Yin, C.C., Su, C., Zhu, X.Q. and Zhao, J.L. (2010). Genetic characterization of *Toxoplasma gondii* isolates from pigs in China. *Journal of Parasitology* **96**, 1027–1029.

Zhou, P., Zhang, H., Lin, R.-Q., Zhang, D.-L., Song, H.-Q., Su, C. and Zhu, X.-Q. (2009). Genetic characterization of *Toxoplasma gondii* isolates from China. *Parasitology International* **58**, 193-195.

Appendices

Appendix A. Expanded PCR-RFLP genotyping reveals new diversity among globally distributed strains of *T. gondii*

Introduction

Toxoplasma gondii is among the most widespread eukaryotic parasites known. It chronically infects approximately one third of the world's human population and has been isolated from an extremely diverse array of mammals and birds from every continent besides Antarctica (Dubey *et al.* 2010). Understanding the structure of the worldwide *T. gondii* population enhances our understanding of the factors that have shaped that structure and led to the proliferation of one of the most evolutionarily successful pathogens on Earth. To this end, numerous studies have been performed in which various aspects of *T. gondii* population genetics have been analyzed using a variety of methods. Genotyping of *T. gondii* isolates has been carried out using intron sequencing, microsatellite DNA analysis, and PCR-RFLP analysis of single-nucleotide polymorphisms (SNPs). Of these, PCR-RFLP has been the most widely used. Though intron sequencing has the advantage of distinguishing strains at higher resolution than PCR-RFLP, the latter technique has the advantage of being less expensive and time-consuming to carry out. It can also be performed in any laboratory in the world equipped with basic molecular biology tools, and does not require access to a sequencing facility. Furthermore, despite providing higher resolution at particular loci, intron sequencing generally does not allow for broad coverage of the genome being examined unless many introns are sequenced, which is likely to become prohibitively costly and time-consuming, particularly for large sample sets. Thus, genotyping information is often limited to a localized portion of the genome. Furthermore, unlike both microsatellite and intron sequence analysis, both of which formulate genotypes based on largely nonfunctional DNA, PCR-RFLP allows for the analysis of more biologically relevant loci, such as surface antigen and virulence genes. It is perhaps for these reasons that most of the genotyping work thus completed has used the PCR-RFLP method.

The use of a standardized method for genotyping is highly desirable because it allows for the collation of data between separate studies so that broader patterns of diversity may be examined. To this end, we recently compiled all of the PCR-RFLP genotype data that had been published as of the end of 2012 using a widely-employed set of ten genetic markers, which included a total of 1,475 isolates comprising 189 distinct genotypes. These isolates were collected from all continents with the exception of Antarctica, and from a variety of animal species, both wild and domestic. Our analysis revealed distinct population structures for each major geographic region examined, with striking contrast between the highly diverse, endemic structure of the South/Central American region and the more clonal populations found in all other areas, wherein only a few dominant genotypes account for the overwhelming majority of isolates. The data obtained using this approach have given us the most complete picture to date of the global *T. gondii* population structure. However, this technique has shortcomings in that several chromosomes are not represented by the ten genetic markers employed, while most of the chromosomes that are included are only represented by a single locus, precluding analysis of chromosomal recombination. Furthermore, the standard markers used were originally designed to differentiate the 3 archetypeal *T. gondii* lineages, and thus may generate some degree of bias towards classifying strains according to this clonal population structure model. In order to better

understand the global diversity of *T. gondii* we have developed 9 additional novel PCR-RFLP markers (12 including ROP5, 16 and 18, see Chapter IV) designed to differentiate strains in a manner independent of the archetypal lineages which, combined with the ten standard markers, provide representation of all 14 nuclear *T. gondii* chromosomes and the apicoplast chromosome, with at least two loci examined on chromosomes of over 40kDa in length, potentially allowing for analysis of chromosomal recombination. These additional PCR-RFLP markers provide an analysis of the worldwide *T. gondii* population structure that is both comprehensive in geographical scope and also of higher resolution than previous RFLP-based studies.

Materials and methods

PCR-RFLP Protocols

PCR-RFLP protocols were designed for 12 genetic loci based on sequence data from 44 strains of *T. gondii*. Loci included Ia-P89, AK2, II-C35, BSR4, AK126, SRS4, UPRT-1, ROP1, MIC5, ROP16, ROP18 and ROP5. Polymorphic sites were identified for each locus and restrictions enzymes were selected to differentially digest PCR amplified DNA. External and internal primers and restriction enzymes for each locus are presented in Table A-1. All DNA samples from *T. gondii* isolates were amplified in 96-well PCR plates via multiplex PCR using all external primers simultaneously according to Su *et al.* (2010). Amplification for multiplex PCR used FastStart DNA Polymerase (Roche). The thermocyler program was as follows: 4 minutes at 95C, 30 cycles of 30 seconds at 95C, 1 minute at 55C, and 2 minutes at 74C. This was followed by nested PCR amplification using PAQ5000 DNA polymerase (Stratagene) with individual marker primers and a thermocycler program of 4 minutes at 95C, 35 cycles of 30 seconds at 95C, 1 minute at 58C, and two minutes at 72C. Restriction enzyme digestions were carried out using PCR amplified DNA in 96-well plates. Digested products were separated via electrophoresis in 2.5% agarose gels stained with ethidium bromide and visualized under UV light. Each gel contained a set of standard *T. gondii* reference strains including GT1, PTG, CTG. MAS, COUG, TgCtBr5, TgCtBr64 and TOUCAN which aided in genotype identification. Each plate also contained multiple no-template negative-controls.

T. gondii isolate DNA

DNA samples were provided by numerous laboratories from around the world. Specific information regarding isolate origins is provided in Shwab *et al.* (2014). Genomic DNA stocks were stored at -20C prior to analysis.

Network Analysis

Allelic data from multilocus analysis of PCR-RFLP markers were used to generate a network of *T. gondii* strain polymorphisms from 330 samples. Multilocus PCR-RFLP typing data were coded for all genetic loci. For a given locus, presence or absence of DNA restriction fragments was coded as either 1 or 0, respectively. SplitsTree v4.4 was used to generate an unrooted network using the neighbor-net method with 1,000 bootstrap replicates.

Table A-1. External and internal primers and restriction enzymes for PCR-RFLP markers

Marker	External Primers (5' to 3')	Internal Primers (5' to 3')	Restriction Enzymes
Ia-P89	Ia-P89-Fext: TCAAACACCGAGTCGTCATC Ia-P89-Rext: GTGCTCAACACGCTCGTAAG	Ia-P89-F: CCGCAAAGTAGTCGTCGTTA Ia-P89-R: AGCACTTTGTCGTTCGGACT	SfaNI+TseI
AK2	AK2-Fext: GGCCGAAGTGAAGCATAAAC AK2-R-extTAAAAGTGCAGAGCGGACAA	AK2F-int: CATGGCTTCGAGAACACTCA AK2R-int: CGTCTGCTGGAATGCCAAG	NheI+BcoDI
II-C35	II-C35-Fext: CGCCGAAAGGACATATGAGT II-C35-Rext: AACGATTTGGAAAGGTGTGC	II-C35-Fint: TTCATTCCCGAGTTCCTGAC II-C35-Rint: GACACGCGCTACAAATCTGA	Fnu4HI+HpyCH4III
BSR4	BSR4-Fext: CAGTTGTTGGCAGTTTGCAT BSR4-Rext: GCGTAGCTGAAGTCCGTTTC	BSR4-Fint: CAAGGCTAACCGTAGCGGGT BSR4-Rint: CTCTTCAGGAACTTGGGGAA	Hpy188I+RsaI
AK126	AK126-Fext: ACAAGGGCACAGCAACAT AK126-Rext: AACAATCGGCAAAGGTAG	AK126-Fint: CATGGCACGAGCTGCGGTAC AK126-Rint: GTTGTCATCGGTCAGTCG	Tsp509I
SRS4	SRS4-Fext: CGAGACCTTCTTTCGTTG SRS4-Rext: CAGGAGGCAGGATGATGT	SRS4-Fint: CGGCAAGGAAAGCAACCC SRS4-Rint: CTTCAAGCCCACCGTGTT	BbsI+MslI
UPRT1	UPRT1extF: TCCACAGGGCTTCTAAAAT UPRT1extR: GAGTTGAGAACAGGCTTCAG	UPRT1intF: CCCGATATTCGACAAACGAC UPRT1intR: GAGCCGTCTGCTTCATGAGC	RsaI+BstUI
ROP1	ROP1-Fext: GCATTCCAGGCAGTCTTAGG ROP1-Rext: CATCCTCTGGAAGCTCTTGG	ROP1-Fint: CGTGACATATACTGCACTG ROP1-Rint: CATCGTGAAACGTGTCATC	TspRI+MspA1I
MIC5	MIC5-Fext: ACAGCGAAGGAACTTCGAGA MIC5-Rext: GTTTGCGTGCTTGCTACAGA	MIC5-Fint: GCGGTGGTCAGATTCCTCTA MIC5-Rint: GCCCAGTGTCGATAGCAAAT	NlaIII+HaeIII
ROP16	ROP16F-ext: ATCTGCTTATCCGGCGACTA ROP16R-ext: TCCGTTGGCATTTATCATCA	ROP16F-int: TACCAAACCCAGCTTTCACC ROP16R-int: TCGTCAACAGCTGACTCCAC	NlaIII+TaqaI
ROP18	ROP18-DelFext: CTCGTCGACCACACAGCTAA ROP18-UPSRext: GAGTGCTTTCTGTCGCTCCT	ROP18-DelFint: AGTTCCCTTCCCTGGTGTCT ROP18-DelRint: ACAAACTGGACTGGGGTGAG Rop18-UPSFint: CACAGCATGAGCTTAAGAGTTG Rop18-UPSRint: CACCGCAAGACAGGCTGTCTTC	None required
ROP5	ROP5F-ext: GGACAGACGCAGGCTTTTAC ROP5R-ext: TCAAACGTCCTGACACTTCG	ROP5F-int: TGTGGCAGTTCAGTCTCAGC ROP5R-int: TCGAAGTTGAGGAACCGTCT	FspBI+HaeII BstUI

Results

Identification of genotypes at novel PCR-RFLP loci

PCR-RFLP protocols were developed in order to identify SNPs present at 12 genetic loci not previously analyzed using this technique. Figure A-1 shows the locations of these markers throughout the 14 nuclear chromosomes and apicoplast chromosome of *T. gondii*. Figure A-2 depicts the RFLP patterns at each of these loci for a set of eight diverse reference strains representing a number of major distinct phylogenetic clusters of *T. gondii* isolates identified previously (Su *et al.* 2010). These reference strains include GT1 (archetypal Type I), PTG (archetypal Type II), CTG (archetypal Type III), MAS, COUG (TgCgCa1), TgCatBr5, TgCatBr64 and TOUC (TgRsCr1). Precise phylogenetic relationships between these strains are illustrated in the network analysis presented in Figure A-3, discussed below. These reference strains capture the majority of the genetic diversity identified at each locus, however in some instances additional alleles were identified that were not present in any of these reference strains. Two alleles were identified for the AK126 and ROP1 markers. Three alleles were identified for the Ia-P89, AK2, SRS4 and ROP16 markers. Four alleles were identified for ROP18. Five alleles were identified for the UPRT-1, BSR4 and MIC5 markers. Seven alleles were identified for II-C35, and eight separate alleles were identified for ROP5. In some cases, alleles were very rare and were limited to a few or even only a single strain out of the 330 analyzed.

Genotyping of T. gondii isolates at 12 novel loci

A total of 330 strains were successfully genotyped using the developed methodology for the 12 markers described. When combined with the genotypic data for the 10 standard loci as described in Su *et. al* (2010), this new genotypic data revealed 183 distinct genotypes, compared to only 142 genotypes from the same set of isolates using the 10 standard markers alone. Table E-3 (Appendix E) shows the complete genotypic data at all 22 loci for each isolate. New diversity was identified among several genotypes that are dominant within major geographic regions. Genotypes #4 and #5, dominant in North America, were divided into three and two subtypes, respectively, genotype #6, dominant in South America, was divided into three subtypes, and genotype #20, identified frequently on the Indian subcontinent and the Middle East, was divided into three subtypes. Interestingly, genotype #9, she most commonly identified type from China and southeast Asia (sometimes referred to as Chinese I) was found to segregate into two subtypes, differing only at the ROP18 locus among the loci examined. One subtype, designated 4a in Table A-1, possesses ROP18 genotype #2, which includes the archetypal Type II allele, associated in previously published studies with a mouse-virulent phenotype, while the second subtype, 4b, possesses ROP18 genotype #3, associated with the avirulent Type III form of the gene.

Comparison of network analyses for a subset of 140 strains representing major phylogenetic groups identified previously (REF) using the set of 10 markers from Su *et al.* (2010) (Figure A-3A) and the full set of those markers combined with the 12 presented here (Figure A-3B) revealed marked rearrangement of the majority of these groups, with the notable exception of the archetypal Type II-related clade (indicated in green), which remained relatively conserved regardless of the marker set used for analysis. Network analysis of all 330 strains genotyped in

this study revealed distinct phylogenetic clusters formed by the 183 genotypes identified, as shown in Figure A-3C. Nine clusters are delineated in this figure, including strains closely associated with the archetypal Types I, II and III (clusters 1,2 and 3, respectively), along with several clusters of strains loosely related to Type I and primarily isolated from South America, including clusters 4, 5 and 9, the latter of which includes a subcluster including highly mouse-virulent strains identified in Guiana (GUY-MAT, GUY-DOS, GUY-BAS1, GUY-KOE, RUB and VAND, Carme *et al.* 2003). Clusters 7 and 8, located between archetypal Types II and III, are less highly resolved from one another phylogenetically which may be an indication of more extensive recombination between these groups.

Discussion

The nature of parasitic relationships generally necessitates a highly nuanced and specific co-evolution between host and symbiont. This is especially true in the case of pathogens that have developed the ability to produce chronic infections, usually through a selective process wherein virulence of the pathogen is modulated by evolution of both parasite pathogenicity factors and the host immune response until an equilibrium is achieved such that the parasite is able to survive indefinitely within the host without being eradicated and without attenuating the fitness of the host to an extent significant enough to impose selective pressure towards stronger anti-parasite defense mechanisms. In such instances, it is unsurprising that particular pathogens have a pronounced tendency to develop a high level of specificity for a particular type of host. It is in this light that the case of *T. gondii* may be viewed as particularly remarkable. Among the pathogens of eukaryotic organisms, *T. gondii* is perhaps the most versatile and ubiquitous. Unlike other members of its protozoan phylum, including the malarial *Plasmodium* species, each of which are known to infect only a very limited range of host species, *T. gondii* has been identified in the tissues of myriad animal hosts from both the mammalian and avian classes, findings which collectively suggest the ability of the parasite to utilize any endothermic organism as a viable host. Moreover, *T. gondii* has been found to be capable of infecting a wide variety of cell types, including muscular, neuronal, epidermal and lymphocytic cells from numerous host species - all cell types yet examined, in fact - further underscoring the pathogenic versatility of the protozoan. Even its closest known relative, *Hammondia hammondi*, though quite similar

Figure A-1. Chromosomal positions of new and standard PCR-RFLP markers. Approximate positions of PCR-RFLP markers among the 14 nuclear *T. gondii* chromosomes and the apicpolast chromosome (APICO marker). Chromosome numbers are identified by roman numerals. Numbers to the left of each chromosome represent genetic distances in centimorgans. Names of new markers are enclosed in boxes while standard markers are not. Adapted from *Khan et al. Nucleic Acids Res.* 2005 May 23;33(9):2980-92.

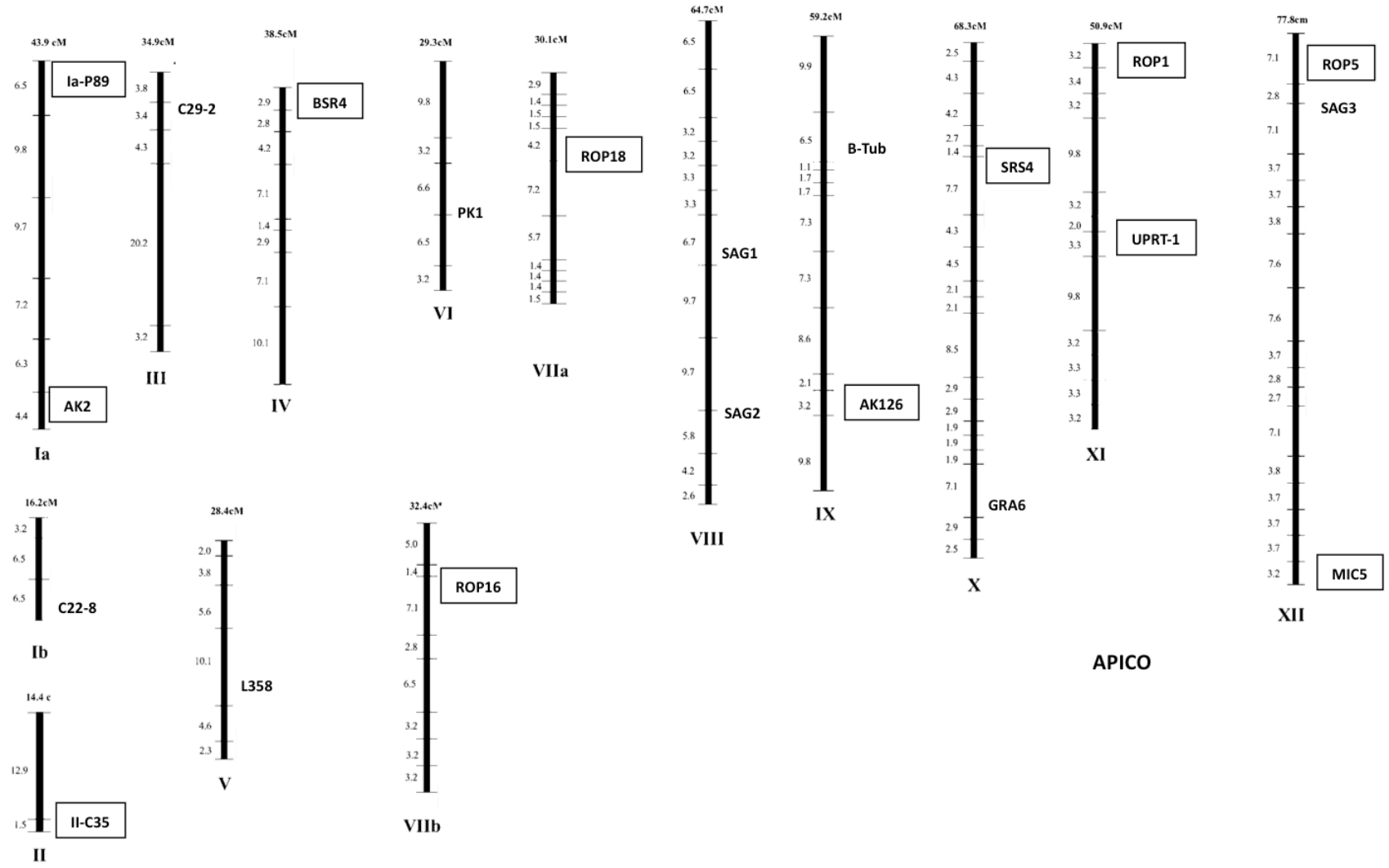


Figure A-1 continued

Figure A-2. Reference strain RFLP patterns for 12 PCR-RFLP markers. Restriction fragments of reference strains GT1, PTG, CTG, MAS, COUG, TgCatBr5, TgCatBr64 and TOUCAN for the 12 described PCR-RFLP markers using primers and restriction enzymes as given in Table 1. Strain IDs are presented above each column of gels and identified genotypes are indicated below each gel lane. Fragments were visualized under UV light following electrophoresis in 2.5% agarose gels containing ethidium bromide.

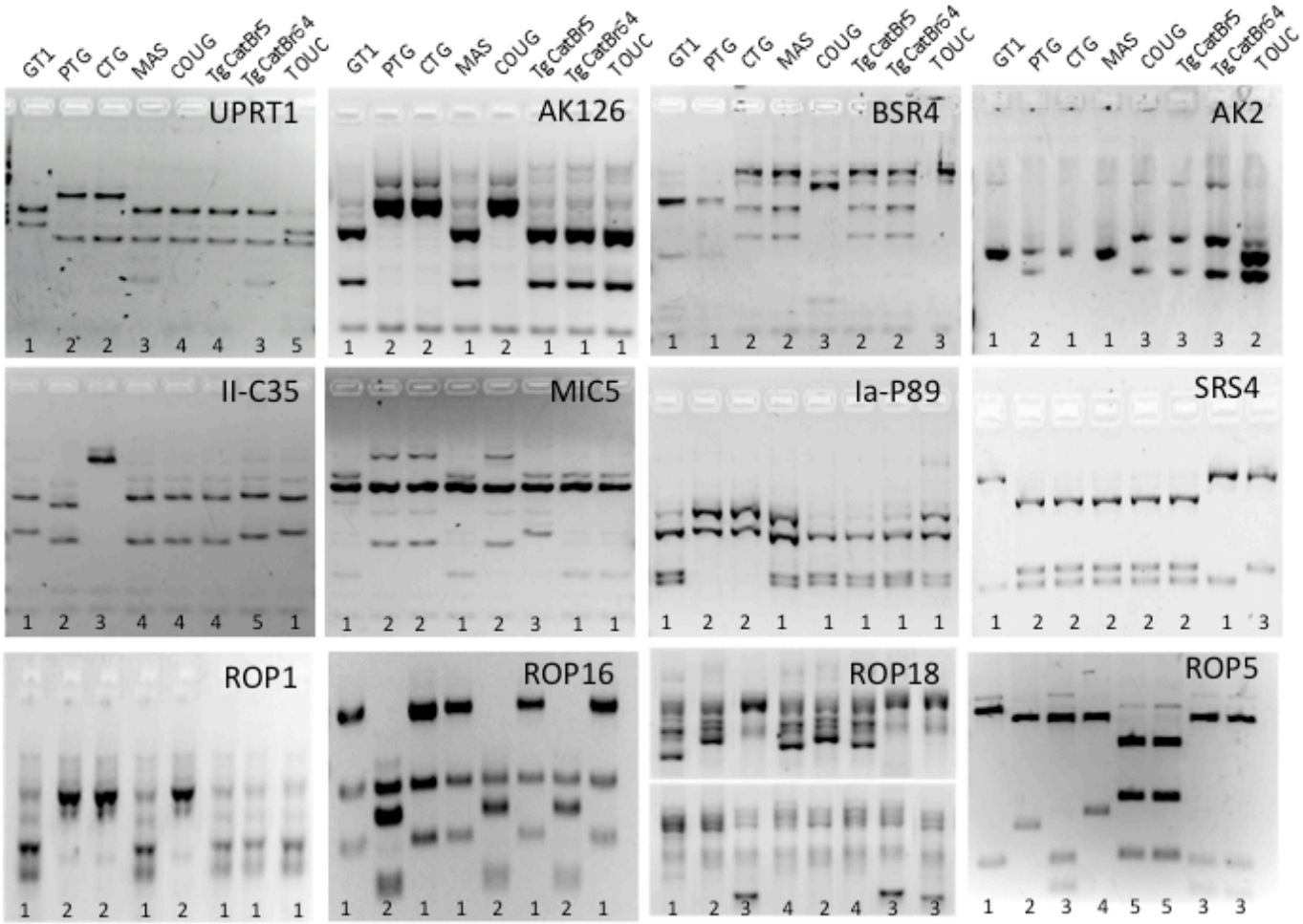


Figure A-2 continued

Figure A-3. Network analysis of strains using standard and expanded PCR-RFLP marker sets. Neighbor-joining networks were generated for strain sets using Splitstree v.2.2. (a) Network of 140 *T. gondii* strains representing known major clades using the 10 PCR-RFLP markers described in Su *et al.* (2010). Strain IDs are color coded by association with major clades. Reference strains are enclosed in boxes. Archetypal I, II and III clades are indicated. (B) Network of the same set of 140 strains from panel B using the 12 PCR-RFLP markers described in the current study in addition to those of Su *et al.* (2010). Color coding is the same as in panel B and reference strains are similarly indicated. (C) Network of 330 strains using the total marker set from Su *et al.* (2010) and the current study. Archetypal Type I, II and III clades are indicated via color coding (red, green and blue, respectively) and reference strains are again boxed. Additionally, 9 distinct clades are indicated via brackets.

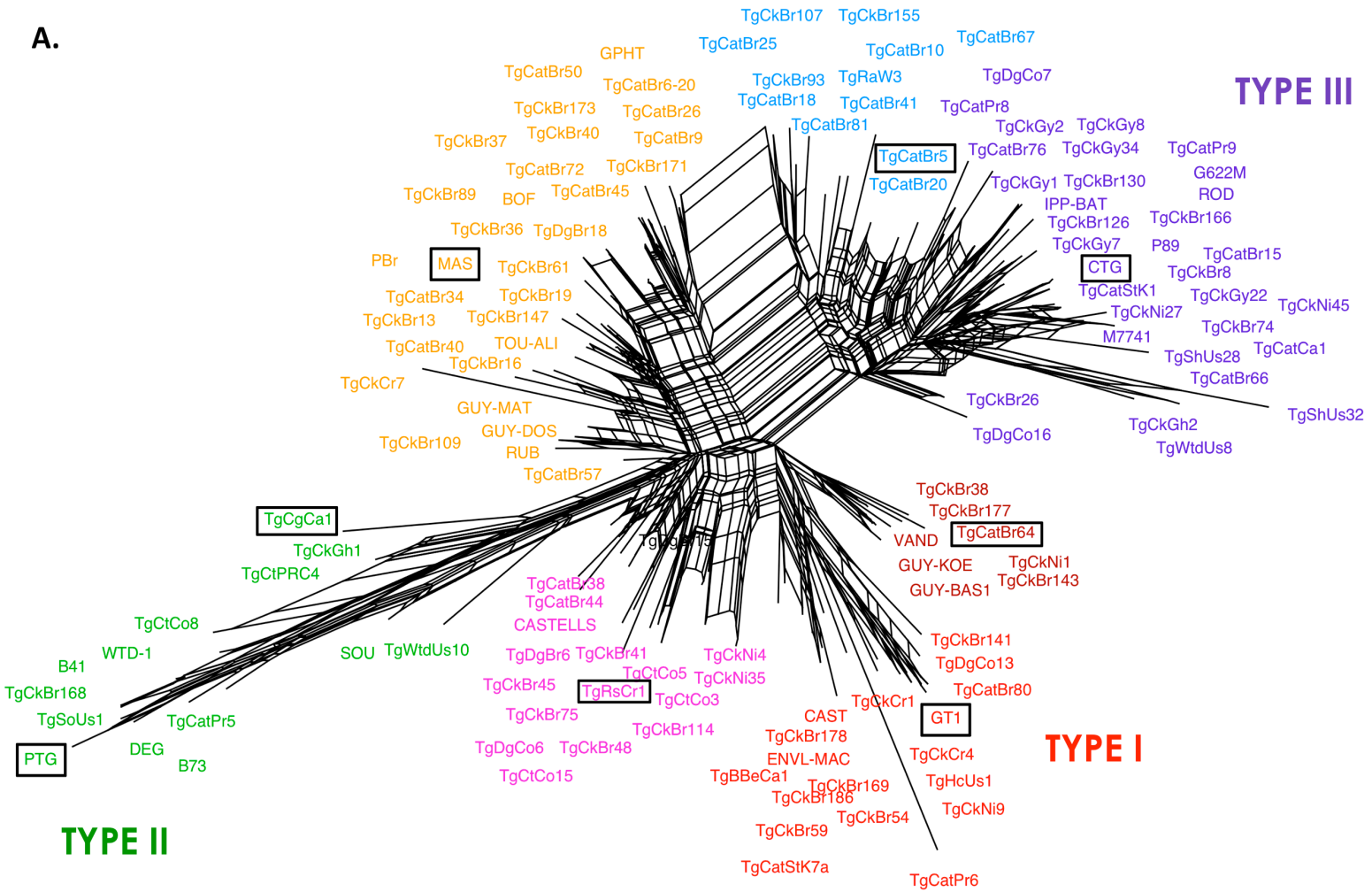


Figure A-3 continued

B.

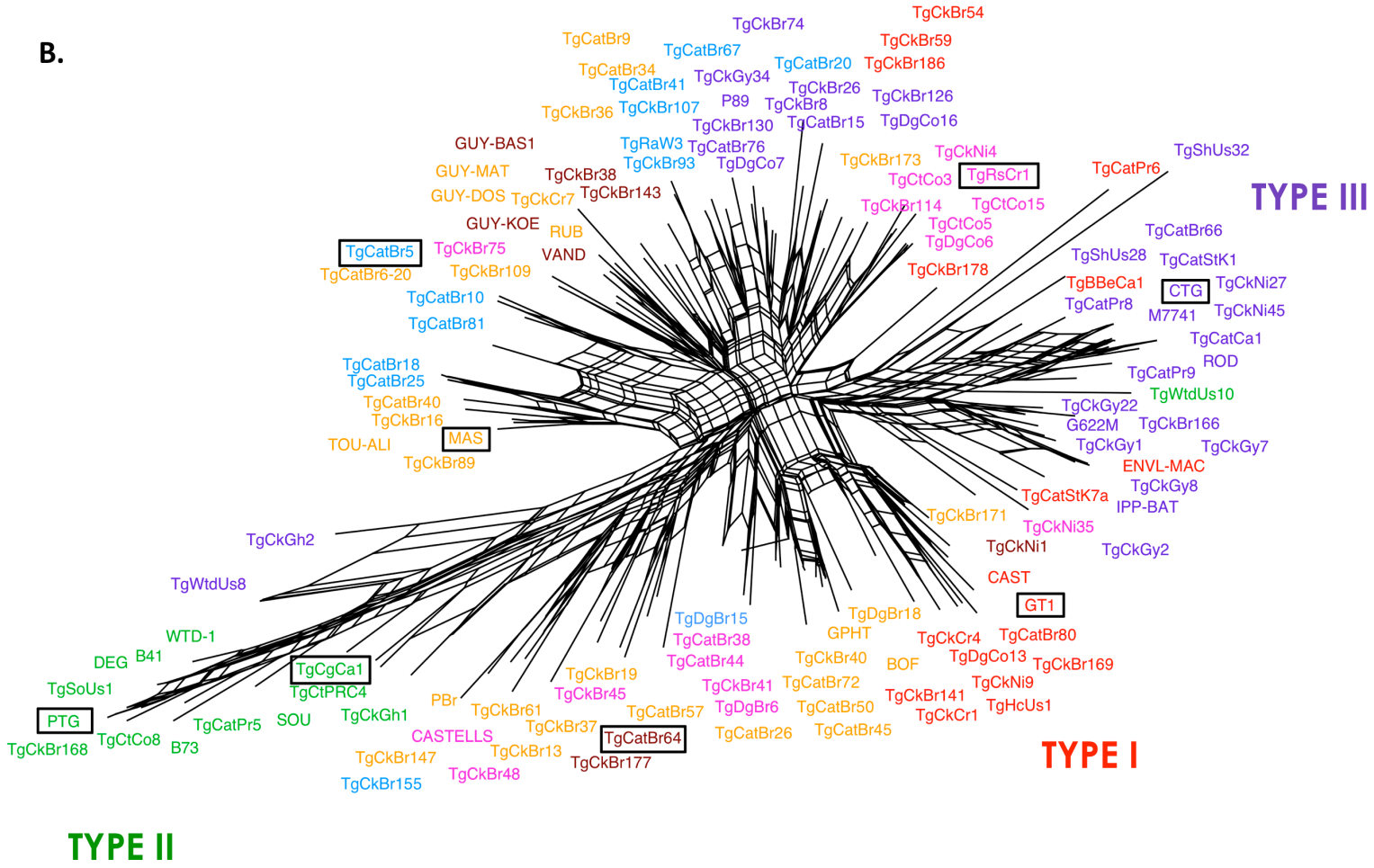


Figure A-3 continued

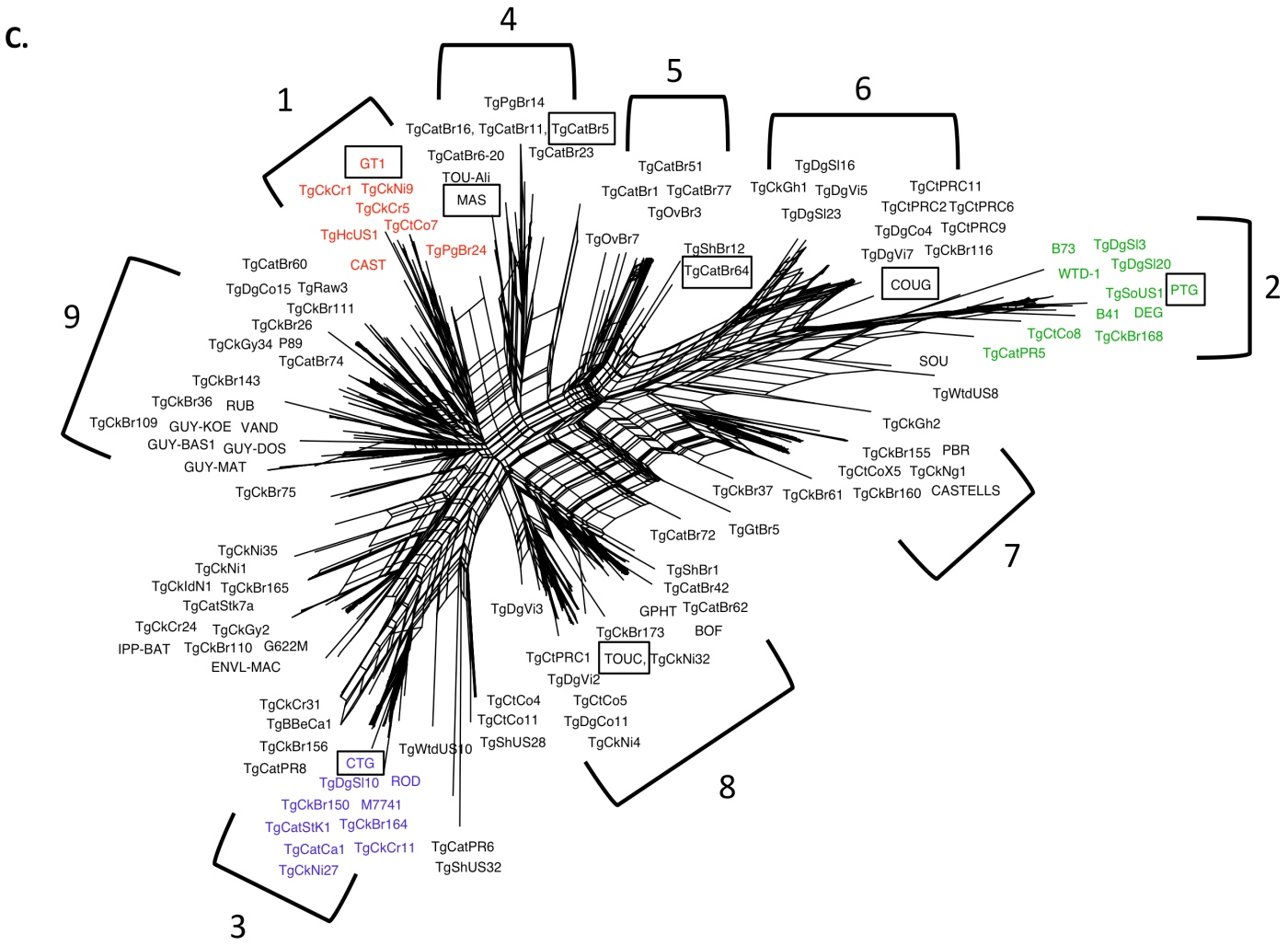


Figure A-3 continued

genetically to *T. gondii*, is much more restricted in host range. The difference here between *T. gondii* and other apicompexans such as *H. Hammondi* appears to lie not in its ability to infect multiple cell types, but in its ability to progress to subsequent infection events within these cell types and thus to proliferate indefinitely. However, the true key to the evolutionary success of *T. gondii* is likely a novel and relatively recent adaptation wherein the parasite is able to be orally transmitted between secondary hosts via carnivory. Prior to the advent of transmission between secondary hosts, the life cycle of the ancestral apicomplexan would necessarily have included the feline definitive host (as in the case of *H. hammondi* and its canine host), since transmission of the parasite from an infected secondary host could only occur through ingestion by this definitive host. Thus, infection of secondary hosts other than those preyed upon by felids would have represented a propagational dead end. Acquiring the possibility of transmission between secondary hosts opened an entirely new and separate life cycle to *T. gondii* which did not require any interaction with feline hosts, and this event combined with the extremely broad host range of the parasite thus allowed for asexual proliferation across virtually any food web involving endotherms. This in turn paved the way for clonal expansion of the parasite on a massive scale, likely facilitated by its introduction into remote areas of the globe by human interaction. Numerous factors have thus contributed to the complex patterns of expansion of *T. gondii* that have shaped the unique global population structure of the parasite as it currently exists.

Combined with the 10 standard markers published in Su *et al.* (2010), the markers developed for this study provide representation of each of the 14 *T. gondii* nuclear chromosomes as well as the apicoplast chromosome, resulting in a simple and inexpensive method for generating relatively high resolution genotypic data. The currently presented set of markers also has the benefit of being designed to distinguish alleles identified from sequence data derived from a set of strains representing a diverse array of genotypes rather than being designed to distinguish the three archetypal lineages, so that a greater degree of accuracy in determining phylogenetic relationships between strains is expected using these markers in combination with the 10 markers presented in Su *et al.* (2010). When comparison is made between network analyses of the strains analyzed in this study using the ten markers from Su *et al.* (2010) and using the combined set of 22 markers described here, we see marked rearrangement of the positioning of the majority of strains. The notable exception to this is in the Type II-related clade, which remains largely unchanged with the addition of the expanded marker set. This suggests a lesser degree of recombination between strains in this and other phylogenetic groups than among the other clades. This is consistent with the hypothesis that parasite expansion has been primarily clonal in regions where Type II-related strains are dominant, including much of the northern hemisphere. Interestingly, the dominant Asian strain type, ToxoDB genotype #9 (Chinese I) was in this study separated into two subtypes, differing only at the ROP18 locus (Table S1). Subtype #9a possesses ROP18 genotype 2, associate with the virulent Type II allele, while subtype #9b possesses ROP18 genotype 3, associated with the avirulent Type III allele. It has been previously noted that genotype #9 strains include both virulent and avirulent forms (Li *et al.* 2014), and the identification here of two subgroups differing at the ROP18 locus seems likely to be relevant to this observation.

Though it is clear from the data presented here and elsewhere that the South American *T. gondii* population differs dramatically in structure from elsewhere in the world, the reasons for this discrepancy are not well understood. It may be considered that the high degree of strain

variability found in South America provides evidence that the parasite originated in this region, and thus has resided there longer than in other regions of the world and has therefore had more time to evolve a diversity of forms. By contrast, *T. gondii* populations found on other continents would likely then be the result of more recent founding events, seeded by limited numbers of strains carried either by wild migratory animals, or by intercontinental transport of domesticated host animals by humans. This scenario would thus explain the reduced diversity observed outside of South and Central America. Additionally, it is possible that the inordinately high host species diversity found in the tropical regions of South and Central America has also promoted the diversification of parasite strains exposed to varying selective pressures within different host organisms. Genotyping information from other geographic regions with high species diversity, such as the tropical rainforests of Africa and southeast Asia, could provide valuable insight as to the influence of host species variability on the *T. gondii* population structure. However, such data is unfortunately quite scarce. The tools presented here should prove useful in future studies aimed at better understanding the diversity that exists within the global *T. gondii* population and also the evolutionary basis for the expansion of this unique pathogen.

Appendix B. Altered gene expression correlates with dramatically increased susceptibility of mice to *T. gondii* infection

Introduction

The pathogenesis of *T. gondii* may vary widely in response to myriad factors relating to the parasite, the host and the environment. In the case of human infections, *T. gondii* typically persists indefinitely and asymptomatically in the muscle and brain tissues of the host (Dubey 2010). In addition to chronic infections, acute *T. gondii* infections may also occur in some human patients, frequently manifesting as ocular toxoplasmosis or encephalitis which can in the worst cases result in blindness and death, respectively (Dubey 2010, Holland 2003). Such cases are most commonly observed in persons with compromised immune systems, such as those suffering from AIDS or in cases of congenital infection. It is also possible for severe acquired infection to occur in immunocompetent adults, however, including both systemic and ocular toxoplasmosis (Darde *et al.* 1998, Grigg *et al.* 2001). The factors that contribute to the manifestation of such a condition, whether related to the genetic background of the host or the parasite or to some external factor, are not well understood.

Variations in disease presentation are also observed within other host species. Mice in particular have been studied extensively as a model host for *T. gondii*. In general, mice have increased susceptibility to the parasite compared to other host species, and may be killed by low doses of strains that would only result in asymptomatic chronic infections in more resistant hosts such as rats (Dubey 2010). Large differences in pathogenesis have also been observed in mice in response to variations in both parasite strain type and mouse lineage, indicating the importance of both host and parasite genetic background in determining disease severity (Williams *et al.* 1978, Sibley and Boothroyd 1992, Khan *et al.* 2009). Research has shown that the host immune response plays an important role in controlling both acute and chronic infection of *T. gondii*. Numerous studies in mice have indicated the importance of cytokines, such IL-12, TNF-α, IFN-γ, and production of reactive nitric oxide radicals (via iNOS) as mediators of host resistance to *T. gondii* infection (Gazinelli *et al.* 1993, Hunter *et al.* 1994, Reichmann *et al.* 2000). IFN-γ is secreted initially by natural killer (NK) cells and subsequently by CD4+ and CD8+ T cells. It is the major effector mechanism that controls both the acute stage of *T. gondii* replication and the reactivation of chronic stage tissue cysts (Dupont *et al.* 2014). IFN-γ mediated resistance is achieved in part by activating macrophages which produce antimicrobial nitric oxide (NO) and activate immunity-related GTPases (IRGs) (Reichmann *et al.* 2000, Howard *et al.* 2011). IL-12, produced primarily by dendritic cells (DCs), is the most important cytokine in inducing and maintaining production of IFN-γ in T cells, and neutralization of endogenously produced IL-12 with monoclonal antibodies has been demonstrated to significantly increase the mortality of mice (Liu *et al.* 2006). However, if dysregulated, the above mentioned mediators may also cause Th1-mediated immunopathology, leading to mortality (Liesenfeld *et al.* 1996, Egan *et al.* 2011). IL-10 has been shown to be the dominant immunoregulatory mechanism preventing Th1-mediated immunopathology (Gazzinelli *et al.* 1996). Th17 cells may also have a potential pathogenic role in CNS inflammation during *T. gondii* encephalitis and this pathogenic effect can be suppressed by IL-27, suggesting IL-27 can complement the immunoregulatory effect of IL-10 during *T. gondii* infection (Stumhofer *et al.* 2006). The balance of immune responses by IL-12, TNF-α,

IFN-γ, and iNOS and counter-inflammatory regulation of IL-10 and IL-27 is important to the resistance and pathogenesis of *T. gondii* infection (Dupont *et al.* 2012). However, at present there is a lack of understanding relating to hierarchical networks and immune pathways that regulate the host immune response to *T. gondii*. Identification of host genes that influence disease severity in response to *T. gondii* is essential for predicting disease outcome and identifying personalized treatment strategies.

In this study, we have compared the host response to *T. gondii* infection between two lines of inbred mice. C57BL/6 mice (C6) mice have been previously demonstrated to be more susceptible to *T. gondii* infection than DBA/2 (D2) mice, though the reasons for this variation have not been adequately explored (McLeod *et al.* 1989, Fux *et al.* 2003). In order to elucidate the genetic basis for the observed differences in susceptibility of the two lines, we have analyzed the gene expression profiles of mice in response to infection and identified differential expression of a relatively small number of immunity-related loci despite observing a high discrepancy between the two mouse lines with regards to rate of disease progression, parasite load and overall mortality. These data indicate that altered regulation of a limited number of host genes may be responsible for large differences in the outcome of toxoplasmosis in mice. Such information raises the possibility that subtle genetic or epigenetic differences may also have a significant impact on disease progression in human hosts.

Materials and Methods

Strain maintenance and inoculum preparation

A Stock of tachyzoites of the archetypal Type II strain TgMe49 (ME49, Howe and Sibley 1995) preserved in liquid nitrogen in a solution of 50% D10 medium (DMEM supplemented with glucose, L-glutamine, and sodium pyruvate with defined fetal bovine serum, non-essential amino acids, HEPES and 10 mM gentamicin) / 50% glycerol were added to 25 mL cell culture flasks containing human foreskin fibroblast (HFF) cells in D10 medium and incubated in a 37.1 C/ 5% CO2 environment and subsequently transferred to new HFF cell culture flasks as necessary until the culture had reached a two day rate of passage. To prepare the parasites for mouse infection, tachyzoites and cell culture medium were transferred to centrifuge tubes and centrifuged at low speed to pellet, followed by removal of the supernatant and resuspension of the pellet in phosphate-buffered saline (PBS), followed by two additional rounds of centrifugation and resuspension to remove excess cellular debris. Concentrations of suspended tachyzoites were then determined via hemocytometer counting.

Establishment of chronic infections in mice for use as inoculum sources, and inoculation of two inbred mice lines for examination of host response to T. gondii infection

Concentration of ME49 tachyzoites was adjusted via dilution with PBS to approximately 1 cell/uL. 200 uL of each strain suspension were then used to infect one six-week-old CD1 outbred mouse via intraperitoneal (IP) injection with a 1 mL syringe and 25-gauge needle. Thus, the mouse was inoculated with an estimated 200 tachyzoites of ME49. Four weeks post-inoculation, the mouse was terminated via cervical dislocation following isoflurane-induced loss of

consciousness. Brain tissue was then extracted from the mouse and homogenized via addition of 2 mL sterile PBS followed by serial passage through increasingly smaller-gauged syringe needles. Homogenate was examined for tissue cysts via microscopy. 10 uL of the homogenate were added to microscope slides in triplicate, and the number of visible tissue cysts on each slide was counted to estimate cyst concentrations. Cyst concentration was then adjusted to 5 cysts/200 uL via dilution in PBS and then used to infect new mice via oral gavage. 5 cysts of ME49 were administered to ten three-month-old C57BL/6 inbred mice, as well as eight three-month-old DBA/2 inbred mice. Three mice from each inbred line were terminated three days post-infection and spleen, ileum and mesenteric lymph nodes were extracted from each. The remaining mice were monitored for four weeks' time (28 days post-infection). After this time period, surviving mice were terminated and brain and spleen tissues were extracted. Mice that displayed severe disease symptoms were euthanized prior to the end of the 28-day period and their brain and spleen tissues were also extracted.

Parasite DNA extraction from infected spleen tissue and determination of parasite load in infected spleen tissue

Parasite load in mouse spleen tissue was determined as described previously (Hill *et al.* 2012) with modifications. Briefly, 100 µl of homogenized spleen tissue at a concentration of 0.1 g/ml in PBS was diluted with 100 µl of PBS, then 50 µl of 10 mg/ml proteinase K was added, and the mixture was incubated at 55°C for 2 h. DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (catalog number 69504; Qiagen, Valencia, CA). For qPCR standard controls, the spleen of an uninfected mouse was homogenized and diluted in PBS, and *T. gondii* tachyzoites were spiked into the homogenized spleen to make a series of concentrations of 1×10^7 to 1×10^3 tachyzoites/ml. All homogenized spleen samples were adjusted to a volume of 0.1 g/ml in PBS, and genomic DNA was purified using the same procedure as detailed above. The concentration of parasites in mouse spleen was estimated by qPCR using a TaqMan probe targeting the ITS1 sequence (GenBank accession number AY143141). The primers for PCR amplification were ITS1-Fx (GAAGGGGCTCAATTTCTGG) and ITS1-Rx (TGTTCCTCAGATTTGTTGTTTGA), which amplify a 117-bp sequence. The ITS1 probe was 5'-5-FAM-CGTGTCTCTGTTGGGATACTGATTTCCAGG- BHQ-1-3', with the 5' end labeled with 6-carboxyfluorescein (FAM) and the 3' end labeled with Black Hole Quencher-1(BHQ-1) (Integrated DNA Technologies, Inc., Coralville, IA). The qPCR mixture had a total volume of 25 µl containing 10.9 µl of H_2O, 12.5 µl of 2×TaqMan Fast Advanced Master Mix(catalog number 4444964; Applied Biosystems, Foster City, CA), 0.15 µl of 50 µM ITS1-Fx and ITS1-Rx primers, 0.3 µl of 50 µM ITS1 probe, and 1 µl of purified DNA. The reaction was carried out using the iCycler thermal cycler (iQ5, Bio-Rad Laboratories, Inc. Hercules, CA) under the following conditions: 94°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec. The CT value of each sample was compared to values of the standard controls to estimate the concentration of parasite per gram of spleen.

Microarray analysis of transcriptomes of infected C57BL/6 and DBA/2 mice

Microarray analysis was performed by the method described previously (Hill *et al.* 2012). In brief, total RNA from peritoneal cells was extracted using Qiagen RNeasy Plus Mini Kit (catalog #74131; Qiagen,Valencia, CA) following the manufacturer's instructions. The quality of RNA

was tested by agarose gel electrophoresis, and the concentration of RNA was determined by reading the absorbance at 260/280 nm. Affymetrix Mouse Exon 1.0 ST array containing 35,556 transcripts was used. RNA samples were processed according to the Affymetrix protocol for one-cycle DNA synthesis using a Message Amp II-Biotin Enhanced Kit (P/N AM1791; Ambion, Austin, TX). Five micrograms of fragmented cRNA was hybridized to the Affymetrix GeneChip. Arrays were processed using the Affymetrix Hyb/Stain kit PN900720. Arrays were immediately scanned using the Affymetrix 7G scanner. Array images were visually inspected for anomalies. The individual chip scans were quality checked for the presence of control genes and background signal values. All microarray procedures were done at the University of Tennessee Affymetrix Core Facility.

Results

Disease symptoms and mortality of orally-infected mice

C57BL/6 mice died more rapidly than DBA/2 following oral infection with 5 tissue cysts of *T. gondii* (Figure B-1), with mortality beginning on day 12 post-infection and ending with termination of the seventh and final mouse on day 17. By contrast, no mortality was observed among DBA/2 mice until day 14. Furthermore, all 7 infected C57BL/6 mice succumbed to disease, whereas only 1 out of 5 DBA/2 mice died before the end of the 28-day course of the experiment. Symptoms also appeared earlier in C57BL/6 mice than in DBA/2 mice. This discrepancy in disease severity between the two mouse lineages also correlated with differences in parasite load (Figure B-2). Three days post-inoculation, parasite DNA concentration was an average of 120 times higher in spleen tissue from infected C57BL/6 mice compared with DBA/2 mice, suggesting that the differences in mortality rate between the two host lines may be due to increased ability of the parasite to proliferate in the more susceptible strain.

Microarray analysis of gene expression in spleen tissue from infected C57BL/6 and DBA/2 mice.

Microarray-based comparison of total mRNA from spleen tissue of infected C57BL/6 and DBA/2 mice was used to analyze 35,556 genes and indicated 523 transcripts that varied in expression level between the two mouse lineages by a factor of 2 or greater, 306 of which were upregulated and 217 of which were downregulated in DBA/2 mice compared to BL/6 mice. Table B-1 indicates 14 genes with the highest degree of differential regulation. These genes include several members of the serpin class of protease inhibitors, a lectin-like receptor expressed by natural killer cells (Klrd1), and a number of genes of unknown function. The largest variation in gene expression between the two mouse lines involved H2-Ea, a gene of the H-2 class II major histocompatibility complex (MHC) family of genes, which had approximately 225-fold higher expression in DBA/2 mice than in C57BL/6 mice. A heat map of overall gene expression differences is shown in Figure B-3.

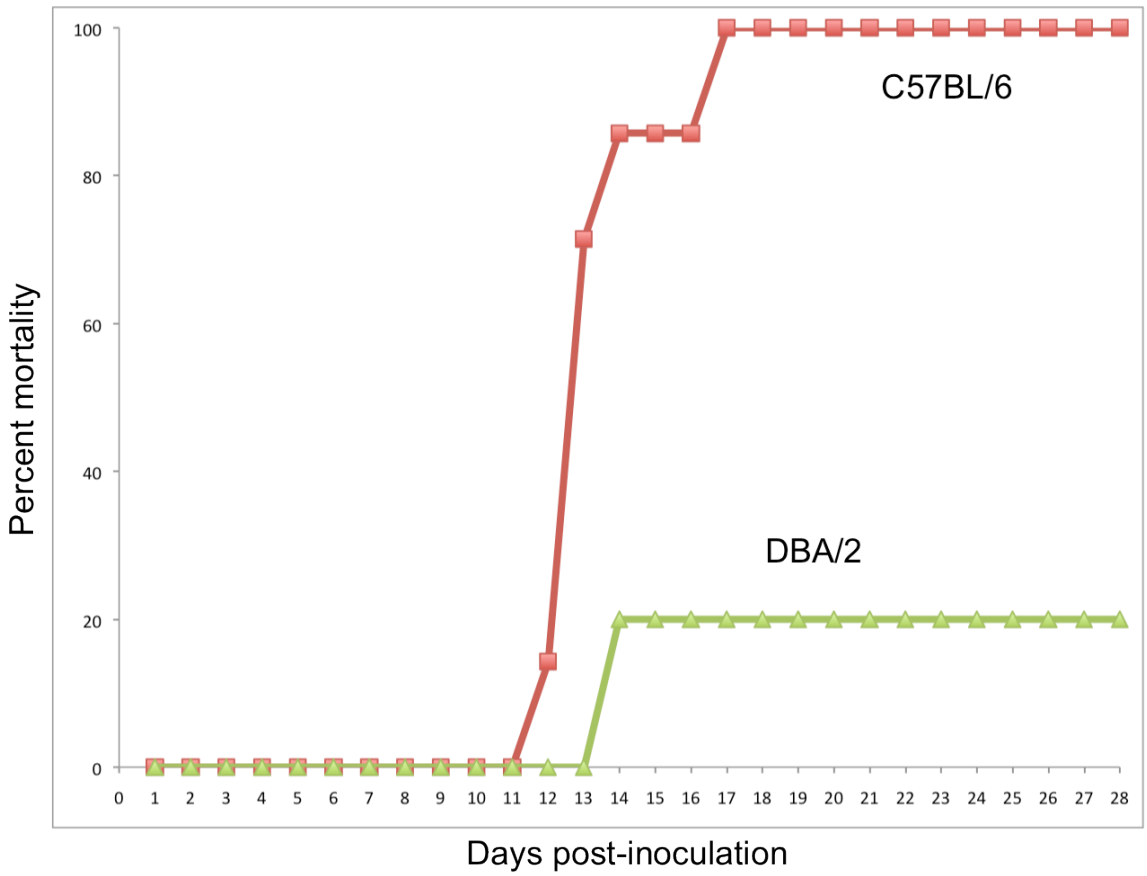


Figure B-1. Mortality of C57BL/6 and DBA/2 mice orally infected with five tissue cysts of *T. gondii.*

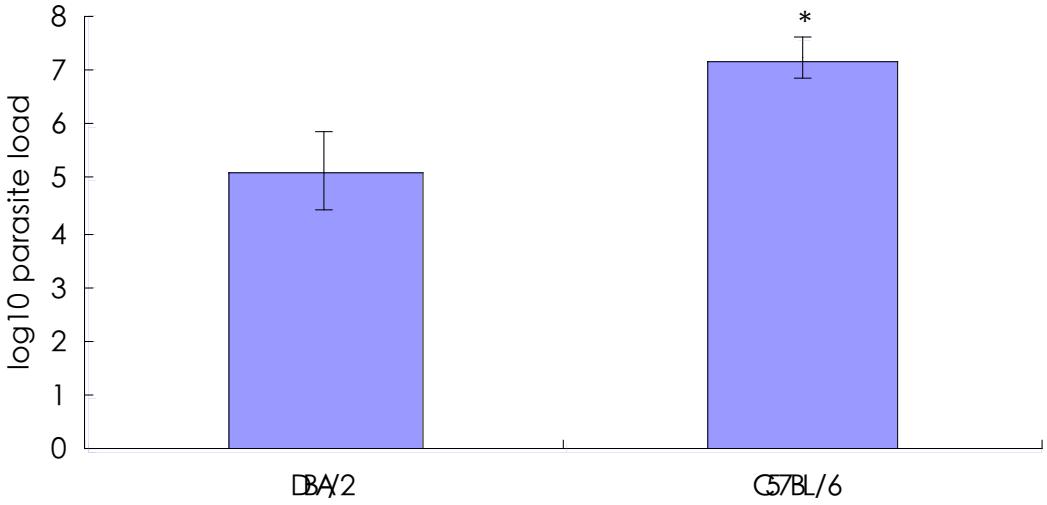


Figure B-2. Parasite load in infected spleen tissue of C57BL/6 and DBA/2 mice. The log parasites/g of spleen for BL2 mice was 7.21± 0.39, 120 times higher than that in DBA/2 mice (5.13± 0.74). Asterisk indicates significant difference (P=0.006).

Discussion

In this study, we have compared gene expression profiles of two mouse lineages undergoing acute infection with *T. gondii*. C57BL/6 mice were found to succumb to *T. gondii* infection at a much higher rate and to have much higher parasite loads in spleen tissue than the more resistant DBA/2 mice. Despite these markedly different responses to infection with regard to rapidity and severity of symptom presentation, parasite load and mortality, the expression of only a small number of genes was found to differ by a factor of two or more between the two lines. This suggests that altered transcription of small numbers of key genes, rather than more sweeping, genome-wide changes in expression profiles, may be responsible for the dramatic variance in resistance to *T. gondii* observed among mouse lineages.

The genes found to exhibit the highest degree of altered expression include several members of the highly diverse serpin class of protease inhibitors. Specifically, SerpinA1A, SerpinA1B and SerpinA1E are downregulated in C57BL/6 mice compared to DBA/2 mice. Of these, Serpins A1A and A1B are known to inhibit neutrophil elastase, involved in the inflammatory response of neutrophils and macrophages in humans and mice (Belaaouaj *et al.* 2000, Shapiro *et al.* 2003). SerpinA1A is essential for murine embryonic development despite the presence of the highly similar A1B paralog in mice (Wang *et al.* 2011). In humans, inactivation of the SerpinA1 ortholog does not result in embryonic lethality, but is the major genetic contributor to chronic pulmonary disease/emphysema (Wang *et al.* 2011). As a group the murine alpha 1 protease inhibitors, clustered on chromosome 12, have an unusually high rate of nonsynonymous mutations, particularly in surface exposed residues, suggesting diversifying selection which may enhance interaction of these proteins with more diverse protease targets (Borriello and Krauter 1991).

Also identified as being differentially expressed was Klrd1, a lectin-like receptor expressed by NK cells. Also known as CD94, this receptor is expressed on NK cell subsets in both mice and humans, and interacts primarily with a nonclassical MHC class I molecule (HLA-E in humans, Qa-1b in mice) (Cho *et al.* 2011). Such interaction may induce functional maturation in NK cells (Yu *et al.* 2009). NK cells with high expression of Klrd1 have been found to have a greater capacity for IFN-g production and for lysis of targets cells (Yu *et al.* 2009), and also suppress the CD8+ T cell response, limiting immunopathology in chronic infections (Cho et al. 2011). These features suggest possible relevance of this protein in the control of *T. gondii* infection.

Another differentially expressed gene was identified as the melanoma antigen MelA, an ecotropic murine leukemia virus (MuLV) envelope protein identified as being highly expressed in melanoma cells (Hayashi *et al.* 1992). Its expression during *T. gondii* infection is of possible interest. Other differentially regulated genes included Trim12a (tripartite motif-containing protein 12A) and the putative protein D730005E14Rik, both of which currently remain uncharacterized. The large difference in expression of H2-Ea, the E-alpha subunit of the MHC Class II E-complex was expected, as this gene has previously been found to be unexpressed in C57BL/6 mice due to a deletion within the promoter sequence, while the functional gene is expressed in DBA/2 mice (Jones *et al.* 1981). Further investigation into the roles played by this and other differentially regulated genes in the defense against *T. gondii* infection is desirable.

Table B-1. Differential gene expression in C57BL/6 and DBA/2 mice infected with *T. gondii*

Transcript ID	Gene Symbol	p-value(B6 vs. D2)	Fold-Change(B6 vs. D2)
10430645	D730005E14Rik	1.25E-05	7.40002
10450161	H2-Ea-ps	2.75E-07	-224.988
10542214	Klrd1	8.42E-06	5.85938
10582545	Mela	1.19E-06	-24.718
10402399	Serpina1a	1.13E-05	-7.33244
10402390	Serpina1b	2.08E-06	-18.3078
10402409	Serpina1e	8.92E-07	-9.88549
10566326	Trim12a	1.07E-05	16.976
10341070	unknown	3.38E-06	14.644
10415081	unknown	1.11E-05	-21.7788
10520121	unknown	1.11E-05	-21.7788
10578405	unknown	1.11E-05	-21.7788
10586076	unknown	1.11E-05	-21.7788
10389717	unknown	1.43E-05	-18.4185

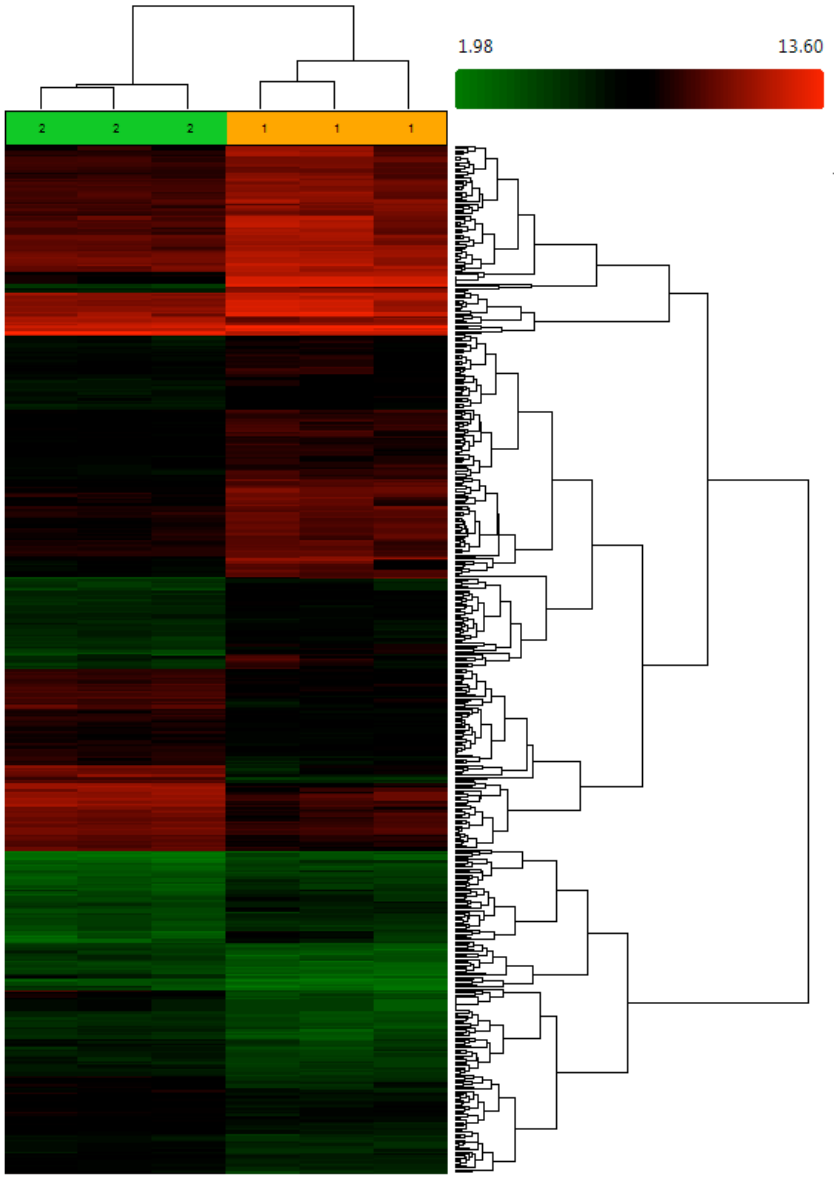


Figure B-3. Microarray gene expression profiles of mice infected with *T. gondii*. Expression of individual genes in spleen tissue of three replicates each of C57BL/6 (left, 2) and DBA/2 (right, 1) mice three days following infected with the ME49 strain of *T. gondii*. Red indicates high and green indicates low gene expression. Lines indicate coordinated clusters of gene expression.

In order to make full use of the data obtained here, it will be necessary first to confirm the microarray-based estimates of expression levels for a subset of genes in order to ascertain that the microarray data are reliable. Assuming this is the case, the full set of 523 differentially regulated genes will need to be analyzed in order to identify those most likely to affect the immune response to *T. gondii*. It will also be of value to obtain and analyze histological data from harvested spleen tissue in order to better assess the differences in the immune response and pathology associated with *T. gondii* infection in these two mouse lineages and better understand the genetic basis for host resistance to the parasite.

Appendix C. Attempted expression of Type III ROP18 using a Type I promoter

Introduction

The ROP18 allele is underexpressed in archetypal Type III strains of *T. gondii*, presumably due to the presence of a 2,242bp upstream promoter sequence (UPS) not found in the Type I and II alleles and without homology to any other sequences in the *T. gondii* genome (Boyle *et al.* 2008, Khan *et al.* 2009). Fusion of the type II and III promoter sequences to a firefly luciferase ORF resulted in approximately 6-fold higher production of luciferase driven by the Type II promoter than by the Type III promoter (Boyle *et al.* 2008). Furthermore, the presence of the UPS appears to reflect the ancestral state of the parasite, as a similar sequence is found in the promoter region of the outgroup *Neospora caninum* ROP18 homologue, suggesting that the UPS was lost at some point through deletion or rearrangement in a common ancestor of the Type I and II alleles (Khan *et al.* 2009). Analysis of the coding sequence of the Type III allele predicts that the gene should maintain functionality due to the intact nature of key sequence elements, suggesting that the gene has continued to play a role in the selective fitness of Type III strains despite the low level of expression, possibly by aiding in establishment of infection in more susceptible host species (Khan *et al.* 2009). In order to ascertain the functionality of the gene product of the Type III ROP18 allele (ROP18III) and to more definitively identify the UPS as the cause of underexpression of this allele, we undertook the development of a transgenic parasite strain which would express ROP18III using the ROP18I promoter sequence.

Methods and results

Plasmid construction

Primers were designed to amplify the 2.8kb genomic region upstream of the ROP18 ORF in the Type I strain GT1, the 1.7kb ORF of ROP18 from the Type III strain CTG, including a 3' 27 bp HA tag sequence in the reverse primer, and the 1.4kb region downstream of the CTG ROP18 ORF (based on ToxoDB genomic sequence). Primers were designed to include restriction enzyme recognition sites for use in plasmid construction. Fragments were amplified using FastStart DNA Polymerase (Roche) and a 35 cycle thermocyler program using a 55C annealing temperature and 72C, 2 minute extension temperature. Primer sequences are shown in Table C-1 (ROP185PGT1F-PsiI, ROP18R1-ApaI, ROP18F2-ApaI, ROP18R2-HA-PspXI, ROP18F3-PspXI, ROP183PCTGR-SpeI). PCR products were purified using a Wizard DNA cleanup kit (Promega) and inserted into the plasmid pDHFR, containing the pyrimethamine resistance gene dihydrofolate reductate (DHFR) and an ampicillin resistance gene (AMP) in a stepwise manner using standard molecular cloning techniques. Plasmids were cloned in E. coli cells. The final plasmid construct is diagrammed in Figure C-1.

Table C-1. Primers used in generation and testing of transgenic strains

Primer ID	Sequence (5' to 3')	Target
ROP185PGT1F-PsiI	TGTATACATATATAGGAGTAAGTCTC	GT1 ROP18 promoter
ROP18R1-ApaI	TTCACACAAACTGCTGTAAAATATGG	
ROP18F2-ApaI	TTTTGTACTCACCCGGTACAGCGGCC	CTG ROP18 ORF
ROP18R2-HA-PspXI	ACGTTCCCCTCTTGATGTTCCTGCTG	
ROP18F3-PspXI	AAACAGAATAAAAGAACGTGGTGGTG	CTG ROP18 3' sequence
ROP183PCTGR-SpeI	ATATGGCGGAAACACAGCTGTAACCC	
ROP18pDHFRF-Check	TTTCGCCACCTCTGACTTGAGC	Full length ROP18I:III fusion construct
ROP18PDHFRR-Check	CGGATATGGTTACACGTGACCACGC	
ROP18QF	TCCCGGTTACGAGCCTCCGG	ROP18 cDNA
ROP18QR	AGTACAAAGTTATACCGAGTTGCC	

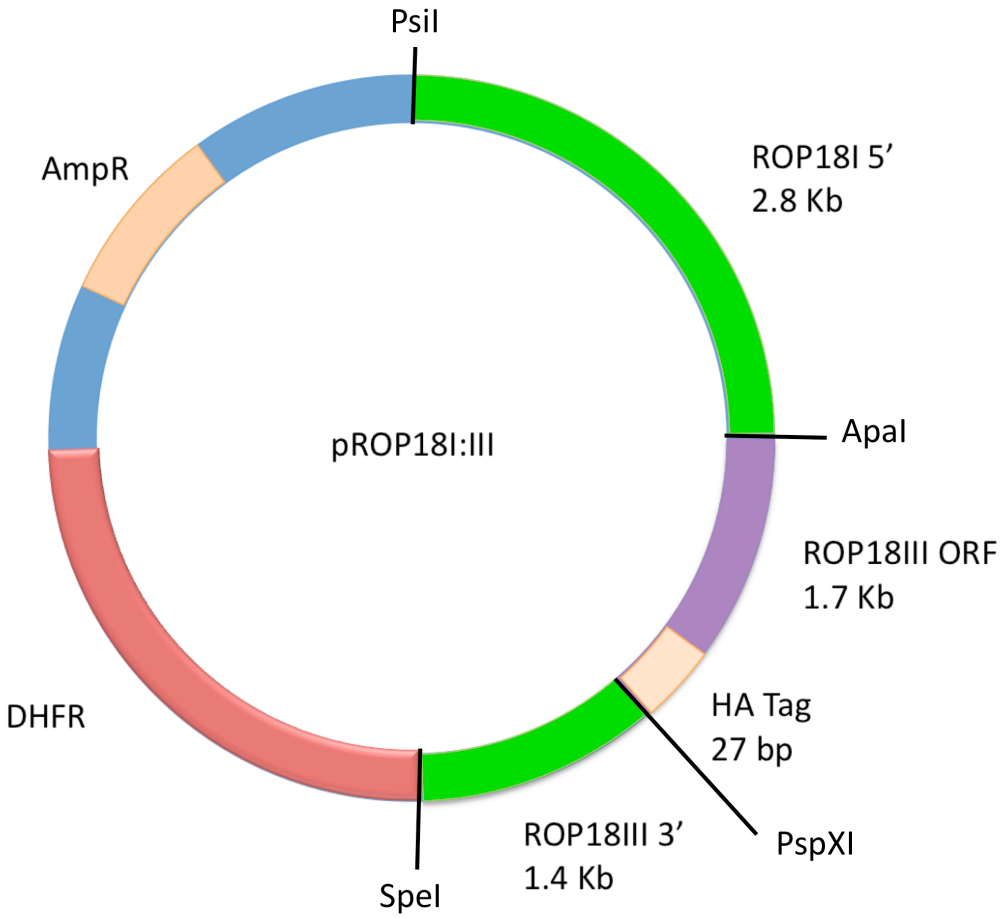


Figure C-1. Map of plasmid construct for transformation of *T. gondii.* Plasmid derived from pDHFR. Contains 2.8kb 5' sequence of ROP18I, 1.7kb ORF of ROP18III with 3' 27bp HA tag sequence, 1.4kb ROP18III 3' sequence, dihydrofolate reductase (DHFR) and ampicillin resistance (AmpR) marker genes.

Transformation of T. gondii cells

Tachyzoites of the Type III strain CTG were grown in cell culture as described. Cells were pelleted by spinning for 15 minutes at 2000xg, followed by resuspension in 250uL of electroporation buffer containing 1mg/mL ATP and 1.54mg/mL glutathione. 150 uL plasmid construct was mixed with tachyzoites in electroporation cuvette on ice, followed by electroporation at 1.5KeV and 25 ohms, followed by incubation for 15 minutes at room temperature. Electroporated cells were then added to a 25mL cell culture flask containing confluent HFF cells in 5mL dialyzed DMEM + 5uL 1000X pyrimethamine. As controls, tachyzoites were also electroporated without the addition of plasmid, and electroporated cells were grown in the absence of pyrimethamine. Parasites receiving the plasmid construct that were able to grow in the presence of pyrimethamine were harvested via pelleting and diluted to a concentration of approximately 1 tachyzoite per 100uL in D10 medium (estimated via hemacytometer counting). 100uL of suspension was then added to each well of two 24-well cell culture plates containing confluent HFF cells in D10 medium + pyrimethamine. Plates were then monitored for parasite grown over the course of one week. Parasites from wells in which only one focus of infection was identified (thus representing isogenic cultures) were passed to separate T25 cell culture flasks containing HFF cells in D10+pyrimethamine. In this manner, 9 pyrimethamine-resistant transgenic strains were isolated. Genomic DNA was extracted from each transformant culture and amplified using primers specific to the Type I ROP18 promoter sequence and Type III ROP18 3' sequence (Table C-1, ROP18pDHFRF-Check, ROP18PDHFRR-Check) in order to verify the presence of the full length construct. In this manner, two positive transgenic strains were identified (Figure C-2).

ROP18 expression in transgenic strains

In order to determine the degree of ROP18 expression in the two positive transgenic strains identified, qRT-PCR was employed. Total RNA was extracted from cultures of the two transgenic strains as well as CTG and GT1 control strains using the Qiagen RNeasy kit. qRT-PCR was carried out using the Verso 1-Step qRT-PCR Kit (Thermo) with primers specific to the ROP18 ORF (Table C-1, ROP18QF, ROP18QR). In this manner, it was determined that expression of ROP18 in the two transgenic strains was not significantly different from that of the wild type CTG strain. All three of these strains produced significantly less ROP18 transcript than did the GT1 strain (Figure C-3). Comparisons between expression levels of strains were done using Student's T-Tests ($P<0.01$).

Mouse virulence of transgenic strains

In order to assess the virulence of mutant strains, triplicate CD1 outbred mice were injected intraperitoneally with 100 or 1000 tachyzoites of transgenic strains 3 and 27 harvested from cell culture. Mice were monitored for one month prior to termination, but no disease symptoms or mortality resulted (data not shown). Following termination after one month, brain tissue was homogenized and examined microscopically for the presence of tissue cysts, which were identified in all mice injected with 1000 tachyzoites and 5 of 6 mice injected with 100 tachyzoites (tissue cysts were not identified in one mouse from the mutant 27 infection group).

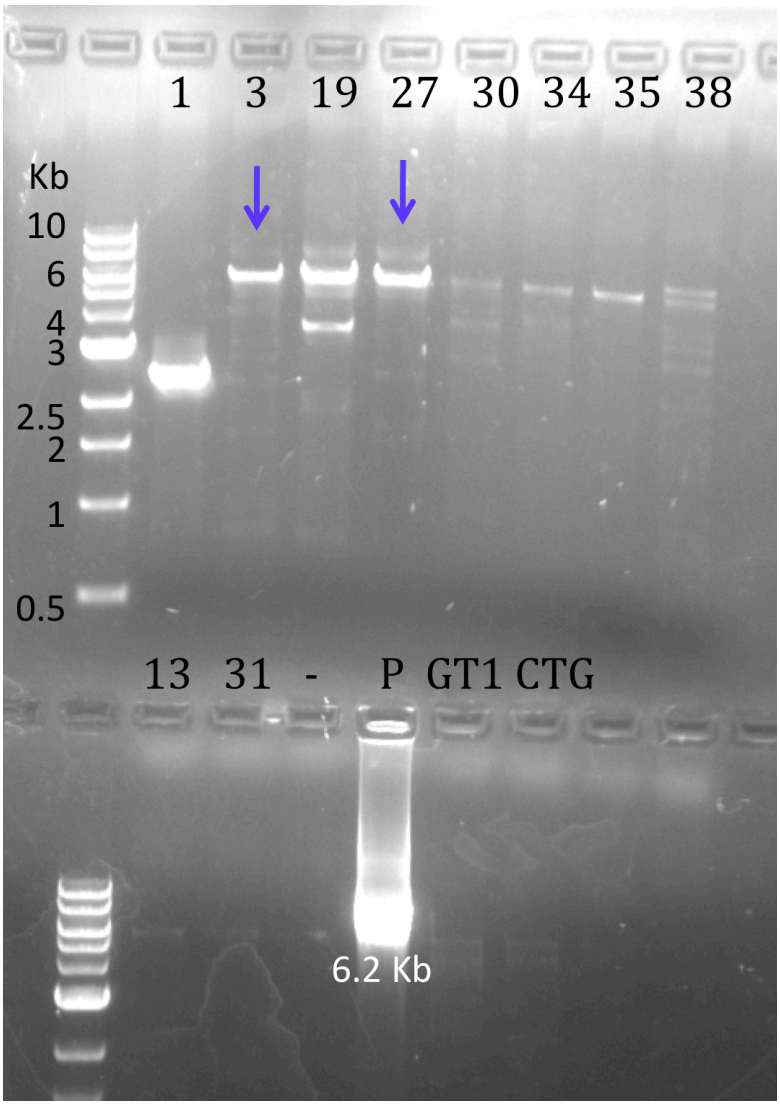


Figure C-2. PCR identification of transgenic *T. gondii* clones. Genomic DNA from 10 pyrimethamine resistant transformant strains was used for template with construct-specific primers. Strain IDs are given above gel lanes. "-" indicates no template negative control, "P" indicates plasmid construct positive control, "GT1" and "CTG" indicate control genomic DNA from wild type *T. gondii* strains. Blue arrows indicate apparent positive results (expected product size 6.2kb)

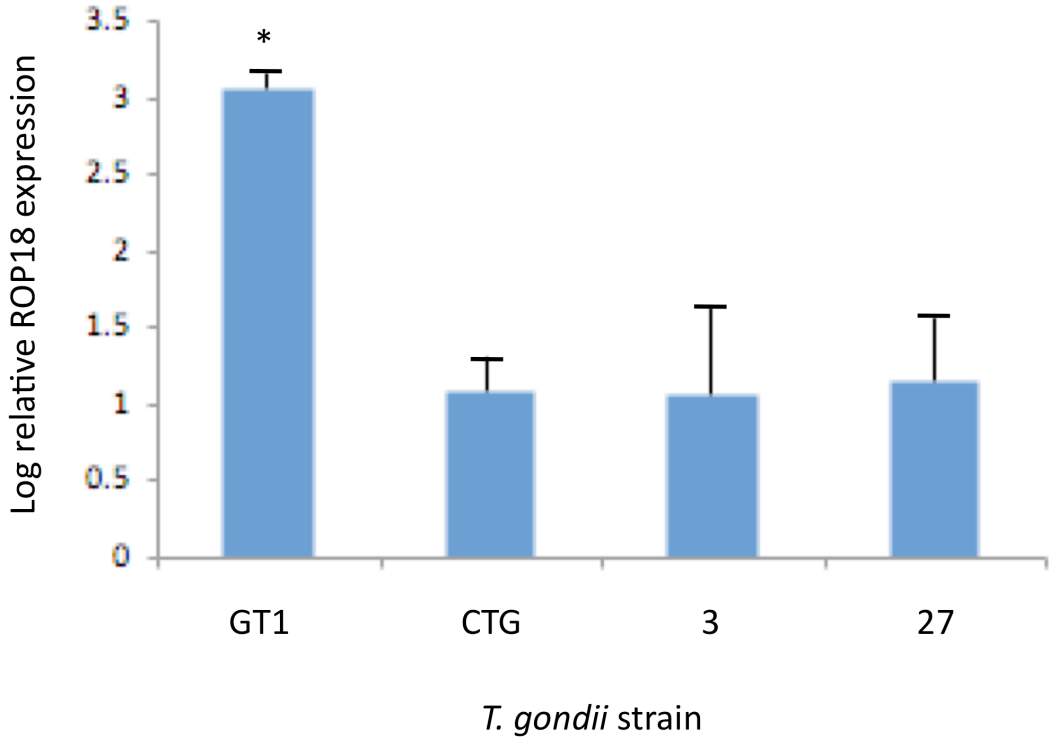


Figure C-3. Relative expression of ROP18 in control and transgenic *T. gondii* lines. ROP18 expression was determined using qRT-PCR on total RNA extracted from tachyzoites of wild type strains GT1 and CTG and transgenic strains 3 and 27. Expression levels are presented relative to expression in CTG, which is designated as 1. Error bars represent one standard deviation from the mean. Asterisk indicates statistically significant difference (Student's T-Test, $P<0.01$).

Discussion

Though the transformants 3 and 27 produced PCR products when amplified with primers targeting the transgenic ROP18 construct, they did not show increased levels of ROP18 expression nor did they prove virulent to mice. This may indicate a problem with the design of the construct, or the PCR results may have been false positives, possibly indicating amplification of the native ROP18 allele rather than the transgenic allele. Alternatively, the genomic context of the insertion may have precluded expression of the transgene, possibly through epigenetic mechanisms. It is also possible that other genetic elements in the vicinity of the native ROP18 locus may be important for efficient expression of the gene. If future studies are conducted to continue this line of research, it may be worthwhile to attempt to perform a targeted replacement of the Type III ROP18 promoter region with that of Type I via homologous recombination rather than simply attempting to effect random insertion of the hybrid gene construct as has been done here. Additionally, expression of a reporter gene such as GFP in place of the ROP18III ORF in the current construct might be attempted prior to this as a simpler and more direct way to test the viability of the construct for gene expression in general. Another possibility is simply to express the ROP18III allele from a different promoter already known to be effective in transgenic *T. gondii* work, simply to ascertain whether or not higher levels of the ROP18III gene product would be effective in conferring a virulent phenotype to a Type III strain, as has been shown to be the case with the Type I and II ROP18 variants (Taylor *et al.* 2006, Saeij *et al.* 2006).

Appendix D: Genotype tables

Table D-1. **A total of 189 *Toxoplasma gondii* genotypes defined by 10 PCR-RFLP markers.**

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
1	177	II	II	II	II	II	II	II	II	II	II	II	Chicken, Striped Dolphin, Raccoon, Coyote, Goose, Human, Sheep, Bear, Cat, Chicken, Rodent, Sea Otter, Pig, Bottlenose Dolphin, White-Tailed Deer, Arctic Fox, Dog, Guinea Fowl, Goat, Mouse, Black Bear, Swainson's Hawk, Bald Eagle	TgCkCh6,10,11,16,TgSdCr1, TgRaW1, TgCyW4,5, TgCkUsIL1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, TgGoUs1, PE, PIH, PTG, B70, C980, R18, 0761, GJL, MUC, PSC, 3440, FUK, Tg132, Tg17, TgCatStK3a, TgCkIt3, TgDoUs2, 3,TgShUs6, 7, 15, 16, 18, 19, 20, 33, 41a, 42b, 44, 46, 48, 49b, 50, 51, 53, TgWtdUs1, 3, 4, 5, TgFox, TgFxSv13, 38, 62, 70, 93, 105, 107, 114, 169, 176,TgDgAu3,TgSoUs 5, 20,TgSoUs27, 36, TgPgUs1, 2, 3, 4, 6, 7, 8, 9, 12, 13, TgPgUs17, 19, 21-24, 28-31, 33-35, 38, 43, 50-54, 56, 62-64, 66-68, 72-74, 77, 81, 83, 85, 86, 88, 91, 94, 95, 98, 99, TgPgUs112, 113, TgPgUs129, 137, 144, 160, 161, 163, 165, 169, 170, 172, 178, 180, 181, TgCatEg 55, 115, TgNmBr1, TgGoatUS1, 11, 12, 14, 15, 16, 18, 19, 21, TgMmUs2, TgCatUs1, TgCoGa1, TgBbPa3, 5, TgCoWI1, TgSoUS41, TgDoUS5, TgBsCoUS1, 2, 3, TgHlUs1, TgVaUS2, TgPigUS183, 184, 185, 186, 187,188, 189, 190, 191, 193, 194	Dubey, Patitucci et al. 2006; Rajendran et al. 2012; Dubey, Morales et al. 2007; Dubey, Sundar, Nolden et al. 2007; Dubey, Webb et al. 2007; Howe and Sibley, 1995; Dubey, Moura et al. 2009; Dubey, Huong et al. 2008; Dubey, Fair et al. 2008; Dubey, Sundar et al. 2008; Dubey, Velmurugan, Ulrich et al. 2008; Prestrud, Dubey et al. 2008; Prestrud, Asbakk et al. 2008; Al-Qassab et al. 2009; Sundar et al. 2008; Dubey, Hill et al. 2008; Velmurugan et al. 2009; Dubey, Thulliez et al. 1995; Gamble et al. 2005; Dubey, Gamble et al. 2002; Dubey, Hill et al. 2005; Al-Kappany et al. 2010; Dubey, Passos et al. 2011; Dubey, Rajendran et al. 2011; Dubey, Velmurugan et al. 2011; Dubey, Hill et al. 2012

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
2	201	II	III	III	III	III	III	III	III	III	III	III	Chicken, Dog, Human, Bear, Cat, Sheep, White-Tailed Deer, Arctic Fox, Pig, Capybara, Goat, Mouse, Raccoon, Wallaby, Striped Skunk, Goose, European Starling, White-footed Mouse, Black Bear, Rough-legged Hawk	TgCkCh3, 15, 18, 20, TgCkCr11, TgCkNi3, 8, 13, 44, 48, 2x, TgCkBr158, 161, 164, TgDgBr11, TgCkBr31, 56, TgDgSl 5, 6, 10, TgCkGy26, 27, VEG, EDZ, STRL, ASH, B74, CTG, F958, C37, C56, K117C, K125B, COR, AFI, P51, LEG-NJA, LGE97-3, TgCatStK2, 3b, 4, 6, TgCkEg12, 13, 14, 16, 17, TgCkDROC-3, 6, 8, 9, TgCkMal-2, 3, 4, TgCkBF-1, TgShUs13, 17, 21, 36, 40b, 41b, 42a, 43, TgWtdUs13, 15, TgFxSv81, 83, 121, 144, TgPgUs18, 41, 61, 70, 71, 87, TgPgUs109, 110, 111, TgPgUs130, 132, 133, 134, 135, 136, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 162, 164, 166, 167, 168, 171, 173, 174, 175, 176, 177, 179	Dubey, Patitucci et al. 2006; Rajendran et al. 2012; Dubey, Su et al. 2006; Dubey, Sundar et al. 2006; Dubey, Sundar et al. 2007; Dubey, Gennari et al. 2007; da Silva et al. 2003; Dubey,Velmurugan et al. 2008; Dubey, Graham et al. 2003; Dubey, Rajapakse et al. 2007; Dubey JP, Applewhaite 2007; Howe and Sibley, 1995; Ajzenberg et al. 2004; Dubey, Moura et al. 2009; Velmurugan et al. 2008; Dubey, Sundar et al. 2008; Dubey, Velmurugan, Ulrich et al. 2008; Prestrud, Asbakk et al. 2008; Dubey,Thulliez et al. 1995; Velmurugan et al. 2009; Gamble et al. 2005; Dubey, Gamble et al. 2002; Dubey, Velmurugan, Alvarado-Esquivel et al. 2009; Yai et al. 2009; Dubey, Rajendran et al. 2010; Al-Kappany et al. 2010; Dubey, Rajendran et al. 2011; Dubey, Velmurugan et al. 2011; Rajendran et al. 2012; Dubey, Hill et al. 2012; Dubey, Graham et al. 2004

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
2 (continued)	201	II	III	III	III	III	III	III	III	III	III	III	Chicken, Dog, Human, Bear, Cat, Sheep, White-Tailed Deer, Arctic Fox, Pig, Capybara, Goat, Mouse, Raccoon, Wallaby, Striped Skunk, Goose, European Starling, White-footed Mouse, Black Bear, Rough-legged Hawk	TgCkMx1, TgCkMx2, TgCkMx3, TgCkMx4, TgCpBr2, TgCpBr4, TgCpBr5, TgCpBr6, TgCpBr7, TgCkBr231, TgCatEg 94, TgCatEg6, 11, 13, 15, 17, 19, 22, 24, 35, 36, 37, 43, 47, 48, 50, 58, 61, 62, 66, 69, 70, 71, 72, 73, 74, 80, 82, 83, 84, 86, 87, 92, 93, 95, 100, 102, 105, 108, 110, 111, 114, TgGoatUS4, 10, 23, 29, TgWtdUs32, TgMmUs6, TgRaGa1, TgWyUs1, 2, 3, TgSkMs1, TgGoMs1, TgEsMt1, TgWfmMt1, 2, TgBbPa1, 4, TgBlUS1, TgCkGr12, 16, 23, 24, 29, TgCkAr2, 6, 24, TgCkPe2, TgPigUS195, 196, 198, 199	Dubey, Patitucci et al. 2006; Rajendran et al. 2012; Dubey, Su et al. 2006; Dubey, Sundar et al. 2006; Dubey, Sundar et al. 2007; Dubey, Gennari et al. 2007; da Silva et al. 2003; Dubey,Velmurugan et al. 2008; Dubey, Graham et al. 2003; Dubey, Rajapakse et al. 2007; Dubey JP, Applewhaite 2007; Howe and Sibley, 1995; Ajzenberg et al. 2004; Dubey, Moura et al. 2009; Velmurugan et al. 2008; Dubey, Sundar et al. 2008; Dubey, Velmurugan, Ulrich et al. 2008; Prestrud, Asbakk et al. 2008; Dubey,Thulliez et al. 1995; Velmurugan et al. 2009; Gamble et al. 2005; Dubey, Gamble et al. 2002; Dubey, Velmurugan, Alvarado-Esquivel et al. 2009; Yai et al. 2009; Dubey, Rajendran et al. 2010; Al-Kappany et al. 2010; Dubey, Rajendran et al. 2011; Dubey, Velmurugan et al. 2011; Rajendran et al. 2012; Dubey, Hill et al. 2012; Dubey, Graham et al. 2004

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
3	184	II	II	II	II	II	II	II	II	II	II	I	Chicken, Coyote, Skunk, Sea Otter, Human, Rabbit, Sheep, Arctic Fox, Pig, Sand Cat, Cat, Goat, Mouse, Arctic Fox, Red Fox	TgCkCh2, 4, 5, 7, 8, 9, 12, 13, 14, 17, 19, 21, 22, TgCyW3, TgSkW1, 0790, DEG, Tg51, BEV, CHA, FUN, Tg96, GANGI, TgCkIt1, 2, TgCkEg15, 19, TgCkMal-5, TgCkKen-1, TgDoCA1, TgShUs4, 9, 10, 12, 14, 23, 24, 25, 26, TgFxSv19, 27, 42, 43, 48, 50, 61, 64, 87, 106, 120, 136, 140, 148, 175, 177, 178, TgSoUs 3, 4, 18, 19, 21, TgSoUs26, 29, 30, 37, TgPgUs27, 32, 36, 37, 39, 44, 48, 49, 59, 69, 93, 97, TgPgUs 100, 101, 102, 103, 104, 105, 106, 107, 108, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, QHO, TgCkBr221, 225, 226, 228, 230, TgSandcatQA1, TgCatEg 1, 2, 3, 4, 5, 8, 9, 10, 12, 14, 16, 20, 21, 23, 25, 26, 28, 29, 30, 31, 32, 33, 38, 39, 41, 42, 44, 45, 46, 49, 51, 52, 54, 56, 59, 60, 63, 64, 68, 75, 76, 77, 78, 79, 81, 85, 90, 91, 96, 97, 98, 99, 101, 103, 104, 107, 109, 112, 113, TgOvBr2, 5, TgGoatUS2, 9, 22, 27, TgMmUs1, 3, 4, TgCatUs8, TgSoUS42, 43, TgVaUS4, TgVvUS1, TgVaUS6, H2, PRU	Dubey, Patitucci et al. 2006; Rajendran et al. 2012; Dubey, Sundar, Nolden et al. 2007; Howe and Sibley 1995; Ajzenberg et al. 2004; Dubey Huong et al. 2008; Velmurugan et al. 2008; Dubey, Mergel et al. 2009; Dubey, Sundar et al. 2008; Prestrud, Asbakk et al. 2008; Sundar et al. 2008; Dubey, Thulliez et al. 1995; Velmurugan et al. 2009; Gamble et al. 2005; Zhou et al. 2009; Dubey, Rajendran et al. 2010; Dubey, Pas et al. 2010; Al-Kappany et al. 2010; da Silva et al. 2011; Dubey, Rajendran et al. 2011; Dubey, Velmurugan et al. 2011; Huang et al. 2012; Su et al. 2012.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
4	66	II	II	II	II	II	II	II	II	I	II	I	Chicken, Dog, Raccoon, Coyote, Bear, Rodent, Sheep, White-Tailed Deer, Sea Otter, Pig, Goat, Mouse, Cat, Black Bear	TgCkNi18, 39, 42, TgDgSl1, 2, 3, 7, 8, 9, 15, 17, 19, 20, 24, TgRaW5, TgCyW1, 2, B41, R961 (LDS), P10, TgShUs3, 8, 11, 29, 30, 31, 34, 38, 45, TgWtdUs2, 6, TgWtdUs7, 9, 11, 12, 14, TgSoUs1, 2, TgSoUs35, TgPgUs 16, 20, 25, 26, 40, 42, 47, 60, 65, 75, 78, 79, 80, 82, 84, 90, 96, TgGoatUS13, 17, 24, TgMmUs5, TgCatUs2, 3, 4, 5, 6, TgBbPa2	Dubey, Sundar et al. 2006; Dubey, Rajapakse et al. 2007; Dubey, Sundar, Nolden et al. 2007; Howe and Sibley 1995; Dubey, Sundar , Hill et al. 2008; Dubey, Velmurugan, Ulrich et al. 2008; Sundar et al. 2008; Dubey, Thulliez et al. 1995; Dubey, Rajendran et al. 2011; Dubey, Velmurugan et al. 2011
5	73	u-1	II	II	II	II	II	II	II	I	II	I	Human, Deer, Turkey, Sea Otter, Cat, Raccoon, Skunk, Sheep, Sea Otter, Pig, Goat, White-Tailed Deer, Bobcat, Wolf, Bottle-nosed Dolphin, Barn Owl, Brown Bear, Arctic Fox	ARI, RAY, WTD-1, WTD-3, Tg61(T61), 0729, 0791, RMS-MAU, TgCatCa2, TgRaCa2, TgSkCa1, TgShUs37, TgSoUs6, 7, 9, 10, 12, 13, 14, 22, 23, 24, 25, TgSoUs8, 11, 15, 16, 17, 32, 33, 34, 31, 38, TgPgUs89, TgGoatUS25, 28, TgWtdUs16, 17, 18, 19, 20, 22, 23, 24, 26, 27, 28, 29, 30, 31, 33, 34, 35, 36, TgCatGa1, 2, 3, 4, TgCatMs1, TgRaTx1, TgRaGa8, TgWolfWI1, TgSoUS40, 44, TgDoUS4, 6, TgTaUS1, TgBbAk1, TgVaUS3, TgWolfMN1, 3, 4, TgVaUS5	Howe and Sibley 1995; Ajzenberg et al. 2004; Dubey, Quirk et al. 2008; Dubey, Sundar, Hill et al. 2008; Sundar, Cole et al. 2008; Dubey, Thulliez et al. 1995; Velmurugan et al. 2009; Dubey, Rajendran et al. 2011; Dubey, Velmurugan et al. 2011; Dubey, Graham et al. 2004

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
6	48	I	I	I	III	I	II	u-1	I	I	I	I	Cat, Chicken, Dog, Human, Capybara, Sheep, Goat	TgCatBr2, 12, 17, 21, 30, TgCkBr144, TgDgBr3, 7, TgCatBr42, 47, 53, 54, 55, 62, 71, 75, TgCkBr123, 124, TgCkBr10, TgCkBr98, 101, 102, 104, TgCkBr55, 79, 86, 87, GPHT, FOU, BOF, TRK1, PSP-KOM, WIK, TgBrCp14, TgShBr8, 9, 10, 11, TgGtBr2, 3, 4, 9, TgCkBr201, 203, 207, 534N, PL8, S1PF2	Dubey, Navarro et al. 2004; Su et al. 2006; Dubey, Sundar et al. 2007; Dubey, Gennari et al. 2007; Pena et al. 2006; Pena et al. 2008; Dubey, Gennari et al. 2006; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2002; Dubey, Navarro et al. 2003; da Silva et al. 2003; Dubey, Graham et al. 2003; Howe and Sibley 1995; Ajzenberg et al. 2004; Yai et al. 2009; Ragozo et al. 2010; Soares et al. 2011; Ferreira et al. 2011; Mercier et al. 2010.
7	38	I	III	III	III	III	III	III	III	III	III	I	Chicken, Raccoon, Dove, Sheep, Pig	TgCkCr8, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, TgCkNi14, 15, 20, 25, 30, TgCkBr111, 112, TgCkBr182, TgCkGy23, 24, TgRaW4, G622M, TgShUs40a, TgPgUs5, 10, 14, TgCkBr196, TgRaGa2, TgCkAr16, 18, TgCkGa6	Dubey, Su et al. 2006; Rajendran et al. 2012; Dubey, Sundar et al. 2006; Dubey, Sundar et al. 2007: de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008; Dubey, Applewhaite et al. 2007; Dubey, Sundar, Nolden et al. 2007; Howe and Sibley 1995; Dubey, Sundar, Hill et al. 2008; Dubey, Hill et al. 2008; Velmurugan et al. 2009; Soares et al. 2011; Dubey, Velmurugan et al. 2011; Rajendran et al. 2012.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
8	34	I	III	III	III	III	III	II	III	III	III	III	Cat, Dog, Chicken, Pig, Capybara, Sheep, Opossum	TgCatBr3, 4, TgDgBr4, 12, TgCatBr58, 59, 60, 73, 74, TgCkBr131,132, 133,134, TgCkBr11, 7, 17, TgPgUs15-a.k.a.P89, 45, 46, 55, 57, 58, 76, 92, TgCpBr36, TgCpBr17, TgCpBr18, TgCpBr20, TgShBr15, TgCkBr194, 195, TgCatUs7, TgOpGa1, TgCkVe1	Dubey, Navarro et al. 2004; Su et al. 2006; Dubey, Gennari et al. 2007; Pena et al. 2006; Pena et al. 2008; Dubey, Gennari et al. 2006; Dubey, Velmurugan 2008; Dubey, Graham et al. 2002; Dubey, Thulliez et al. 1995; Velmurugan et al. 2009; Yai et al. 2009; Ragozo et al. 2010; Soares et al. 2011; Dubey et al. 2011; Rajendran et al. 2012
9	55	u-1	II	II	III	III	II	II	III	II	II	I	Dog, Cat, Sheep, Human, Pig, Wolf, Pigeon	TgDgCo4, TgCtPRC2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, TgDgVi5, 6, 7, 8, TgDgSl 21, TgShUs1, TgCatMx2, TgCatMx5, TgC1, 2, 3, 4, 5, 6, 7, 8, ZS, GYS, PYS, ZC, FS, NY5, NY19, NY11, NY8, NY13, TgWolfAK1, TgCatBj1-11, TgPigeonMx1	Dubey, Cortés-Vecino et al. 2007; Dubey, Zhu et al. 2007; Dubey, Huong et al. 2007; Dubey, Rajapakse et al. 2007; Dubey, Sundar, Hill et al. 2008; Dubey, Velmurugan, Alvarado-Esquivel et al. 2009; Zhou et al. 2009; Zhou et al. 2010; Dubey, Velmurugan et al. 2011; Qian et al. 2012; Alvarado-Esquivel et al. 2011.
10	31	I	I	I	I	I	I	I	I	I	I	I	Cat, Chicken, Human, Cow, Pig	TgCtCo2, 7, TgCkBr146, RH88, VEL, GT1, CT-1, OH3, S11, BK, ENT, MOR, FR-93, SH, CN, NT, TgPHs1, TgPXx, TgPXd, TgpNx, TgPLh, TgPxda, TgPxdb1, Tgpyh, TgPNY, TgPGZ, ATIH, P, PiL, GiL, S10	Dubey, Su et al. 2006; Rajendran et al. 2012; Dubey, Sundar, Gennari et al. 2007; Howe and Sibley. 199; Zhou et al. 2009; Zhou et al. 2010; Mercier et al. 2010; Ajzenberg et al. 2004.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
11	20	I	I	II	III	III	III	I	III	I	II	III	Cat, Chicken, Capybara, Sheep, Rabbit, Wood Rat	TgCatBr1, 7, TgCatBr39, 51, 52, 56, 61, 68, 77, 78, TgCkBr97, TgCkBr57, 64, TgCpBr11, TgCpBr23, TgCpBr24, TgOvBr3, TgRabbitBr2, TgNlTX1, TgCkAr1	Dubey, Navarro et al. 2004; Su et al. 2006; Pena et al. 2006; Pena et al. 2008; Dubey, Navarro et al. 2003; Dubey, Velmurugan et al. 2008; da Silva et al. 2003; Dubey, Graham et al. 2003; Yai et al. 2009; da Silva et al. 2011; Dubey, Passos et al. 2011; Dubey, Velmurugan et al. 2011; Rajendran 2012; Huang et al. 2012.
12	13	I	III	III	I	I	III	II	III	III	I	III	Chicken, Human	TgCkGy2, 3, 5, 6, 9, 12, 16, 19, 20, 25, 28, 32, IPP-BAT	Dubey, Applewhaite et al. 2007; Ajzenberg et al. 2004.
13	16	I	I	I	I	I	III	II	III	III	I	III	Chicken, Human, Cat, Goat, Red-Handed Howler Monkey	TgCkBr165, TgCkBr167, 170, TgCkBr174, 176, TgCkBr179, 180, TgCkBr183, TgCkBr184, 185, PSP-ERO, TgCatStK7, TgGtBr10, TgRhHmBr1, TgCkGr25, 26	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008; Ajzenberg et al. 2004; Dubey, Moura et al. 2009; Ragozo et al. 2010; Pena et al. 2011; Rajendran et al. 2012.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
14	14	I	III	III	III	III	III	III	I	III	III	III	Chicken, Cat, Dog, Coyote	TgCkCh1, TgCtCo14, TgCatBr15, TgCkBr153, TgDgBr19, TgCkBr82, 90, TgDgCo9, 12, 15, 18, TgCyW6, TgCkVe3, TgCkCo2	Dubey, Patitucci et al. 2006; Rajendran et al. 2012; Dubey, Su et al. 2006; Dubey, Navarro et al. 2004; Su et al. 2006; Dubey, Sundar, Gennari et al. 2007; Dubey, Gennari et al. 2007; da Silva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; Dubey, Cortés-Vecino et al. 2007; Dubey, Sundar, Nolden et al. 2007; Rajendran et al. 2012.
15	16	I	I	II	III	III	III	I	III	I	II	III	Dog, Chicken, Sheep, Bottlenose Dolphin, Red-tailed Hawk	TgDgBr1, 13, TgCkBr119, 120, 122, 129, 135, 137,140, CASTELLS, TgCkPl1,2, TgCkNg1, TgDoUs1, TgBjUS1, TgCkAr25	Dubey, Gennari et al. 2007; Howe and Sibley 1995; Dubey, Huong et al. 2008; Velmurugan et al. 2008; Dubey, Fair et al. 2008; Dubey, Velmurugan et al. 2011; Rajendran et al. 2012.
16	11	I	I	II	III	III	I	III	I	III	III	I	Chicken	TgCkNi1, 11, 22, 23, 26, 29, 33, 36, 38, 46, 47y	Dubey, Sundar, Pineda et al. 2006; Rajendran et al. 2012.
17	16	u-1	I	II	III	III	III	u-1	I	I	III	I	Chicken, Human, Sheep	TgCkBr147, 148, 151, 154, 160, 162, 163, TgCkBr81, MAS, IPP-URB, TgOvBr1, 6, TgCkAr27, 28, TgCkPe4, 6.	Dubey, Sundar, Gennari et al. 2007; da Silva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; Howe and Sibley 1995; Ajzenberg et al. 2004; da Silva et al. 2011; Rajendran et al. 2012.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
18	9	I	I	I	III	I	III	II	I	III	III	I	Dog, Cat	TgCtCo12, 13, TgDgCo3, TgCtPRC1, 3, TgDgVi1, 2, 3, 4	Dubey, Su et al. 2006; Rajendran et al. 2012; Dubey, Cortés-Vecino et al. 2007; Dubey, Zhu et al. 2007; Dubey, Huong et al. 2007.
19	16	I	III	III	III	III	III	I	I	I	u-1	I	Cat, Chicken, Capybara, Sheep, Rabbit, Mouse	TgCatBr5, 11, 16, TgCatBr84, TgCkBr28, 33, 50, 52, 58, TgCpBr10, TgCpBr31, TgOvBr4, TgRabbitBr1, TgMmBr03 (MM03), TgCkBr205, 209	Dubey, Navarro et al. 2004; Su et al. 2006; Pena et al. 2006; Pena et al. 2008; da Silva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; Yai et al. 2009; da Silva et al. 2011; Dubey, Passos et al. 2011; Araújo et al. 2010; Soares et al. 2011.
20	15	u-1	II	II	III	III	II	II	III	II	u-2	I	Dog, Sandcat,Cat	TgDgSl 4, 11, 12, 13, 14, 16, 18, 22, 23, TgSandcatUAE1, 3, TgCatEg7,18,34,65	Dubey, Rajapakse et al. 2007; Dubey, Pas et al. 2010; Al-Kappany et al. 2010.
21	10	I	III	III	III	III	III	I	I	I	III	III	Cat, Chicken, Capybara, Rat	TgCatBr10, 22, 23, 28, 31, 32, 37, TgCkBr95, TgCpBr 29, TgRrBr09 (RR09)	Dubey, Navarro et al. 2004; Su et al. 2006; Dubey, Navarro et al. 2003; Dubey, Velmurugan et al. 2008; Yai et al. 2009; Araújo et al. 2010.
22	8	u-1	I	II	III	III	III	u-1	I	III	III	III	Chicken	TgCkBr38, 27, 44, 51, 65, 66, 78, 80	da Silva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
23	8	I	I	II	III	I	III	II	I	III	u-1	I	Chicken, Dog	TgCkNi4, 6, 10, 17, 21, 34, 37, TgDgCo19	Dubey, Sundar et al. 2006; Dubey, Cortés-Vecino et al. 2007
24	6	I	I	I	I	III	I	I	I	I	I	III	Chicken	TgCkCr2, 28, 29, 30, 31, 32	Dubey, Su, et al. 2006; Rajendran et al. 2012
25	6	I	III	III	I	III	III	III	III	III	I	I	Chicken	TgCkBr110, TgCkGy8, 10, 14, 15, 35	Dubey, Sundar et al. 2007; Dubey, Applewhaite et al. 2007.
26	6	II	III	III	III	III	III	I	III	III	I	III	Chicken, Cat	TgCkBr149, 150, 152, 157, TgCatBr65, 66	Dubey, Sundar, Gennari et al. 2007; Pena et al.2006; Pena et al. 2008
27	5	I	I	I	I	I	I	I	III	I	I	I	Chicken	TgCkNi7, 9, 40, 41, 43	Dubey, Sundar, Pineda et al. 2006
28	6	I	I	I	I	I	I	II	I	III	I	III	Cat, Chicken, Human	TgCtCo1, TgCkBr115, 142, 145, CAST, TgCkCo5	Dubey, Su et al. 2006; Rajendran et al. 2012; Dubey, Sundar, Gennari et al. 2007; Howe and Sibley 1995
29	6	I	I	II	III	I	III	II	I	III	III	I	Chicken, Dog	TgCkBr114, TgDgCo1, 2, 10, 20, TgCkCo20	Dubey, Sundar, Gennari et al. 2007; Dubey, Cortés-Vecino et al. 2007; Rajendran et al. 2012
30	5	I	III	III	I	III	III	III	III	III	I	III	Chicken	TgCkBr113, TgCkGy7, 11, 13, 31	Dubey, Sundar, Gennari et al. 2007; Dubey, Applewhaite et al. 2007
31	5	I	III	III	III	I	III	III	III	III	I	III	Chicken	TgCkGy1, 4, 29, 30, 33	Dubey, Applewhaite et al. 2007;

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
32	5	I	III	III	III	III	II	I	I	I	I	I	Dog, Raccoon	TgDgBr8, 9, 10, TgRaW2, 3	Dubey, Gennari et al. 2007; Dubey, Sundar, Nolden et al. 2007
33	7	u-1	I	II	III	I	III	u-1	I	I	I	I	Chicken, Capybara	TgCkBr41, 42, 49, 60, 62, TgCpBr34, TgCpBr35	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; Yai et al. 2009
34	8	u-1	I	II	III	III	III	II	I	I	u-1	I	Dog, Cat, Capybara	TgDgBr5, TgCatBr44, 48, 69, 70, TgCpBr8,13, 15	Dubey, Gennari et al. 2007; Pena et al. 2006; Pena et al. 2008; Yai et al. 2009
35	4	I	I	I	I	I	I	I	III	I	I	III	Chicken	TgCkCr3, 4, 5, 6	Dubey, Su et al. 2006; Rajendran et al. 2012
36	4	I	I	I	III	I	III	II	I	III	I	III	Chicken	TgCkBr59, 30, 34, 67	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
37	4	I	II	II	III	III	III	u-1	I	I	III	I	Chicken	TgCkBr36, 32, 84, 85	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
38	13	I	III	III	III	I	I	I	III	I	I	III	Cat, Dog, Chicken	TgCtCo4,10,11, TgDgCo17, TgCkCo6, 10, 12, 13, 21, 23, 24, 8, 15	Dubey, Su et al. 2006; Rajendran et al. 2012; Dubey, Cortés-Vecino et al. 2007;
39	3	II	II	II	II	II	II	II	II	I	II	II	Sheep, Goat	TgShUs47, 52, TgGoatUS7	Dubey, Sundar, Hill et al. 2008; Dubey, Rajendran et al. 2011

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
40	4	u-1	I	II	III	III	III	III	III	I	III	I	Cat, Chicken	TgCtCo5x, TgCkBr75, 76, 92	Dubey, Su et al. 2006; Rajendran et al. 2012; daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
41	5	I	I	I	III	I	II	I	I	I	I	I	Chicken, Capybara	TgCkBr136, 138, 139, TgCpBr9, TgCpBr12	Dubey, Gennari et al. 2006; Dubey, Velmurugan et al. 2008; Yai et al. 2009
42	2	I	I	I	III	III	II	I	I	I	u-1	I	Cat	TgCatBr9,19	Dubey, Navarro et al. 2004; Su et al. 2006
43	3	I	I	II	I	II	I	I	I	I	u-1	I	Chicken	TgCkCr7, 9,10	Dubey, Su et al. 2006; Rajendran et al. 2012
44	3	I	I	II	III	I	III	II	I	I	u-3	I	Dog	TgDgCo5, 6, 11	Dubey, Cortés-Vecino et al. 2007
45	3	I	III	III	III	I	II	II	III	I	I	III	Chicken	TgCkBr126, 127, 117	Dubey Gennari et al. 2006; Dubey, Velmurugan et al. 2008
46	3	I	III	III	III	I	III	II	I	III	III	I	Dog	TgDgCo8, 14, 16	Dubey, Cortés-Vecino et al. 2007

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
47	3	I	III	III	III	III	II	u-1	I	I	II	I	Cat, Chicken	TgCatBr25, TgCkBr99, 100	Dubey, Navarro et al. 2004; Su et al. 2006; Dubey, Navarro et al. 2003; Dubey, Velmurugan et al. 2008
48	7	I	III	III	III	III	III	III	III	III	III	III	Chicken	TgCkBr181, TgCkGy21, 22, TgCkVe 4, 5, 10, TgCkAr7	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008; Dubey, Applewhaite et al. 2007; Rajendran et al. 2012
49	3	II	II	II	I	II	II	II	II	III	II	nd	Cat	TgCatPr5, 7, 11	Dubey, López-Torres et al. 2007
50	3	II	III	III	I	III	I	III	III	III	III	III	Chicken	TgCkNi16, 45, 7x	Dubey, Sundar, Pineda et al. 2006
51	3	u-1	I	II	III	I	III	II	I	I	I	I	Dog, Chicken	TgDgBr6, 17, TgCkBr46	Dubey, Gennari et al. 2007; daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
52	3	u-1	I	II	III	I	III	u-2	I	I	III	I	Chicken, Toucan	TgCkNi12, 32, TgRsCr1	Dubey, Sundar, Pineda et al. 2006; Dubey, Velmurugan, Morales et al. 2009

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
53	6	u-1	I	II	III	III	III	II	I	I	III	I	Dog, Chicken, Capybara	TgDgBr14, 15, TgCkBr96, TgCpBr19, TgCpBr21, TgCpBr22	Dubey, Gennari, Sundar et al. 2007; Dubey, Navarro et al. 2003; Dubey, Velmurugan et al. 2008; Yai et al. 2009
54	3	II	II	II	III	III	III	III	III	III	III	II	Sheep, White-tailed deer, Sea otter	TgShUs2, TgWtdUs10, TgSoUs39	Dubey, Sundar, Hill et al. 2008; Dubey, Velmurugan, Ulrich et al. 2008; Sundar, Cole et al. 2008
55	2	I	I	I	I	III	I	u-1	I	I	I	I	Cat	TgCatBr79, 80	Pena et al. 2006; Pena et al. 2008
56	2	I	I	I	III	I	II	u-1	I	III	I	I	Cat	TgCatBr45, 46	Pena et al. 2006; Pena et al. 2008
57	2	I	I	I	III	I	II	u-1	I	III	II	III	Chicken	TgCkBr171, 172	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
58	2	I	I	I	III	I	III	u-1	III	III	I	I	Dog, Cat	TgDgBr2, TgCatBr83	Dubey, Gennari et al. 2007; Pena et al. 2006; Pena et al. 2008
59	2	I	I	I	III	III	II	u-1	I	I	I	I	Chicken	TgCkBr40, 47	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
60	2	I	I	II	I	III	III	III	I	III	III	I	Human	GUY-KOE, VAND	Howe and Sibley 1995
61	2	I	I	II	III	I	III	II	III	I	u-2	I	Cat	TgCtCo5, 6	Dubey, Su et al. 2006; Rajendran et al. 2012
62	2	I	I	II	III	I	III	II	III	III	III	I	Cat	TgCtCo3, 9	Dubey, Su et al. 2006; Rajendran et al. 2012
63	2	I	I	II	III	III	III	I	III	I	II	I	Chicken	TgCkBr13, 23	Dubey, Graham et al. 2002; Dubey, Velmurugan et al. 2008
64	2	I	I	II	III	III	III	u-1	I	I	u-2	I	Chicken	TgCkBr19, 24	Dubey, Graham et al. 2002; Dubey, Velmurugan et al. 2008
65	20	I	I	II	III	III	III	u-1	I	I	III	I	Cat, Chicken, Human	TgCatBr82, TgCkBr89, 477Co, 244N, 262N, 331N, 384N, 581N, 692N, 704N, 527N, 176Co, 539O, 571O, 441N, 508N, 358N, 607O, 616O, 622O	Pena et al. 2006; Pena et al. 2008; daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; Ferreira et al. 2011

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
66	2	I	II	II	III	II	II	II	u-1	I	u-2	I	Cougar	TgCgCa1(Cougar), TgCgCa2	Howe and Sibley 1995; Dubey, Quirk et al. 2008
67	3	I	III	III	III	I	III	I	III	III	u-1	III	Dog, Cat, Capybara	TgDgBr16, TgCatBr76, TgCpBr33	Dubey, Gennari et al. 2007; Pena et al. 2006; Pena et al. 2008; Yai et al. 2009
68	2	I	III	III	III	I	III	III	III	III	III	III	Chicken	TgCkGy17, 18	Dubey, Applewhaite et al. 2007
69	2	I	III	III	III	III	II	I	III	I	II	I	Chicken	TgCkBr93, 94	Dubey, Navarro et al. 2003; Dubey, Velmurugan et al. 2008
70	2	I	III	III	III	III	II	u-1	I	I	I	III	Chicken	TgCkBr107, 108	Dubey, Sundar, Gennari et al. 2007
71	3	I	III	III	III	III	III	II	I	III	III	I	Chicken, Human	TgCkBr26, 69, 180N	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; Ferreira et al. 2011
72	2	I	III	III	III	III	III	III	III	III	u-2	III	Human, Sheep	ROD, TgShUs5	Howe and Sibley, 1995; Dubey, Sundar, Hill et al. 2008.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
73	3	II	III	III	I	I	I	III	III	III	I	I	Sheep, Dog	TgShUs22, 28, TgDgMx2	Dubey, Sundar, Hill et al. 2008; Dubey, Velmurugan, Alvarado-Esquivel et al. 2009
74	5	II	III	III	III	II	II	II	III	II	II	I	Sheep, White-tailed Deer, Dog, Cat	TgShUs39, TgWtdUs8, TgDgMx1, TgDgMx3, TgCatMx1	Dubey, Sundar, Hill, et al. 2008; Dubey, Velmurugan, Ulrich et al 2008; Dubey, Velmurugan, Alvarado-Esquivel et al. 2009
75	2	u-1	I	II	III	III	III	II	I	I	III	III	Chicken	TgCkBr48, 88	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
76	2	u-1	III	III	III	III	III	u-1	I	I	III	I	Chicken	TgCkBr155, 159	Dubey, Sundar, Gennari et al. 2007
77	1	I	I	I	I	I	I	u-1	I	I	III	III	Chicken	TgCkBr141	Dubey, Sundar, Gennari et al. 2007
78	1	I	I	I	I	I	III	II	I	III	I	III	Chicken	TgCkBr169	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; de Oliveira et al. 2009
79	1	I	I	I	I	III	I	I	III	III	I	III	Dog	TgDgCo13	Dubey, Cortés-Vecino et al. 2007
80	1	I	I	I	III	I	II	I	I	I	u-1	I	Cat	TgCatBr26	Dubey, Navarro et al. 2004; Su et al. 2006

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
81	1	I	I	I	III	I	II	u-1	I	I	III	III	Chicken	TgCkBr173	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008
82	1	I	I	I	III	I	III	II	I	I	I	III	Chicken	TgCkBr54	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
83	1	I	I	I	III	I	III	II	III	III	I	III	Monkey	ENVL-MAC	Ajzenberg et al. 2004
84	1	I	I	I	III	I	III	u-1	I	I	III	I	Dog	PBr	Howe and Sibley, 1995
85	1	I	I	I	III	III	II	u-1	I	I	II	I	Cat	TgCatBr72	Pena et al. 2006; Pena et al. 2008
86	1	I	I	I	III	III	II	u-1	I	I	III	I	Cat	TgCatBr50	Pena et al. 2006; Pena et al. 2008
87	1	I	I	I	III	III	III	I	I	III	I	III	Chicken	TgCkBr156	Dubey, Sundar, Gennari et al. 2007
88	1	I	I	I	III	III	III	II	I	III	I	III	Chicken	TgCkBr186	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008
89	1	I	I	I	III	III	III	II	III	III	III	III	Chicken	TgCkId1	Dubey, Huong et al. 2008
90	1	I	I	I	III	III	III	III	III	III	I	III	Black Bear	TgBBeCa1	Dubey, Quirk et al. 2008

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
91	1	I	I	II	I	I	I	II	I	I	I	I	Chicken	TgCkCr1	Dubey, Su et al. 2006; Rajendran et al. 2012
92	1	I	I	II	I	III	II	II	I	I	II	I	Cat	TgCatBr40	Pena et al. 2006; Pena et al. 2008
93	1	I	I	II	I	III	II	u-1	I	I	III	I	Chicken	TgCkBr61	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
94	1	I	I	II	I	III	III	I	I	I	II	I	Chicken	TgCkBr16	Dubey, Graham et al. 2002; Dubey, Velmurugan et al. 2008
95	1	I	I	II	I	III	III	II	I	I	III	I	Human	GUY-MAT	Howe and Sibley, 1995
96	1	I	I	II	I	III	III	II	III	I	III	III	Chicken	TgCkBr109	Dubey, Sundar, Gennari et al. 2007
97	1	I	I	II	I	III	III	II	III	I	III	I	Human	GUY-DOS	Howe and Sibley, 1995
98	1	I	I	II	I	III	III	III	III	I	III	III	Human	RUB	Howe and Sibley, 1995
99	1	I	I	II	I	III	III	u-1	I	I	III	I	Human	TOU-ALI	Ajzenberg et al. 2004
100	1	I	I	II	I	III	III	u-1	I	III	III	I	Human	GUY-BAS1	Ajzenberg et al. 2004
101	1	I	I	II	III	I	III	II	I	I	u-2	I	Cat	TgCtCo15	Dubey, Su et al. 2006; Rajendran et al. 2012
102	1	I	I	II	III	I	III	II	I	III	u-1	III	Chicken	TgCkNi35	Dubey, Sundar, Pineda et al. 2006
104	1	I	I	II	III	I	III	u-1	I	I	III	I	Cat	TgCatBr34	Dubey, Navarro et al. 2004; Su et al. 2006

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
105	1	I	I	II	III	III	II	u-1	III	III	III	I	Chicken	TgCkBr143	Dubey, Sundar, Gennari et al. 2007
106	1	I	I	II	III	III	II	u-1	I	I	II	I	Dog	TgDgBr18	Dubey, Gennari et al. 2007
107	1	I	I	II	III	III	II	u-1	I	I	III	I	Chicken	TgCkBr37	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003
108	1	I	I	II	III	III	III	II	I	I	III	I	Cat	TgCatBr57	Pena et al. 2006; Pena et al. 2008
109	1	I	I	II	III	III	III	III	I	III	III	III	Chicken	TgCkBr177	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008
110	1	I	I	II	III	III	III	u-1	III	I	III	I	Sheep	TgShUs49a	Dubey, Sundar, Hill et al. 2008
111	6	I	I	u-1	III	III	III	u-1	I	III	III	I	Cat, Sheep, Rat	TgCatBr64, TgShBr6, 7, 16, TgOvBr12, TgRtBr1	Pena et al. 2006; Pena et al. 2008; Ragozo et al. 2010; da Silva et al. 2011; Muradian et al. 2012
112	1	I	II	II	I	I	I	III	II	III	I	nd	Cat	TgCatPr6	Dubey, López-Torres et al. 2007
113	1	I	II	II	III	II	II	II	nd	III	II	nd	Pig	TgPgUs11	Dubey, Hill et al. 2008; Velmurugan et al. 2009

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
114	1	I	III	III	I	III	III	III	I	III	I	I	Chicken	TgCkBr166	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008
115	1	I	III	III	I	III	III	III	III	III	III	I	Cat	TgCatPr9	Dubey, López-Torres et al. 2007
116	3	I	III	III	III	I	III	II	III	III	III	III	Chicken	TgCkBr130, TgCkVe11, TgCkPe3	Dubey, Gennari et al. 2006; Dubey, Velmurugan et al. 2008; Rajendran et al. 2012
117	1	I	III	III	III	I	III	u-1	I	I	u-1	III	Cat	TgCatBr41	Pena et al. 2006; Pena et al. 2008
118	2	I	III	III	III	III	I	I	III	III	I	nd	Cat, Goat	TgCatPr8, TgGoatUS6	Dubey, López-Torres et al. 2007. Dubey, Rajendran, Ferreira et al. 2011
119	1	I	III	III	III	III	II	u-1	I	I	u-1	I	Cat	TgCatBr18	Dubey, Navarro et al. 2004; Su et al. 2006
120	1	I	III	III	III	III	III	I	I	III	III	III	Cat	TgCatBr20	Dubey, Navarro et al. 2004; Su et al. 2006.
121	1	I	III	III	III	III	III	I	III	I	III	III	Cat	TgCatBr67	Pena et al. 2006; Pena et al. 2008

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
122	1	I	III	III	III	III	III	I	III	III	I	III	Dog	TgDgCo7	Dubey, Cortés-Vecino et al. 2007
123	1	I	III	III	III	III	III	I	III	III	III	III	Chicken	TgCkGy34	Dubey JP, Applewhaite et al. 2007
124	1	I	III	III	III	III	III	II	III	I	u-1	I	Cat	TgCatBr81	Pena et al. 2006; Pena et al. 2008
125	1	I	III	III	III	III	III	II	III	III	u-2	III	Chicken	TgCkBr8	Dubey, Graham et al. 2002; Dubey, Velmurugan et al. 2008
126	1	I	nd	I	III	III	II	u-1	I	I	u-1	I	Cat	TgCatBr6-20	Dubey, Navarro et al. 2004; Su et al. 2006
127	1	II	II	II	II	II	II	II	II	III	III	III	Bear	B73	Howe and Sibley, 1995
128	1	II	II	II	II	II	II	II	III	II	II	I	Cat	TgCtCo8	Dubey, Su et al. 2006; Rajendran et al. 2012
129	1	II	II	II	II	III	II	II	II	II	II	II	Chicken	TgCkBr168	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008
130	1	II	III	III	I	III	III	II	I	III	III	III	Cat	TgCatCa1	Dubey, Quirk et al. 2008
131	1	II	III	III	II	II	I	III	III	II	II	I	Sheep	TgShUs32	Dubey, Sundar, Hill et al. 2008.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
132	1	II	III	III	III	II	II	II	III	III	II	III	Chicken	TgCkGh2	Dubey, Huong et al. 2008.
133	1	II	III	III	III	III	I	III	III	III	III	III	Sheep	M7741	Howe and Sibley, 1995.
134	1	u-1	I	II	III	I	III	II	III	III	I	III	Chicken	TgCkBr178	de Oliveira et al. 2009; Dubey, Velmurugan et al. 2008.
135	2	u-1	I	II	III	III	III	II	I	III	III	I	Chicken, Goat	TgCkBr45, TgGtBr8	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003; Ragozo et al. 2010
136	1	u-1	I	u-1	III	III	III	II	I	I	III	I	Cat	TgCatBr38	Pena et al. 2006; Pena et al. 2008
137	1	u-1	II	II	III	III	II	II	III	II	III	I	Chicken	TgCkGh1	Dubey, Huong et al. 2008
138	1	u-1	III	III	III	III	III	III	I	III	III	III	Chicken	TgCkBr74	daSilva et al. 2003; Dubey, Velmurugan et al. 2008; Dubey, Graham et al. 2003.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
139	1	II	II	II	III	III	III	II	II	III	III	I	Human	SOU	Howe and Sibley 1995
140	1	II	III	III	III	III	III	II	III	III	I	III	Chicken	TgCkNi27	Dubey, Sundar et al. 2006
141	1	II	III	III	III	III	III	III	III	III	I	III	Cat	TgCatStK1	Dubey, Moura et al. 2009
142	1	I	I	I	I	I	II	II	II	II	I	I	Chicken	TgCkBr222	Dubey, Rajendran, Costa et al. 2010
143	1	I	I	I	III	I	III	III	I	III	I	III	Goat	TgGoatUS5	Dubey, Rajendran et al. 2011
144	2	I	I	I	III	I	III	u-1	III	I	I	I	Sheep	TgShBr1, 2	Ragozo et al. 2010
145	1	I	I	I	III	I	II	I	I	I	I	III	Black-Eared Opposum	TgOpBr1	Pena et al. 2011
146	15	I	I	I	III	II	II	I	III	III	II	III	Chicken	TgCkBr210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 223, 224, 227, 229, 233	Dubey, Rajendran, Costa et al. 2010
147	1	I	I	II	I	III	III	I	I	I	III	I	Alaska Black Bear	TgBbUS1	Dubey, Rajendran, Ferreira et al. 2010
148	1	I	I	II	III	I	III	II	I	I	III	I	Capybara	TgCpBr26	Yai et al. 2009

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
149	4	I	I	II	III	III	II	II	I	I	I	I	Goat	TgGtBr1, 6, 7, 12	Ragozo et al. 2010
150	3	I	I	II	III	III	II	u-1	III	I	I	I	Sheep, Goat	TgShBr3, 4, TgGtBr5	Ragozo et al. 2010
152	3	I	I	u-1	III	III	III	II	I	I	III	I	Sheep	TgShBr12, 13, 14	Ragozo et al. 2010
153	1	I	II	I	III	I	III	I	III	III	II	III	Chicken	TgCkBr232	Dubey, Rajendran, Costa et al. 2010
154	1	I	II	II	III	II	II	II	u-1	III	II	I	Goat	TgGoatUS20	Dubey, Rajendran et al. 2011
155	1	I	III	III	I	I	III	I	III	III	I	nd	Cat	TgCatMx1b	Dubey, Velmurugan, Alvarado-Esquivel et al. 2009
156	1	I	III	III	III	I	I	III	III	III	I	III	Goat	TgGoatUS3	Dubey, Rajendran et al. 2011
157	3	I	III	III	III	I	III	I	III	III	III	III	Chicken, American Kestrel	TgCkBr202, 204, TgFsUS1	Soares et al. 2011; Dubey, Velmurugan, et al. 2011
158	1	I	III	III	III	I	III	u-1	III	III	III	I	Chicken	TgCkBr206	Soares et al. 2011

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
159	1	I	III	III	III	III	I	I	III	I	III	III	Chicken	TgCkBr200	Soares et al. 2011
160	1	I	III	III	III	III	III	I	III	III	u-1	III	Sheep	TgShBr5	Ragozo et al. 2010
161	1	I	III	III	III	III	III	I	I	III	III	I	Chicken	TgCkBr199	Soares et al. 2011
162	1	I	III	III	III	III	III	II	I	I	III	I	Capybara	TgCpBr1	Yai et al. 2009
163	1	I	III	III	III	III	III	II	I	III	III	III	Chicken	TgCkBr220	Dubey, Rajendran, Costa et al. 2010
164	1	I	III	III	III	III	III	III	I	I	III	I	Chicken	TgCkBr208	Soares et al. 2011
165	1	I	III	III	III	III	III	u-1	I	I	III	III	Capybara	TgCpBr27	Yai et al. 2009
166	1	I	III	III	III	III	III	u-1	I	III	III	III	Jaguarundi	TgJagBr1	Pena et al. 2011
167	2	II	I	I	I	I	I	I	III	III	III	III	Goat, Arctic Fox	TgGoatUS26, TgBsCoUS1, TgVaUS1	Dubey, Rajendran et al. 2011; Dubey, Velmurugan et al. 2011
168	1	II	II	II	II	III	II	II	II	III	II	I	Cat	TgCatEg57	Al-Kappany et al. 2010

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
169	2	II	II	II	III	III	III	III	III	II	II	III	Cat	TgCatEg53,67	Al-Kappany et al. 2010
170	1	II	III	III	III	I	III	III	III	III	III	III	Goat	TgGoatUS8	Dubey, Rajendran et al. 2011
171	2	u-1	I	I	III	III	I	u-1	III	III	I	III	Sheep	TgOvBr7, 8	da Silva et al. 2011
172	6	u-1	I	II	I	III	II	u-1	III	III	III	I	Chicken	TgCkBr188, 189, 190, 191, 192, 193	Soares et al. 2011
173	1	u-1	I	II	III	III	II	u-1	I	I	III	I	Capybara	TgCpBr3	Yai et al. 2009
174	1	u-1	I	II	III	III	II	u-1	III	I	III	I	Chicken	TgCkBr197	Soares et al. 2011
175	2	u-1	I	II	III	III	III	III	I	I	u-1	I	Capybara	TgCpBr25, TgCpBr30	Yai et al. 2009
176	1	u-1	II	II	II	II	II	II	III	II	III	I	Cat	TgCatEg88	Al-Kappany et al. 2010
177	2	I	I	I	I	I	I	I	III	III	I	III	Hawaiian crow	TgHcUS1, 2	Dubey *et al.* 2011c.
178	1	I	I	I	III	I	III	II	I	I	III	I	Chicken	TgCkCo4	Rajendran *et al.* 2012.

Table D-1 continued

ToxoDB PCR-RFLP Genotype	N	SAG1	SAG2	Alt.SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Host Animals	Strain IDs	References
179	2	I	I	II	III	I	III	I	III	III	III	III	Chicken	TgCkCo17, 22	Rajendran et al. 2012
180	1	I	III	III	I	I	I	II	III	III	I	I	Striped Skunk	TgSKMs3	Dubey, Velmurugan et al. 2011
185	1	II	II	II	III	III	II	III	III	III	u-2	III	Chicken	TgCkVe6	Rajendran et al. 2012
186	1	II	III	III	III	III	I	I	I	III	I	III	Wallaby	TgWyUs4	Dubey, Velmurugan et al. 2011
187	2	II	III	III	III	III	III	II	III	III	III	III	Chicken	TgCkGr17, 18	Rajendran et al. 2012
188	1	II	III	III	III	III	III	III	III	I	I	III	Chicken	TgCkCo9	Rajendran et al. 2012
189	1	u-1	I	II	I	I	I	I	I	I	III	I	Chicken	TgCkPe5	Rajendran et al. 2012
190	1	I	III	III	III	I	I	II	III	III	I	III	Chicken	TgCkGa01	Rajendran et al. 2012
191	1	I	I	I	I	I	I	II	III	III	I	III	Chicken	TgCkGa04	Rajendran et al. 2012
197	1	I	II	II	II	II	II	II	II	I	II	I	Jaguar	GUY-2004-JAG1	Su et al. 2012
202	1	u-1	I	II	III	III	II	III	I	I	III	I	Human	LGE-2007-CUV	Su et al. 2012
203	4	I	I	I	III	I	II	II	III	III	I	III	Human	P24, PF26, PL15, PM2	Mercier et al. 2010
223	1	I	III	III	III	II	III	II	II	II	III	II	Pig	P105 WGA	Howe and Sibley 1995

Note: N= the number of isolates belonging to the same genotype. At locus SAG1, type II and III alleles are indistinguishable.
nd: no data
Some genotype numbers are assigned to potential new PCR-RFLP types but not yet confirmed or published, therefore not listed in this table.

Table D-2. ROP18, ROP5 and ROP16 genotypes of *T. gondii* strains

Strain ID	ToxoDB Genotype#	ROP18 type	ROP5 type	ROP16 type	Genotype combination	Mouse mortality	Lethality
TgCtCo2	10	1	1	1	1	75	nonlethal
GT1	10	1	1	1	1	100	lethal
TgCtCo7	10	1	1	1	1	100	lethal
TgCkBr146	10	1	1	1	1	ND	ND
TgCkNi9	27	1	1	1	1	100	lethal
TgCkCr3	35	1	1	1	1	100	lethal
TgCkCr4	35	1	1	1	1	100	lethal
TgCkCr5	35	1	1	1	1	100	lethal
TgCkCr6	35	1	1	1	1	ND	ND
TgCatBr80	55	1	1	1	1	100	lethal
TgShUS28	73	1	1	1	1	100	lethal
TgCkBr169	78	1	1	1	1	100	lethal
TgDgCo13	79	1	1	1	1	50	nonlethal
TgCkCr1	91	1	1	1	1	67	nonlethal
TgShUS32	131	1	2	1	2	0	nonlethal
TgCatBr54	6	1	3	1	3	75	nonlethal
TgCatBr12	6	1	3	1	3	100	lethal
TgCatBr17	6	1	3	1	3	100	lethal
TgCatBr2	6	1	3	1	3	100	lethal
TgCatBr21	6	1	3	1	3	100	lethal
TgCatBr30	6	1	3	1	3	100	lethal
TgCatBr42	6	1	3	1	3	100	lethal
TgCatBr47	6	1	3	1	3	100	lethal
TgCatBr53	6	1	3	1	3	100	lethal
TgCatBr55	6	1	3	1	3	100	lethal
TgCatBr62	6	1	3	1	3	100	lethal
TgCatBr71	6	1	3	1	3	100	lethal
TgCatBr75	6	1	3	1	3	100	lethal
TgCkBr144	6	1	3	1	3	100	lethal
GPHT	6	1	3	1	3	ND	ND
TgCkBr59	36	1	3	1	3	100	lethal
TgCtCo10	38	1	3	1	3	100	lethal
TgCtCo11	38	1	3	1	3	100	lethal
TgCtCo4	38	1	3	1	3	100	lethal
TgDgCo17	38	1	3	1	3	100	lethal
TgCkBr136	41	1	3	1	3	80	nonlethal
TgCkBr126	45	1	3	1	3	100	lethal
TgDgBr6	51	1	3	1	3	100	lethal
TgCatBr45	56	1	3	1	3	100	lethal
TgCatBr46	56	1	3	1	3	100	lethal
TgDgBr2	58	1	3	1	3	ND	ND
TgCkBr40	59	1	3	1	3	100	lethal
TgCkGy18	68	1	3	1	3	0	nonlethal
TgCkBr141	77	1	3	1	3	100	lethal

Table D-2 continued

Strain ID	ToxoDB Genotype#	ROP18	ROP5	ROP16	Genotype combination	Mouse mortality	Lethality
TgCkBr54	82	1	3	1	3	100	lethal
TgCatBr50	86	1	3	1	3	0	nonlethal
TgCkBr186	88	1	3	1	3	100	lethal
TgBBeCa1	90	1	3	1	3	50	nonlethal
TgDgBr18	106	1	3	1	3	75	nonlethal
TgCatPR8	118	1	3	1	3	100	lethal
TgShBr1	144	1	3	1	3	ND	ND
TgGtBr1	149	1	3	1	3	ND	ND
TgPgBr24	nd	1	3	1	3	ND	ND
TgOvBr7	171	1	3	2	4	ND	ND
TgCatBr6-20	126	1	4	1	5	ND	ND
BOF	6	1	6	1	6	8.3	nonlethal
PTG	1	2	2	2	7	40	nonlethal
DEG	3	2	2	2	7	0	nonlethal
TgDgSl1	4	2	2	2	7	0	nonlethal
TgDgSl15	4	2	2	2	7	0	nonlethal
TgDgSl17	4	2	2	2	7	0	nonlethal
TgDgSl19	4	2	2	2	7	0	nonlethal
TgDgSl2	4	2	2	2	7	0	nonlethal
TgDgSl20	4	2	2	2	7	0	nonlethal
TgDgSl24	4	2	2	2	7	0	nonlethal
TgDgSl3	4	2	2	2	7	0	nonlethal
TgDgSl7	4	2	2	2	7	0	nonlethal
TgDgSl8	4	2	2	2	7	0	nonlethal
TgDgSl9	4	2	2	2	7	0	nonlethal
B41	4	2	2	2	7	71.4[a]	nonlethal
TgSoUS8	5	2	2	2	7	ND	ND
WTD-1	5	2	2	2	7	ND	ND
TgSoUS1	39	2	2	2	7	ND	ND
TgCtCo8	128	2	2	2	7	0	nonlethal
TgCkBr168	129	2	2	2	7	0	nonlethal
TgWtdUS8	74	2	3	2	8	0	nonlethal
SOU	139	2	3	2	9	ND	ND
TgCkBr116	9	2	5	2	10	0	nonlethal
TgCtPRC10	9	2	5	2	10	0	nonlethal
TgCtPRC11	9	2	5	2	10	0	nonlethal
TgCtPRC12	9	2	5	2	10	0	nonlethal
TgCtPRC16	9	2	5	2	10	0	nonlethal
TgCtPRC2	9	2	5	2	10	0	nonlethal
TgCtPRC4	9	2	5	2	10	0	nonlethal
TgCtPRC6	9	2	5	2	10	0	nonlethal
TgCtPRC7	9	2	5	2	10	0	nonlethal
TgCtPRC8	9	2	5	2	10	0	nonlethal
TgCtPRC9	9	2	5	2	10	0	nonlethal
TgDgCo4	9	2	5	2	10	ND	ND

Table D-2 continued

Strain ID	ToxoDB Genotype#	ROP18	ROP5	ROP16	Genotype combination	Mouse mortality	Lethality
TgDgSl21	9	2	5	2	10	ND	ND
TgDgVi5	9	2	5	2	10	ND	ND
TgDgVi7	9	2	5	2	10	ND	ND
TgDgVi8	9	2	5	2	10	ND	ND
TgCgCa1	66	2	5	2	10	90	nonlethal
TgCkGy2	12	3	1	1	11	50	nonlethal
IPP-BAT	12	3	1	1	11	ND	ND
TgCatStk7a	13	3	1	1	11	ND	ND
TgCkCr2	24	3	1	1	11	0	nonlethal
TgCkCr29	24	3	1	1	11	ND	ND
TgCkCr30	24	3	1	1	11	ND	ND
TgCkCr31	24	3	1	1	11	ND	ND
TgCkCr32	24	3	1	1	11	ND	ND
TgCkGy8	25	3	1	1	11	0	nonlethal
TgCkBr110	25	3	1	1	11	25	nonlethal
TgCkBr142	28	3	1	1	11	25	nonlethal
TgCkGy7	30	3	1	1	11	0	nonlethal
TgCkBr113	30	3	1	1	11	75	nonlethal
TgCkNi45	50	3	1	1	11	0	nonlethal
TgCkBr166	114	3	1	1	11	0	nonlethal
TgCatCa1	130	3	1	1	11	ND	ND
TgHcUS1	177	3	1	1	11	ND	ND
TgCkBrCr28	nd	3	1	1	11	ND	ND
B73	127	3	2	2	12	ND	ND
CTG	2	3	3	1	13	0	nonlethal
TgCkBr161	2	3	3	1	13	0	nonlethal
TgDgSl6	2	3	3	1	13	0	nonlethal
TgDgSl5	2	3	3	1	13	ND	ND
G622M	7	3	3	1	13	66.7[b]	nonlethal
TgCkCr13	7	3	3	1	13	ND	ND
TgCkCr14	7	3	3	1	13	ND	ND
TgCkCr15	7	3	3	1	13	ND	ND
TgCkCr16	7	3	3	1	13	ND	ND
TgCkCr17	7	3	3	1	13	ND	ND
TgCkCr18	7	3	3	1	13	ND	ND
TgCkCr19	7	3	3	1	13	ND	ND
TgCkCr20	7	3	3	1	13	ND	ND
TgCkCr21	7	3	3	1	13	ND	ND
TgCkCr22	7	3	3	1	13	ND	ND
TgCkCr24	7	3	3	1	13	ND	ND
TgCkCr25	7	3	3	1	13	ND	ND
TgCkCr26	7	3	3	1	13	ND	ND
TgCkCr27	7	3	3	1	13	ND	ND
TgCatBr3	8	3	3	1	13	0	nonlethal
TgCatBr4	8	3	3	1	13	0	nonlethal

Table D-2 continued

Strain ID	ToxoDB Genotype#	ROP18	ROP5	ROP16	Genotype combination	Mouse mortality	Lethality
TgCatBr58	8	3	3	1	13	0	nonlethal
TgCatBr59	8	3	3	1	13	0	nonlethal
TgCatBr60	8	3	3	1	13	0	nonlethal
TgCatBr73	8	3	3	1	13	0	nonlethal
TgCatBr74	8	3	3	1	13	0	nonlethal
P89	8	3	3	1	13	76	nonlethal
TgCkNi1	16	3	3	1	13	50	nonlethal
TgCtCo13	18	3	3	1	13	0	nonlethal
TgCtCo12	18	3	3	1	13	43	nonlethal
TgCkNi4	23	3	3	1	13	100	lethal
TgCkBr114	29	3	3	1	13	0	nonlethal
TgDgCo20	29	3	3	1	13	30	nonlethal
TgDgCo1	29	3	3	1	13	ND	ND
TgDgCo10	29	3	3	1	13	ND	ND
TgDgCo2	29	3	3	1	13	ND	ND
TgCkGy1	31	3	3	1	13	0	nonlethal
TgDgCo14	46	3	3	1	13	0	nonlethal
TgDgCo16	46	3	3	1	13	0	nonlethal
TgDgCo8	46	3	3	1	13	100	lethal
TgCkGy22	48	3	3	1	13	25	nonlethal
TgCkNi32	52	3	3	1	13	0	nonlethal
TgRsCr1	52	3	3	1	13	0	nonlethal
TgWtdUS10	54	3	3	1	13	0	nonlethal
TgCtCo3	62	3	3	1	13	0	nonlethal
TgCkBr26	71	3	3	1	13	ND	ND
ROD	72	3	3	1	13	90[b]	nonlethal
TgCkBr173	81	3	3	1	13	0	nonlethal
ENVL-MAC	83	3	3	1	13	ND	ND
TgCkBr156	87	3	3	1	13	0	nonlethal
TgCkNi35	102	3	3	1	13	25	nonlethal
TgCatPr9	115	3	3	1	13	0	nonlethal
M7741	133	3	3	1	13	11	nonlethal
TgCatBr38	136	3	3	1	13	40	nonlethal
TgCkNi27	140	3	3	1	13	0	nonlethal
TgCatStK1	141	3	3	1	13	ND	ND
TgShBr3	150	3	3	1	13	ND	ND
TgCatBr65	186	3	3	1	13	0	nonlethal
TgCatBr66	186	3	3	1	13	0	nonlethal
TgCkBr149	186	3	3	1	13	0	nonlethal
TgCkBr150	186	3	3	1	13	0	nonlethal
TgCkBr157	186	3	3	1	13	0	nonlethal
TgCkBr152	186	3	3	1	13	25	nonlethal
TgCatBr64	111	3	3	2	14	100	lethal
TgCkGh2	132	3	3	2	14	0	nonlethal
TgShBr12	152	3	3	2	14	ND	ND

Table D-2 continued

Strain ID	ToxoDB Genotype#	ROP18	ROP5	ROP16	Genotype combination	Mouse mortality	Lethality
TgCkNg1	15	3	4	2	15	0	nonlethal
CASTELLS	15	3	4	2	15	100	lethal
TgCkBr160	17	3	4	2	15	25	nonlethal
TgCkBr163	17	3	4	2	15	50	nonlethal
TgCkBr162	17	3	4	2	15	67	nonlethal
TgCkBr155	76	3	4	2	15	0	nonlethal
TgCkBr159	76	3	4	2	15	0	nonlethal
PBR	84	3	4	2	15	43.1[c]	nonlethal
TgCtPRC13	9	3	5	2	16	0	nonlethal
TgCtPRC15	9	3	5	2	16	0	nonlethal
TgCtPRC17	9	3	5	2	16	0	nonlethal
TgCtPRC5	9	3	5	2	16	0	nonlethal
TgDgVi6	9	3	5	2	16	ND	ND
TgDgSl12	20	3	5	2	16	0	nonlethal
TgDgSl13	20	3	5	2	16	0	nonlethal
TgDgSl14	20	3	5	2	16	0	nonlethal
TgDgSl16	20	3	5	2	16	0	nonlethal
TgDgSl22	20	3	5	2	16	0	nonlethal
TgDgSl23	20	3	5	2	16	0	nonlethal
TgDgSl4	20	3	5	2	16	ND	ND
TgCkGh1	137	3	7	2	17	0	nonlethal
CAST	28	4	1	1	18	100	viruelnt
TgCtCo1	28	4	1	1	18	100	viruelnt
TgCkBr112	7	4	3	1	19	50	nonlethal
TgCkBr111	7	4	3	1	19	75	nonlethal
TgDgCo9	14	4	3	1	19	67	nonlethal
TgCatBr15	14	4	3	1	19	100	lethal
TgCkBr153	14	4	3	1	19	100	lethal
TgCtCo14	14	4	3	1	19	100	lethal
TgDgCo12	14	4	3	1	19	100	lethal
TgDgCo15	14	4	3	1	19	100	lethal
TgDgCo18	14	4	3	1	19	100	lethal
TgCtPRC1	18	4	3	1	19	0	nonlethal
TgDgCo3	18	4	3	1	19	ND	ND
TgDgVi1	18	4	3	1	19	ND	ND
TgDgVi2	18	4	3	1	19	ND	ND
TgDgVi3	18	4	3	1	19	ND	ND
TgDgVi4	18	4	3	1	19	ND	ND
TgCkBr38	22	4	3	1	19	100	lethal
TgRaw3	32	4	3	1	19	75	nonlethal
TgCkBr41	33	4	3	1	19	ND	ND
TgCatBr44	34	4	3	1	19	60	nonlethal
TgCatBr69	34	4	3	1	19	75	nonlethal
TgCatBr70	34	4	3	1	19	80	nonlethal
TgCatBr48	34	4	3	1	19	100	lethal

Table D-2 continued

Strain ID	ToxoDB Genotype#	ROP18	ROP5	ROP16	Genotype combination	Mouse mortality	Lethality
TgCkBr36	37	4	3	1	19	ND	ND
TgCkBr75	40	4	3	1	19	100	lethal
TgCatBr19	42	4	3	1	19	100	lethal
TgCatBr9	42	4	3	1	19	100	lethal
TgCkCr7	43	4	3	1	19	60	nonlethal
TgDgCo11	44	4	3	1	19	100	lethal
TgDgCo5	44	4	3	1	19	100	lethal
TgDgCo6	44	4	3	1	19	100	lethal
TgDgBr15	53	4	3	1	19	100	lethal
TgCtCo5	61	4	3	1	19	89	nonlethal
TgCtCo6	61	4	3	1	19	100	lethal
TgCatBr76	67	4	3	1	19	100	lethal
TgCkBr93	69	4	3	1	19	ND	ND
TgCkBr107	70	4	3	1	19	75	nonlethal
TgCkBr108	70	4	3	1	19	100	lethal
TgCatBr26	80	4	3	1	19	80	nonlethal
TgCkBr109	96	4	3	1	19	100	lethal
TgCtCo15	101	4	3	1	19	100	lethal
TgCatBr34	104	4	3	1	19	100	lethal
TgCkBr143	105	4	3	1	19	75	nonlethal
TgCatBr41	117	4	3	1	19	100	lethal
TgCatBr20	120	4	3	1	19	100	lethal
TgCatBr67	121	4	3	1	19	40	nonlethal
TgDgCo7	122	4	3	1	19	100	lethal
TgCkGy34	123	4	3	1	19	50	nonlethal
TgCkBr8	125	4	3	1	19	ND	ND
TgCkBr74	138	4	3	1	19	ND	ND
TgCPBr26	148	4	3	1	19	100	lethal
TgCPBr27	165	4	3	1	19	100	lethal
TgCPBr25	175	4	3	1	19	100	lethal
TgCatBr61	11	4	3	2	20	0	nonlethal
TgCatBr39	11	4	3	2	20	50	nonlethal
TgCatBr77	11	4	3	2	20	60	nonlethal
TgCatBr68	11	4	3	2	20	75	nonlethal
TgCatBr51	11	4	3	2	20	80	nonlethal
TgCatBr1	11	4	3	2	20	100	lethal
TgCatBr52	11	4	3	2	20	100	lethal
TgCatBr56	11	4	3	2	20	100	lethal
TgCatBr7	11	4	3	2	20	100	lethal
TgCatBr78	11	4	3	2	20	100	lethal
TgOvBr3	11	4	3	2	20	ND	ND
TgCkBr13	63	4	3	2	20	100	lethal
TgCkBr19	64	4	3	2	20	100	lethal
TgCkBr37	107	4	3	2	20	100	lethal
TgCatBr57	108	4	3	2	20	100	lethal

Table D-2 continued

Strain ID	ToxoDB Genotype#	ROP18	ROP5	ROP16	Genotype combination	Mouse mortality	Lethality
TgCkBr177	109	4	3	2	20	100	lethal
TgCkBr45	135	4	3	2	20	100	lethal
TgGtBr5	150	4	3	2	20	ND	ND
TgCatBr72	85	4	3	3	21	100	lethal
MAS	17	4	4	1	22	100	lethal
TgCatBr11	19	4	4	1	22	100	lethal
TgCatBr16	19	4	4	1	22	100	lethal

Mortality data is from references in Shwab *et al.* (2014) except where indicated.
[a] (Khan *et al.* 2011),
[b] (Howe *et al.* 1996)
[c] (Fux *et al.* 2003)

ND = No data

Table D-3. Expanded RFLP genotypes of 330 *T. gondii* strains.

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	II-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
PTG	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2
CTG	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	3	3
TgCkBr161	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	3	3
TgDgSl5	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	3	3
TgDgSl6	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	3	3
TgDgSl10	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	N	3	3
TgCkBr158	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	N	3
TgCkBr164	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	N	3
TgCkCr11	2	2	2	3	3	3	3	3	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	N	3
DEG	3	3	2	2	2	2	2	2	2	2	2	2	1	1	1	2	1	2	2	2	2	2	2	2	2
TgDgSl1	4	4a	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	2	2	2	2
TgDgSl15	4	4a	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	2	2	2	2
TgDgSl2	4	4a	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	2	2	2	2
TgDgSl20	4	4a	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	2	2	2	2
TgDgSl3	4	4a	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	2	2	2	2
TgDgSl7	4	4a	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	2	2	2	2
TgDgSl9	4	4a	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	2	2	2	2
TgDgSl19	4	4b	2	2	2	2	2	2	2	2	1	2	1	3	3	6	1	2	2	2	2	2	2	2	2
TgDgSl17	4	4b	2	2	2	2	2	2	2	2	1	2	1	3	3	N	1	2	2	2	2	2	2	2	2
TgDgSl8	4	4b	2	2	2	2	2	2	2	2	1	2	1	3	3	N	1	2	2	2	2	2	2	2	2
TgDgSl24	4	4c	2	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	?	2	2	2	2	2
B41	4	4d	2	2	2	2	2	2	2	2	1	2	1	2	2	2	1	2	2	2	2	3	2	2	2
TgSoUS8	5	5a	4	2	2	2	2	2	2	2	1	2	1	2	2	2	1	2	2	2	2	2	2	2	2
WTD-1	5	5b	4	2	2	2	2	2	2	2	1	2	1	3	3	2	1	2	2	2	2	3	2	2	2
TgSoUS6	5	5b	4	2	2	2	2	2	2	2	1	2	1	3	N	N	1	N	N	N	2	3	N	N	2
GPHT	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr12	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr17	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr2	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr21	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	II-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCatBr30	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr42	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr47	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr53	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr54	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr62	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr71	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCatBr75	6	6a	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCkBr144	6	6b	1	1	1	3	1	2	4	1	1	1	1	3	3	3	3	1	3	4	1	1	1	1	3
TgCatBr55	6	6c	1	1	1	3	1	2	4	1	1	1	1	1	1	1	2	1	2	5	1	1	1	1	3
BOF	6	6d	1	1	1	3	1	2	4	1	1	1	1	1	1	1	3	1	3	4	1	1	1	1	6
TgCkCr12	7	7a	1	3	3	3	3	3	3	3	3	3	1	1	2	1	2	2	1	2	2	2	1	N	1
TgCkCr18	7	7b	1	3	3	3	3	3	3	3	3	3	1	3	2	3	2	1	1	2	2	2	1	3	3
G622M	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr13	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr14	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr15	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr16	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr17	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr19	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr20	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr21	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr22	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr24	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr25	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr26	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr27	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	3	3
TgCkCr23	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	N	3
TgCkCr8	7	7c	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	1	2	2	2	1	N	3
TgCkBr112	7	7d	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	3	4	1	1	1	4	3

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	II-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCkBr111	7	7e	1	3	3	3	3	3	3	3	3	3	1	3	3	3	2	1	3	5	1	1	1	4	3
P89	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgCatBr3	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgCatBr4	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgCatBr58	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgCatBr59	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgCatBr60	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgCatBr73	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgCatBr74	8	8	1	3	3	3	3	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	3	3
TgDgVi7	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	2	2	2	5
TgCtPRC11	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC12	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC16	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC2	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC4	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC7	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC8	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC9	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgDgCo4	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgDgSl21	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgDgVi8	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	2	5
TgCtPRC10	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	N	3	2	2	5
TgCtPRC6	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	N	3	2	2	5
TgDgVi5	9	9a	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	N	3	2	2	5
TgCkBr116	9	9a	4	2	2	3	3	2	2	N	2	2	1	3	3	4	1	2	2	4	N	3	2	2	5
TgCtPRC14	9	9a/b	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	N	3	2	N	5
TgCtPRC13	9	9b	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	3	5
TgCtPRC5	9	9b	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	2	3	2	3	5
TgCtPRC15	9	9b	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	N	3	2	3	5
TgCtPRC17	9	9b	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	N	3	2	3	5

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	IL-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgDgVi6	9	9b	4	2	2	3	3	2	2	3	2	2	1	3	3	4	1	2	2	4	N	3	2	3	5
GT1	10	10a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TgCkBr146	10	10a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TgCtCo7	10	10a	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TgCtCo2	10	10b	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	4	1	1	1
TgCatBr1	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr39	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr51	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr52	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr56	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr61	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr68	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr7	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr77	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgCatBr78	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
TgOvBr3	11	11	1	1	2	3	3	3	1	3	1	2	3	3	3	4	2	1	1	3	1	1	2	4	3
IPP-BAT	12	12	1	3	3	1	1	3	2	3	3	1	3	3	3	3	2	2	2	2	2	2	1	3	1
TgCkGy2	12	12	1	3	3	1	1	3	2	3	3	1	3	3	3	3	2	2	2	2	2	2	1	3	1
TgCatStk7a	13	13	1	1	1	1	1	3	2	3	3	1	3	3	3	3	2	2	2	2	2	1	1	3	1
TgCkBr165	13	13	1	1	1	1	1	3	2	3	3	1	3	3	3	3	2	2	2	2	2	1	1	N	1
TgCatBr15	14	14	1	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	3	4	1	5	1	4	3
TgCkBr153	14	14	1	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	3	4	1	5	1	4	3
TgCtCo14	14	14	1	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	3	4	1	5	1	4	3
TgDgCo12	14	14	1	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	3	4	1	5	1	4	3
TgDgCo15	14	14	1	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	3	4	1	5	1	4	3
TgDgCo18	14	14	1	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	3	4	1	5	1	4	3
TgDgCo9	14	14	1	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	3	4	1	5	1	4	3
TgCkNg1	15	15a	4	1	2	3	3	3	3	1	1	3	1	3	2	1	1	1	2	3	1	3	2	3	4
CASTELLS	15	15b	4	1	2	3	3	3	3	1	1	3	1	1	2	1	3	1	2	3	1	3	2	3	4
TgCkNi1	16	16	1	1	2	3	3	1	3	1	3	3	1	3	2	3	4	2	1	2	2	4	1	3	3

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	IL-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCkBr160	17	17a	4	1	2	3	3	3	4	1	1	3	1	3	2	1	1	1	2	3	1	3	2	3	4
TgCkBr162	17	17a	4	1	2	3	3	3	4	1	1	3	1	3	2	1	1	1	2	3	1	3	2	3	4
TgCkBr163	17	17a	4	1	2	3	3	3	4	1	1	3	1	3	2	1	1	1	2	3	1	3	2	3	4
MAS	17	17b	4	1	2	3	3	3	4	1	1	3	1	1	1	4	2	1	2	3	1	1	1	4	4
TgCkBr147	17	17c	4	1	2	3	3	3	4	1	1	3	1	1	1	4	2	1	2	3	1	3	2	4	4
TgCkBr148	17	17c	4	1	2	3	3	3	4	1	1	3	1	1	1	4	2	1	2	3	1	3	2	4	4
TgCkBr151	17	17c	4	1	2	3	3	3	4	1	1	3	1	1	1	4	2	1	2	3	1	3	2	4	4
TgCkBr154	17	17c	4	1	2	3	3	3	4	1	1	3	1	1	1	4	2	1	2	3	1	3	2	4	4
TgCtCo12	18	18a	1	1	1	3	1	3	2	1	3	3	1	3	2	4	3	1	3	4	2	4	1	3	3
TgCtCo13	18	18b	1	1	1	3	1	3	2	1	3	3	1	3	N	4	3	1	3	4	2	4	1	3	3
TgDgVi3	18	18c	1	1	1	3	1	3	2	1	3	3	1	3	2	4	3	1	3	4	1	2	1	4	3
TgDgVi4	18	18d	1	1	1	3	1	3	2	1	3	3	1	3	2	4	3	1	2	4	1	4	1	4	3
TgCtPRC1	18	18e	1	1	1	3	1	3	2	1	3	3	1	3	2	4	3	1	3	4	1	4	1	4	3
TgDgCo3	18	18e	1	1	1	3	1	3	2	1	3	3	1	3	2	4	3	1	3	4	1	4	1	4	3
TgDgVi2	18	18e	1	1	1	3	1	3	2	1	3	3	1	3	2	4	3	1	3	4	1	4	1	4	3
TgDgVi1	18	18f	1	1	1	3	1	3	2	1	3	3	1	3	2	4	3	N	3	4	1	4	1	4	3
TgCatBr11	19	19	1	3	3	3	3	3	1	1	1	4	1	3	3	5	2	1	1	3	1	1	1	4	4
TgCatBr16	19	19	1	3	3	3	3	3	1	1	1	4	1	3	3	5	2	1	1	3	1	1	1	4	4
TgCatBr5	19	19	1	3	3	3	3	3	1	1	1	4	1	3	3	5	2	1	1	3	1	1	1	4	4
TgDgSl4	20	20a	4	2	2	3	3	2	2	3	2	5	1	3	2	4	5	2	2	4	2	3	2	3	5
TgDgSl13	20	20b	4	2	2	3	3	2	2	3	2	5	1	3	2	4	2	2	N	4	2	3	2	3	5
TgDgSl16	20	20b	4	2	2	3	3	2	2	3	2	5	1	3	2	4	2	2	N	4	2	3	2	3	5
TgDgSl22	20	20b	4	2	2	3	3	2	2	3	2	5	1	3	2	4	2	2	N	4	2	3	2	3	5
TgDgSl23	20	20b	4	2	2	3	3	2	2	3	2	5	1	3	2	4	2	2	N	4	2	3	2	3	5
TgDgSl12	20	20b	4	2	2	3	3	2	2	3	2	5	1	3	2	4	N	2	N	4	2	3	2	3	5
TgDgSl14	20	20b	4	2	2	3	3	2	2	3	2	5	1	3	2	4	N	2	N	4	2	3	2	3	5
TgDgSl18	20	20b/c	4	2	2	3	3	2	2	3	2	5	1	3	2	4	2	2	N	4	2	3	2	N	5
TgDgSl11	20	20c	4	2	2	3	3	2	2	3	2	5	1	3	N	4	N	2	N	4	N	3	2	1	N
TgCatBr10	21	21	1	3	3	3	3	3	1	1	1	3	3	3	3	5	2	1	1	4	1	1	1	4	4
TgCatBr22	21	21	1	3	3	3	3	3	1	1	1	3	3	3	3	5	2	1	1	4	1	1	1	4	4

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	IL-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCatBr23	21	21	1	3	3	3	3	3	1	1	1	3	3	3	3	5	2	1	1	4	1	1	1	4	4
TgCatBr28	21	21	1	3	3	3	3	3	1	1	1	3	3	3	3	5	2	1	1	4	1	1	1	4	4
TgCatBr31	21	21	1	3	3	3	3	3	1	1	1	3	3	3	3	5	2	1	1	4	1	1	1	4	4
TgCatBr32	21	21	1	3	3	3	3	3	1	1	1	3	3	3	3	5	2	1	1	4	1	1	1	4	4
TgCatBr37	21	21	1	3	3	3	3	3	1	1	1	3	3	3	3	5	2	1	1	4	1	1	1	4	4
TgCkBr38	22	22	4	1	2	3	3	3	4	1	3	3	3	3	3	1	2	1	2	5	1	1	1	4	3
TgCkNi4	23	23	1	1	2	3	1	3	2	1	3	4	1	3	2	4	3	1	3	4	2	4	1	3	3
TgDgCo19	23	23	1	1	2	3	1	3	2	1	3	4	1	3	2	4	3	1	3	4	2	4	1	N	3
TgCkCr2	24	24	1	1	1	1	3	1	1	1	1	1	3	1	1	1	2	2	1	2	2	2	1	3	1
TgCkCr29	24	24	1	1	1	1	3	1	1	1	1	1	3	1	1	1	2	2	1	2	2	2	1	3	1
TgCkCr31	24	24	1	1	1	1	3	1	1	1	1	1	3	1	1	1	2	2	1	2	2	2	1	3	1
TgCkCr32	24	24	1	1	1	1	3	1	1	1	1	1	3	1	1	1	2	2	1	2	2	2	1	3	1
TgCkCr30	24	24	1	1	1	1	3	1	1	1	1	1	3	1	1	1	2	2	1	2	N	2	1	3	1
TgCkBr110	25	25	1	3	3	1	3	3	3	3	3	1	1	3	3	3	2	2	2	2	2	2	1	3	1
TgCkGy8	25	25	1	3	3	1	3	3	3	3	3	1	1	3	3	3	2	2	2	2	2	2	1	3	1
TgCkNi9	27	27	1	1	1	1	1	1	1	3	1	1	1	1	1	1	2	1	3	1	1	4	1	1	1
TgCkBr142	28	28a	1	1	1	1	1	1	2	1	3	1	3	3	3	1	2	1	2	4	1	3	1	3	1
CAST	28	28b	1	1	1	1	1	1	2	1	3	1	3	1	1	4	2	1	3	4	1	1	1	4	1
TgCtCo1	28	28b	1	1	1	1	1	1	2	1	3	1	3	1	1	4	2	1	3	4	1	1	1	4	1
TgCkBr114	29	29a	1	1	2	3	1	3	2	1	3	3	1	3	2	4	3	1	3	1	1	1	1	3	3
TgDgCo1	29	29b	1	1	2	3	1	3	2	1	3	3	1	3	2	4	3	1	3	1	1	4	1	3	3
TgDgCo10	29	29b	1	1	2	3	1	3	2	1	3	3	1	3	2	4	3	1	3	1	1	4	1	3	3
TgDgCo2	29	29b	1	1	2	3	1	3	2	1	3	3	1	3	2	4	3	1	3	1	1	4	1	3	3
TgDgCo20	29	29b	1	1	2	3	1	3	2	1	3	3	1	3	2	4	3	1	3	1	1	4	1	3	3
TgCkBr113	30	30	1	3	3	1	3	3	3	3	3	1	3	3	3	3	2	2	1	3	1	2	1	3	1
TgCkGy7	30	30	1	3	3	1	3	3	3	3	3	1	3	3	3	3	2	2	1	3	1	2	1	3	1
TgCkGy1	31	31	1	3	3	3	1	3	3	3	3	1	3	3	3	3	2	1	2	2	2	2	1	3	3
TgRaw3	32	32	1	3	3	3	3	2	1	1	1	1	1	3	3	5	2	1	3	3	1	1	1	4	3
TgCkBr41	33	33	4	1	2	3	1	3	4	1	1	1	1	1	1	1	2	1	2	4	1	1	1	4	3
TgCatBr44	34	34	4	1	2	3	3	3	2	1	1	4	1	1	1	5	2	1	2	3	1	1	1	4	3

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	II-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCatBr48	34	34	4	1	2	3	3	3	2	1	1	4	1	1	1	5	2	1	2	3	1	1	1	4	3
TgCatBr69	34	34	4	1	2	3	3	3	2	1	1	4	1	1	1	5	2	1	2	3	1	1	1	4	3
TgCatBr70	34	34	4	1	2	3	3	3	2	1	1	4	1	1	1	5	2	1	2	3	1	1	1	4	3
TgCkCr3	35	35	1	1	1	1	1	1	1	3	1	1	3	3	2	1	2	1	3	1	1	4	1	1	1
TgCkCr4	35	35	1	1	1	1	1	1	1	3	1	1	3	3	2	1	2	1	3	1	1	4	1	1	1
TgCkCr5	35	35	1	1	1	1	1	1	1	3	1	1	3	3	2	1	2	1	3	1	1	4	1	1	1
TgCkCr6	35	35	1	1	1	1	1	1	1	3	1	1	3	3	2	1	2	1	3	1	1	4	1	1	1
TgCkBr59	36	36	1	1	1	3	1	3	2	1	3	1	3	3	3	1	2	1	3	4	1	5	1	1	3
TgCkBr36	37	37	1	2	2	3	3	3	4	1	1	3	1	3	3	1	4	1	2	5	1	1	1	4	3
TgCtCo10	38	38	1	3	3	3	1	1	1	3	1	1	3	2	1	1	2	2	1	1	1	2	1	1	3
TgCtCo11	38	38	1	3	3	3	1	1	1	3	1	1	3	2	1	1	2	2	1	1	1	2	1	1	3
TgCtCo4	38	38	1	3	3	3	1	1	1	3	1	1	3	2	1	1	2	2	1	1	1	2	1	1	3
TgDgCo17	38	38	1	3	3	3	1	1	1	3	1	1	3	2	1	1	2	2	1	1	1	2	1	1	3
TgSoUS1	39	39	2	2	2	2	2	2	2	2	1	2	2	2	2	2	1	2	2	2	2	2	2	2	2
TgCkBr75	40	40a	4	1	2	3	3	3	3	3	1	3	1	3	3	5	2	1	1	3	1	5	1	4	3
TgCtCoX5	40	40b	4	1	2	3	3	3	3	3	1	3	1	3	2	1	1	1	2	3	1	3	2	N	4
TgCkBr136	41	41	1	1	1	3	1	2	1	1	1	1	1	3	3	4	2	1	3	4	1	1	1	1	3
TgCatBr19	42	42	1	1	1	3	3	2	1	1	1	4	1	3	3	5	2	1	3	4	1	1	1	4	3
TgCatBr9	42	42	1	1	1	3	3	2	1	1	1	4	1	3	3	5	2	1	3	4	1	1	1	4	3
TgCkCr7	43	43a	1	1	2	1	2	1	1	1	1	4	1	3	2	4	1	1	1	4	1	4	1	4	3
TgCkCr9	43	43b	1	1	2	1	2	1	1	1	1	4	1	3	2	4	1	1	1	4	1	4	1	4	5
TgCkCr10	43	43c	1	1	2	1	2	1	1	1	1	4	1	3	2	4	3	1	1	4	1	4	1	4	5
TgDgCo5	44	44a	1	1	2	3	1	3	2	1	1	6	1	3	2	4	3	1	3	1	1	4	1	4	3
TgDgCo11	44	44b	1	1	2	3	1	3	2	1	1	6	1	3	2	4	3	1	3	4	1	4	1	4	3
TgDgCo6	44	44b	1	1	2	3	1	3	2	1	1	6	1	3	2	4	3	1	3	4	1	4	1	4	3
TgCkBr126	45	45	1	3	3	3	1	2	2	3	1	1	3	3	3	1	2	1	3	4	1	1	1	1	3
TgDgCo14	46	46a	1	3	3	3	1	3	2	1	3	3	1	3	3	4	2	1	3	4	1	1	1	3	3
TgDgCo16	46	46b	1	3	3	3	1	3	2	1	3	3	1	3	3	4	3	1	3	4	1	1	1	3	3
TgDgCo8	46	46c	1	3	3	3	1	3	2	1	3	3	1	3	2	4	3	1	3	4	1	4	1	3	3
TgCatBr25	47	47	1	3	3	3	3	2	4	1	1	2	1	3	1	4	2	1	3	3	1	3	3	4	4

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	II-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCkGy22	48	48	1	3	3	3	3	3	3	3	3	3	3	3	3	3	2	1	1	3	1	2	1	3	3
TgCatPR5	49	49	2	2	2	1	2	2	2	2	3	2	N	1	1	4	1	2	2	2	2	2	2	4	5
TgCkNi45	50	50	2	3	3	1	3	1	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	3	1
TgDgBr6	51	51	4	1	2	3	1	3	2	1	1	1	1	1	1	1	2	1	2	4	1	1	1	1	3
TgCkNi32	52	52	4	1	2	3	1	3	5	1	1	3	1	3	2	1	3	1	3	5	1	1	1	3	3
TgRsCr1	52	52	4	1	2	3	1	3	5	1	1	3	1	3	2	1	3	1	3	5	1	1	1	3	3
TgDgBr15	53	53	4	1	2	3	3	3	2	1	1	3	1	3	3	3	2	1	2	3	1	1	1	4	3
TgWtdUS10	54	54	2	2	2	3	3	3	3	3	3	3	2	1	1	2	2	2	2	2	2	2	1	3	3
TgCatBr80	55	55	1	1	1	1	3	1	4	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
TgCatBr45	56	56	1	1	1	3	1	2	4	1	3	1	1	1	1	4	2	1	3	4	1	1	1	1	3
TgCatBr46	56	56	1	1	1	3	1	2	4	1	3	1	1	1	1	4	2	1	3	4	1	1	1	1	3
TgCkBr171	57	57	1	1	1	3	1	2	4	1	3	2	3	3	3	3	2	N	1	2	2	1	1	N	3
TgDgBr2	58	58	1	1	1	3	1	3	4	3	3	1	1	3	3	3	3	1	3	4	1	1	1	1	3
TgCkBr40	59	59	1	1	1	3	3	2	4	1	1	1	1	1	1	1	3	1	3	3	1	1	1	1	3
GUY-KOE	60	60a	1	1	2	1	3	3	3	1	3	3	1	3	2	7	1	1	2	5	1	1	1	4	8
VAND	60	60b	1	1	2	1	3	3	3	1	3	3	1	3	3	4	2	1	1	3	1	3	1	4	8
TgCtCo5	61	61	1	1	2	3	1	3	2	3	1	5	1	3	2	4	3	1	3	4	1	4	1	4	3
TgCtCo6	61	61	1	1	2	3	1	3	2	3	1	5	1	3	2	4	3	1	3	4	1	4	1	4	3
TgCtCo3	62	62	1	1	2	3	1	3	2	3	3	3	1	3	2	4	3	1	3	1	1	1	1	3	3
TgCtCo9	62	62	1	1	2	3	1	3	2	3	3	3	1	3	2	4	3	1	3	N	1	1	1	N	3
TgCkBr13	63	63	1	1	2	3	3	3	1	3	1	2	1	3	3	3	2	1	1	4	1	3	2	4	3
TgCkBr19	64	64	1	1	2	3	3	3	4	1	1	5	1	3	3	3	2	1	3	3	1	1	2	4	3
TgCkBr89	65	65	1	1	2	3	3	3	4	1	1	3	1	1	1	1	4	1	2	5	1	1	1	4	4
TgCgCa1	66	66	1	2	2	3	2	2	2	4	1	5	1	1	3	4	3	2	2	4	2	3	2	2	5
TgCatBr76	67	67	1	3	3	3	1	3	1	3	3	4	3	3	3	4	2	1	1	3	1	1	1	4	3
TgCkGy18	68	68	1	3	3	3	1	3	3	3	3	3	3	N	3	3	2	2	2	2	2	2	1	1	3
TgCkBr93	69	69	1	3	3	3	3	2	1	3	1	2	1	3	3	5	2	1	3	3	1	1	1	4	3
TgCkBr107	70	70	1	3	3	3	3	2	4	1	1	1	3	3	3	3	2	1	3	4	1	1	1	4	3
TgCkBr108	70	70	1	3	3	3	3	2	4	1	1	1	3	3	3	3	2	1	3	4	1	1	1	4	3
TgCkBr26	71	71	1	3	3	3	3	3	2	1	3	3	1	3	3	5	2	1	3	3	1	5	1	3	3

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	IL-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
ROD	72	72	1	3	3	3	3	3	3	3	3	5	3	2	1	3	2	2	1	2	2	2	1	3	3
TgShUS28	73	73	2	3	3	1	1	1	3	3	3	1	1	2	1	1	2	2	1	4	1	2	1	1	1
TgWtdUS8	74	74	2	3	3	3	2	2	2	3	2	2	1	2	2	2	1	2	2	2	2	2	2	2	3
TgCkBr48	75	75	4	1	2	3	3	3	2	1	1	3	3	3	3	5	2	1	2	3	1	1	2	4	4
TgCkBr155	76	76	4	3	3	3	3	3	4	1	1	3	1	2	1	3	2	2	2	3	1	2	2	3	4
TgCkBr159	76	76	4	3	3	3	3	3	4	1	1	3	1	2	1	3	2	2	2	3	1	2	2	3	4
TgCkBr141	77	77	1	1	1	1	1	1	4	1	1	3	3	1	1	1	2	1	1	4	1	3	1	1	3
TgCkBr169	78	78	1	1	1	1	1	3	2	1	3	1	3	1	1	3	2	1	2	4	1	1	1	1	1
TgDgCo13	79	79	1	1	1	1	3	1	1	3	3	1	3	1	1	1	1	2	2	2	1	4	1	1	1
TgCatBr26	80	80	1	1	1	3	1	2	1	1	1	4	1	1	1	1	2	1	3	4	1	1	1	4	3
TgCkBr173	81	81	1	1	1	3	1	2	4	1	1	3	3	3	3	1	2	1	3	4	1	1	1	3	3
TgCkBr54	82	82	1	1	1	3	1	3	2	1	1	1	3	3	3	1	3	1	2	4	1	1	1	1	3
ENVL-MAC	83	83	1	1	1	3	1	3	2	3	3	1	3	3	3	3	2	2	2	2	2	2	1	3	3
PBR	84	84	1	1	1	3	1	3	4	1	1	3	1	2	1	1	3	1	2	4	1	2	2	3	4
TgCatBr72	85	85	1	1	1	3	3	2	4	1	1	2	1	1	1	1	3	1	3	4	1	N	3	4	3
TgCatBr50	86	86	1	1	1	3	3	2	4	1	1	3	1	1	1	1	3	1	3	4	1	1	1	1	3
TgCkBr156	87	87	1	1	1	3	3	3	1	1	3	1	3	2	1	1	2	2	1	2	2	2	1	3	3
TgCkBr186	88	88	1	1	1	3	3	3	2	1	3	1	3	3	3	1	2	1	3	4	1	5	1	1	3
TgCkId1	89	89	1	1	1	3	3	3	2	3	3	3	3	N	N	3	N	N	3	N	2	2	1	N	3
TgBBeCa1	90	90	1	1	1	3	3	3	3	3	3	1	3	2	1	1	2	2	1	2	2	2	1	1	3
TgCkCr1	91	91	1	1	2	1	1	1	2	1	1	1	1	3	2	1	4	1	1	4	1	4	1	1	1
TgCatBr40	92	92	1	1	2	1	3	2	2	1	1	2	1	1	1	4	2	1	2	3	1	3	3	4	4
TgCkBr61	93	93	1	1	2	1	3	2	4	1	1	3	1	1	1	4	3	1	1	3	1	1	2	4	4
TgCkBr16	94	94	1	1	2	1	3	3	1	1	1	2	1	1	1	5	2	1	1	3	1	1	1	4	4
GUY-MAT	95	95	1	1	2	1	3	3	2	1	1	3	1	3	3	1	1	1	2	5	1	1	1	4	8
TgCkBr109	96	96	1	1	2	1	3	3	2	3	1	3	3	3	3	4	4	1	1	3	1	5	1	4	3
GUY-DOS	97	97	1	1	2	1	3	3	2	3	1	3	1	3	3	4	1	1	2	3	1	1	1	4	8
RUB	98	98	1	1	2	1	3	3	3	3	1	3	3	3	2	3	3	1	1	4	1	3	1	4	8
TOU-Ali	99	99	1	1	2	1	3	3	4	1	1	3	1	1	1	4	2	1	2	3	1	3	1	4	4
GUY-BAS1	100	100	1	1	2	1	3	3	4	1	3	3	1	3	2	4	3	1	1	5	1	1	1	4	8

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	IL-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCtCo15	101	101	1	1	2	3	1	3	2	1	1	5	1	3	2	4	3	1	3	4	1	4	1	4	3
TgCkNi35	102	102	1	1	2	3	1	3	2	1	3	4	3	3	2	3	3	1	3	2	2	2	1	3	3
TgCatBr34	104	104	1	1	2	3	1	3	4	1	1	3	1	3	3	1	2	1	3	3	1	3	1	4	3
TgCkBr143	105	105	1	1	2	3	3	2	4	3	3	3	1	3	3	3	4	1	1	4	1	1	1	4	3
TgDgBr18	106	106	1	1	2	3	3	2	4	1	1	2	1	1	1	4	5	1	2	3	1	3	1	1	3
TgCkBr37	107	107	1	1	2	3	3	2	4	1	1	3	1	1	1	3	3	1	1	3	1	3	2	4	3
TgCatBr57	108	108	1	1	2	3	3	3	2	1	1	3	1	3	3	4	2	1	2	4	1	3	2	4	3
TgCkBr177	109	109	1	1	2	3	3	3	3	1	3	3	3	3	3	4	2	1	3	4	1	1	2	4	3
TgCatBr64	111	111	1	1	4	3	3	3	4	1	3	3	1	3	3	4	2	1	2	4	1	3	2	3	3
TgCatPR6	112	112	1	2	2	1	1	1	3	2	3	1	N	2	1	4	1	2	1	2	2	2	1	4	5
TgCkBr166	114	114	1	3	3	1	3	3	3	1	3	1	1	3	3	3	2	1	1	4	1	2	1	3	1
TgCatPr9	115	115	1	3	3	1	3	3	3	3	3	3	1	2	2	3	2	2	2	2	2	2	1	3	3
TgCkBr130	116	116	1	3	3	3	1	3	2	3	3	3	3	3	3	3	2	1	1	4	1	1	1	N	3
TgCatBr41	117	117	1	3	3	3	1	3	4	1	1	4	3	3	3	4	2	1	3	4	1	1	1	4	3
TgCatPR8	118	118	1	3	3	3	3	1	1	3	3	1	N	2	1	1	2	2	1	2	2	2	1	1	3
TgCatBr18	119	119	1	3	3	3	3	2	4	1	1	4	1	3	1	4	2	1	3	3	1	3	3	4	N
TgCatBr20	120	120	1	3	3	3	3	3	1	1	3	3	3	3	3	3	2	1	1	4	1	1	1	4	3
TgCatBr67	121	121	1	3	3	3	3	3	1	3	1	3	3	3	3	4	2	1	1	3	1	1	1	4	3
TgDgCo7	122	122	1	3	3	3	3	3	1	3	3	1	3	3	3	1	2	2	1	3	1	2	1	4	3
TgCkGy34	123	123	1	3	3	3	3	3	1	3	3	3	3	3	3	4	2	2	1	4	1	2	1	4	3
TgCatBr81	124	124	1	3	3	3	3	3	2	3	1	4	1	3	3	4	3	1	1	3	1	3	1	4	4
TgCkBr8	125	125	1	3	3	3	3	3	2	3	3	5	3	3	3	3	2	2	1	2	2	5	1	4	3
TgCatBr6-20	126	126	1	N	1	3	3	2	4	1	1	4	1	3	3	5	2	1	1	3	1	1	1	1	4
B73	127	127	2	2	2	2	2	2	2	2	3	3	3	2	1	2	1	2	2	2	2	2	2	3	2
TgCtCo8	128	128	2	2	2	2	2	2	2	3	2	2	1	1	1	2	1	2	2	2	2	2	2	2	2
TgCkBr168	129	129	2	2	2	2	3	2	2	2	2	2	2	1	1	6	1	2	2	2	2	3	2	2	2
TgCatCa1	130	130	2	3	3	1	3	3	2	1	3	3	3	2	1	3	2	2	1	2	2	2	1	3	1
TgShUS32	131	131	2	3	3	2	2	1	3	3	2	2	1	2	2	2	1	2	1	4	1	2	1	1	2
TgCkGh2	132	132	2	3	3	3	2	2	2	3	3	2	3	2	1	3	2	2	2	2	2	2	2	3	3
M7741	133	133	2	3	3	3	3	1	3	3	3	3	3	2	1	3	2	2	2	2	2	2	1	3	3

Table D-3 continued

Strain ID	ToxoDB #	Expanded Type	SAG1	SAG2	SAG2 alt.	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Ia-P89	AK2	II-C35	BSR4	AK126	SRS4	UPRT1	ROP1	MIC5	ROP16	ROP18	ROP5
TgCkBr178	134	134	4	1	2	3	1	3	2	3	3	1	3	3	3	1	2	2	2	4	1	2	1	N	1
TgCkBr45	135	135	4	1	2	3	3	3	2	1	3	3	1	3	3	3	2	1	3	4	1	5	2	4	3
TgCatBr38	136	136	4	1	4	3	3	3	2	1	1	3	1	1	1	5	2	1	2	4	1	3	1	3	3
TgCkGh1	137	137	4	2	2	3	3	2	2	3	2	3	1	3	2	4	5	2	N	4	2	3	2	3	7
TgCkBr74	138	138	4	3	3	3	3	3	3	1	3	3	3	3	3	3	2	1	2	4	1	1	1	4	3
SOU	139	139	2	2	2	3	3	3	2	2	3	3	1	1	1	2	2	2	2	2	2	2	2	2	3
TgCkNi27	140	140	2	3	3	3	3	3	2	3	3	1	3	2	1	3	2	2	2	2	2	2	1	3	3
TgCatStK1	141	141	2	3	3	3	3	3	3	3	3	1	3	2	1	3	2	2	2	2	2	2	1	3	3
TgShBr1	144	144	1	1	1	3	1	3	4	3	1	1	1	1	1	4	4	1	2	5	1	1	1	1	3
TgCPBr26	148	148	1	1	2	3	1	3	2	1	1	3	1	3	3	1	2	1	2	4	1	3	1	4	3
TgGtBr1	149	149	1	1	2	3	3	2	2	1	1	1	1	1	1	5	3	1	2	4	1	1	1	1	3
TgShBr3	150	150a	1	1	2	3	3	2	4	3	1	1	1	N	3	N	N	1	3	4	1	N	1	3	3
TgGtBr5	150	150b	1	1	2	3	3	2	4	3	1	1	1	1	1	1	4	1	3	4	1	1	2	4	3
TgShBr12	152	152	1	1	4	3	3	3	2	1	1	3	1	3	3	5	2	1	2	3	1	3	2	3	3
TgShBr5	160	160	1	3	3	3	3	3	1	3	3	4	3	3	3	5	2	1	1	4	1	1	1	N	3
TgCPBr1	162	162	1	3	3	3	3	3	2	1	1	3	1	3	3	5	2	1	1	3	1	1	1	4	4
TgCPBr27	165	165	1	3	3	3	3	3	4	1	1	3	3	3	3	5	2	1	3	4	1	1	1	4	3
TgOvBr7	171	171	4	1	1	3	3	1	4	3	3	1	3	3	3	1	2	1	3	4	1	3	2	1	3
TgCPBr25	175	175	4	1	2	3	3	3	3	1	1	4	1	1	1	5	2	1	2	5	1	5	1	4	3
TgHcUS1	177	177	1	1	1	1	1	1	1	3	3	1	3	1	1	1	2	1	3	2	1	1	1	3	1
TgCatBr65	186	186	2	3	3	3	3	3	1	3	3	1	3	2	1	1	2	2	1	2	2	2	1	3	3
TgCatBr66	186	186	2	3	3	3	3	3	1	3	3	1	3	2	1	1	2	2	1	2	2	2	1	3	3
TgCkBr149	186	186	2	3	3	3	3	3	1	3	3	1	3	2	1	1	2	2	1	2	2	2	1	3	3
TgCkBr150	186	186	2	3	3	3	3	3	1	3	3	1	3	2	1	1	2	2	1	2	2	2	1	3	3
TgCkBr152	186	186	2	3	3	3	3	3	1	3	3	1	3	2	1	1	2	2	1	2	2	2	1	3	3
TgCkBr157	186	186	2	3	3	3	3	3	1	3	3	1	3	2	1	1	2	2	1	2	2	2	1	3	3
TgCkBrCr28	N	a	N	N	N	N	N	N	N	N	N	N	N	1	1	1	N	2	1	N	2	2	1	3	1
TgPgBr24	N	b	N	N	N	N	N	N	N	N	N	N	N	1	1	4	2	1	3	4	1	3	1	1	3
TgCatBr27	N	c	N	N	N	N	N	N	N	N	N	N	N	3	3	5	2	1	1	4	1	1	1	4	4
TgPgBr14	N	d	N	N	N	N	N	N	N	N	N	N	N	3	3	5	N	1	1	3	1	1	N	4	4

N= No data

Vita

Elliot Keats Cullen Shwab was born in Knoxville, Tennessee, to Ellen Barrentine and Clay Davidson Shwab. He is the second of three children, including an elder sister, Linden Blue Branton, and a younger brother, Reilly Oliver Winston Shwab. He attended Nature's Way Montessori School through fourth grade, then attended Webb School of Knoxville from the fifth through twelfth grades. After graduating high school, he earned a Bachelor of Arts degree from The College of Wooster in Wooster, OH. He then worked as a research assistant at the Center for Environmental Biotechnology and University of Tennessee Medical Center in Knoxville before attending The University of Wisconsin in Madison and obtaining a Master of Science degree in Plant Pathology. He then enrolled in the Microbiology program at The University of Tennessee, where he earned a Doctor of Philosophy degree in the field. He plans to begin a post-doctoral research fellowship at Duke University in August of 2015.