Transmission pattern of major clonal lineages of *Toxoplasma gondii*

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Pooja Saraf

August 2017

DEDICATION

I dedicate this dissertation to my parents. Your words of encouragement and your love have always been an inspiration for me to achieve all I can.

ACKNOWLEDGEMENT

First, I would like to thank my advisor Dr.Chunlei Su for his constant support and patience during my time in his lab. He always welcomed new ideas and encouraged discussing them at any given time. Because of his enthusiasm towards science and confidence in me, I was able to learn new techniques and think critically. He has been an excellent mentor throughout and with his guidance and constructive criticism; I could push through some really tough times during my Ph.D. Thank you for being such a great mentor and making me a better scientist.

I am also thankful to other members on my advisory committee; Dr. Elizabeth Fozo for always being there for me. I enjoyed working with her during my time as a rotation student in her lab and was inspired by her attitude towards science. She has always been supportive and guided me throughout my Ph.D. I appreciate her honesty and constant encouragement as my committee member. Dr. Todd Reynolds for his excellent questions during the committee meetings and his cheerful attitude. Despite his busy schedule, he has always been accommodating to answer my questions and help me in every possible way. He not only encouraged me but also guided me during my application process. Dr. Rick Gerhold for his constant support during my Ph.D. I appreciate his enthusiasm, suggestions and comments that guided my research in the right direction. Dr.Erik Zinser for his insightful comments and encouragement and hard questions that made me dig deeper into the literature. I would also like to thank Dr. Jeffrey M. Becker for being an excellent head of the department and giving me the opportunity to work in his lab as a rotation student. His caring nature towards students and thoughtfulness is really inspiring and encouraging. Thank you all for your constant support, patience, and encouragement during my journey of research. I would also like to express my gratitude towards Dr.Melinda Hauser and Sarah Kauffman for teaching me new techniques and being patient with me, despite your busy schedules.

I would also like to thank my friends; Arkadipta, Khushboo, Shantanu, Keerthana, Ujjal, Dr. Siddheshvar Bhela, Dr. Fernanda Gimenez, Punita, and Aditya for being there every step of the way. Thanks are also due to the other current/past members of the lab and my friends Keats, Tiantian, Matt, Aldo and Chase for their constant help whenever needed and making the lab a fun place.

I would also like to acknowledge Elizabeth McPherson for her belief in me as a teacher, and her guidance and encouragement to do better. Thank you for your love towards me and

Pranay, and making us feel special and loved. I am also very thankful to the members of the microbiology administrative staff Rachelle, Brietta, Kelly, Misty, and Kyra for taking care of all the paper work and answering all the naïve questions. Without you all, life in graduate school would not have been easy.

The acknowledgements would be incomplete without thanking my husband Dr.Pranay Dogra, without him I would have been lost. Thank you for pushing me to my limits and guiding me as I worked on achieving this goal. And last but not the least, I would also like to pay my gratitude towards my parents, my brother, my sister-in-law, my in laws and Oreo for your love and constant prayers throughout my Ph.D. Without your support, encouragement and blessings I could not have accomplished my goal.

ABSTRACT

Toxoplasma gondii is the most successful zoonotic pathogen known today. One-third of people are chronically infected worldwide. Different strains of *T. gondii* shows variability in mouse virulence which may potentially correlate with disease manifestation in humans. As a result, mouse is used as the model organism to study the virulence of *T. gondii* strains. To study the virulence on a global scale, it is necessary to establish a standardized approach for mice virulence assays. Thus, we **review the methodologies used in different labs and put forth standardized approaches to study the *T. gondii* virulence in mice (Chapter 1).** Recent advances in diversity and population structure of *T. gondii* had shown that even though the dominant type II strain is spread globally, it is particularly dominant in the region of Europe. Thus, to understand the origin and transmission pattern of the type II dominant lineage of *T. gondii* **(Chapter 2),** we tested the hypothesis that **the current distribution structure and dominance of type II *T. gondii* in Europe is because the most recent common ancestor (MRCA) of type II originates in the continent of Europe**. Based on our study we found that type II dominant lineage originated in the Old World and has transmitted to the New World (America). To further examine the migration pattern of second most dominant type III *T. gondii*, diversity among isolates was analyzed. Based on previous knowledge, in **Chapter 3** we tested the hypothesis that, **despite the diversity of genotypes in South America, the higher frequency of type III lineage is because the most recent common ancestor (MRCA) of type III originated on this continent.** Our study revealed that origin of type III lineage is in the New World close to Central America and that the dissemination pattern is from New World to the Old World. In **Chapter 4** we studied the seroprevalence rates for 471 and genotyped (n=19) wild life samples of *T. gondii* in the southeastern United States**.** We found **high seroprevalence rates amongst game meat animals (white-tailed deer and feral hogs)** which could be a potential risk for humans consuming contaminated meat.

TABLE OF CONTENTS

INTRODUCTION 1

1. Overview 2

2. Life Cycle 2

3. Virulence 3

4. Genetic Basis for Differences in Virulence 5

5. Human Toxoplasmosis 6

6. Population Structure 8

7. Immune Responses 10

8. Interconversion and Disease Pathogenesis 12

9. The Goal of Current Research 19

CHAPTER 1: ON THE DETERMINATION OF *TOXOPLASMA GONDII* VIRULENCE IN MICE 22

1. Abstract 23

2. Introduction 24

3. Current methods for virulence assessment of *T. gondii* in mice 25

4. Proposed methodology for virulence assessment of *T. gondii* in mice 29

5. Future perspective 34

CHAPTER 2: TO REVEAL THE MOST RECENT ANCESTOR AND TRANSMISSION PATTERN OF TYPE II LINEAGE OF *TOXOPLASMA GONDII* 35

1.Abstract 36

2. Introduction 37

3. Materials and Methods 38

4. Results 40

5. Conclusions 47

6. Future Perspectives 50

CHAPTER 3: TO REVEAL GLOBAL TRANSMISSION OF TYPE III *TOXOPLASMA GONDII* LINEAGE 52

1.Abstract 53

2. Introduction 54

3. Materials and Methods 54

4. Results 55

5. Conclusions 60
6. Future Perspectives 61
CHAPTER 4: *TOXOPLASMA GONDII* SEROPREVALENCE AND GENOTYPE DIVERSITY IN SELECT WILDLIFE SPECIES FROM THE SOUTHEASTERN UNITED STATES 62
1.Abstract 63
2. Introduction 64
3. Materials and Methods 65
4. Results 66
5. Discussion 71
6. Conclusion 75
7. Future perspectives 75
CONCLUSIONS 76
REFERENCES 79
APPENDICES 99
APPENDIX A: MODERN DAY *T. GONDII* GENETIC DIVERSITY IS DERIVED FROM RECOMBINATION OF FIVE ANCESTRAL GENOMES 100
1. Introduction 100
2. Materials and Methods 100
3. Results 101
4. Conclusions 102
5. Future perspectives 107
APPENDIX B: TO ESTABLISH AN EXPERIMENTAL PROTOCOL TO STUDY PHASE INTERCONVERSION IN *TOXOPLASMA GONDII* 108
1. Introduction 108
2. Materials and Methods 111
3. Results 112
4. Conclusion 113
5. Future perspectives 113
VITA 119

LIST OF TABLES

Table 2.1: Calculation of cumulative mortality rate (Example 1)……..…32
Table 2.2: Calculation of cumulative mortality rate (Example 2)…….……33
Table 3.1: Location and frequency of samples for 296 types II *T. gondii* isolates…….……...45
Table 3.4: Basic statistics for type II *T. gondii* populations from different geographical regions49
Table 4.3: Basic statistics for type III *T. gondii* populations from different geographical region 59
Table 5.1: Seroprevalence rates of *T. gondii* categorized by State and County..……..67
Table 5.2 Seroprevalence rate of *T. gondii* categorized by species ……..69
Table 5.3 Results for bioassay in mice for *T. gondii*……..72
Table 5.4: Genotyping data for *T. gondii* isolates from wildlife……...74

LIST OF FIGURES

Figure 1.1: Life cycle of *T. gondii*……..4
Figure 1.2: Population structure and diversity of *T. gondii* on a global scale…….9
Figure 2.1: Proposed methodology to test virulence of *T. gondii* strains in mice……..31
Figure 3.1: Phylogenetic network tree constructed for 435 isolates of *T. gondii*……....41
Figure 3.2: Phylogenetic rooted tree for 296 isolates of *T. gondii* analyzed with 15 microsatellite markers……...42
Figure 4.1: Phylogenetic rooted tree constructed for type III (n=123) *T. gondii* isolates…….56
Figure 4.2: Phylogenetic neighbor joining tree (NJ) tree constructed for 437 *T. gondii* isolates (123 type III + 296 type II + 18outgroup)…….57
Figure A.1: Chromosome painting of 160 *T. gondii* strains……103
Figure A.2: Analysis of present day *T. gondii* population structure using genome-wide SNPs data for 160 strains using STRUCTURE……104
Figure B.1: Examples of network structure of predicted genes analyzed using Gene net analysis……110
Figure B.2 (a-d): Expression of Bag1 (Green) antigen following the *in vitro* differentiation of ME49 strain of *T. gondii*……114
Figure B.3: Western blot analysis of *in vitro* Bag 1 protein expression of ME49 strain of *T. gondii*……117

LIST OF ATTACHMENTS

Table 3.2: Microsatellite data for 435 isolates of *T. gondii*……Table 3.2.xlxs

Table 3.3: Microsatellite data for 296 type II isolates of *T. gondii*……Table 3.3.xlxs

Table 4.1: Microsatellite data for 123 type III isolates of *T. gondii*……..Table 4.1.xlxs

Table 4.2: Microsatellite data for 437 type II isolates of *T. gondii.* ……..Table 4.2.xlxs

Table A.1: Concatenated SNPs for 160 strains of *T. gondii*……..Table A.1.xlxs

Attachment 1- Concatenated sequence data for 160 *T. gondii* strains… ……...Attachment 1.txt

INTRODUCTION

1. Overview

Toxoplasma gondii is one of the most successful pathogens on earth, capable of infecting a broad range of mammals and birds [1]. It belongs to the phylum apicomplexan which also includes other medically significant members such as *Plasmodium, Cryptosporidium,* and *Babesia*. Felids are the only known definitive hosts in which sexual replication of the parasite occurs, resulting in the dissemination of oocysts into the environment via defecation, thus felids play a critical role in the transmission of *T. gondii*. Mammals and birds are intermediate hosts in which asexual replication of *T. gondii* occurs, which generates a large number of fast-replicating tachyzoites during acute phase infection. This phase is followed by a growth switch to slow-growing bradyzoites in tissue cysts. It is estimated that one-third of the world's human population is chronically infected with this parasite [1]. Humans become infected by ingesting oocysts from contaminated food and water, consuming undercooked meat containing the tissue cysts, or vertical transmission of tachyzoites from mother to fetus [1, 2]. Primary infection in immunocompetent individuals is mostly asymptomatic but in some cases can lead to ocular toxoplasmosis [3]. In immunocompromised individuals, such as AIDS patients, reactivation of chronic infection can cause life-threatening encephalitis [1, 4].

2. Life Cycle

Toxoplasma gondii has three distinct forms: 1) sexually-reproduced sporozoites, 2) the fast-replicating tachyzoites, and 3) slow-replicating bradyzoites. Sexual replication of *T. gondii* is restricted to felids. Felids can ingest oocysts from environmental sources or tissue cysts from chronically infected prey [1, 2] **(Figure 1.1).** The cyst wall is ruptured due to acid pepsins present in the felids stomach. Once released, the sporozoites or bradyzoites invade the epithelial cells of the intestine and differentiate into schizonts. The parasite then forms merozoites which later differentiate into male and female gametes. The gametes fuse together to form diploid oocysts and are shed in the feces of the felines. The time between the infection and shedding of oocysts is generally 3-10 days [1, 5]. A single cat can shed up to 20 million oocysts per day in its feces [6]. When released in the environment, these oocysts can sporulate under ambient temperatures producing eight haploid sporozoites enclosed in the wall of the cyst. The oocysts can survive in moist soil and water for up to 18 months which prolongs their survival in the harsh environments [7].

Almost all warm-blooded animals and birds can serve as an intermediate hosts for *T. gondii*. Intermediate hosts are infected via sporozoites in oocysts from contaminated water sources as well as via tissue cysts in contaminated meat sources **(Figure 1.1).** On ingestion of oocysts, the sporozoites are detectable in the small intestine within hours of infection [8]. The sporozoites then differentiate into tachyzoites and replicate rapidly [1]. Upon consumption of contaminated meat containing tissue cysts, the cyst wall is dissolved due to acid-pepsin in the host's stomach and bradyzoites are released. In mice, bradyzoites differentiate into tachyzoites within one hour and enter into a rapidly- replicating form by two hours post infection. Fast replicating tachyzoites enter the lymph nodes by 48 hours and can disseminate by infecting migratory cells like dendritic cells and macrophages[1]. Eventually, the tachyzoite form is converted to a more slowly-replicating, dormant form of bradyzoites. Bradyzoites enclosed in the cyst wall are resistant to immune responses and are retained primarily in brain and muscle for the lifespan of the infected host [8, 9].

3. Virulence

Susceptibility differences to *T. gondii* infection have been reported among closely related species [1]. For examples, rats (genus *Rattus*) are generally resistant (form chronic infection) to toxoplasmosis, whereas house mice (*Mus musculus*) are highly susceptible compared to most other hosts[1]. Mice are generally selected as the model organism for testing the virulence of *Toxoplasma*. As house mice are more susceptible to *T. gondii*, a difference in the susceptibility is observed among different strains of the parasite. *Toxoplasma* strains can, therefore, be classified into virulent and avirulent categories. Parasite strains causing 100% mortality in mice at a dosage of as low as one parasite are considered to be virulent. Some strains, on the other hand, require a high dosage to achieve mortality, and subsequent infection with low dosage leads to chronic infection in mice [10, 11]. In the early 1990's using PCR-RFLP (Polymerized chain reaction-Restriction fragment length polymorphism) based genotyping of strains it was observed that all the virulent strains fall in the

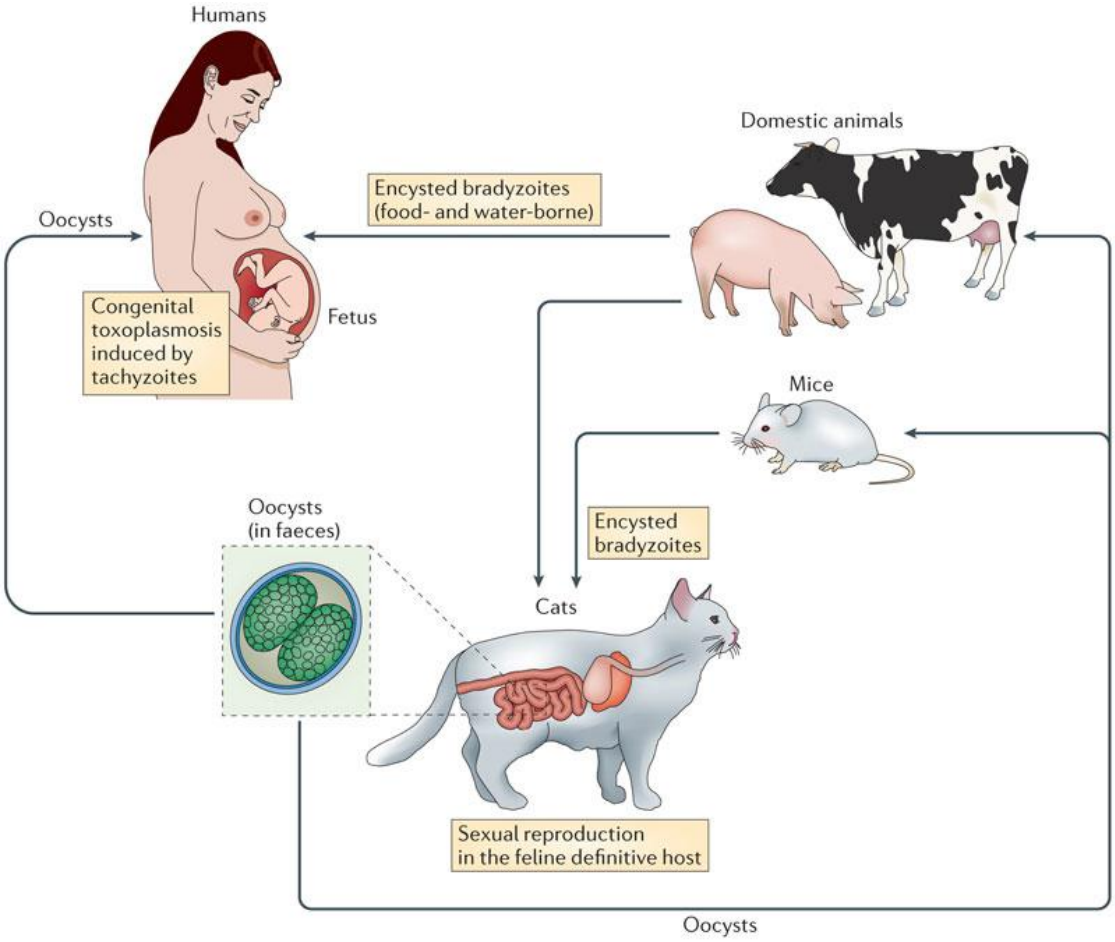


Figure 1.1: Life cycle of *T. gondii* [222].

category of type I lineage and non-virulent strains belonged to either the type II or III lineages of the parasite [10, 12]. Later, PCR-RFLP based genotyping study on *T. gondii* samples from Europe and North America suggested that the population structure of *Toxoplasma* was dominated by the three clonal lineages type I, II and III [11]. This observation led to the investigation of the genetic differences among the lineages and how they lead to differences in mouse virulence.

4. Genetic Basis for Differences in Virulence

The first evidence for virulence differences emerged from mapping the progeny from sexually crossed type I, II and III clonal lineages using Quantitative Trait locus (QTL) analysis [13] [14]. From these studies, two major virulence genes rhoptry protein 18 (ROP18) and ROP5 were identified in *T. gondii* [13-15]. The first key virulence factor identified was the ROP18 kinase [14]. The main function of ROP18 is to interact with immunity-related GTPases (IRGs) and p65 guanylate binding proteins (GBPs) that are known to localize to the parasitophorous vacuole membrane (PVM), ultimately causing disruption of the PVM and leaving the parasites vulnerable to clearance by other host defense mechanisms [16-19]. Thus, functional ROP18 is required by *T. gondii* for evasion of the host immune system. In the crosses generated, it was revealed that types I and II express a functional ROP18 allele and are thus included in the virulent category. In contrast, the ROP18 allele of type III clonal lineage has an insertion in the promoter region that attenuates expression of the gene, making it non-functional [20]. ROP5 is a rhoptry-secreted pseudo kinase, non-functional kinase by itself due to a mutation in the catalytic site and is correlated even more strongly with virulence than ROP18 [21]. ROP5 plays a role in the suppression of the immune system via formation of a complex with ROP18 and murine IRGs [21, 22]. Functional ROP5 alleles are expressed by clonal types I and III. In the case of type I strains, the expression of both functional ROP5 and ROP18 alleles appears to account for the hyper virulence of this lineage. On the other hand, the ROP5 allele of type II is nonfunctional, so that this strain type is avirulent despite expression of functional ROP18 [15, 22]. ROP17 is another rhoptry protein that was found to interact with ROP5. The combination of ROP17 and ROP5 also determines virulence similar to RO18 and ROP5 interaction but the interaction is less significant in comparison to the combination of ROP5 and ROP18 [21]. Recently, using PCR – RFLP technique, genotyping was performed for four loci ROP5, ROP18, ROP 16 and ROP17 for

240 strains collected globally [23]. Comparing the genotyping data with the previously published mouse virulence data for these strains, it was shown that there is indeed an association between the genotypes of the ROP5 and ROP18 allele and virulence in mice. ‘

5. Human Toxoplasmosis

One-third of the human population is infected with *T. gondii* [24]. For immunocompetent individuals, the general symptoms of the acute infection include fatigue, fever, muscle and joint pain [1]. Approximately two weeks after acute infection, the parasites enter into the chronic phase and maintain infection for the entire life of infected host [25, 26]. In the United States, the seroprevalence rate ranges between 10-38%. In other regions like China, UK and Korea the seroprevalence percentage is lower and estimated to be 11, 8 and 4% respectively [1, 3]. These geographical differences in the infection rate are the reflection of the dietary habits in that particular region. However, severe cases of toxoplasmosis are typically reported in immunocompromised people like AIDS, cancer or organ transplant patients. The majority of AIDS patients infected with *Toxoplasma* suffer from formation of necrotic lesions in the brain, which are associated with encephalitis as well as pneumonia and other systemic forms of the disease. These severe cases of toxoplasmosis are a result of reactivation of latent infection when the immune system is compromised or suppressed [1]. However, recent studies have also reported severe cases of toxoplasmosis in immunocompetent people [3, 27-29].

5.1 Ocular Toxoplasmosis

Ocular toxoplasmosis (OT) is one of the most common infections observed in nearly 18% of adults in Southern Brazil compared to 2% of adults in Europe and the United States [3]. The infection can result in scarring of the ocular tissue leading to vision impairment. A large number of ocular toxoplasmosis cases in South America manifest more severe clinical symptoms. For example, eye lesion and impaired vision cases are more common in children and the lesions formed are much larger in size and multi focal when compared to cases from Europe. This relatively high number and increased severity in South America is hypothesized to be linked to the atypical strains of *T. gondii* dominant in regions like Brazil. Similarly, another study in 2011 reported that 18 out of 20 tissue samples collected from toxoplasmosis patients in Sao Paulo, Brazil belonged to genotype # 65 which comprises a small fraction of total *T. gondii* strains

found in this region [30]. Additionally, the majority of Colombian OT patients were found to be infected with atypical or type I virulent strains of *T. gondii* [30]. All this evidence indicates that the strains circulating in the environment determine the disease manifestation in humans residing in that region.

5.2 Congenital Toxoplasmosis

Toxoplasma can also be vertically transmitted from mother to fetus and, as a result, is a major health concern for pregnant women. The higher seroprevalence rates reported in pregnant women also highlights this threat. Seroprevalence rates as high as 61% and 84% were documented in France and Madagascar respectively. In regions like Brazil where undercooked meat is consumed daily, the seroprevalence can range between 61-92% [3, 31]. In general, pregnant women infected for the first time during pregnancy can vertically transmit the parasite to the developing fetus during the acute infection stage. Even so, few cases of vertical transmission have been reported due to chronic infection or preexisting infection before pregnancy [32]. Thus, women infected during pregnancy are likely at a greater risk for abnormalities in the fetus. The severity of the symptoms due to the vertical transmission varies depending on the gestational stage of the pregnancy. More severe outcomes of infection are reported during the first trimester of pregnancy, while infections occurring during the later stages of pregnancy have less severe symptoms, but are more prone to congenital infection. The manifestation of the infection during pregnancy can include ocular disease, congenital defects and pregnancy loss [1].

5.3 Severe Disseminated Toxoplasmosis

Severe cases of toxoplasmosis have also been reported in the population of French Guiana [28]. Of the 44 cases of severe primary toxoplasmosis reported from the period of 1998-2004, one-third were hospitalized for respiratory distress. Genotyping studies revealed that the majority of strains infecting these patients belonged to the atypical category. In addition, 11 cases of multi-visceral toxoplasmosis were reported from Patam village in French Guiana from December 2003 to January 2004. Eight out of 11 patients were found to be immunocompetent and genotyping studies revealed one atypical genotype infecting all the patients [28]. Organ failure was also reported in 11 immunocompetent adults of French Guiana, thus requiring intensive care. This

severe form of systemic toxoplasmosis called 'Amazonian toxoplasmosis' and is speculated to be a result of spillage of strains circulating among wild animals to intermediate hosts like humans and domestic animals in that region [28, 33]. Wild definitive hosts are generally more resistant to *T. gondii* which leads to the selection of more virulent strains causing more severe infection in the intermediate hosts in close proximity. In other regions like Europe, where type II strains are found to be most dominant, the majority of OT cases were found to be caused by the virulent type I or atypical strains of *T. gondii* [34, 35].

6. Population Structure

Early genotyping studies of 106 isolates from Europe and North America using 6 PCR-RFLP markers divided *T. gondii* isolates into three clonal types denoted as types I, II and III [11]. It was suggested that the global population structure of *T. gondii* was largely clonal with three archetypical lineages (type I, II and III) dominating the entire world. However, recent advances have revealed that the population structure of *T. gondii* is more complex than previously thought [36, 223]. The most recent data on diversity structure of *Toxoplasma* have been obtained from a study performed on 1457 isolates using 10 PCR-RFLP markers **(Figure 1.2)** [36]**.** A total of 189 ToxoDB PCR-RFLP genotypes were found from these isolates of *T. gondii* collected worldwide. The population structure in the northern hemisphere appears to be largely clonal, whereas the southern hemisphere samples show a diverse population structure. In Europe, the majority of the isolates studied belonged to ToxoDB genotypes #1 and #3 that together are recognized as type II lineage [223]. The second dominant lineage in Europe was found to be #2 that is known as type III lineage. However, genotype #10 (type I) [223] constituted a relatively lower percentage of the total population. This shows that the European population structure is mostly clonal which is in agreement with previous report [11]. In North America, the majority of the population is comprised of genotype #1 or #3 (type II) [223] strains, whereas genotype #2 (type III) strains were also found in relatively higher frequency. Interestingly, genotypes # 4 and # 5, together known as type 12 [223] were found in high frequency in wildlife. In Eastern Asia, genotype #9 (Chinese type I) was found to be widespread followed by #10 [223]. Other genotypes identified in this region were genotypes #4, #18 and #20 [223]. Overall, the population structure in Asia was recognized to be clonal. In Africa, #2 and #3 accounted for the majority of the isolates.

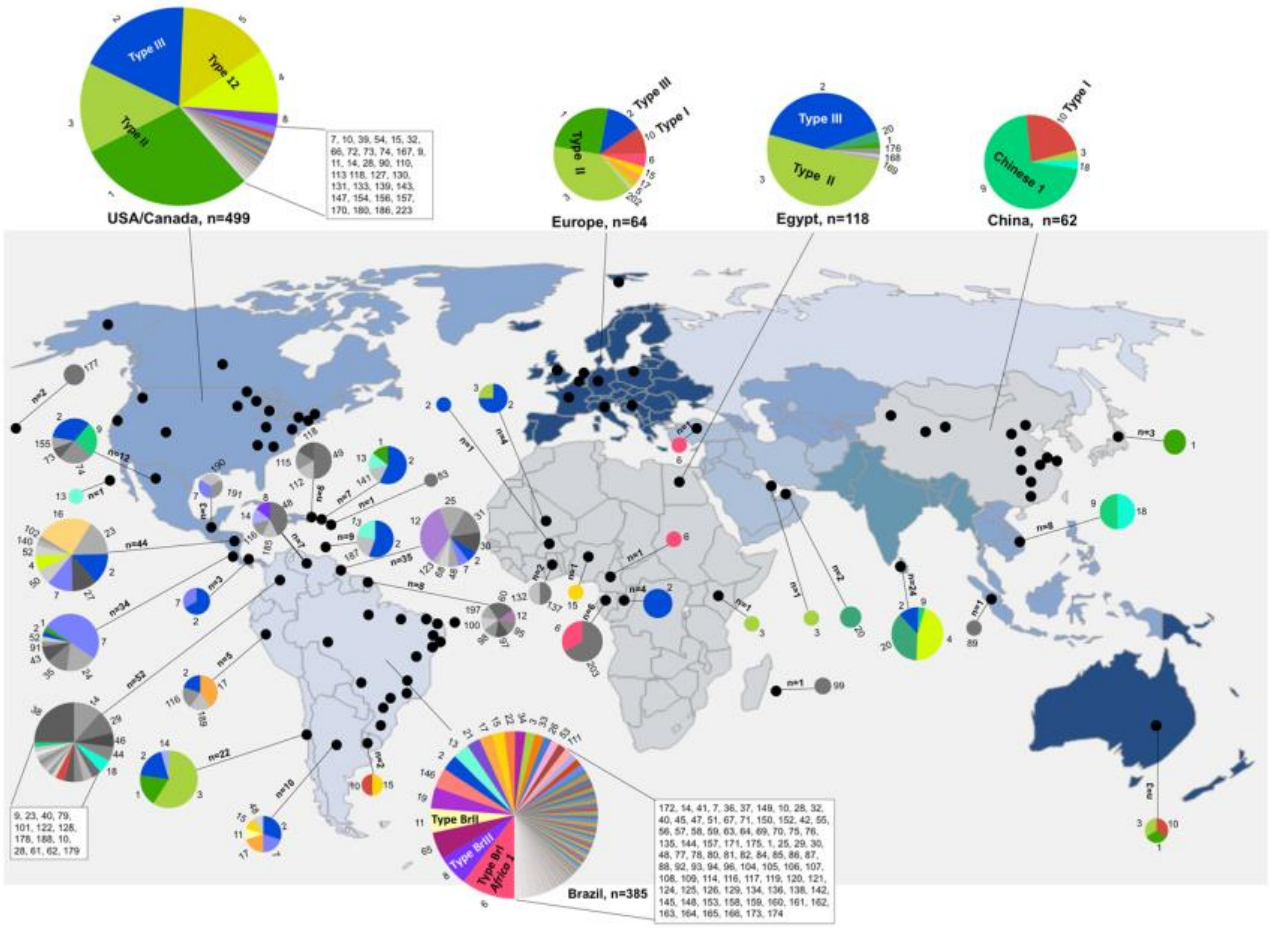


Figure 1.2: Population structure and diversity of *T. gondii* on a global scale [36].

Additionally, genotype #6 was observed [223]. Unlike other regions, the population structure in Central and South America is highly diverse and there is a lack of predominant lineages. Commonly found genotypes included in decreasing order, #2, #6, #7, #8, #11, #3, #65, #13, #19 and #146. However, in the geographical region which includes Chile, the three lineages including ToxoDB genotype #1, #2 and #3 were found to be predominant **(Figure 1.2)** [36**].** Overall, the current population structure for *Toxoplasma* appears to be much more complex with the northern hemisphere showing a clonal structure, compared to the southern hemisphere which is more diverse.

7. Immune Responses

7.1 Interferon Gamma

Interferon gamma (IFNγ) is one of the most important Th1 cell-mediated cytokines critical for the control of *T. gondii* infection. Mice lacking the IFNγ gene have been shown to succumb to *T. gondii* infection. The major cells producing IFNγ include neutrophils, T cells and NK cells [37-39]. The main function of IFNγ is the expression of immunity related GTPases (IRGs) and guanylate binding proteins (GBPs) [40-42]. The role of IRGs and GBPs is to disrupt the integrity of the PV membrane leading to the release of the parasites in the host cytoplasm [43]. IRG proteins can be divided into two groups (GKS and GMS) depending on their specific role. Effector proteins (GKS) bind to the parasite membrane and are responsible for mechanically disrupting the membrane. On the other hand, regulatory proteins (GMS) are required for protection of the host intracellular membrane to prevent disruption via GKS proteins [18, 44]. The role of GBPs in parasite clearance is rather indirect and requires the recruitment of other IRG proteins [42]. In addition to the expression of IRGs and GBPs, IFNγ also aids in limiting the replication of the parasite by inhibiting tryptophan production. Interferon gamma induces the production of indoleamine 2, 3- dioxygenase that converts tryptophan into an unusable form. As *T. gondii* is a tryptophan auxotroph, the absence of tryptophan limits the parasite growth [45, 46]. IFNγ can also play a role in parasite inhibition via production of reactive oxygen and nitrogen species (ROS and RNS) that have antimicrobial properties. It does so by inducing the NO synthase gene using L-arginine as the substrate [47, 48].

7.2 Tumor Necrosis Factor Alpha

Tumor necrosis factor Alpha (TNFα) alone cannot trigger parasite clearance and requires the help of IFNγ [49]. Activated macrophages when primed with IFNγ and TNFα show enhanced antimicrobial activity [50]. TNFα in conjunction with IFNγ can lead to parasite control by fusion of PV to the lysosomes causing their degradation [51]. In macrophages, TNFα also plays a role in the production of RNS having antimicrobial activity [52, 53]. TNFα can also act as a co-stimulatory molecule in NK cells. It has been shown that NK cells exposed to parasite antigen along with TNFα produce IFNγ [37].

7.3 Interleukin -12 (IL-12)

Th1 cell-mediated response is known to be critical for parasite clearance; this is in part mediated by upregulation of IL-12 which is required for the production of IFNγ [39, 54]. Mice infected with lethal doses of the parasite shows enhanced survival on treatment with exogenous IL-12 [55]. The major circulatory cells involved in the IL-12 production are dendritic cells, monocytes and neutrophils [56, 57]. In response to the secretion of chemokines like macrophage inflammatory proteins 1 and 2 on infection with *T. gondii,* macrophages and neutrophils are recruited to the lumen of the small intestine in mice [58-60]. This leads to the clearance of parasites via processes like phagocytosis, antigen presentation and involvement of other immune cells [57, 61-65]. The production of IL-12 is dependent on myeloid differentiation factor 88, which is recruited to TLRs on the binding of pathogen-specific molecules [66].

7.4 Adaptive Immune Response

T and B cell mediated response is critical for the survival of humans as well as mice, especially during the chronic infection stage. CD4+T cells are involved in the production of IFNγ as well as mediation of B and CD8+T cell responses. CD8+ T cells, in turn, offer protection via production of IFNγ and CD40L as well as perforin-mediated lysis of infected cells [67, 68]. CD4+T cells are activated by dendritic cells (DCs) which are one of the most important antigen presenting cells (APCs), in addition to other cells like B cells and macrophages [69]. B cell activation, in turn, produces antibodies that provide protection via activation of complement pathway and opsonization of the pathogen. CD8+ T cells are activated by *T. gondii* antigens like surface antigen 1 (SAG1), dense granule proteins (GRA4 and GRA6) and rhoptry protein ROP7 [70].

8. Interconversion and Disease Pathogenesis

The life cycle of *Toxoplasma* consists of three stages sporozoites, tachyzoites, and bradyzoites. Infections in immunocompetent individuals are mostly asymptomatic [1]. However, the key to disease pathogenesis of this parasite is in part linked to its ability to convert into a dormant, slowly replicating bradyzoite enclosed within a tissue cyst. Tissue cysts can hide from the immune system and remain in the host indefinitely [71]. However, in immunocompromised people such as AIDS patients or chemotherapy patients, tissue cysts can rupture and release bradyzoites. Transformation of bradyzoites into tachyzoites can lead to parasite reactivation and in some cases severe disseminated toxoplasmosis and life-threatening encephalitis. Therefore, understanding the mechanisms behind the ability of tachyzoites to convert to bradyzoites is of utmost importance. The combination of drugs like sulfonamide and pyrimethamine are commonly used to control the active infection. Sulfonamide is known to interfere with the folic acid biosynthesis pathway by preventing the addition of para-amino benzoic acid (PABA). Pyrimethamine, on the other hand, is a dihydrofolate reductase that blocks the biosynthesis of purine and pyrimidines. Although drugs which control the tachyzoites in the active stage of infection currently exist, no successful drugs have been developed to control the chronic infection bradyzoite form [1, 72]. Hence, knowledge of the molecular prerequisites for interconversion can be used to force the parasite into tachyzoites stage and then be targeted by using known drugs.

8.1 Bradyzoites

Toxoplasma in the form of cysts can be maintained indefinitely in almost all the intermediate hosts thereby sustaining its infectivity [8]. Within 2 hours of ingestion of oocysts, sporozoites are detectable in the epithelial cells of the small intestine [1]. By 12 hours post infection, sporozoites differentiate into tachyzoites and enter into a rapidly replicating stage by 48 hours [1, 73]. The other route of infection in intermediate hosts is the ingestion of cysts in the contaminated meat. Once tissue cysts are ingested by intermediate hosts, the cyst wall is digested by acids present in the stomach. In mice, tachyzoites are detectable in epithelial cells 2 hours after infection. When migratory cells like dendritic and macrophages are infected, tachyzoites spread in the host [74]. Eventually, factors like immune pressure or spontaneous conversion lead to conversion of tachyzoites to the dormant bradyzoite form enclosed within the cyst wall [8]. Tissue cysts are

formed within 6 to 7 days post infection. Cysts can be harbored for decades primarily in the brain, skeletal muscle, and cardiac tissues. The reason why cysts are predominantly found in these cells is not well understood. Bradyzoites are generally believed to be dormant however, they can rupture periodically during the life time of an individual for reasons not yet known, leading to reactivation of the disease [1].

8.2 Bradyzoites and Human Health

It was 50 years after the discovery of *Toxoplasma* when it was recognized that the parasite can disseminate via contaminated meat sources [75]. This highlighted the fact that consumption of undercooked meat may prove as one of the major routes of infection in humans and other intermediate hosts. Upon ingestion of the tissue cysts via contaminated meat, bradyzoites are released in the intestinal epithelial cells of the intermediate host and the asexual life cycle begins. Later in the life cycle, the parasites enter into a slowly replicating dormant stage of bradyzoites enclosed in tissue cysts[1]. Once an infection is established the parasite can maintain its infectivity as tissue cysts, for the entire life span of the host. Even though bradyzoites are believed to be dormant, in the conditions of immune suppressed conditions, e.g. cancer and AIDS, the parasite can emerge from latency and convert back to the tachyzoites form and reinvade other nucleated cells [76]. Thus, in 1980 when AIDS emerged, *Toxoplasma* became a major threat [77]. Since bradyzoites can reside in different organs in humans, it is an additional cause of concern during organ transplant procedures. These findings led to the beginning of intense research for the understanding of important regulatory factors for switch between the acute and chronic infection stages.

8.3 Surface Proteins and Heat Shock Proteins

Stage conversion from tachyzoite to bradyzoite is associated with changes in the stage-specific surface antigens (SAG) [78]. The SAG superfamily of antigens can be divided into SAG1 and SAG2 family of surface antigens [79]. The SAG1 family of proteins plays a role in surface attachment of the parasite before the invasion. The SAG1 family comprises of SAG3, bradyzoite specific recombinant BSR4, SAG related sequences (SRS) 1-4 proteins, SAG 5, SAG5.1 and SAG5.2 [79]. SRS1-3 is specific to the tachyzoite stage, BSR4 is specific to bradyzoites and SAG3 is found in both stages. Similarly, the SAG2 family consists of SAG2A and SAG2B-D.

SAG2A and SAG2B are antigens specific to tachyzoite stage. On the other hand, SAG2C and D are specifically expressed in the bradyzoite stage [80]. These differentially expressed surface proteins can be used as a marker for the identification of parasite stages in *in vitro* assays.

Heat shock proteins (HSPs) are another family of stress-related proteins required for bradyzoite development. Experiments using the drug Quercetin which specifically inhibit HSPs have shown the suppression of the stage conversion process. Bradyzoite specific-antigen (BAG1), previously known as HSP30, is a bradyzoite stage specific HSP commonly used to identify the cyst form in cell culture experiments [81]. Studies have shown that deletion of the BAG1 gene reduces the number of cysts in *in vitro* assays [82].

8.4 Triggers and Determinants of Bradyzoite Formation

In vivo determinants of tachyzoites to bradyzoite conversion

Various *in vivo* factors such as parasite cell cycle, stage specific metabolites, host cell type, manipulation of host immune responses, translation and transcription regulation, cyclic nucleotides, etc. are known to play a role in the differentiation process of *T. gondii*. These factors are discussed below in more detail.

Bradyzoite development and maintenance is dependent on transcription of the required genes which in turn is tightly regulated with the cell cycle stage of the parasite. Abrupt cessation of the cell cycle does not lead to bradyzoite development of the parasite, which suggests that the parasite requires the completion of the cycle in order to enter the G0 phase for completion of differentiation process [83]. Mature bradyzoites isolated from the brains of mice infected with oocysts were found to be stalled in the G0 phase of the cell cycle containing 1N DNA. On the other hand, differentiating parasite population contained 1.8 to 2 N DNA and was discovered to be in the S/G2 phase of the cell cycle [84, 85]. This shows that commitment to bradyzoite formation requires an entry into the pre-mitotic phase.

The metabolism profile of the parasite is linked to the stage of life cycle. Metabolomics studies have identified stage-specific isoforms of enolases (ENO 1 and 2) and lactate dehydrogenases (LDH 1 and 2) [86, 87]. The LDH2 form of the enzyme LDH is found to be specific to the bradyzoite stage and is resistant to acidic pH, making it functional during the catabolism of amylopectin in bradyzoites [87]. This is in accordance with electron microscopy

studies that show an increase in the number of amylopectins during the bradyzoite stage [88]. The enzyme enolase also shows stage dependent differences in the specificity and stability in the interconversion phenomenon. This enzyme catalyzes the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in glycolysis [86]. The specificity of the bradyzoite stage-specific ENO1 form is three-fold greater than ENO2, even though both the isoforms have the same Michalis constant (km). This indicates that stage-specific glycolytic flux can be regulated via expression of different isoforms of enzymes [86].

The role of host cell type in differentiation is still unclear. Given that cysts predominate in brain and muscle cells and that the yield of tachyzoites varies between different host cell types, identifies the need of the elucidation of this process. The host cell type differences could be attributed to several factors. It is possible that as neuron cells in the brain and muscles are long-lived, the chances of finding cysts in these cells for a longer period of time is increased [9]. In addition, the slowing of parasites replication, directed by host cell microenvironment, could also promote the differentiation. Studies using Compound 1(4-[2(4-flurophenyl)-5-(1-methyl piperidine-4-yl)-1 H pyrrol-3-yl] pyridine), support this hypothesis [89, 90]. It was observed that only Hela cells treated with compound 1 increase the expression of the human cell division auto antigen 1(CDA1), which slows the parasite replication, thereby increasing the number of cysts [91]. Parasites also exhibit spontaneous differentiation in certain cell types such as rat-isolated CNS cells including microglia, neurons, and astrocytes, primary and continuous muscle cells for type III VEG strain of *T. gondii* [92, 93]. This further supports the possible role of the host cells in the differentiation process.

T. gondii has been shown to develop multiple strategies to evade the host immune responses, as well as manipulate the cytokines required by hosts for its own benefit. It is known that IFNγ dependent cell- mediated immunity is critical for killing the parasites during the acute infection phase [41, 42]. Additionally, prolonged IFNγ treatment is required to kill the rapidly dividing reactivated tachyzoites. However, *in vivo* experiments using mice infected in the presence of IFNγ have shown that IFNγ promotes differentiation into bradyzoites [94]. It is speculated that this effect is due to the induction of the nitric oxide synthase gene to form nitric oxide [95, 96]. Treatment of cells with NO donor (sodium nitroprusside) causes an increase in the number of cysts formed *in vitro* [81, 82, 92]. IL-12 is another key cytokine required for protection of the host. Mice lacking IL12 succumb to the parasite infection due to uncontrolled

replication [39, 55]. IL-12 is produced on the translocation of NF-kB from the cytoplasm to the nucleus when pathogen -specific molecules bind to toll-like receptors (TLRs) and MyD88 is recruited to the TLRs. Type I and III strains of *Toxoplasma* have been shown to block the NF-kB translocation, thereby hindering IL-12 production [97-99]. In contrast, type II strains of *T. gondii* promote the expression and translocation of NF-kB via secretion of GRA15, thereby increasing the proinflammatory response [100]. Type II strains adapted to the proinflammatory response increase in the host to maintain chronic infections for a longer period of time. *Toxoplasma* also avoids killing by suppressing the production of NO, especially in macrophages. NO is produced by nitric oxide synthase using arginine as the substrate. *Toxoplasma* secretes rhoptry protein ROP16 that activates the STAT6 pathway in the host leading to the expression of arginase 1. Arginase 1 is known to degrade arginine, thereby reducing NO production [47, 101]. As NO has an antimicrobial property, limited NO availability proves beneficial for the parasite. Furthermore, *Toxoplasma* being an arginine auxotroph requires exogenous arginine, and so, limited NO availability decreases the rate of parasite replication, which then helps in the differentiation process [101]. NO production is also blocked by a patatin like phospholipase (TgPL1) [102, 103]. Lower levels of cytokines were found at 8 weeks post infection in mice vulnerable to toxoplasmic encephalitis (TE) [104]. On the other hand, mice infected with a mutated strain lacking the TgPL1 gene maintained high cytokine levels, protecting them from TE symptoms. Thus TgPL1 is required for suppression of NO, leading to lower cytokine levels, and formation of cysts in the brain [104].

Translation regulation can also play a role in regulating the differentiation process. Eukaryotic initiation factor-2 (eIF2) is one of the most characterized factors known to play a role under different stress conditions [105]. Similarly, in *T. gondii*, protein translation is blocked when the alpha subunit of TgIF2α is phosphorylated by protein kinases TgIF2K-A to D [106, 107]. This reduced protein translation helps *Toxoplasma* to stall the parasite at the tachyzoite stage in order to promote the induction of bradyzoite stage genes.

Transcriptome analysis of *T. gondii* at different stages of the life cycle revealed that stage differentiation in *T. gondii* is a transcriptionally governed process. Recent studies have identified a role of the plant AP2-like domain containing *T. gondii* protein (TgAP2XI-4) in acting as a transcriptional regulator to control the expression of bradyzoite stage-specific genes [108]. This AP2 domain containing protein localizes in the nucleus, and binds to the ‘CACACAC’ sequence

specific DNA motif, and its expression peaks after cytokinesis [109]. Transcript levels of this protein were higher in cysts isolated from the brain than in rapidly dividing tachyzoites. Thus, transcription regulation of stage-specific genes can govern the parasite differentiation.

The role of cyclic nucleotide pathways in differentiation was studied in two *T. gondii* strains ME49 and PLK. The numbers of bradyzoites were increased *in vitro* when HFF cells, infected with *T. gondii* strains, were treated with cGMP and cAMP nucleotides [110, 111]. Additionally, infection conducted using forskolin, an agent used to transiently increase cAMP levels, induced tachyzoite differentiation [110]. Further investigations revealed the involvement of the protein kinase catalytic subunit TgPKAc3 in the bidirectional regulation of differentiation [110]. This suggests that cyclic nucleotide signaling pathways are involved in stress- induced differentiation and are dependent on protein kinase subunit -3 [110].

Bradyzoite pseudo kinase 1 (BPK1) expressed during early stages of cyst formation, was found to be influential in the infectivity and development of cysts [112, 113]. Using immunoprecipitation techniques, BPK1 was found to interact with four associated proteins; MAG1, MCP4, GRA8 and 9. Deletion of the BPK1 gene resulted in the formation of cysts that were smaller in size with lower oral infectivity [113].

Some strains of *T. gondii* show spontaneous stage conversion *in vitro*. Sporozoites or bradyzoites of the VEG strain (ToxoDB genotype #2, type III) were inoculated in HFF cells and were found to readily convert into tachyzoites stage. After approximately 20 divisions, the sporozoites slowed their growth uniformly leading to bradyzoite formation [84, 85]. Specific *T. gondii* strains are therefore able to follow a defined course of development without the need for any external factors. Serial analysis of gene expression profile (SAGE) also revealed that a 15-day sporozoites culture contained a mixed population of tachyzoite and bradyzoite stage parasites, with a gene expression profile similar to pH stressed parasites [85]. Together, these data suggest that parasites are programmed to slow their replication in order to enter into the bradyzoite stage.

In vitro Stresses Inducing Bradyzoite Formation

Exogenous stress can trigger differentiation, leading to cysts formation *in vitro*. Immunological factors are therefore not necessary to trigger bradyzoite development. Discussed below are the

commonly used external stress inducers and stress related changes in the parasite which lead to the conversion of tachyzoite to bradyzoite.

Growing *Toxoplasma* under alkaline conditions is one of the most commonly used strategies for bradyzoite development [114-116]. Experiments using human fibroblast cells (HFF) infected with the ME49 strain of *T. gondii* were shown to readily form cysts (3-4 days) by using an alkaline pH medium [116]. Changing the pH of the medium after infection or preconditioning of the HFF cells in alkaline pH for an hour before invasion leads to bradyzoite formation [114]. Use of alkaline pH conditions, however, is not considered to be a physiologically relevant stress factor [114]. This is of concern when trying to identify the molecular mechanisms behind the conversion process.

Tachyzoites can be converted to bradyzoites *in vitro* using heat stress. Studies have identified a role of heat shock family proteins (HSPs) in the conversion of tachyzoites following heat stress [81, 114]. HSP30/Bag1, HSP72, and HSP90 are a few HSP family proteins that have been identified to play a role in cysts production [81, 117, 118]. When parasites are allowed to invade preconditioned thermotolerant human fibroblast cells (HFF) cells at 37°C and then are heat shocked for 2 hours at 43°C, they readily form cysts *in vitro* [119, 120]. It currently remains unknown whether fever/high- temperature conditions symptoms during acute toxoplasmosis in humans induces the bradyzoite formation.

Of all the methods currently used to study conversion of tachyzoites to bradyzoites, use of NO is most physiologically relevant [81, 95, 110]. *In vivo*, NO is produced in response to the production of IFNγ and acts on the iron-sulfur center of proteins involved in the parasite respiratory chain [95, 121]. Sodium nitroprusside (SNP) is the most commonly used NO donor drug in *in vitro* experiments and triggers bradyzoite differentiation in almost all host cell lines [81, 92, 95].

Tachyzoites replicate faster than bradyzoites and as a result, require a higher concentration of nutrients. Recent studies have shown that deprivation of the amino acid arginine induces the formation of bradyzoites [122]. *Toxoplasma*, an arginine auxotroph, relies on the host arginine supply [122]. Thus, in the absence of external arginine, replication of the parasite is blocked, leading to the formation of bradyzoites. Additionally, inhibition of pyrimidine de novo biosynthesis and the salvage pathway has also shown to induce conversion of tachyzoites. *T. gondii* has a truncated pyrimidine salvage pathway and thus requires uracil phosphoribosyl

transferase gene (UPRT), as well as CO_2, for the de novo synthesis of pyrimidines [123]. Altogether, deprivation of specific nutrients can be used as a strategy to study the molecular perquisites for the differentiation of the parasite.

9. The Goal of Current Research

Susceptibility to *T. gondii* infection varies between intermediate hosts. House mouse (*Mus musculus*) being more sensitive, variability in mouse virulence exists for different strains of *T. gondii* [1]. Thus, in order to study the virulence of strains of *T. gondii*, the house mouse is the preferred model. Several studies have also shown that a correlation exists between disease severity in humans and mouse virulence [33]. However, when studying virulence in mice, many factors such as route of infection, life stage of the parasite, the number of passages of the parasite in mice or cell culture, and the mouse host line used gives rise to variable results. This inconsistency in the protocol makes the comparison of results between labs difficult. Thus, in chapter 1, we discuss important factors to be considered when conducting *T. gondii* murine virulence assays and propose a standardized methodology to facilitate the integration of *T. gondii* virulence data throughout the research community. This will enable more efficient and effective analysis of genetic and virulence patterns for this important parasite.

The most recent study performed to understand the diversity of *T. gondii* on a global scale has shown that the population structure of *T. gondii* is more complex than thought originally. This study showed that type II and III are the two dominant *T. gondii* lineages [36]. Despite the dominance of different strains in different regions in the world, type II stands out as the most dominant lineage globally. Furthermore, type II *T. gondii* was found to be most dominant in Europe compared to other regions like Africa, North America or South America. Additionally, congenital toxoplasmosis cases in Europe were caused by type II *T. gondii* [124]. Therefore, it is critical to understand the origin and transmission pattern of the type II lineage to other regions of the world. In order to study this, 299 type II samples were collected globally and analyzed to study the diversity among these isolates. Furthermore, the current population structure suggests that the second most dominant lineage, distributed globally, is the type III lineage of *T. gondii* [36]. Based on the mouse virulence studies, almost all the strains belonging to the type III lineage are avirulent and infections often result in the establishment of chronic infection in mice. This could be one explanation for the global spread of type III *T. gondii*. However, despite its

global spread, type III strains are more dominant in South America, and therefore, would be interesting to investigate whether type III MRCA originated in South America, or if it is transmitted and recently expanded in this region.

Toxoplasma gondii is a wide spread pathogen capable of infecting almost all warm-blooded animals [1]. One-third of the human global population is infected with *T. gondii*. As *T. gondii* can be transmitted to humans via consumption of contaminated meat, the study of the seroprevalence rate among the wildlife is of importance [2]. Furthermore, the resistance of wildlife to *T. gondii* favors the selection of more virulent strains [27, 28, 30]. Even though the number of epidemiological studies is increasing, the reports for seroprevalence rate in wildlife from certain regions have been limited. To, therefore, study the infection rate of *T. gondii* and the genotypes circulating among wildlife from the southeastern region of United States, we collected 471 wildlife sera samples from six southeastern states namely, Tennessee, Kentucky, North Carolina, South Carolina, Alabama, and Georgia.

Even though several of the pathways discussed above that are known to control the bradyzoite differentiation/ maintenance have been studied, the master regulatory gene or pathway for developmental switching is still unknown. As described previously, studies have predicted the possible genes/enzymes that contribute to bradyzoite differentiation under different stress conditions such as NO, heat stress and nutrient starvation [95, 119, 122]. Although these studies add to the pool of knowledge on type switching, they fail to establish a cohesive representation of genes necessary for bradyzoite conversion pathways. Our current understanding of genes involved comes from studying the conversion of tachyzoites to bradyzoite stage of the parasite which fails to paint the complete picture. In order to detect the main regulatory gene(s) or pathway(s), it is undeniably important to study the interconversion process between the two stages. A recent study using cDNA microarray analysis of tachyzoite to bradyzoite conversion defective mutants (TBD), predicted the hierarchy of genes involved in bradyzoite development [125]. This study supported the notion that multiple bradyzoites inducing conditions and stresses should have a common pathway/genes leading to the stage switching of *Toxoplasma*. In order to accurately characterize the most important genes, our future study will focus on identifying the gene(s) that plays a major role in the conversion of tachyzoites to bradyzoites and vice versa. The strategy involves the use of the physiologically relevant NO donor drug, SNP as the stress model and the subsequent use of RNA sequencing at

different time points throughout the interconversion process *in vitro*. This study focused on establishing an experimental setup for converting tachyzoites into bradyzoites and vice versa under external stress conditions. Further use of this experimental strategy could lead to the identification of gene(s) that occupy the key position(s) in the bradyzoite development pathway. This could be done by comparison of upregulated/downregulated genes over the time during differentiation to bradyzoites. The reverse should be true when parasites revert to the tachyzoite stage of the life cycle.

CHAPTER 1: ON THE DETERMINATION OF *TOXOPLASMA GONDII* VIRULENCE IN MICE

This chapter is a publication by the same title published in *Experimental Parasitology*, 2017 by Pooja Saraf, E. Keats Shwab, Jitender P. Dubey, Chunlei Su and has been reproduced here with permission from the journal .

Pooja Saraf, M.S.; Elliot K Shwab, Ph.D.; Jitender P Dubey, Ph.D. On the determination of *Toxoplasma gondii* virulence in mice. 2017, *Experimental Parasitology*, 174: 25-30
PubMed PMID: 28153801.

My use of we in this chapter refers to my coauthors and myself. My primary contributions to this paper include (1) researching the topic and (2) writing of this review article.

1. Abstract

Toxoplasma gondii is one of the most successful pathogens on earth, capable of infecting an extremely broad range of mammals and birds and causing potentially fatal disease in humans. The house mouse (*Mus musculus*) has been used as the primary laboratory animal model for determining the virulence of *T. gondii* strains. Epidemiological evidence also suggests a potential association between virulence in mice and disease severity in human toxoplasmosis. However, many factors can affect virulence measurements, including route of infection, life stage of the parasite, number of passages of the parasite in mice or cell culture, and the mouse host line used. Variability among these factors makes it difficult to compare results between different studies in different laboratories. Here, we discuss important factors that should be considered when carrying out *T. gondii* murine virulence assays and propose a standardized methodology that should facilitate integration of *T. gondii* virulence data throughout the research community in future studies and thereby enable more efficient and effective analysis of genetic and virulence patterns for this important parasite.

2. Introduction

Toxoplasma gondii is an obligate intracellular parasite capable of infecting almost all warm-blooded animals, including humans [1]. Felids are the only known hosts, in which sexual reproduction of the parasite occurs, resulting in the dissemination of oocysts into the environment via defecation, and thus play a critical role in the transmission of *T. gondii*. It is estimated that one third of the world's human population is chronically infected with this parasite [1]. Humans acquire infection by ingesting oocysts from contaminated food and water, consuming undercooked meat containing tissue cysts, or by vertical transmission from mother to fetus [1, 2]. The primary infection in immunocompetent individuals is mostly asymptomatic but in some cases it can lead to ocular toxoplasmosis [3]. In immunocompromised individuals, such as AIDS patients, reactivation of chronic infection can cause life threatening encephalitis [1, 4].

Susceptibility to *T. gondii* infection varies among different hosts: cattle, horses, rats, Old World monkeys and humans are resistant to infection, while Australian marsupials and New World monkeys are much more susceptible. The reasons for such differences are not clear, but one reason may be a consequence of host-parasite co-evolution [1]. Laboratory mice are generally sensitive to *T. gondii* infection and are often used as the preferred animal model to determine the virulence of the parasite.

Virulence of *T. gondii* strains in mice varies with the genetic background of the parasites. Some genotypes of *T. gondii* are lethal to all strains of mice regardless of the dose of parasites administered, whereas other genotypes are non-lethal with a low dose of inoculation and can readily establish chronic infection in mice [10, 11, 124, 126, 127].

Recent analysis of *T. gondii* genetic diversity has revealed geographical patterns of genotype distribution [36]. Archetypal type II and type III strains are dominant in Europe and North Africa, while types II, III, and 12 are dominant in North America, and Chinese type 1 is most prevalent in East Asia. In contrast, *T. gondii* strains in South America are highly diverse with no clear dominance of any particular genotypes. The genotypes prevalent in Europe, North America, North Africa and Asia are non-lethal to mice at low infection dose, whereas a large proportion of *T. gondii* strains identified in South America are highly virulent and lethal to mice [126]. Population genetics and epidemiological studies have indicated a correlation between the geographic variations of *T. gondii* genotype and disease manifestation in humans. For example, severe symptoms associated with ocular toxoplasmosis are more frequently reported in Brazil

than in European countries [3, 128], and numerous incidences of severe systemic toxoplasmosis in immunocompetent adults from French Guiana have been reported, in some cases resulting in the deaths of the afflicted individuals [27, 28, 129]. Taken together, virulence of different *T. gondii* strains in mice appears to be generally correlated with disease manifestations in human cases [33]. Therefore, determination of *T. gondii* virulence in mice could be invaluable in predicting the potential outcome of human infections.

To obtain a better understanding of large-scale patterns of *T. gondii* virulence, it is essential to have a standardized methodology for parasite virulence determination which allows for direct comparison of the results obtained from different studies. Currently, a variety of mouse strains, different life cycle stages of the parasite, and different routes of inoculation are used in *T. gondii* virulence assays. This variation hinders meaningful comparisons and complicates integration of data. In order to address this issue, in this review we summarize the common methodologies used to determine *T. gondii* virulence in laboratory mice, and put forth a simple standardized methodology that will facilitate more productive comparisons for future studies. Establishment of a cohesive database for studying the relationship between *T. gondii* genotype and virulence in mice should greatly enhance our understanding of parasite virulence patterns and aid in predicting the outcome of *T. gondii* infection in humans.

3. Current methods for virulence assessment of *T. gondii* in mice

3.1 Virulence of tachyzoites, bradyzoites and oocysts of *T. gondii*

Toxoplasma gondii has a complex life cycle, and the specific stages of the parasite used for inoculation may lead to marked differences in the outcomes of virulence in mice. The three infectious forms of the parasite include: the rapidly dividing tachyzoite, responsible for systemic invasion during primary infection; the slowly growing bradyzoite, associated with chronic infection; and the sporozoite, sexually produced in mature oocysts. All three forms may be used to infect mice, but infection with different forms may have varied results in terms of virulence. For example, mice inoculated orally with a single oocyst of the strain M-7741 were found to exhibit 100% mortality after approximately two weeks, whereas 10^3 bradyzoite-containing tissue cysts were required to produce this same mortality rate, and mice infected orally with 10^4 tachyzoites failed even to establish infection [130]. Higher pathogenicity of oocysts is also

evident from a previous study in which mice infected orally with 10 oocysts died within two weeks but mice orally infected with 10 tissue cysts failed to cause any infection [131]. Among the three infectious stages of the parasite, oocysts in general are more virulent [130-132]. In addition, oocysts are environmentally resistant, highly infectious, and thus hazardous to work with, whereas tachyzoites and bradyzoites are readily killed even in water.

3.2 Change of *T. gondii* strain phenotypes after multiple passages in mice and cell culture

Changes in biological characteristics occur in *T. gondii* strains after passage in mice or cell culture [127, 133-137]. *Toxoplasma gondii* strain M-7741, initially isolated from a sheep in 1950, was found to have lost the capacity to produce oocysts in cats after 30-35 continuous passages in mice [134]. Similarly, after maintained in cell culture for 40 passages, the type I strain GT1 lost the ability to produce oocysts in cats [135]. The most commonly used *T. gondii* RH strain which was isolated from a six-year old child in 1939, has been found to no longer produce oocysts in cats following prolonged maintenance through passage in laboratory mice or cell culture, presumably due to unknown biological changes in parasites over time [137]. A variety of phenotypic changes among several RH-derived clonal lineages were also observed [127]. Differences among these lineages included larger plaque formation, enhanced survival outside the cells, faster growth, and decreased differentiation. Enhanced virulence in mice for *T. gondii* strains maintained in cell culture for several passages has been reported previously [138] In that study, 28 out of 31 original isolates from Costa Rica established asymptomatic infection in mice with only 3 isolates were highly virulent to mice. However, after five to ten passages, 7 of the 31 isolates were highly virulent and killed the mice [138]. Genetic variability among RH lineages in different laboratories has been reported [139]; however, the cause-effect relationship between genetic variations and phenotypes is unclear. Taken together, previous studies highlighted the phenomenon of phenotypic changes during continuous passages in mice or cell culture. Therefore any comparison of phenotypes among *T. gondii* isolates should be conducted using the original or low passage stocks.

3.3 Routes of infection and virulence of *T. gondii*

The three common routes in determining *T. gondii* virulence in mice include intraperitoneal (IP) injection, subcutaneous (SC) injection and per oral inoculation [130, 140]. The IP injection

involves direct deposition of *T. gondii* parasites into the peritoneal cavity, whereas SC injection deposits the parasites below the dermis. In oral infections, parasites are deposited into the stomach of mice. Differences in infectivity and pathogenicity in mice have been observed between IP and oral route of infections with *T. gondii* [130]. Mice infected orally with 10 tissue cysts failed to establish infection, whereas the IP infection resulted in successful infection in 33% of mice. In the same study, results for the SC inoculation were comparable to the IP injection [130]. Similar results were obtained in a separate study, wherein all mice orally infected with 100 tissue cysts of a *T. gondii* isolate survived, while all mice infected with same number of cysts by IP injection died an average of 12 days [131]. Another study showed that C57BL/6 mice were highly sensitive to oral infection, but resistant to IP injection [140]. As evident from these observations, the route of infection has an important effect on *T. gondii* virulence in mice and thus a standardized inoculation method is necessary in order for direct comparisons between different studies.

3.4 Variable susceptibility of different mouse lines to *T. gondii* infection

A high degree of variation in susceptibility to *T. gondii* has been observed among different lines of laboratory mice [140-143]. The DBA/1, DBA/2, BALB/c, C57B1/6J, B10.D2, SW/SIM and C3H/Bi mice showed markedly different outcomes when infected IP with tachyzoites of the C56 strain of *T. gondii* [141]. At a higher dosage ($1x10^5$ parasites), all DBA/2 and BALB/c mice died within 12 days of inoculation, all B10.D2 mice died within 18 days, and the other mouse strains showed mortality rates ranging from 60 to 90 percent at 30 days post infection. Outbred mice (SW/SIM) were considerably resistant, with only 67% mortality at day 30 [141]. Mouse-line dependent susceptibility was also observed in C57BL/6, LACA and BALB/c mice [140]. When 10 brain tissue cysts were used for IP inoculation, LACA mice were highly sensitive, whereas C57BL/6 mice were resistant. However, by oral infection, the LACA mice were resistant and the C57BL/6 mice were sensitive. BALB/c mice were resistant to both routes of infection [140]. A more recent study observed that when oocysts were administered orally, the ME49 strain of *T. gondii* was more pathogenic for transgenic (KO, HLA3.11, HLA2.1 and Hlab7), inbred (C57/black and BALB/c) and outbred (SW) mouse lines in decreasing order [143]. It has been demonstrated that BALB/c mice had a higher mortality rate than that of CBA/Ca mice during acute infection; however, the inverse was true for chronic infection [142](. McLeod and others

observed differences among the inbred mouse strains (C57BL/6 and A/J) infected per orally with 100 cysts of the ME49 strain [144]. C57BL/6 mice were found to be consistently susceptible, whereas A/J mice were resistant to infection. Susceptible mouse lines had a weakened gastrointestinal barrier, a lower splenic cytotoxic count, a weak IgM protective immune response and activation of Kupffer cells. To further investigate the differences between the aforementioned mouse lines (A/J and C57BL/6), McLeod and others studied the survival rates and brain cyst numbers of recombinant strains obtained by crossing the two lines, and found that survival was controlled by H-2 complex and a set of at least five genetic loci [145]. The H-2a haplotype was linked to resistance in a dominant manner, whereas the H-2b haplotype was found to be recessively associated with susceptibility. Mice with the H-2a haplotype were found to regulate the number of tissue cysts during chronic infection. Together, these studies demonstrate the strong effect that genetic variability of laboratory mouse lines has on their susceptibility to *T. gondii* infection. It illustrates the importance of selecting appropriate mouse lines with similar resistance when making virulence comparisons.

3.5 Methods of preparing tachyzoites for infection

Most studies on *T. gondii* virulence in laboratory mice have employed the tachyzoites due to its relative ease of preparation. The two most common ways of propagating tachyzoites include the use of mice and cell culture [1]. For the former, *T. gondii* bradyzoites, tachyzoites or sporozoites can be inoculated into the mice by IP injection. Within a week post infection, tachyzoites can be harvested from the peritoneal exudates in the abdominal cavities of mice [146]. *Toxoplasma gondii* strains that are highly virulent usually propagate well in mice and release a large amount of free tachyzoites within 3-7 days post infection. However, *T. gondii* strains of low virulence usually replicate slowly and free tachyzoites are difficult to obtain. To overcome this problem, it is advisable to administer 10 µg/ml dexamethasone phosphate in drinking water, which will suppress the murine immune response and facilitate tachyzoites production [1].

In laboratories with access to cell culture, *T. gondii* tachyzoites can be propagated and maintained readily. Tachyzoites can proliferate in almost any mammalian cell lines, with fibroblasts being the most commonly used [147]. Whether obtained from cell culture or mouse peritoneal exudate, tachyzoites will often be mixed with a large amount of host cells. Therefore, it is necessary to remove these cells and enrich tachyzoites. There are several methods to purify

tachyzoites, including density gradients, sonication and trypsin digestion, differential centrifugation, hemolysin digestion, filtration through glass wool, cellulose columns or sintered glass, and polycarbonate filtration [146]. With the exception of sonication and trypsin digestion, all of these methods are capable of removing greater than 90% of the mouse leukocytes without altering parasite viability [146]. Recently, a comparison of tachyzoite purification by trypsin digestion, filtration by 3-μm polycarbonate membrane, filtration by CF-11 cellulose, and separation by percoll solution showed similar results [148]. Therefore, tachyzoites prepared by trypsin digestion should be avoided for virulence testing in mice. In our laboratory, purification by filtration through a 3-μm polycarbonate membrane is routinely conducted due to its ease of use.

3.6 Dose-independent and dose-dependent mortality in mice

Earlier studies of mortality in mice have shown that some *T. gondii* strains such as those belonging to the type I lineage are highly virulent to mice and kill all infected mice within two weeks after infection regardless of inoculation dosage [10, 149]. For these acutely virulent *T. gondii* strains, a single viable parasite is lethal to mice; therefore the mortality is dose-independent. In contrast, the majority of *T. gondii* strains can readily establish chronic infection in mice if inoculated at low dosages, and mortality in mice is dose-dependent. The type II and III *T. gondii* lineages belong to this group [10, 12, 149, 150]. Therefore, to accurately determine the virulence of *T. gondii* strains, it is necessary to test a series of parasite concentrations ranging from low to high inoculation doses. Any single dose inoculation will not be able to appropriately assess the virulence of a particular *T. gondii* strain.

4. Proposed methodology for virulence assessment of *T. gondii* in mice

Virulence of *T. gondii* strains has been commonly defined by the mortality rate in laboratory mice. However, due to the lack of a standard method, comparing results among published reports is challenging. To make measurements of virulence of *T. gondii* in mice comparable across different studies, it is imperative to establish a standardized method. Examination of the cumulative mortality in mice infected via IP injection with serial dosages of tachyzoites is arguably the most suitable method for accomplishing this goal. This method has been previously used for the quantitative trait locus (QTL) analysis of *T. gondii* virulence determinants using

genetic crosses that led to the discovery of the major parasite virulence factor ROP18 [13, 14, 151].

Here we propose a standard protocol to determine *T. gondii* virulence in mice (**Figure 2.1**):

(1). Propagate low-passage *T. gondii* strains in mice or cell culture. If propagating in mice, administer 10 μg/ml dexamethasone in drinking water starting two days before inoculating the parasites to suppress mouse immune response.

(2). Collect tachyzoites from a two-day passage of cell culture or from the peritoneal cavity of infected mice within 3-7 days of infection. Purify tachyzoites by filtering through a 3-μm polycarbonate filter.

(3). Collect the parasites in 15-ml centrifuge tube, centrifuge at 1000**x** g for 10 min at room temperature. Discard supernatant and resuspend the parasites in 5 ml phosphate-buffered saline (PBS).

(4). Determine the concentration of tachyzoites using a hemocytometer.

(5). Prepare ten-fold serial dilutions of $2x10^4$ to $2x10^1$ tachyzoites/ml using PBS.

(6). IP or SC inoculate 500 μl of parasites to 5 outbred mice (CD-1, CF-1 or Swiss-Webster) for each dilution. Observe mice daily for four weeks. Euthanize severely ill mice.

(7). Record daily mortality. For those mice survived the infection for 4 weeks, collect blood samples and determine seroconversion by modified agglutination test (MAT) test. The cutoff value for positive infection is 1:25.

(8). Determine cumulative mortality in mice. To calculate cumulative mortality, three sequential inoculation dosages should be analyzed; with the lowest dose resulting in only partial infection of the mice (**Table 2.1** and **Table 2.2**).

Below is the suggested protocol for the MAT test. It is slightly modified from previously published protocol [1, 152].

(1). Materials and reagents needed are: 1x PBS pH 7.2; alkaline buffer [7.02 g NaCl, 3.09 g boric acid, 24 ml of 1N NaOH, 4 g of bovine plasma albumin in 1 liter of distilled water, adjust pH to 8.7, add 1 g sodium azide (0.1% final) as a preservative, final pH 8.2-8.4]; *Toxoplasma* whole cell antigen (RH strain prepared in cell culture, formalin-fixed, $2x10^8$ tachyzoites/ml); 2-mercaptoethanol; Evans blue dye (2 mg/ml in H_2O); 96-well U-bottom microtiter plate; positive serum control (1:200 titer); and negative serum control.

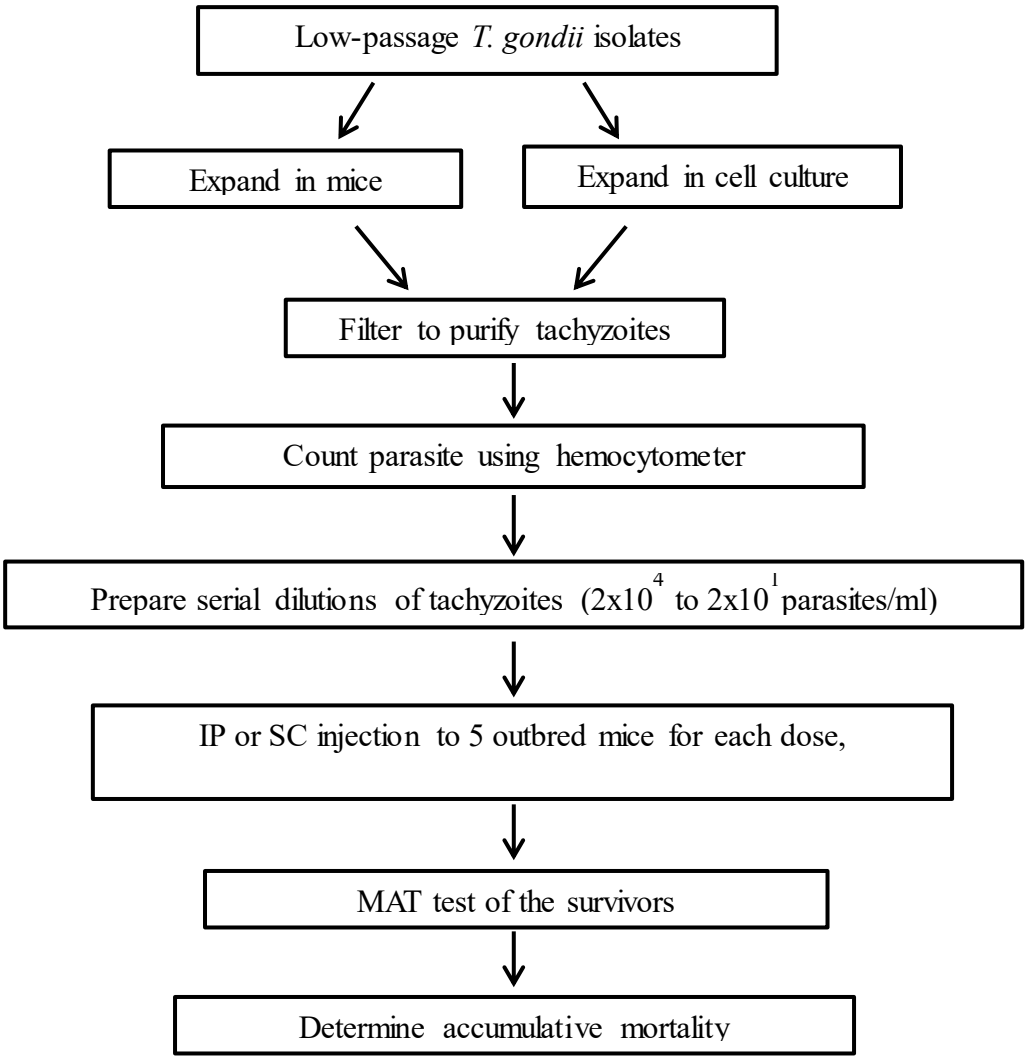


Figure 2.1: Proposed methodology to test virulence of *T. gondii* strains in mice.

Table 2.1: Calculation of cumulative mortality rate (Example 1).

Dosages	Mice survived	Mice seronegative	Mice seropositive	No. mice died	No. mice infected
$1x10^4$	1	0	1	4	5
$1x10^3$	3	0	3	2	5
$1x10^2$	4	2	2	1	3
$1x10^1$	5	5	0	0	0

The number of mice injected per group is 5.

The dosages $1x10^4$, $1x10^3$, and $1x10^2$ will be included for calculation. The cumulative mortality is calculated based on the number of mice died divided by the number of mice infected. The number of infected mice includes mice that have died and surviving mice that are MAT test positive. The cumulative mortality for this example is (4+2+1) / (5+5+3) = 7/13 = 54%.

Table 2.2: Calculation of cumulative mortality rate (Example 2).

Dosages	Mice survived	Mice seronegative	Mice seropositive	No. mice died	No. mice infected
$1x10^4$	0	0	0	5	5
$1x10^3$	0	0	0	5	5
$1x10^2$	2	1	1	3	4
$1x10^1$	4	3	1	1	2

The number of mice injected per group is 5.

The dosages $1x10^3$, $1x10^2$, and $1x10^1$ are included for calculation. The cumulative mortality is (5+3+1) / (5+4+2) = 9/11 = 82%.

(2). Mix 3.0 µl of serum sample with 72.0 µl PBS (1:25 dilution).

(3). Transfer 50 µl of diluted serum samples to the first row a U-bottom 96-well microtiter plate (10 samples, plus one negative and one positive controls).

(4). Add 25 µl of PBS to the rest of wells using multichannel pipette.

(5). Make serial dilution to 1:3200 for each serum samples including the negative and positive controls. Discard the last 25 µl of diluted serum samples.

(6). Prepare antigen mixture (for each 96-well plate) by mixing the following reagents: 2.5 ml alkaline buffer, 35 µl 2-mercaptoethanol, 50 µl Evans blue dye (2 mg/ml in H_2O), and 150 µl *T. gondii* MAT antigen.

(7). Mix antigen well by pipetting, immediately transfer 25 µl to each well using multichannel pipette.

(8). Cover the plate with sealing tape and incubate at 37°C for 16 hours (overnight) Read results. A pellet at the bottom of the well means negative. Samples without pellets are positive.

5. Future perspective

In recent years, two highly polymorphic rhoptry proteins (ROP18 and ROP5) have been identified as the major virulence determinants in *T. gondii* [13-15, 22]. Together, the ROP18 and ROP 5 play a role in the suppression of host innate immune response via formation of a complex with murine immunity related GTPases, preventing destruction of the parasitophorous vacuole [15, 21, 22]. Recently, using PCR-RFLP technique, it was shown that ROP18 and ROP5 allele types are associated with the virulence of *T. gondii* in mice [126]. Therefore, we suggest performing the above *in vivo* virulence test in mice and the genotyping test of ROP18 and ROP5 in parallel in the future. Over time, this will accumulate a large data set to further evaluate the strength of the association between virulence in mice with the genotypes of ROP18 and ROP5 genes. If a strong association is confirmed, then genotyping of these genes can be used as a simple test to predict *T. gondii* virulence in the future.

CHAPTER 2: TO REVEAL THE MOST RECENT ANCESTOR AND TRANSMISSION PATTERN OF TYPE II LINEAGE OF *TOXOPLASMA GONDII*

E. Keats Shwab[a], P. Saraf[a], Xing-Quan Zhu[b], Dong-Hui Zhou[b], Brent M. McFerrin[c], Daniel Ajzenberg[d], Gereon Schares[e], Kenneth Hammond-Aryee[f], Steve Higgins[a], Richard Gerhold[g], Benjamin M. Rosenthal[h], Xiaopeng Zhao[c],1, J.P. Dubey[h],1, Chunlei Sua,[c],1

a. Department of Microbiology, The University of Tennessee, Knoxville, TN 37996. USA;

b. State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province 730046, P.R. China;

c. Department of Mechanical, Aerospace, and Biomedical Engineering, The University of Tennessee, Knoxville, TN 37996. USA;

d. Service de Parasitologie-Mycologie, CHU Dupuytren, 87042 Limoges, France;

e. Friedrich Loeffler Institute, Institute of Epidemiology, Greifswald, Mecklenburg-Vorpommern, Germany;

f. Department of Biomedical Sciences, Faculty of Health Sciences, Stellenbosch University, Tygerberg 7505, South Africa;

g. Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996. USA;

h. Animal Parasitic Diseases Laboratory, United States Department of Agriculture, Beltsville, MD 20705, USA

1.Abstract

Toxoplasma gondii is one of the most successful pathogens on earth, capable of infecting all mammals and birds. Recent population genetics studies in *T. gondii* revealed low diversity in North America, Europe, Africa and Asia but high diversity in South America. Among the many genotypes identified so far, the type II lineage is widespread and dominant in Europe. To elucidate the most recent ancestor (MRCA) of type II and to study its transmission pattern, we genotyped 296 type II isolates using 15 microsatellite markers. Our results showed that the type

II strains from Europe have the highest diversity, followed by those from Africa, South America, and North America. Type II isolates from North America are largely clustered together to the tip of the phylogenetic tree. These results suggest that the MRCA of type II *T. gondii* may have originated from Old World (Europe or Africa), and expanded and transmitted globally in the past several hundred years.

2. Introduction

Toxoplasma gondii is the most successful zoonotic pathogen known today [1]. It has a complex life cycle that propagates sexually in a definitive host (cats) and asexually in the intermediate host (mammals and birds) [1]. Cats contaminate the environment by shedding *T. gondii* oocysts in the feces. Both intermediate and definitive hosts can acquire the infection by oral ingestion of oocysts from the environment, oral ingestion of tissue cysts in contaminated meat from infected animals or by transplacental transmission via tachyzoites. One-third of people are chronically infected worldwide [1, 153]. Infection in humans can cause ocular, congenital, or severe acute disseminated toxoplasmosis. In immunocompromised patients, reactivation of latent infection can cause life-threatening encephalitis [4].

Early genotyping studies on 106 isolates from Europe and North America using 6 PCR-RFLP markers divided *T. gondii* isolates into three clonal types denoted as types I, II and III. The three archetypical lineages of *Toxoplasma* showed variability in mouse virulence [10, 11]. Isolates belonging to type I were found to be highly virulent causing mortality during the acute phase of infection even at very low doses [10, 12]. Type II and III strains cause chronic infections at lower dosages. However, type II strains at moderate dosages can cause mortality in mice [11]. The most recent data on diversity structure of *Toxoplasma* have been obtained from a study carried on 1457 isolates using 10 PCR-RFLP markers. A total of 189 haplotypes were found from 1457 isolates of *T. gondii* collected worldwide. Use of these markers revealed that the type II strain is most dominant worldwide and comprises of 64.1% of the *T. gondii* population in Europe compared to 45.4% and 43.9% in Egypt and North America respectively [36]. Previously, type II has also been linked with congenital toxoplasmosis in Europe [124]. Even though type II is the most dominant clonal lineage of *T. gondii* worldwide, little is known about its origin or transmission.

Based on use of 10 PCR-RFLP markers all the strains included in this study are considered to be type II with little genetic variability. However, use of markers designed for microsatellite regions gives a higher discriminatory power to differentiate isolates belonging to the same lineage. Microsatellite regions are found in eukaryotes with a mutation rate of 10^{-2} to $10^{-5,}$ which is 1000 times higher than the SNPs. The MS regions undergo length polymorphism as a result of strand slippage process during DNA replication. The number of repeat units differs due to addition or deletion, thus creating multiple alleles at an MS locus [154]. In this study, we utilized 15 previously designed microsatellite markers to obtain a better genetic differentiation among the 299 type II isolates available from around the world [155]. In this work, we attempted to comprehend the diversity of type II *T. gondii* strains and track its transmission on a global scale. The genetic variability among the type II isolates collected from different geographical regions can shed some light on the origin and the transmission pattern of the type II clonal lineage of *T. gondii*. We also discuss the factors such as agriculture and European expansion that may have played a role in the transmission and expansion of type II in the last few hundred years.

3. Materials and Methods

DNA samples

DNA samples were collected from previous studies in our laboratory **(Table 3.2 and Table 3.2)** [36]. Information about the location and host for these isolates were obtained from previously published papers. DNA for all the isolates was either obtained from heart or brain tissues of experimentally infected mice or tissue culture. Ethiopian feral cat isolates (n=14) included in our study were collected from feral cats from Addis Ababa region in Ethiopia [156]. Egyptian feral cat samples (n=26) were also isolated from Abourawash, Giza, Egypt [157]. Egypt dog samples (n=8) were obtained from stray dogs supplied by a contractor from the region of Abourawash, Giza, Egypt [158]. One Mali chicken sample was obtained from an infected chicken collected from Mali [159]. Isolates from Norway fox (n=6) from the continent of Europe were obtained from an arctic fox (*Vulpes lagopus*) population from the high arctic archipelago of Svalbard[160]. Samples from Austria (n=30), Portugal (n=4) and Israel (n=4) included in our study were obtained from free range/backyard chickens [161]. *T. gondii* isolates from pigs (n=16) were all derived from adult pigs (sows) from Iowa [162]. Samples collected from Maryland (n=19) were

isolated from lambs from a slaughter house in Baltimore, Maryland [163]. Lambs were between 6 to 12 months old and were raised in Maryland, Virginia and West Virginia. One sample from New Jersey was obtained from a white-tailed deer during the 2011-2012 hunting season. Samples acquired from Brazil (n=2) were collected from sheep from two slaughter houses in Botucatu region, Sao Paulo state [164]. *T. gondii* DNA samples obtained from Chile (n=16) were collected from naturally infected chicken from rural farms spread around 85 different properties [165]. Four samples (PSC, 3440, FUN and CHA) included in this study were obtained from different AIDS patients with acute toxoplasmic encephalitis [11]. Two samples (DEG and Tg132) were obtained from non-AIDS- related human infections [11]. One sample was isolated from sheep from the Huzhu city of Qinghai province of China [166]. The information of hosts and location for previously unpublished samples are included in the study **(Table 3.2 and Table 3.3).**

Genotyping with MS markers

Genotyping of *T. gondii* isolates was performed using 15 MS markers located on 11 different chromosomes in a single multiplex PCR reaction [155]. Primers were used at the working concentration of 29.4 µM, except primers B18, N82, AA, and XI.I. Markers B18 and N82 had more PCR products than other markers; therefore, the concentration was reduced to 17.64 µM. Alternatively, markers AA and XI.1 had significantly less PCR product and so the concentration in the multiplex mix was increased to 82.3 µM and 58.82 µM respectively. The microsatellite sequences were amplified by multiplex PCR carried out in 25 µl reactions using the reagents provided in a Qiagen multiplex PCR kit in 96 well plates. Non-type II reference strains included 5 isolates; GT1 (type I, ToxoDB PCR-RFLP genotype #10), CTG (type III, ToxoDB genotype #2), MAS (ToxoDB genotype #17), TgCgCa1 (ToxoDB genotype #66) and TgCatBr5 (ToxoDB genotype #19). A PCR reaction without DNA was used as a negative control to check for any DNA contamination during the procedure. PCR products were diluted in deionized formamide and the dilution factor was optimized based on the quantity of PCR product estimated from running a 2.5% agarose gel. 1µl of each diluted PCR product was mixed with 0.5 µl of a dye-labeled internal size standard (ROX 500; Applied Bio-systems) and deionized formamide [155]. This mixture was later denatured and electrophoresed using an automatic sequencer (3730 DNA analyzer; Applied Bio-systems). The size of the base pairs was determined using Peak-Scanner analysis software (version 2.0; Applied Bio-systems).

Data analysis

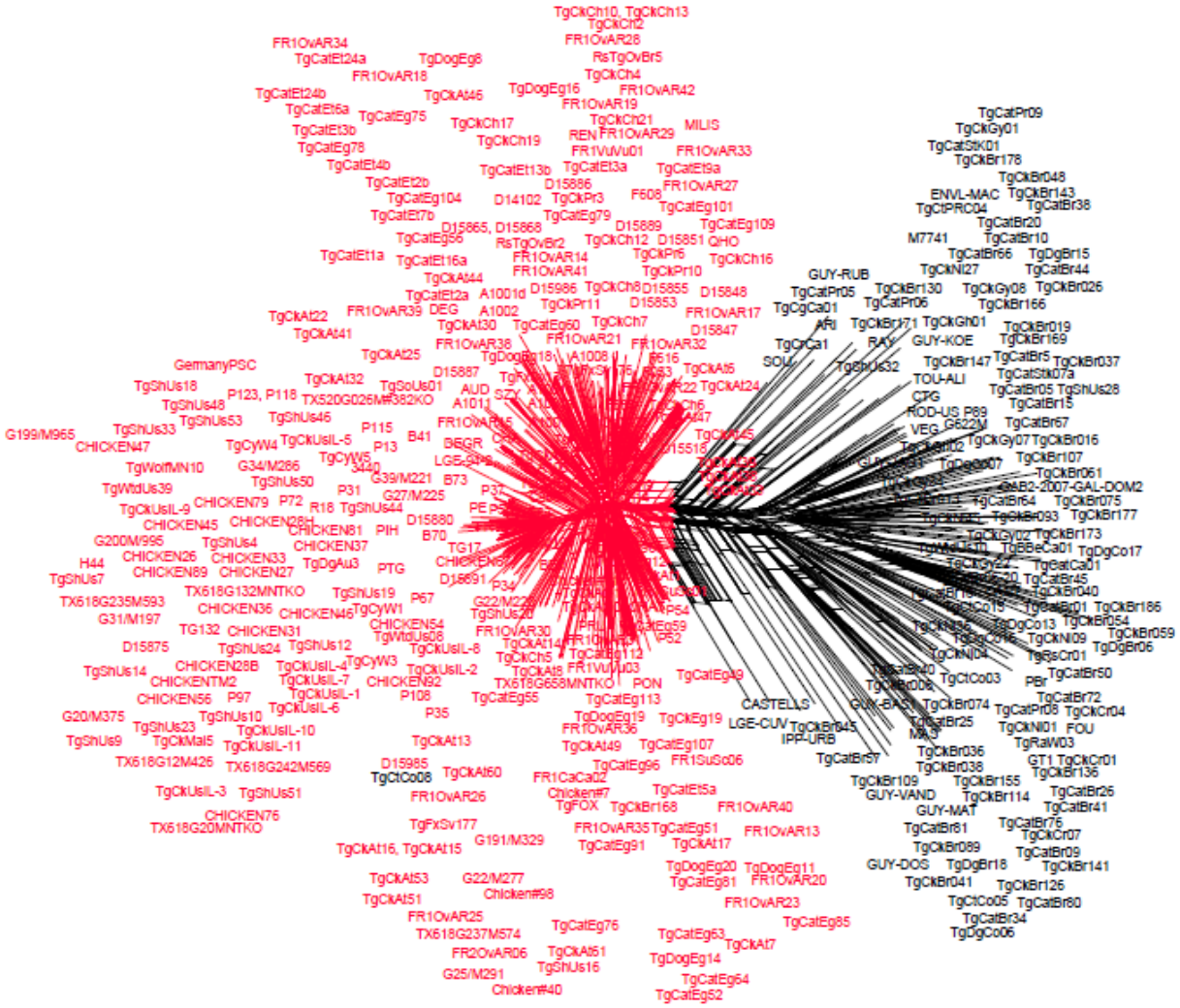
The multilocus microsatellite typing data was coded for all genetic loci. For a given locus, the DNA banding pattern was coded with a string of 1s and 0s. Phylogenetic network and neighbor-joining tree were generated by SplitsTree 4.8 [167]. A network tree was generated for 433 samples that included 296 type II and 139 non-type II samples collected from different regions of the world **(Table 3.2).** Additionally, a rooted NJ tree for 301 samples (296 type II + 5 non type II as outgroup) was constructed using Fig tree v1.4.2 (Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh, 2006) **(Table 3.3).** Basic statistics for quantitative gene diversity within populations were calculated using Arlequin v 3.5 [168] **(Table 3.4).**

4. Results

In the phylogenetic network tree generated for 435 samples, type II samples (n=296) were found to be clustered together. Non-type II samples (n=139) were found clustered with longer branch lengths **(Table 3.1 and Figure 3.1).** A total of 296 type II *T. gondii* isolates were analyzed by 15 Microsatellite markers (**Table 3.3**). These samples were divided into five regional groups, including Africa (n=59), Europe (n=116), North America (n=99), South America (n=18), and others (n=4). The majority of these samples were reported in previous publications **(Table 3.3 and Figure 3.2)** [36]. Based on PCR-RFLP typing results, all 296 samples belong to the Toxo DB genotype #1 or # 3 that together are considered to be type II. Five non-type II samples; including GT1 (type I), CTG (type III), MAS, TgCrCa1 and TgCatBr5 (atypical) were included as an outgroup for phylogenetic analysis.

In total, 205 unique microsatellite (MS) types were identified among the 296 type II samples **(Table 3.3).** This includes 90 MS genotypes from 116 European isolates, 43 MS genotypes from 59 African samples, 2 MS genotypes from 4 Asian isolates, 59 MS genotypes from 99 North America isolates, and 11 MS genotypes from 18 South America isolates. Overall, samples from the same geographical region appear to cluster together.

The branch length for the European type II isolates appeared to be much longer in comparison to isolates from other regions (**Figure 3.2**). Four samples collected from Germany (D15875, D15891, 3440 and PSC) were found to be quite different from other isolates from the



Color	Type of *T. gondii*
Red	Type II (n=296)
Black	Non-type II (n=139)

Figure 3.1: Phylogenetic network tree constructed for 435 isolates of *T. gondii* using Splits tree v 4.3.1.

Figure 3.2: Rooted tree for 285 isolated of *T. gondii* analyzed with 15 microsatellite markers constructed using Fig tree v1.4.2.

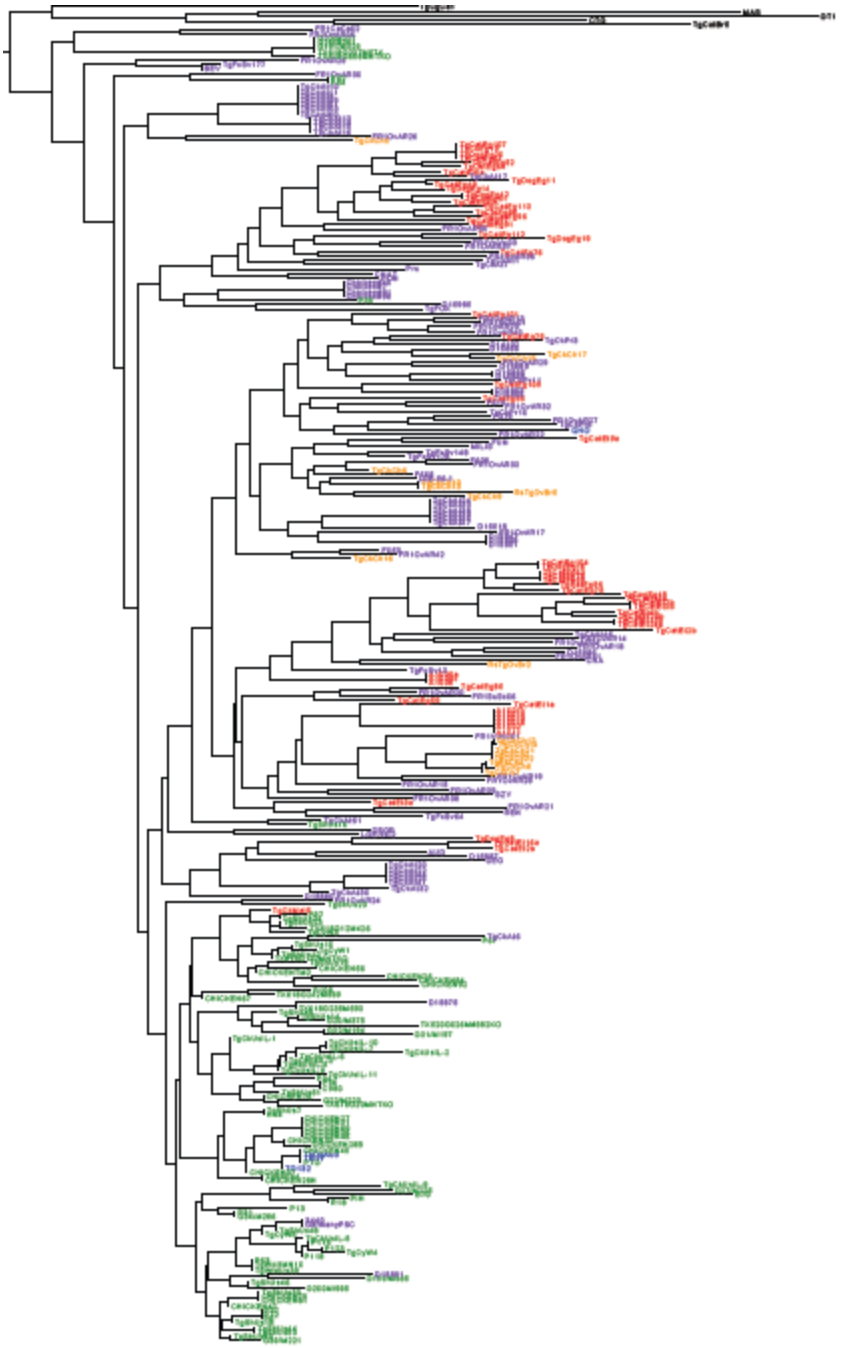


Figure 3.2 (continued)

Color	Region
Purple	Europe (n=116)
Green	North America (n=99)
Orange	South America (n=18)
Red	Africa (n=59)
Blue	Others (n=4)
Black	Non-type II (n=5)

Figure 3.2 (continued)

Table 3.1: Location and frequency of samples for 296 types II *T. gondii* isolates.

Region of *T. gondii* isolate	Number of Samples
Ethiopia	14
Egypt	35
Mali	1
South Africa	9
France	43
Austria	30
Portugal	13
Belgium	9
Norway	7
Israel	4
Romania	2
Germany	19
England	1
UK	1
Iowa	16
NewEngland	20
Maryland	19

Table 3.1 (continued)

Region of *T. gondii* isolate	**Number of Samples**
Illinois	11
Cleaveland	19
USA	10
Minnesota	5
Wisconsin	4
Grenada	2
Colorado	1
Chile	16
Brazil	2
China	1
Japan	2
Australia	1

European continent. These samples appeared to be separated from the European cluster and were embedded into the cluster of North American isolates.

In Africa, a total of 59 samples had 43 unique genotypes. The branch lengths appeared to be longer when compared to North American isolates. Samples from North Africa (Ethiopia and Egypt) were found to be quite diverse and were distributed within the European isolates. On the other hand, isolates from South Africa were found to be clustered together (**Figure 3.2**). Overall the diversity of samples from Africa was comparable to European isolates.

In North America there appeared to be a high degree of genetic uniformity. Out of 99 samples obtained, 59 unique genotypes were identified. Despite the diverse sampling of isolates, branch length appeared to be shorter and samples were clustered together forming an ingroup within the old world isolates of Europe and Africa. A few isolates from Cleveland (G25/M291, G22/M277, G191/M329, TX618G237M574, and TX618G658MNTKO) and Iowa (P35, P52 and P54) were separated from the North American cluster and were emebedded within European isolates **(Figure 3.2).**

Even though the available South American isolates were limited (n=18), the diversity was reasonably high, with 11 unique genotypes identified. Samples from Chile and Brazil were also found to be rooted within Old World isolates but did not belong to one cluster. Rather, isolates were divided into several groups dispersed within the Old World samples **(Figure 3.2).**

The diversity within Type II *T. gondii* populations from different geographical areas was quantified using Arlequin v 3.5 [168]. The results are summarized in (**Table 3.4)**. The gene diversity observed among the four populations from high to low was in the following order; Europe > Africa > South America > North America.

5. Conclusions

The compiled microsatellite genotyping results for 296 isolates revealed high genetic diversity for isolates from Europe and Africa in comparison to the New World isolates from North and South America **(Figure 3.2 and Table 3.3).** In addition, all type II strains from North and South America shared common relatives towards the Old World isolates. Thus, our findings indicate that the most common recent ancestor (MCRA) of the type II *T. gondii* may have originated from the Old World (Europe and Africa) and likely to have transmitted to the New World (North and South America) from the Old World. Understanding the origin and transmission of the type II

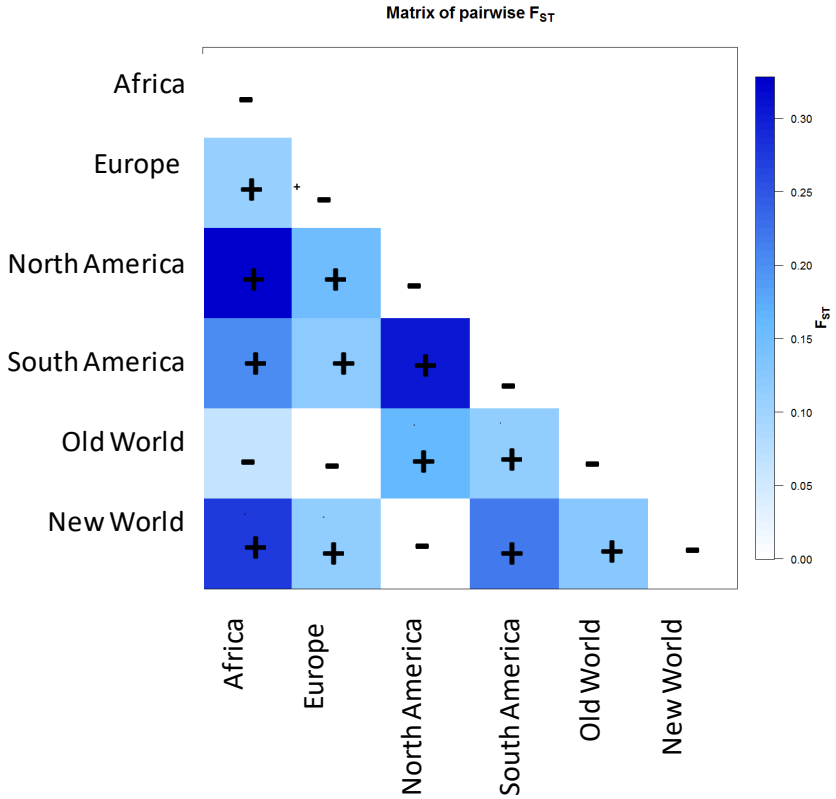
lineage is of interest as it is most dominant lineage spread globally, and knowledge gathered from the study on this lineage may provide information regarding the spread of other zoonotic diseases.

In the network tree generated for 433 isolates, type II samples clustered at one end of the phylogenetic tree with smaller branch lengths, indicating that type II samples may have experienced recent expansion **(Figure 3.1).** A large number of unique MS genotypes (n=90) were found among type II samples (n=116) collected from diverse locations in Europe. Further, the phylogenetic rooted tree for the type II samples showed deeper branch lengths for the European isolates and situates European samples in an ancestral position **(Table 3.3 and Figure 3.2).** This suggests that type II isolates from Europe are older. Moreover, quantitative comparison of genetic diversity among the four populations also revealed an overall higher (0.3686 ± 0.1975) diversity for the European isolates **(Table 3.4).** Similar to the European isolates, the branch length and average gene diversity per locus (0.3504± 0.1903) was found to be high for African isolates. Together, these results suggest that the MRCA for type II *T. gondii* may have originated in the Old World, comprised of Europe and Africa (average gene diversity of 0.3775 ± 0.2012). In addition to the shorter branch lengths and lower gene diversity (0.2632 ± 0.1469), the majority of isolates from North and South America appeared to be embedded within the Old World isolates suggesting that type II may have transmitted from the Old World continents to the New World of America. Several major factors could have played a role in the transmission of type II lineage. Agriculture began approximately 11,000 years ago in the fertile crescent area near Europe and Africa [169]. Agriculture as an occupation conferred enormous advantages to farmers, and this resulted in expansion and dispersal of the farming population [170]. This is also reflected in a sudden rise in the human population during this period. Agriculture leads to an increase in the availability of grain crops that attract house mice and cats, which in turn increases their population density and proximity humans [171, 172]. Altogether, the advent of agriculture could have served as a factor in the expansion of *T. gondii* in Europe and Africa. Furthermore, European expansion (approximately within the last 500 years) may have introduced type II to the new world via maritime trading of goods, transportation of pets (including cats), slave trade routes, or accidental transport of infected rodents from Europe [173].

Table 3.4: Basic statistics for type II *T. gondii* populations from different geographical regions.

	Europe	Africa	North America	South/Central America	Old World	New World
Number of isolates	116	59	99	18	175	117
Number of MS genotypes	90	43	59	11	133	70
Average Gene diversity	0.3686 ±0.1975	0.3504 ±0.1903	0.2193 ± 0.1259	0.2641 ± 0.1544	0.3775 ± 0.2012	0.2632 ± 0.1469

Due to small sample size of only four isolates; samples from Japan (2), China (1), and Australia (1) were not included in analysis.



The genetically clonal population of type II isolates in North America could also be attributed to the 'Founder Effect'. A change in landscape owing to the encroachment of forest areas for the purpose of farming leads to the replacement of wild animals by domestic animals [173]. This combined with the agricultural revolution may have led to the expansion of a limited number of successful strains from Europe, eventually reducing their diversity. In contrast to North American isolates, which cluster together, isolates from South America appeared to be spread within the Old World cluster and showed a higher gene diversity over loci. This suggests that transmission of isolates to South America from the Old World may have occurred several times independently. From the limited number of samples available from South America for this study, the majority were derived from Chile. The west coast of Chile is encompassed by the Pacific Ocean which serves as the major body of water used for the transportation of goods [36]. The maritime transport and travel between the continents could have served as the medium for multiple events of type II *T. gondii* transmission from Europe and could also explain the high diversity of isolates seen in South America [174]. Another possible explanation could be the dominance of wild animals in areas like the Amazon forest which serves as the hotspot for the diversity of *T. gondii* [175]. The expansion of human populated areas could result in a spillover of diverse type II strains from wildlife species leading to higher recombination rates and genetically diverse populations. However, the limitation of our samples to human environments fails to support the latter hypothesis. Better sampling from wildlife areas, as well as human-dominated areas, is required in the future to draw a better conclusion for the higher diversity observed in this region.

6. Future Perspectives

Our current study reveals that the MRCA for type II *T. gondii* exists in the Old World and that type II is likely to have transmitted to the New World (America) from the Old World (Europe and Africa). However, little is known regarding the origin of other clonal lineages (type I and III). Thus, it will be interesting to further study the origin for other types of *T. gondii* and determine whether the transmission pattern appears to follow the pattern of type II clonal lineage. Based on our study, agriculture could be predicted as the main cause of transmission; however, our understanding of the role of migratory birds and animals is limited. In some regions like Brazil, pigeons can be infected with the parasite on a large scale and may thus play a

critical role in the transmission in regions with large populations of the migratory birds. Other species like sea otters and dolphins are also known to be infected and may play a role in the transmission of *T. gondii* as well [219]. Therefore, in order to obtain a deeper understanding of other factors, sampling from different hosts is necessary. This will also lead to a better understanding of the transmission and evolution of other zoonotic pathogens. A recent study by Mercier showed that human environments play a role in decreasing the diversity of *T. gondii* isolates [175]. As the human population continues to rise, it will be fascinating to unearth and predict the diversity structure of *T. gondii* in the coming future. The current global diversity structure of *T. gondii* shows the dominance of certain types in different pockets of the world. It will be of interest to unravel the factors behind this dominance in order to gain a better insight about the important host or players in different regions of the world. In the future, this knowledge will aid in controlling the spread of *T .gondii* in different regions of the world in the event of an epidemic.

CHAPTER 3: TO REVEAL GLOBAL TRANSMISSION OF TYPE III *TOXOPLASMA GONDII* LINEAGE

Pooja Saraf[a], J.P.Dubey[b] and Chunlei Su[a]

a. Department of Microbiology, The University of Tennessee, Knoxville, TN 37996. USA.

b. Animal Parasitic Diseases Laboratory, United States Department of Agriculture, Beltsville, MD 20705, USA

1.Abstract

Toxoplasma gondii is one of the major zoonotic pathogens infecting a third of the world population. Previous studies have shown that population of *T. gondii* in northern hemisphere is clonal, whereas many genotypes co-exist in the southern hemisphere. Despite the diversity in South America, a large proportion of genotypes belong to type III lineage of *T. gondii*. Thus, to unveil the origin and transmission pattern of type III lineage, genotyping for 15 microsatellite markers was carried out for a total of 128 isolates. Furthermore, to study the expansion time, Microsatellite (MS) data for type II and type III (n=437) was analyzed. Out study revealed that type III lineage most recent common ancestor (MRCA) belongs to the New World and may have disseminated from the New World (America) to the Old World (Europe and Africa).

2. Introduction

The current population structure suggests that type III lineage of *T. gondii* is the second most dominant lineage found globally [36]. Based on the mouse virulence studies, almost all the strains belonging to type III lineage are avirulent and infections often result in establishing a chronic infection in mice [10, 12]. Previous studies showed that type III lineage is wide spread and frequently isolated in South America, which is in striking contrast to the most dominant type II lineage. Thus, it would be interesting to investigate global transmission pattern of type III lineage, and potential factors that facilitate its transmission.

3. Materials and Methods

DNA samples for MS analysis

DNA samples were collected from previous studies in our laboratory [36]. Location and host information for these isolates were obtained from previously published papers **(Table 4.1)**. DNA for all the isolates was either obtained from the heart or brain tissues of experimentally infected mice or cell culture. A total of 137 type III isolates were collected from 13 mammalian species; including badger, bear, cat, chicken, dog, deer, dove, goat, goose, human, mongoose, sheep and white-tailed deer. The isolates belong to five regions; including Africa(n=48), Europe(n=7), North America(n=57), South America(n=16), Central America(n=9).

Genotyping using 15 MS markers

Genotyping of *T. gondii* isolates was performed using 15 MS markers located on 11 different chromosomes in a single multiplex PCR reaction [155]. Primers were used at the working concentration of 29.4 μM, except primers B18, N82, AA, and XI.I. Markers B18 and N82 had more PCR product than other markers; therefore, the amount was reduced to 17.64 μM. On the other hand markers AA and XI.1 had less PCR product and therefore their concentration in the multiplex mix was increased to 82.3 μM and 58.82 μM respectively. The microsatellite sequences were amplified by multiplex PCR carried out in 25 μl reactions using the reagents provided in a Qiagen multiplex PCR kit in 96 well plates. Non-type III reference strains included 5 isolates; GT1 (ToxoDB PCR-RFLP genotype #10, type I), PTG (ToxoDB genotype #1, type II), MAS (ToxoDB genotype #17), TgCgCa1 (ToxoDB genotype #66) and TgCatBr5 (ToxoDB genotype

#19). A PCR reaction without any DNA was used as a negative control to check for any DNA contamination. PCR products were diluted in deionized formamide and the dilution factor was optimized based on the quantification of the PCR products estimated from running a 2.5% Agarose gel. 1 µl of each diluted PCR product was mixed with 0.5 µl of a dye-labeled internal size standard (ROX 500; Applied Bio-systems) and deionized formamide. This mixture was later denatured and electrophoresed using an automatic sequencer (3730 DNA analyzer; Applied Bio-systems). The size of the base pairs was determined using Peak-Scanner analysis software (version 2.0; Applied Bio-systems).

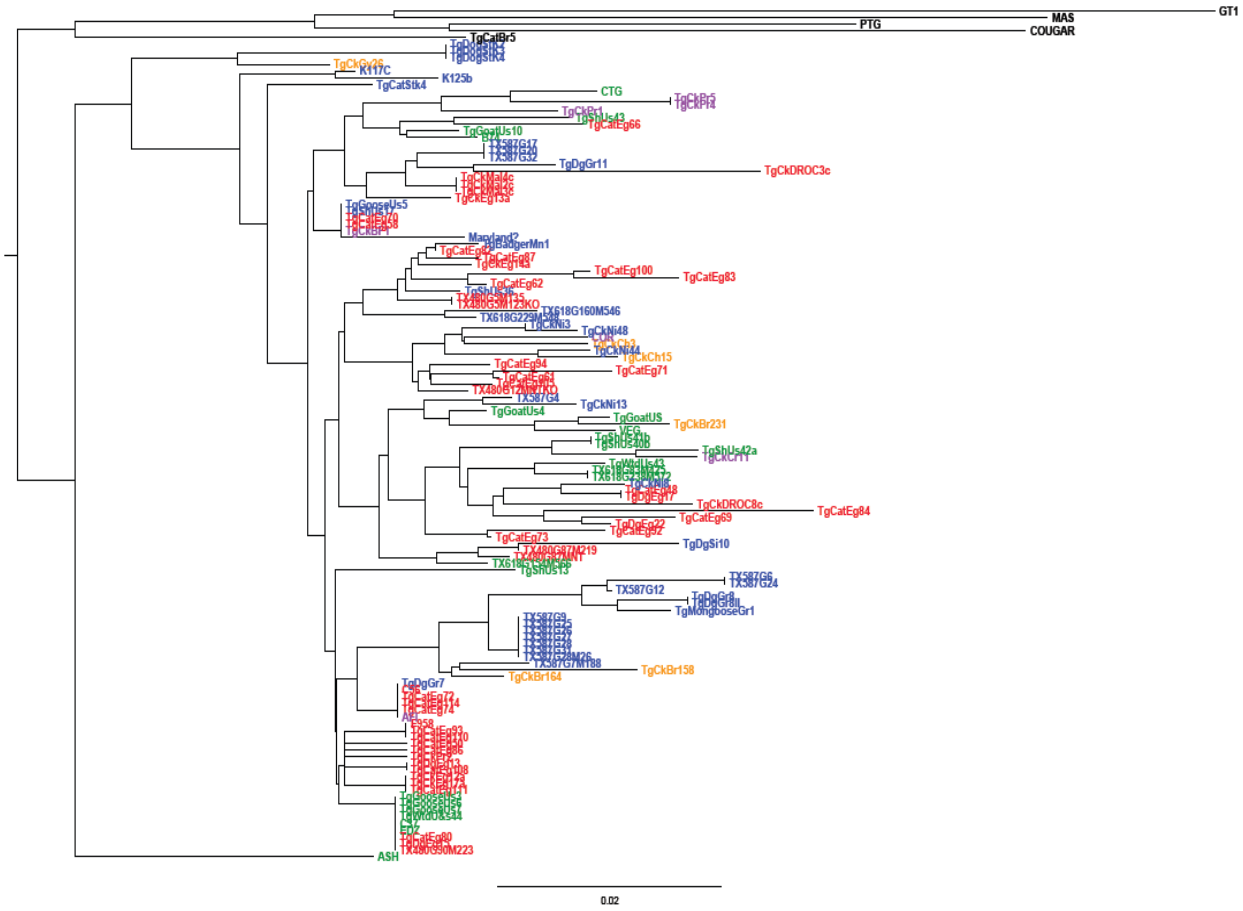
Data analysis

The multilocus microsatellite typing data for 123 type III isolates was coded for all genetic loci. For a given locus, the DNA banding pattern was coded with a string of 1s and 0s. Phylogenetic network and neighbor joining tree were generated by SplitsTree 4.8 [167]. A rooted NJ tree was constructed for 142 samples (137 type III + 5 non-type III as outgroup) using Fig tree v1.4.2 (Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh, 2006). A rooted phylogenetic tree was generated using the non-type III reference strains as outgroup. Similarly, a neighbor joining tree based on MS data was constructed for 437 (137 type III + 296 type II + 4 outgroups) isolates. Basic statistics for quantitative gene diversity within populations were calculated using Arlequin v 3.5 [168].

4. Results

Based on the MS data, a rooted phylogenetic tree was constructed for type III isolates (n=123) using 5 non-type III isolates as the outgroup **(Figure 4.1).** No geographical clustering of isolates was observed for type III lineage isolates. Irrespective of the location or host, branch length appeared to be more or less similar for all the isolates indicating similar divergence time. This was also evident when the number of unique genotypes within each population were found to be comparable. Furthermore, isolates from Central America were placed at the basal position, close to the root.

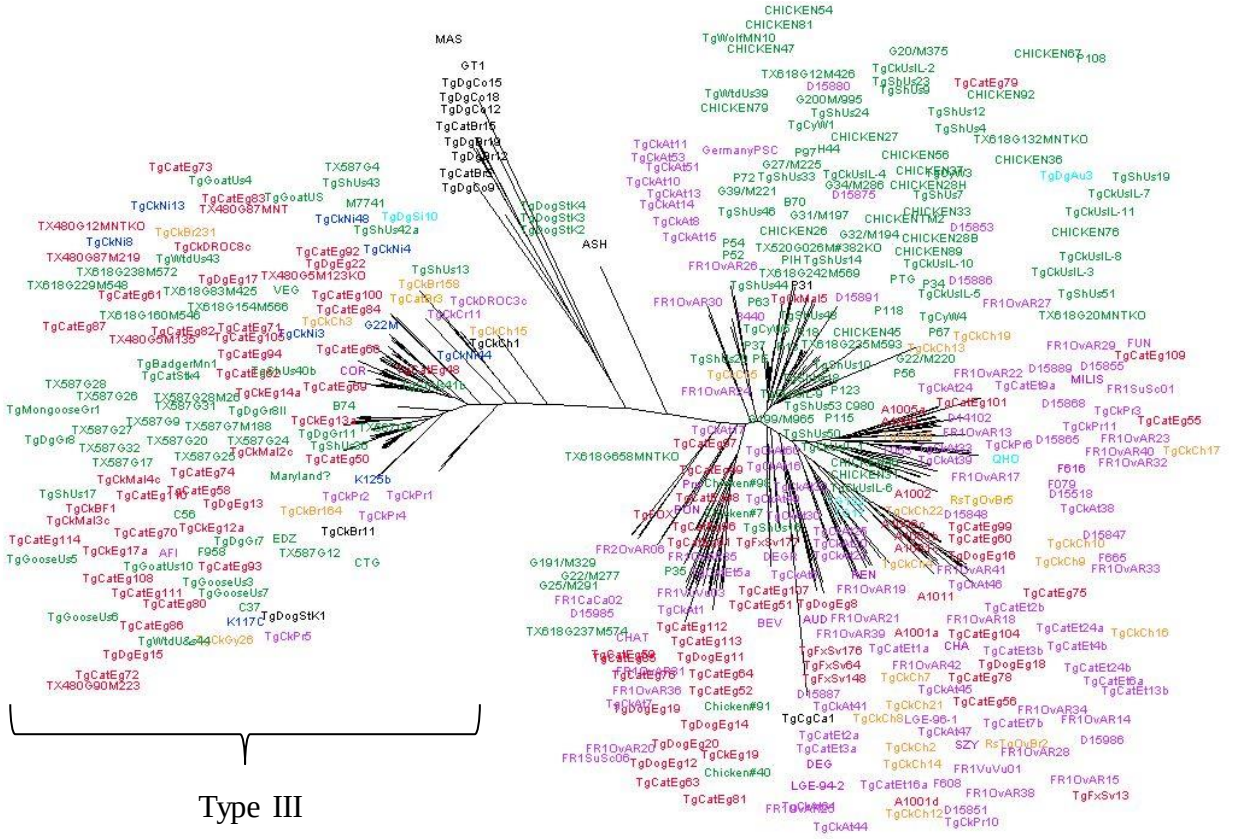
To compare relative expansion time between type III and type II lineages, neighbor joining network tree was constructed for 437 isolates; including 123 type III + 296 type II + 18 outgroups **(Figure 4.2 and Table 4.2).**



Color	Region
Purple	Europe (n= 6)
Green	North America (n=30)
Orange	South America (n=6)
Red	Africa (n=48)
Blue	Others (n=31)
Black	Non-type II (n=5)

Figure 4.1: Phylogenetic rooted tree constructed for type III (n=123) *T. gondii* isolates.

Figure 4.2: Phylogenetic neighbor joining tree (NJ) tree constructed for 437 *T. gondii* isolates (123 type III + 296 type II + 18 outgroup).



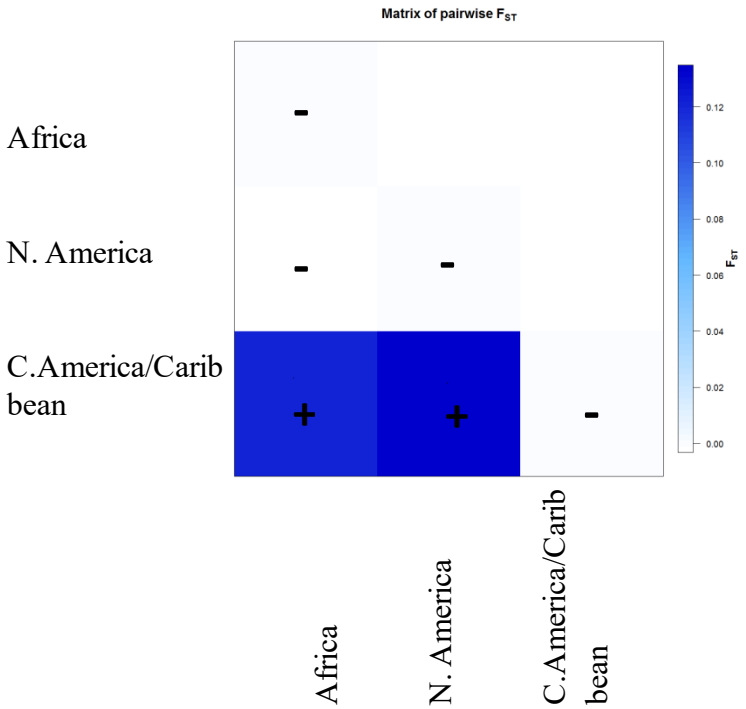
Color	Region
Purple	Europe
Green	North America
Orange	South America
Red	Africa
Blue	Others
Black	Non-type II (outgroup)

Figure 4.2 (continued)

Table 4.3: Basic statistics for type III *T. gondii* populations from different geographical regions.

	Africa	North America	Caribbean/Central America
Number of isolates	48	30	31
Number of MS genotypes	33	22	18
Average Gene diversity	0.2166 ±0.1259	0.1645 ± 0.1016	0.2698 ± 0.1536

Due to small sample size; samples from Europe and South America were not included in analysis.



It was observed that type II and III isolates formed two distinct clusters, different from the outgroups. Overall, type III lineage was less diverse than the type II lineages. However, the overall branch lengths of type III strains was similar to that of type II strains in North America type II isolates from North American were clustered together comparison to type III isolates. Type III isolates from North America were distributed within the type III population and lacked clustering **(Figure 4.2).**

5. Conclusions

We examined genetic diversity of the type III lineage by microsatellite analysis. Our results suggest that the MRCA of type III lineage is likely originated in the New World and in recent history, this lineage has been rapidly radiated to the Old World. Given that neighbor-joining tree for 123 type III strains showed no association of genotypes with geographical locations, the type III lineage may have spread from New World to the Old World many times independently in recent history. This intense transmission could be explained by the fact that most type III strains are avirulent to house mice. This may enable them to readily establish chronic infection in house mice and be efficiently transmitted in the house mice – domestic cats life cycle. Furthermore, Central America isolates placed near the root also suggests that the isolates from this region are older and may speak for the origin of type III in this region **(Figure 4.2).**

NJ tree constructed for combined data for type II and III isolates showed some interesting results for North American isolates. The obvious difference in topology between North American isolates clusters suggests that unlike type II North American strains, type III isolates have been transmitted to North America in multiple independent events. The most surprising information based on our results was the fact that type III transmission from the New World to the Old World, is opposite to that of type II lineage. The difference in the dissemination pattern for the two most dominant lineages suggests that these lineages may have undergone different selective pressures in the history of evolution. It is possible that dated back, type II may have migrated from the Old World to the New World, however in recent times the modern day type III strains maybe able to outcompete the type II strains and are being transmitted to the Old World. Our genetic analysis showed that type III strains from America are placed closer to the root of the phylogenetic tree, whereas samples from Africa and Europe are closer to the tip of the branches. A previous study suggests the type III lineage was a recombinant from ancestral type II and type III lineages [220].

Such recombination may provide the modern day type III lineage selective advantages. One biological trait that could attribute to the widespread of type III strain is that they are non-virulent to house mice, which may allow them to be easily transmistted by the domestic life cycle with house mice (*mus musculus*) and cats (*Felis*). Therefore, it can be expected type III will further expand and potentially become the most dominant lineage globally in the future.

6. Future Perspectives

Given that *T. gondii* can infect a variety of host species including mammals and birds, and different hosts may contribute at different levels to the transmission of this parasite, predicting the direction of evolution of *T. gondii* will be challenging. However, future studies focusing on ecology of *T. gondii* infection, together with mathmatical modeling, will help us to better understand the main animal species and environmental factors that influence spread of this parasite, providing us necessary information to control or reduce transmission of *T. gondii* in humans.

CHAPTER 4: *TOXOPLASMA GONDII* SEROPREVALENCE AND GENOTYPE DIVERSITY IN SELECT WILDLIFE SPECIES FROM THE SOUTHEASTERN UNITED STATES

Richard W. Gerhold[1, 2], Pooja Saraf[3], Aly Chapman[1], Xuan Zou[3], Graham Hickling[2] and Chunlei Su[3, 4]

[1] Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN 37996

[2]Center for Wildlife Health, Department of Forestry, Wildlife, and Fisheries, the University of Tennessee, Knoxville, TN 37996

[3]Department of Microbiology, the University of Tennessee, Knoxville, TN 37996

[4]Corresponding author (e-mail: csu1@utk.edu)

1. Abstract

Background: *Toxoplasma gondii* is a wide spread protozoan parasite that infects humans and other animals. Previous studies indicate some genotypes of *T. gondii* are more frequently isolated in wildlife than agricultural animals, suggesting a wild/feral animal diversity model. To determine seroprevalence and genetic diversity of *T. gondii* in southeastern US wildlife, we collected sera from 471 wild animals, including 453 mammals and 18 birds between 2011 and 2014. These serum samples were assayed for *T. gondii* infection using the modified agglutination test (MAT). Heart or tongue tissues from 66 seropositive animals were bioassayed in mice and 19 isolates were obtained. The isolated parasites were genotyped by the PCR-RFLP method employing 10 genetic markers.

Results: One hundred and ninety-six of the 471 samples (41.6%) had a titer ≥ 1:32 and were considered positive for *T. gondii* infection. Of the 453 mammals, 195 (43%) were seropositive, whereas only one (5.6%) of the 18 birds was seropositive. Mammalian hosts with adequate samples size (≥ 20) comprised white-tailed deer (n=241), feral hogs (n=100), raccoons (n=34), and coyotes (n=22), with seroprevalence of 41.0%, 51.0%, 50.0%, and 72.7%, respectively. Genotyping revealed five distinct genotypes, including the ToxoDB PCR-RFLP genotype #5 (a.k.a type 12) for 15 isolates, genotype #3 (a.k.a. type II) for 1 isolate, genotypes #154, #167 and #216, each for 1 isolate. The results showed moderate to high infection rates of *T. gondii* in white-tailed deer, feral hogs, raccoons, and coyotes. Genotyping results indicated limited genetic

diversity with dominance of genotype #5, which has been reported as a major type in wildlife in North America.

Conclusion: We conclude that *T. gondii* infection is common in game animals (white-tailed deer and feral hogs) in the southeastern US, which may pose a food safety risk to human. Further research is necessary to understand *T. gondii* transmission from wildlife to farm animals and humans.

2. Introduction

Toxoplasmosis, caused by *Toxoplasma gondii*, is zoonotic and considered a leading cause of human morbidity attributed to food borne illness in the United States [176]. One-third of the total population in the world is infected by this pathogen [1]. Women infected with *T. gondii* during pregnancy can have variable consequences including pregnancy complications, stillbirths, and abortions. In immunocompromised patients, such as those with AIDS, encephalitis may occur, which is often fatal [1]. Toxoplasmosis is one of five neglected parasitic infections, a group of parasitic diseases that has been targeted by the Centers for Disease Control and Prevention (CDC) for public health action. Infection with *T. gondii* can occur by ingestion of microscopic oocysts in contaminated food or water, or by ingestion of tissue cysts in undercooked or raw meat [2], making it an important foodborne zoonotic pathogen.

Toxoplasma gondii infection occurs in many species of wild mammals and birds, particularly those that are carnivorous or ground dwelling. Clinical toxoplasmosis occurs in a wide variety of US wildlife including threatened and endangered terrestrial mammals, avians, and marine mammals [195, 196]. Epidemiology studies in white-tailed deer populations have reported high seroprevalence rates (30-76%) in areas like Pennsylvania, Minnesota, Mississippi, New Jersey, Iowa and Ohio [177-182]. A high seroprevalence (15-84%) was also observed in raccoons from Iowa, New Jersey, Ohio, Kansas, Illinois, Florida, Pennsylvania, Virginia and Wisconsin [183-185]. A high seroprevalence rate in red and gray foxes (85.9%) has also been reported in Kentucky, Indiana, Michigan and Ohio [178, 186]. Wild hogs from California and black bears from Pennsylvania also show high seroprevalence rates of 17% and 75-80% respectively [187]. Antibodies against *T. gondii* (7-17%) have also been found in wolves from remote areas in Alaska [188, 189]. Genotyping of isolates from wildlife suggests that wild animals maintain a much greater diversity of *T. gondii* genotypes than agricultural animals [190, 191]. There is no

reported association between *T. gondii* genotypes and disease manifestation, but some evidence suggests a relationship. For example, in South America where wild animal populations are more dominant, severe cases of human toxoplasmosis were reported even in immunocompetent adults [27, 28, 30, 192] and the majority of these infections were attributed to unique genotypes. Recent studies have reported the presence of numerous genotypes in wildlife populations in North America. Currently, ToxoDB PCR-RFLP genotypes #4 and #5, also known as type 12, are recognized as the dominant type in North America wildlife [193, 194]. It is likely that some of these *T. gondii* strains from wildlife are highly virulent, posing a potential wildlife health risk and a higher risk for severe toxoplasmosis if transmitted in human populations.

The role of wildlife in the transmission of *T. gondii* demands increased efforts to catalog the major sources of human *T. gondii infection*. Continued characterization is critical to understanding the potential risks of *T. gondii* to wildlife populations and its zoonotic implications. Seroprevalence and genotyping data from the southeast region have been insufficient to determine the pattern of *T. gondii* transmission in the area. Hence, in this study, we focused on determining seroprevalence and characterizing strains isolated from wildlife in the southeastern United States.

3. Materials and Methods

Serum with or without corresponding fresh heart or tongue tissue samples was collected from hunter-killed, road killed, nuisance killed (i.e. feral hogs), or research collected animals from multiple southeastern states **(Table 5.3).** Tissue samples were refrigerated until serological screening was completed.

Screening for *T. gondii* was performed at the clinical parasitology laboratory at the University of Tennessee, College of Veterinary Medicine using the Modified Agglutination Test (MAT) performed as previously described [152, 197] This assay is used to detect anti-*T. gondii* antibodies in blood, serum, and other bodily fluids from a wide variety of wildlife and domestic species. Animals were considered *Toxoplasma* positive if IgG antibodies were detected at $\geq$ 1:32 dilution on MAT. Three to 5 grams of heart or tongue tissues from some seropositive hosts were processed and used in bioassays of mice to propagate *T. gondii* [5]. Isolated *T. gondii* strains were genotyped by multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) employing 10 genetic markers [154].

4. Results

Seroprevalence of *T. gondii*

A total of 471 serum/plasma samples were collected from 31 wildlife species (16 mammal and 15 bird species) from 2011 –2014 **(Table 5.1)**. These samples originated in six southeast states comprising Alabama, Georgia, Kentucky, North Carolina, South Carolina, and Tennessee **(Table 5.1).** From the 471 samples, 41.6% (196/471) had MAT titers ≥ 1:32 and were considered positive for *T. gondii* infection **(Table 5.2).** Nine mammalian (white-tailed deer, opossum, raccoon, coyote, feral hog, woodchuck, elk, gray fox, and mink) and 1 bird species (rock pigeon) collected from five southeastern states had seropositive individuals **(Table 5.1, Table 5.2).** The mammal hosts with samples size ≥ 10 individuals comprised white-tailed deer (n=241), feral hogs (n=100), raccoons (n=34), coyotes (n=22), opossum (n=12), and gray squirrels (n=14) with seroprevalence of 41%, 51%, 50%, 73%, 50%, and 0% respectively.

In Tennessee, 309 serum samples from 29 animal species were collected from 10 counties/sites and tested **(Table 5.1).** Overall, 37.5% (116/309) were positive for *T. gondii* infection. A total of 167 white-tailed deer samples were collected from various counties including Loudon, Fayette (Ames plantation), Anderson (Oakridge National Laboratories) and Coffee (Arnold Engineering Development Complex) area; 39.5% (66/167) were positive. Thirty-four raccoon samples were collected from Knox, Fayette, and Kingston counties, 50% (17/34) were positive. Of the 27 feral hog serum samples collected from the Tennessee side of Great Smoky Mountains National Park (GSMNP), 40.7% (11/27) were positive. From 17 coyote samples collected in Fayette County, 11 (64.7%) were positive. Additionally, 50% (6/12) of opossum from Knox and Fayette counties were positive. Overall, the results from Tennessee indicate *T. gondii* infection is common in wildlife **(Table 5.1).**

In North Carolina, 74 serum samples (73 from feral hogs, 1 from a black bear) were collected from the GSMNP **(Table 5.1)**, 54.1% (40/74) were positive. For the feral hogs, 54.8% (40/73) were positive to *T. gondii* infection. In South Carolina, 74 serum samples from white-tailed deer in Laurens County were tested and 44.5% (33/74) were seropositive. In Georgia, 6 serum samples were collected from 5 coyotes and 1 gray fox in Jefferson and Putnam counties **(Table 5.1)**. The 5 samples from coyotes in Putnam County were all seropositive. The 1 sample from the gray fox in Jefferson County tested seronegative. Four serum samples from 4 animal species

Table 5.1: Seroprevalence rates of *T. gondii* categorized by State and County.

State of Origin	County/Site	Species	Seropositive/ total	Seroprevalence (County)	Seroprevalence (State)
Tennessee	Loudon	white tailed deer	5/9	55.6%	37.5% (116/309)
	Knox	Virginia opossum	1/2	22.4% (13/58)	
		woodchuck	1/4		
		mink	1/1		
		raccoon	5/12		
		gray fox	2/3		
		opossum	2/3		
		pigeon	1/1		
		22 other wildlife species*	0/32		
	Coffee	white tailed deer	1/3	8.3% (1/12)	
		gray squirrel	0/9		
		coyote	11/17		
	Ames Plant	white tailed deer	40/77	54.1% (59/109)	
		raccoon	5/8		
		opossum	3/7		
	GSMNP	black bear	0/1	39.2% (11/28)	
		feral hog	11/25		
	GSMP	feral hog	0/2		
	Kingston	raccoon	7/13	53.8%	
	Oak Ridge	white tailed deer	18/64	28.1%	
	AEDC, Decherd	white tailed deer	2/14	14.2%	
	Jefferson	raccoon	0/1	0%	
	Roane	mink	0/1	0%	
South Carolina	Laurens	white tailed deer	33/74	44.5%	44.5%
North Carolina	Cataloochee	feral hog	0/2	0%	54% (40/74)
	GSMNP	feral hog	40/71	55.5%	
		black bear	0/1		
Georgia	Jefferson	gray fox	0/1	0%	83.3% (5/6)
	Putnam	coyote	5/5	100%	
	Brent	woodchuck	0/1	0%	

Table 5.1 (continued)

State of Origin	County/Site	Species	Seropositive/ total	Seroprevalence (County)	Seroprevalence (State)
Alabama	Hale	eastern cottontail	0/1	0%	0% (0/3)
		armadillo	0/1		
		gray squirrel	0/1		
Kentucky	Perry	elk	2/3	66.6%	50% (2/4)
	Knott	elk	0/1	0%	

Overall positive rate = 196/471 (41.6%)

GSMP= Great Smoky mountain Parkway, GSMNP=Great Smoky mountain National Park, AEDC= Arnold Engineering development complex

*One samples from each of the following wildlife: American Crow, American robin, Beaver, Belted Kingfisher, Chickadee, Chimney Swift, Chipmunk, Eastern Chipmunk, Fox squirrel, Gray Catbird, Hermit Thrush, House Sparrow, Oven Bird, Pileated Woodpecker, Rock Pigeon, Tufted Titmice, Turkey Vulture.
Two samples from: Blue Jay and Mourning Dove.
Three samples from: Cotton-tailed Rabbit
Four samples from: Eastern Cottontail and gray squirrel

Table 5.2: Seroprevalence rate of *T. gondii* categorized by species.

Host	No. of samples	MAT titers					Sero-prevalenc
		<1:32	1:32 – 1:128	1:256-1:1024	1:2048 – 1:8192	>1:8192	
Mammals							
White tailed deer	241	142	59	11	11	18	41.0
Feral hog	100	49	33	13	4	1	51.0
Raccoon	34	17	6	7	0	4	50.0
Coyote	22	6	5	4	4	3	72.7
Opossum	10	5	2	2	1	0	50.0
Woodchuck	5	4	1	0	0	0	20.0
Elk	4	2	1	1	0	0	50.0
Gray fox	4	2	2	0	0	0	50.0
Mink	2	1	0	0	1	0	50.0
Virginia Opossum	2	1	1	0	0	0	50.0
Gray squirrel	14	14	0	0	0	0	0.0
Eastern cottontail	5	5	0	0	0	0	0.0
Cotton-tailed rabbit	3	3	0	0	0	0	0.0
Black bear	2	2	0	0	0	0	0.0
Armadillo	1	1	0	0	0	0	0.0
Beaver	1	1	0	0	0	0	0.0
Chipmunk	1	1	0	0	0	0	0.0
Eastern chipmunk	1	1	0	0	0	0	0.0
Fox squirrel	1	1	0	0	0	0	0.0
Birds							
Pigeon	1	0	1	0	0	0	100.0
Blue jay	2	2	0	0	0	0	0.0
Mourning dove	2	2	0	0	0	0	0.0
American crow	1	1	0	0	0	0	0.0
American robin	1	1	0	0	0	0	0.0
Belted kingfisher	1	1	0	0	0	0	0.0
Chickadee	1	1	0	0	0	0	0.0
Chimney Swift	1	1	0	0	0	0	0.0
Gray Catbird	1	1	0	0	0	0	0.0
Hermit thrush	1	1	0	0	0	0	0.0
House sparrow	1	1	0	0	0	0	0.0
Oven bird	1	1	0	0	0	0	0.0
Pileated woodpecker	1	1	0	0	0	0	0.0

Table 5.2 (continued)

Host	No. of samples	MAT titers					Sero-prevalence%
		<1:32	1:32 – 1:128	1:256-1:1024	1:2048 – 1:8192	>1:8192	
Rock pigeon	1	1	0	0	0	0	0.0
Tufted Titmice	1	1	0	0	0	0	0.0
Turkey Vulture	1	1	0	0	0	0	0.0
Total	471	275	111	38	21	26	41.6% (196/471)

in Alabama were all negative **(Table 5.1).** Two of 4 samples from elk in Kentucky were positive (50%).

Isolation and genotyping of *T. gondii* strains

The tissue samples (hearts and tongues) from 66 seropositive wildlife bioassayed in mice are listed in **Table 5.3.** These samples comprised: 33 from white-tailed deer, 11 from feral hogs, 8 from raccoons, 8 from coyotes, 2 from elk, 2 from opossums, 1 from mink and 1 from gray fox. Nineteen *T. gondii* isolates were obtained by bioassay (13 white-tailed deer, 3 feral hogs, 2 coyotes and 1 mink). All samples were genotyped by the 10 PCR-RFLP markers and the results are summarized in **Table 5.4**. Five distinct genotypes were identified: ToxoDB PCR-RFLP genotype #5 (15 isolates), #3 (1 isolate), #154 (1 isolate), #167 (1 isolate) and #216 (1 isolate). Of the 13 isolates obtained from white-tailed deer, 9 were from South Carolina and 4 from Tennessee.

5. Discussion

The present study demonstrates that *T. gondii* infection is widespread in wild mammals from the southeastern United States. We collected sera from 471 wild animals, including 453 mammals and 18 birds between 2011 and 2014. Overall, 41.6% were positive for *T. gondii* infection, however, only one of the 18 birds was seropositive, whereas mammal hosts with samples size ≥ 10 individuals all had high prevalence >40%. Type 12 (a. k. a. Toxo DB #5) is the most common circulating genotype in wildlife in this region of the US, which is in agreement with previous studies reporting the prevalence of type 12 genotype in white-tailed deer populations [36, 193]. Genotypes #156 and #167 have been previously reported from goats in the USA [199]. Two isolates from coyote (TgWtdTn 122 and 142) and 1 mink isolate (TgMnkTn17) obtained from Tennessee, belong to genotype #5 and #3 respectively. Genotype #3 belongs to type II clonal lineage of *T. gondii* which is the most dominant lineage distributed globally. Furthermore, the 2 feral hog isolates (TgHogNc 194 and 227) from North Carolina also belonged to genotype #5, which is commonly distributed in North America [193].

Table 5.3: Results for bioassay in mice for *T. gondii*.

Sample ID	Host	Location	Date Sample collected	MAT titre	Days between collection and inoculation	Mice bioassayed	Isolate ID
13	Elk	KY	10/8/2011	128	6	2	Nd
14	Elk	KY	10/8/2011	512	6	3	Nd
16	WTD	TN	9/19/2011	128	6	2	Nd
17	Mink	TN	10/8/2011	>8192	6	3	TgMnkTn17
37	WTD	SC	10/15/2011	≥8192	16	2	Nd
40	WTD	SC	10/15/2011	≥8192	16	3	TgWtdSc40
43	WTD	SC	10/15/2011	≥8192	16	3	TgWtdSc43
60	WTD	SC	10/15/2011	≥8192	16	3	TgWtdSc60
77	Gray fox	TN	10/17/2011	32	14	3	Nd
78	WTD	SC	11/13/2011	≥8192	9	2	TgWtdSc78
79	WTD	SC	11/13/2011	2048	9	2	Nd
80	WTD	SC	11/13/2011	2048	9	2	Nd
88	WTD	SC	11/13/2011	≥8192	9	2	TgWtdSc88
95	WTD	SC	11/13/2011	2048	9	2	Nd
98	WTD	SC	11/13/2011	2048	9	2	TgWtdSc98
99	WTD	SC	11/13/2011	2048	9	2	TgWtdSc99
102	WTD	SC	11/13/2011	≥8192	9	2	Nd
110	WTD	SC	11/13/2011	≥8192	9	2	TgWtdSc110
113	WTD	SC	11/13/2011	≥8192	9	2	TgWtdSc113
119	Raccoon	TN	1/21/2012	2048	12	2	Nd
121	Raccoon	TN	1/21/2012	2048	12	1	Nd
122	Coyote	TN	1/21/2012	512	12	1	TgCyTn122
124	Raccoon	TN	1/21/2012	2048	12	2	Nd
126	Coyote	TN	1/21/2012	≥8192	12	2	Nd
133	Raccoon	TN	1/27/2012	≥8192	20	2	Nd
136	Opossum	TN	2/2/2012	512	14	2	Nd
138	Opossum	TN	2/2/2012	512	14	2	Nd
142	Coyote	TN	2/2/2012	4096	14	2	TgCyTn142
151	Coyote	GA	3/28/2012	≥8192	6	3	Nd
152	Coyote	GA	3/28/2012	4096	6	3	Nd
173	Raccoon	TN	6/13/2012	128	5	3	Nd
174	Raccoon	TN	6/13/2012	2048	5	3	Nd
176	Raccoon	TN	6/13/2012	2048	5	3	Nd
194	FH	NC	1/28/2013	4096	16	3	TgHogNc194

Table 5.3 (continued)

Sample ID	Host	Loc a-tion	Date Sample collected	MAT titre	Days between collection and inoculation	Mice bioassayed	Isolate ID
195	FH	NC	1/28/2013	128	16	2	Nd
197	FH	NC	1/31/2013	128	16	2	Nd
201	FH	NC	2/8/2013	512	20	2	Nd
210	Raccoon	TN	1/17/2013	2048	42	2	Nd
211	Coyote	TN	1/22/2013	2048	37	3	Nd
212	Coyote	TN	1/22/2013	≥8192	35	2	Nd
215	Coyote	TN	1/24/2013	2048	35	2	Nd
227	FH	NC	4/9/2013	128	16	3	TgHogNc227
300	WTD	TN	11/10/2013	32	10	2	Nd
313	WTD	TN	11/10/2013	128	10	2	Nd
323	WTD	TN	11/10/2013	32	10	2	Nd
334	WTD	TN	11/12/2013	128	8	2	Nd
335	WTD	TN	11/12/2013	128	9	2	TgWtdTn335
345	WTD	TN	11/12/2013	128	8	2	Nd
352	WTD	TN	11/12/2013	128	8	2	Nd
354	WTD	TN	11/12/2013	128	8	2	Nd
355	WTD	TN	11/12/2013	128	8	2	Nd
372	WTD	TN	12/9/2013	≥8192	4	2	TgWtdTn372
373	WTD	TN	12/9/2013	512	4	2	Nd
383	WTD	TN	12/9/2013	512	4	2	Nd
385	WTD	TN	12/9/2013	512	4	2	Nd
387	WTD	TN	12/9/2013	≥8192	4	2	TgWtdTn387
389	WTD	TN	12/9/2013	2048	4	2	Nd
399	WTD	TN	12/15/2013	2048	8	2	TgWtdTn399
400	WTD	TN	12/15/2013	128	8	2	Nd
402	WTD	TN	12/15/2013	128	8	2	Nd
452	FH	NC	1/16/2014	128	18	3	TgHogNc452
458	FH	NC	1/24/2014	1024	10	3	Nd
462	FH	NC	1/31/2014	128	20	3	Nd
466	FH	NC	2/6/2014	128	14	3	Nd
480	FH	NC	2/12/2014	128	8	3	Nd

WTD=white tailed deer, FH=feral hog.

SC=South Carolina, NC=North Carolina, TN=Tennessee, GA= Georgia, KY=Kentucky

Table 5.4 Genotyping data for *T. gondii* isolates from wildlife.

ID	Host	Location	SAG1	5'-3' SAG2	alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Genotype
TgWtdSc40	WTD	SC	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdSc43	WTD	SC	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdSc60	WTD	SC	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdSc78	WTD	SC	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdSc99	WTD	SC	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdSc113	WTD	SC	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdSc88	WTD	SC	I	II	II	III	II	II	II	u-1	III	II	I	#154
TgWtdSc98	WTD	SC	II or III	I	I	I	I	I	I	III	III	III	III	#167
TgWtdSc110	WTD	SC	I	I	I	III	III	I	III	III	III	I	III	#216
TgHogNc194	FH	NC	u-1	II	II	II	II	II	II	nd	I	nd	nd	#5
TgHogNc227	FH	NC	u-1	II	II	II	II	II	II	nd	I	nd	nd	#5
TgHogNc452	FH	NC	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgMnkTn17	Mink	TN	II or III	II	II	II	II	II	II	II	II	II	I	#3
TgCyTn122	Coyote	TN	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgCyTn142	Coyote	TN	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdTn335	WTD	TN	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdTn372	WTD	TN	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdTn387	WTD	TN	u-1	II	II	II	II	II	II	II	I	II	I	#5
TgWtdTn399	WTD	TN	u-1	II	II	II	II	II	II	II	I	II	I	#5

WTD=white tailed deer, FH=feral hog.

SC=South Carolina, NC=North Carolina, TN=Tennessee

nd = no data.

6. Conclusion

In addition to the commonly observed genotypes, we also isolated several non-clonal types circulating in sampled populations. This is of interest, as previous epidemiological studies have reported a link between the prevalence of non-clonal genotypes and cases of congenital ocular and severe disseminated toxoplasmosis in the area such Brazil [3]. White-tailed deer is one of the most dominant wildlife species found in North America as well as venison is a common game meat. Thus, the high seroprevalence in this species indicates that deer could serve as an important source of human infection. Hence, people consuming wild venison should be advised to cook the meat properly and use proper caution while handling the raw meat. Future genotyping and seroprevalence studies in wildlife hosts and their role in the transmission cycle will increase the understanding of risks associated with *T. gondii* in human populations.

7. Future perspectives

In this study, we measured the seroprevalence rate among wildlife from six southeastern United States; including Tennessee, Georgia, North Carolina, South Carolina, Kentucky, and Alabama and observed a high seroprevalence rate among the animals consumed by humans as game meat. Even though the seroprevalence studies are rising, some areas remain underrepresented and may prove as a risk to humans in the close vicinity. Thus, in future, more seroprevalence studies need to be attempted, especially among wildlife, as consumption of game meat is a common practice. Additionally, previous studies have shown that isolates circulating among wildlife may cause severe cases of toxoplasmosis in humans. Thus, expansion of the existing knowledge regarding strains of *T. gondii* infecting wildlife is of importance and should be a part of future goals.

CONCLUSIONS

Toxoplasma is one of the pathogens included in the category of the neglected parasitic infections, a group of parasitic diseases that has been targeted by the Centers for Disease Control and Prevention (CDC) for public health action. This disease can be spread by contaminated food and water sources as well as can be vertically transmitted. Furthermore, *Toxoplasma* has the capacity to remain infectious in humans as well as almost all warm-blooded animals and can reactivate under immunocompromised conditions causing a severe form of infection and in some cases death. This asserts the importance of studying the transmission pattern, seroprevalence rates as well as the genes that play a role in the maintenance of infection.

At present, the studies to understand the virulence of the parasite *Toxoplasma* have been carried out using the mouse as the model organism. However, the methodology used between labs varies considerably, making it difficult to compare results between different labs. Thus, it is essential to establish a standard method in order to make results comparable and create a database for future studies. Hence, in **Chapter 1** we focused on discussing the different factors that may lead to variability in results; including the route of infection, life stage of the parasite, and the number of passages of the parasite in mice or cell culture, and the mouse host lines. Further, we put forth a protocol to facilitate the integration of *T. gondii* virulence data throughout the research community.

Recent studies using PCR-RFLP technique have established that the population and diversity structure of *Toxoplasma* is complex. This study also showed that *T. gondii* is spread globally, however; the dominance of certain lineages varies geographically. Type II even though spread globally, was found to be most dominant in Europe compared to other regions; including Africa, North and South America. Additionally, cases of congenital toxoplasmosis infected with type II *T. gondii* were reported from the European continent. Thus, it is of interest to study the transmission pattern and origin of this dominant lineage. Our study **(Chapter 2)** based on 299 type II isolates collected globally revealed that diversity of type II lineage is most in Europe and Africa than any other continent. Furthermore, isolates from Europe and Africa were found to be older based on the phylogenetic tree. Taken together, in this study we identified that the type II lineage may have originated in the Old World comprise of Europe and Africa and have transmitted to the New World of America from the Old World.

Similar to type II lineage, type III lineage was found out to be the second most common lineages among all others. Unlike type II, type III is found in higher numbers in South America.

It is therefore, interesting to investigate whether type III MRCA originated in South America, or if it is transmitted and recently expanded in this region. Results of our study **(Chapter 3)** showed that type III strains may have originated in the New World near Central America and are being transmitted back to the Old World (Europe and Africa) from the New World (America). Additionally, type III strains from North America were not clustered together when compared to type II lineages from this region. This together suggests that type III lineage may have originated in the New World and transmitted to North America independently during multiple events in the history.

Toxoplasma gondii is capable of infecting almost all warm-blooded animals [1]. As *T. gondii* can be transmitted to humans via consumption of contaminated meat, the study of the seroprevalence rate among the wildlife is of importance [2]. Furthermore, severe cases of toxoplasmosis have been reported to be infected with the strains found to be circulating among wildlife areas [27, 28, 30]. Hunting is a common practice in the southern region of US, however; the seroprevalence and genotyping reports of *Toxoplasma* are limited in this area. In **Chapter 4** we focused on reporting the seroprevalence rate and genotyping of strains among the wildlife of six regions of Tennessee, Georgia, North Carolina, South Carolina, Kentucky, and Georgia. Our study on a collection of 471 wildlife sera samples showed high seroprevalence rate among game meat animals including white-tailed deer. Furthermore, one previously unidentified genotype and other atypical genotypes were found to be circulating among the wildlife belonging to these regions.

REFERENCES

1. Dubey, J.P., *Toxoplasmosis of Animals and Humans* 2010. Second edition, CRC press, Taylor and Francis group.
2. Tenter, A.M., A.R. Heckeroth, and L.M. Weiss, *Toxoplasma gondii: from animals to humans.* Int J Parasitol, 2000. **30**(12-13): p. 1217-58.
3. Holland, G.N., *Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease.* Am J Ophthalmol, 2003. **136**(6): p. 973-88.
4. Montoya, J.G. and O. Liesenfeld, *Toxoplasmosis.* Lancet, 2004. **363**(9425): p. 1965-76.
5. Dubey, J.P., *Refinement of pepsin digestion method for isolation of Toxoplasma gondii from infected tissues.* Vet Parasitol, 1998. **74**(1): p. 75-7.
6. Fayer, R., *Toxoplasmosis update and public health implications.* Can Vet J, 1981. **22**(11): p. 344-52.
7. Dumetre, A., et al., *Mechanics of the Toxoplasma gondii oocyst wall.* Proc Natl Acad Sci U S A, 2013. **110**(28): p. 11535-40.
8. Dubey, J.P., D.S. Lindsay, and C.A. Speer, *Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts.* Clin Microbiol Rev, 1998. **11**(2): p. 267-99.
9. Skariah, S., M.K. McIntyre, and D.G. Mordue, *Toxoplasma gondii: determinants of tachyzoite to bradyzoite conversion.* Parasitol Res, 2010. **107**(2): p. 253-60.
10. Sibley, L.D. and J.C. Boothroyd, *Virulent strains of Toxoplasma gondii comprise a single clonal lineage.* Nature, 1992. **359**(6390): p. 82-5.
11. Howe, D.K. and L.D. Sibley, *Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease.* J Infect Dis, 1995. **172**(6): p. 1561-6.
12. Khan, A., et al., *Selection at a single locus leads to widespread expansion of Toxoplasma gondii lineages that are virulent in mice.* PLoS Genet, 2009. **5**(3): p. e1000404.
13. Saeij, J.P., et al., *Polymorphic secreted kinases are key virulence factors in toxoplasmosis.* Science, 2006. **314**(5806): p. 1780-3.
14. Taylor, S., et al., *A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii.* Science, 2006. **314**(5806): p. 1776-80.

15. Behnke, M.S., et al., *Virulence differences in Toxoplasma mediated by amplification of a family of polymorphic pseudokinases.* Proc Natl Acad Sci U S A, 2011. **108**(23): p. 9631-6.
16. Fentress, S.J., et al., *Phosphorylation of immunity-related GTPases by a Toxoplasma gondii-secreted kinase promotes macrophage survival and virulence.* Cell Host Microbe, 2010. **8**(6): p. 484-95.
17. Steinfeldt, T., et al., *Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent Toxoplasma gondii.* PLoS Biol, 2010. **8**(12): p. e1000576.
18. Howard, J.C., J.P. Hunn, and T. Steinfeldt, *The IRG protein-based resistance mechanism in mice and its relation to virulence in Toxoplasma gondii.* Curr Opin Microbiol, 2011. **14**(4): p. 414-21.
19. Virreira Winter S1, N.W., Jensen KD, Rosowski EE, Julien L, Spooner E, Caradonna K, Burleigh BA, Saeij JP, Ploegh HL, Frickel EM., *Determinants of GBP recruitment to Toxoplasma gondii vacuoles and the parasitic factors that control it*. PLoS One, 2011. **6**: p. 24434.
20. Boyle, J.P., et al., *Expression quantitative trait locus mapping of toxoplasma genes reveals multiple mechanisms for strain-specific differences in gene expression.* Eukaryot Cell, 2008. **7**(8): p. 1403-14.
21. Etheridge, R.D., et al., *The Toxoplasma pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice.* Cell Host Microbe, 2014. **15**(5): p. 537-50.
22. Reese, M.L., N. Shah, and J.C. Boothroyd, *The Toxoplasma pseudokinase ROP5 is an allosteric inhibitor of the immunity-related GTPases.* J Biol Chem, 2014. **289**(40): p. 27849-58.
23. Shwab, E.K., et al., *The ROP18 and ROP5 gene allele types are highly predictive of virulence in mice across globally distributed strains of Toxoplasma gondii.* Int J Parasitol, 2016. **46**(2): p. 141-6.
24. Beattie, D.a., *Toxoplasmosis of Animals and Man.* CRC Press, 1988.

25. Pusch, L., et al., *Persistent toxoplasma bradyzoite cysts in the brain: incidental finding in an immunocompetent patient without evidence of a toxoplasmosis.* Clin Neuropathol, 2009. **28**(3): p. 210-2.
26. Remington, J.S. and E.N. Cavanaugh, *Isolation of the encysted form of Toxoplasma gondii from human skeletal muscle and brain.* N Engl J Med, 1965. **273**(24): p. 1308-10.
27. Darde, M.L., et al., *Severe toxoplasmosis caused by a Toxoplasma gondii strain with a new isoenzyme type acquired in French Guyana.* J Clin Microbiol, 1998. **36**(1): p. 324.
28. Demar, M., et al., *Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical, and parasitological aspects.* Clin Infect Dis, 2007. **45**(7): p. e88-95.
29. Carme, B., et al., *Severe acquired toxoplasmosis caused by wild cycle of Toxoplasma gondii, French Guiana.* Emerg Infect Dis, 2009. **15**(4): p. 656-8.
30. Gilbert, R.E., et al., *Ocular sequelae of congenital toxoplasmosis in Brazil compared with Europe.* PLoS Negl Trop Dis, 2008. **2**(8): p. e277.
31. Dubey, J.P., *Toxoplasmosis of Animals and Humans, Second Edition*. 2010: Taylor & Francis.
32. Dubey, J.P., et al., *Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology.* Parasitology, 2012. **139**(11): p. 1375-424.
33. Xiao, J. and R.H. Yolken, *Strain hypothesis of Toxoplasma gondii infection on the outcome of human diseases.* Acta Physiol (Oxf), 2015. **213**(4): p. 828-45.
34. Bossi, P. and F. Bricaire, *Severe acute disseminated toxoplasmosis.* Lancet, 2004. **364**(9434): p. 579.
35. Delhaes, L., et al., *Severe congenital toxoplasmosis due to a Toxoplasma gondii strain with an atypical genotype: case report and review.* Prenat Diagn, 2010. **30**(9): p. 902-5.
36. Shwab, E.K., et al., *Geographical patterns of Toxoplasma gondii genetic diversity revealed by multilocus PCR-RFLP genotyping.* Parasitology, 2014. **141**(4): p. 453-61.
37. Sher, A., et al., *Toxoplasma gondii induces a T-independent IFN-gamma response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor-alpha.* J Immunol, 1993. **150**(9): p. 3982-9.

38. Sturge, C.R., et al., *TLR-independent neutrophil-derived IFN-gamma is important for host resistance to intracellular pathogens.* Proc Natl Acad Sci U S A, 2013. **110**(26): p. 10711-6.

39. Gazzinelli, R.T., et al., *Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts.* Proc Natl Acad Sci U S A, 1993. **90**(13): p. 6115-9.

40. Cheng, Y.S., et al., Yin, *Interferon induction of fibroblast proteins with guanylate binding activity.* J Biol Chem, 1983. **258**(12): p. 7746-50.

41. Boehm, U., et al., *Two families of GTPases dominate the complex cellular response to IFN-gamma.* J Immunol, 1998. **161**(12): p. 6715-23.

42. Yamamoto, M., et al., *A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii.* Immunity, 2012. **37**(2): p. 302-13.

43. Ling, Y.M., et al., *Vacuolar and plasma membrane stripping and autophagic elimination of Toxoplasma gondii in primed effector macrophages.* J Exp Med, 2006. **203**(9): p. 2063-71.

44. Khaminets, A., et al., *Coordinated loading of IRG resistance GTPases on to the Toxoplasma gondii parasitophorous vacuole.* Cell Microbiol, 2010. **12**(7): p. 939-61.

45. Pfefferkorn, E.R., *Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan.* Proc Natl Acad Sci U S A, 1984. **81**(3): p. 908-12.

46. Pfefferkorn, E.R., S. Rebhun, and M. Eckel, *Characterization of an indoleamine 2,3-dioxygenase induced by gamma-interferon in cultured human fibroblasts.* J Interferon Res, 1986. **6**(3): p. 267-79.

47. Green, S.J., et al., *Cellular mechanisms of nonspecific immunity to intracellular infection: cytokine-induced synthesis of toxic nitrogen oxides from L-arginine by macrophages and hepatocytes.* Immunol Lett, 1990. **25**(1-3): p. 15-9.

48. Hibbs, J.B., Jr., et al., *Nitric oxide: a cytotoxic activated macrophage effector molecule.* Biochem Biophys Res Commun, 1988. **157**(1): p. 87-94.

49. De Titto, E.H., J.R. Catterall, and J.S. Remington, *Activity of recombinant tumor necrosis factor on Toxoplasma gondii and Trypanosoma cruzi.* J Immunol, 1986. **137**(4): p. 1342-5.

50. Sibley, L.D., et al., *Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages.* J Immunol, 1991. **147**(7): p. 2340-5.

51. Andrade, R.M., et al., *CD40 induces macrophage anti-Toxoplasma gondii activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes.* J Clin Invest, 2006. **116**(9): p. 2366-77.

52. Adams, L.B., et al., *Microbiostatic effect of murine-activated macrophages for Toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine.* J Immunol, 1990. **144**(7): p. 2725-9.

53. Langermans, J.A., et al., *IFN-gamma-induced L-arginine-dependent toxoplasmastatic activity in murine peritoneal macrophages is mediated by endogenous tumor necrosis factor-alpha.* J Immunol, 1992. **148**(2): p. 568-74.

54. O'Garra, A., *Cytokines induce the development of functionally heterogeneous T helper cell subsets.* Immunity, 1998. **8**(3): p. 275-83.

55. Khan, I.A., T. Matsuura, and L.H. Kasper, *Interleukin-12 enhances murine survival against acute toxoplasmosis.* Infect Immun, 1994. **62**(5): p. 1639-42.

56. Muller, W.A. and G.J. Randolph, *Migration of leukocytes across endothelium and beyond: molecules involved in the transmigration and fate of monocytes.* J Leukoc Biol, 1999. **66**(5): p. 698-704.

57. Bliss, S.K., B.A. Butcher, and E.Y. Denkers, *Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection.* J Immunol, 2000. **165**(8): p. 4515-21.

58. Brenier-Pinchart, M.P., et al., *Toxoplasma gondii induces the secretion of monocyte chemotactic protein-1 in human fibroblasts, in vitro.* Mol Cell Biochem, 2000. **209**(1-2): p. 79-87.

59. Robben, P.M., et al., *Recruitment of Gr-1+ monocytes is essential for control of acute toxoplasmosis.* J Exp Med, 2005. **201**(11): p. 1761-9.

60. Ohtsuka, Y., et al., *MIP-2 secreted by epithelial cells increases neutrophil and lymphocyte recruitment in the mouse intestine.* Gut, 2001. **49**(4): p. 526-33.

61. Bliss, S.K., et al., *Human polymorphonuclear leukocytes produce IL-12, TNF-alpha, and the chemokines macrophage-inflammatory protein-1 alpha and -1 beta in response to Toxoplasma gondii antigens.* J Immunol, 1999. **162**(12): p. 7369-75.

62. Del Rio, L., et al., *CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during Toxoplasma gondii infection.* J Immunol, 2001. **167**(11): p. 6503-9.

63. Bliss, S.K., Y. Zhang, and E.Y. Denkers, *Murine neutrophil stimulation by Toxoplasma gondii antigen drives high level production of IFN-gamma-independent IL-12.* J Immunol, 1999. **163**(4): p. 2081-8.

64. Robben, P.M., et al., *Production of IL-12 by macrophages infected with Toxoplasma gondii depends on the parasite genotype.* J Immunol, 2004. **172**(6): p. 3686-94.

65. Reis e Sousa, C., et al., *In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas.* J Exp Med, 1997. **186**(11): p. 1819-29.

66. Scanga, C.A., et al., *Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells.* J Immunol, 2002. **168**(12): p. 5997-6001.

67. Gazzinelli, R., et al., *Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii.* J Immunol, 1992. **149**(1): p. 175-80.

68. Reichmann, G., et al., *The CD40/CD40 ligand interaction is required for resistance to toxoplasmic encephalitis.* Infect Immun, 2000. **68**(3): p. 1312-8.

69. Jenkins, M.K., et al., *In vivo activation of antigen-specific CD4 T cells.* Annu Rev Immunol, 2001. **19**: p. 23-45.

70. Dupont, C.D., D.A. Christian, and C.A. Hunter, *Immune response and immunopathology during toxoplasmosis.* Semin Immunopathol, 2012. **34**(6): p. 793-813.

71. Rougier, S., J.G. Montoya, and F. Peyron, *Lifelong Persistence of Toxoplasma Cysts: A Questionable Dogma?* Trends Parasitol, 2017. **33**(2): p. 93-101.

72. Eyles, D.E. and N. Coleman, *Tests of 2,4-diaminopyrimidines on toxoplasmosis.* Public Health Rep, 1952. **67**(3): p. 249-52.

73. Dubey, J.P., et al., *Oocyst-induced murine toxoplasmosis: life cycle, pathogenicity, and stage conversion in mice fed Toxoplasma gondii oocysts.* J Parasitol, 1997. **83**(5): p. 870-82.

74. Ueno, N., et al., *Real-time imaging of Toxoplasma-infected human monocytes under fluidic shear stress reveals rapid translocation of intracellular parasites across endothelial barriers.* Cell Microbiol, 2014. **16**(4): p. 580-95.

75. Desmonts, G., et al., *[Epidemiological study on toxoplasmosis: the influence of cooking slaughter-animal meat on the incidence of human infection]*. Rev Fr Etud Clin Biol, 1965. **10**(9): p. 952-8.

76. Pereira-Chioccola, V.L., J.E. Vidal, and C. Su, *Toxoplasma gondii infection and cerebral toxoplasmosis in HIV-infected patients.* Future Microbiol, 2009. **4**(10): p. 1363-79.

77. Luft, B.J., et al., *Toxoplasmic encephalitis in patients with acquired immune deficiency syndrome.* JAMA, 1984. **252**(7): p. 913-7.

78. Lyons, R.E., R. McLeod, and C.W. Roberts, *Toxoplasma gondii tachyzoite-bradyzoite interconversion.* Trends Parasitol, 2002. **18**(5): p. 198-201.

79. Manger, I.D., A.B. Hehl, and J.C. Boothroyd, *The surface of Toxoplasma tachyzoites is dominated by a family of glycosylphosphatidylinositol-anchored antigens related to SAG1*. Infect Immun, 1998. **66**(5): p. 2237-44.

80. Lekutis, C., D.J. Ferguson, and J.C. Boothroyd, *Toxoplasma gondii: identification of a developmentally regulated family of genes related to SAG2.* Exp Parasitol, 2000. **96**(2): p. 89-96.

81. Weiss, L.M., et al., *Bradyzoite development in Toxoplasma gondii and the hsp70 stress response.* Infect Immun, 1998. **66**(7): p. 3295-302.

82. Zhang, Y.W., et al., *Disruption of the Toxoplasma gondii bradyzoite-specific gene BAG1 decreases in vivo cyst formation.* Mol Microbiol, 1999. **31**(2): p. 691-701.

83. Gubbels, M.J., M. White, and T. Szatanek, *The cell cycle and Toxoplasma gondii cell division: tightly knit or loosely stitched?* Int J Parasitol, 2008. **38**(12): p. 1343-58.

84. Jerome, M.E., et al., *Toxoplasma gondii bradyzoites form spontaneously during sporozoite-initiated development*. Infect Immun, 1998. **66**(10): p. 4838-44.

85. Radke, J.R., et al., *A change in the premitotic period of the cell cycle is associated with bradyzoite differentiation in Toxoplasma gondii.* Mol Biochem Parasitol, 2003. **131**(2): p. 119-27.

86. Dzierszinski, F., et al., *Differential expression of two plant-like enolases with distinct enzymatic and antigenic properties during stage conversion of the protozoan parasite Toxoplasma gondii.* J Mol Biol, 2001. **309**(5): p. 1017-27.

87. Yang, S. and S.F. Parmley, *Toxoplasma gondii expresses two distinct lactate dehydrogenase homologous genes during its life cycle in intermediate hosts.* Gene, 1997. **184**(1): p. 1-12.

88. Ferguson, D.J. and W.M. Hutchison, *An ultrastructural study of the early development and tissue cyst formation of Toxoplasma gondii in the brains of mice.* Parasitol Res, 1987. **73**(6): p. 483-91.

89. Gurnett, A.M., et al., *Purification and molecular characterization of cGMP-dependent protein kinase from Apicomplexan parasites. A novel chemotherapeutic target.* J Biol Chem, 2002. **277**(18): p. 15913-22.

90. Nare, B., et al., *Evaluation of a cyclic GMP-dependent protein kinase inhibitor in treatment of murine toxoplasmosis: gamma interferon is required for efficacy.* Antimicrob Agents Chemother, 2002. **46**(2): p. 300-7.

91. Radke, J.R., et al., *Changes in the expression of human cell division autoantigen-1 influence Toxoplasma gondii growth and development.* PLoS Pathog, 2006. **2**(10): p. e105.

92. Luder, C.G., et al., *Toxoplasma gondii in primary rat CNS cells: differential contribution of neurons, astrocytes, and microglial cells for the intracerebral development and stage differentiation.* Exp Parasitol, 1999. **93**(1): p. 23-32.

93. Ferreira da Silva Mda, F., et al., *Stress-related and spontaneous stage differentiation of Toxoplasma gondii.* Mol Biosyst, 2008. **4**(8): p. 824-34.

94. Bohne, W., U. Gross, and J. Heesemann, *Differentiation between mouse-virulent and -avirulent strains of Toxoplasma gondii by a monoclonal antibody recognizing a 27-kilodalton antigen.* J Clin Microbiol, 1993. **31**(6): p. 1641-3.

95. Bohne, W., J. Heesemann, and U. Gross, *Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion.* Infect Immun, 1994. **62**(5): p. 1761-7.

96. Luder, C.G., et al., *Reduced expression of the inducible nitric oxide synthase after infection with Toxoplasma gondii facilitates parasite replication in activated murine macrophages.* Int J Parasitol, 2003. **33**(8): p. 833-44.

97. Shapira, S., et al., *Suppression of NF-kappaB activation by infection with Toxoplasma gondii.* J Infect Dis, 2002. **185 Suppl 1**: p. S66-72.

98. Shapira, S., et al., *Initiation and termination of NF-kappaB signaling by the intracellular protozoan parasite Toxoplasma gondii.* J Cell Sci, 2005. **118**(Pt 15): p. 3501-8.

99. Butcher, B.A., et al., *Toxoplasma gondii tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-kappa B.* J Immunol, 2001. **167**(4): p. 2193-201.

100. Rosowski, E.E., et al., *Strain-specific activation of the NF-kappaB pathway by GRA15, a novel Toxoplasma gondii dense granule protein.* J Exp Med, 2011. **208**(1): p. 195-212.

101. Butcher, B.A., et al., *Toxoplasma gondii rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control.* PLoS Pathog, 2011. **7**(9): p. e1002236.

102. Mordue, D.G., et al., *A patatin-like protein protects Toxoplasma gondii from degradation in activated macrophages.* Mol Microbiol, 2007. **63**(2): p. 482-96.

103. Tobin, C.M. and L.J. Knoll, *A patatin-like protein protects Toxoplasma gondii from degradation in a nitric oxide-dependent manner.* Infect Immun, 2012. **80**(1): p. 55-61.

104. Tobin Magle, C., et al., *A toxoplasma patatin-like protein changes localization and alters the cytokine response during toxoplasmic encephalitis.* Infect Immun, 2014. **82**(2): p. 618-25.

105. Wek, R.C., H.Y. Jiang, and T.G. Anthony, *Coping with stress: eIF2 kinases and translational control.* Biochem Soc Trans, 2006. **34**(Pt 1): p. 7-11.

106. Sullivan, W.J., Jr., et al., *Parasite-specific eIF2 (eukaryotic initiation factor-2) kinase required for stress-induced translation control.* Biochem J, 2004. **380**(Pt 2): p. 523-31.

107. Narasimhan, J., et al., *Translation regulation by eukaryotic initiation factor-2 kinases in the development of latent cysts in Toxoplasma gondii.* J Biol Chem, 2008. **283**(24): p. 16591-601.

108. Behnke, M.S., et al., *Coordinated progression through two subtranscriptomes underlies the tachyzoite cycle of Toxoplasma gondii.* PLoS One, 2010. **5**(8): p. e12354.

109. Walker, R., et al., *The Toxoplasma nuclear factor TgAP2XI-4 controls bradyzoite gene expression and cyst formation.* Mol Microbiol, 2013. **87**(3): p. 641-55.

110. Kirkman, L.A., L.M. Weiss, and K. Kim, *Cyclic nucleotide signaling in Toxoplasma gondii bradyzoite differentiation.* Infect Immun, 2001. **69**(1): p. 148-53.

111. Donald, R.G., et al., *Toxoplasma gondii cyclic GMP-dependent kinase: chemotherapeutic targeting of an essential parasite protein kinase.* Eukaryot Cell, 2002. **1**(3): p. 317-28.

112. Buchholz, K.R., et al., *Identification of tissue cyst wall components by transcriptome analysis of in vivo and in vitro Toxoplasma gondii bradyzoites.* Eukaryot Cell, 2011. **10**(12): p. 1637-47.

113. Buchholz, K.R., P.W. Bowyer, and J.C. Boothroyd, *Bradyzoite pseudokinase 1 is crucial for efficient oral infectivity of the Toxoplasma gondii tissue cyst*. Eukaryot Cell, 2013. **12**(3): p. 399-410.

114. Soete, M., D. Camus, and J.F. Dubremetz, *Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of Toxoplasma gondii in vitro.* Exp Parasitol, 1994. **78**(4): p. 361-70.

115. Soete, M. and J.F. Dubremetz, *Toxoplasma gondii: kinetics of stage-specific protein expression during tachyzoite-bradyzoite conversion in vitro.* Curr Top Microbiol Immunol, 1996. **219**: p. 76-80.

116. Weiss, L.M., et al., *A cell culture system for study of the development of Toxoplasma gondii bradyzoites.* J Eukaryot Microbiol, 1995. **42**(2): p. 150-7.

117. Echeverria, P.C., et al., *Toxoplasma gondii Hsp90 is a potential drug target whose expression and subcellular localization are developmentally regulated.* J Mol Biol, 2005. **350**(4): p. 723-34.

118. Bohne, W., et al., *Targeted disruption of the bradyzoite-specific gene BAG1 does not prevent tissue cyst formation in Toxoplasma gondii.* Mol Biochem Parasitol, 1998. **92**(2): p. 291-301.

119. Selawry, O.S., M.N. Goldstein, and C.T. Mc, *Hyperthermia in tissue-cultured cells of malignant origin.* Cancer Res, 1957. **17**(8): p. 785-91.
120. Henle, K.J. and L.A. Dethlefsen, *Heat fractionation and thermotolerance: a review.* Cancer Res, 1978. **38**(7): p. 1843-51.
121. Tomavo, S. and J.C. Boothroyd, *Interconnection between organellar functions, development and drug resistance in the protozoan parasite, Toxoplasma gondii.* Int J Parasitol, 1995. **25**(11): p. 1293-9.
122. Fox, B.A., J.P. Gigley, and D.J. Bzik, *Toxoplasma gondii lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation.* Int J Parasitol, 2004. **34**(3): p. 323-31.
123. Bohne, W. and D.S. Roos, *Stage-specific expression of a selectable marker in Toxoplasma gondii permits selective inhibition of either tachyzoites or bradyzoites.* Mol Biochem Parasitol, 1997. **88**(1-2): p. 115-26.
124. Ajzenberg, D., et al., *Microsatellite analysis of Toxoplasma gondii shows considerable polymorphism structured into two main clonal groups.* Int J Parasitol, 2002. **32**(1): p. 27-38.
125. Singh, U., J.L. Brewer, and J.C. Boothroyd, *Genetic analysis of tachyzoite to bradyzoite differentiation mutants in Toxoplasma gondii reveals a hierarchy of gene induction.* Mol Microbiol, 2002. **44**(3): p. 721-33.
126. Shwab, E.K., et al., *Corrigendum to "The ROP18 and ROP5 gene allele types are highly predictive of virulence in mice across globally distributed strains of Toxoplasma gondii" [Int. J. Parasitol. 46 (2016) 141-146]*. Int J Parasitol, 2016.
127. Khan, A., et al., *Phenotypic and gene expression changes among clonal type I strains of Toxoplasma gondii.* Eukaryot Cell, 2009. **8**(12): p. 1828-36.
128. Grigg, M.E., J.P. Dubey, and R.B. Nussenblatt, *Ocular toxoplasmosis: lessons from Brazil.* Am J Ophthalmol, 2015. **159**(6): p. 999-1001.
129. Ajzenberg, D., et al., *Genotype of 88 Toxoplasma gondii isolates associated with toxoplasmosis in immunocompromised patients and correlation with clinical findings.* J Infect Dis, 2009. **199**(8): p. 1155-67.
130. Dubey, J.P. and J.K. Frenkel, *Experimental toxoplasma infection in mice with strains producing oocysts.* J Parasitol, 1973. **59**(3): p. 505-12.

131. Dubey, J.P., et al., *Characterization of Toxoplasma gondii isolates from an outbreak of toxoplasmosis in Atlanta, Georgia.* Am J Vet Res, 1981. **42**(6): p. 1007-10.
132. Dubey, J.P., *Comparative infectivity of oocysts and bradyzoites of Toxoplasma gondii for intermediate (mice) and definitive (cats) hosts.* Vet Parasitol, 2006. **140**(1-2): p. 69-75.
133. Jacobs, L. and M.L. Melton, *Modifications in virulence of a strain of Toxoplasma gondii by passage in various hosts.* Am J Trop Med Hyg, 1954. **3**(3): p. 447-57.
134. Frenkel, J.K., J.P. Dubey, and R.L. Hoff, *Loss of stages after continuous passage of Toxoplasma gondii and Besnoitia jellisoni.* J Protozool, 1976. **23**(3): p. 421-4.
135. Lindsay, D.S., et al., *Examination of tissue cyst formation by Toxoplasma gondii in cell cultures using bradyzoites, tachyzoites, and sporozoites.* J Parasitol, 1991. **77**(1): p. 126-32.
136. Villard, O., et al., *Loss of oral infectivity of tissue cysts of Toxoplasma gondii RH strain to outbred Swiss Webster mice.* Int J Parasitol, 1997. **27**(12): p. 1555-9.
137. Dubey, J.P., et al., *Infection and immunity with the RH strain of Toxoplasma gondii in rats and mice.* J Parasitol, 1999. **85**(4): p. 657-62.
138. Frenkel, J.K. and P. Ambroise-Thomas, *Genomic drift of Toxoplasma gondii.* Parasitol Res, 1997. **83**(1): p. 1-5.
139. Howe, D.K. and L.D. Sibley, *Toxoplasma gondii: analysis of different laboratory stocks of the RH strain reveals genetic heterogeneity.* Exp Parasitol, 1994. **78**(2): p. 242-5.
140. Johnson, A.M., *Strain-dependent, route of challenge-dependent, murine susceptibility to toxoplasmosis.* Z Parasitenkd, 1984. **70**(3): p. 303-9.
141. Araujo, F.G., et al., *Strain-dependent differences in murine susceptibility to toxoplasma.* Infect Immun, 1976. **13**(5): p. 1528-30.
142. Suzuki, Y., et al., *Susceptibility to chronic infection with Toxoplasma gondii does not correlate with susceptibility to acute infection in mice.* Infect Immun, 1993. **61**(6): p. 2284-8.
143. Dubey, J.P., et al., *Oral oocyst-induced mouse model of toxoplasmosis: effect of infection with Toxoplasma gondii strains of different genotypes, dose, and mouse strains (transgenic, out-bred, in-bred) on pathogenesis and mortality.* Parasitology, 2012. **139**(1): p. 1-13.

144. McLeod, R., et al., *Immune responses associated with early survival after peroral infection with Toxoplasma gondii.* J Immunol, 1989. **142**(9): p. 3247-55.
145. McLeod, R., et al., *Genetic regulation of early survival and cyst number after peroral Toxoplasma gondii infection of A x B/B x A recombinant inbred and B10 congenic mice.* J Immunol, 1989. **143**(9): p. 3031-4.
146. Dempster, R.P., *Toxoplasma gondii: purification of zoites from peritoneal exudates by eight methods.* Exp Parasitol, 1984. **57**(2): p. 195-207.
147. Derouin, F., M.C. Mazeron, and Y.J. Garin, *Comparative study of tissue culture and mouse inoculation methods for demonstration of Toxoplasma gondii.* J Clin Microbiol, 1987. **25**(9): p. 1597-600.
148. Wu, L., et al., *Separation and purification of Toxoplasma gondii tachyzoites from in vitro and in vivo culture systems.* Exp Parasitol, 2012. **130**(1): p. 91-4.
149. Darde, M.L., B. Bouteille, and M. Pestre-Alexandre, *Isoenzyme analysis of 35 Toxoplasma gondii isolates and the biological and epidemiological implications.* J Parasitol, 1992. **78**(5): p. 786-94.
150. Howe, D.K., B.C. Summers, and L.D. Sibley, *Acute virulence in mice is associated with markers on chromosome VIII in Toxoplasma gondii.* Infect Immun, 1996. **64**(12): p. 5193-8.
151. Su, C., et al., *Identification of quantitative trait loci controlling acute virulence in Toxoplasma gondii.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10753-8.
152. Dubey, J.P. and G. Desmonts, *Serological responses of equids fed Toxoplasma gondii oocysts.* Equine Vet J, 1987. **19**(4): p. 337-9.
153. Dubey, J.P., *Toxoplasmosis - a waterborne zoonosis.* Vet Parasitol, 2004. **126**(1-2): p. 57-72.
154. Su, C., et al., *Moving towards an integrated approach to molecular detection and identification of Toxoplasma gondii.* Parasitology, 2010. **137**(1): p. 1-11.
155. Ajzenberg, D., et al., *Genotyping of Toxoplasma gondii isolates with 15 microsatellite markers in a single multiplex PCR assay.* J Clin Microbiol, 2010. **48**(12): p. 4641-5.
156. Dubey, J.P., et al., *Genetic diversity of Toxoplasma gondii isolates from Ethiopian feral cats.* Vet Parasitol, 2013. **196**(1-2): p. 206-8.

157. Al-Kappany, Y.M., et al., *Genetic diversity of Toxoplasma gondii isolates in Egyptian feral cats reveals new genotypes.* J Parasitol, 2010. **96**(6): p. 1112-4.
158. El Behairy, A.M., et al., *Genetic characterization of viable Toxoplasma gondii isolates from stray dogs from Giza, Egypt*. Vet Parasitol, 2013. **193**(1-3): p. 25-9.
159. Velmurugan, G.V., J.P. Dubey, and C. Su, *Genotyping studies of Toxoplasma gondii isolates from Africa revealed that the archetypal clonal lineages predominate as in North America and Europe.* Vet Parasitol, 2008. **155**(3-4): p. 314-8.
160. Prestrud, K.W., et al., *First isolate of Toxoplasma gondii from arctic fox (Vulpes lagopus) from Svalbard.* Vet Parasitol, 2008. **151**(2-4): p. 110-4.
161. Verma, S.K., et al., *Genetic characterization of Toxoplasma gondii isolates from Portugal, Austria and Israel reveals higher genetic variability within the type II lineage.* Parasitology, 2015. **142**(7): p. 948-57.
162. Mondragon, R., et al., *Genotypic analysis of Toxoplasma gondii isolates from pigs*. J Parasitol, 1998. **84**(3): p. 639-41.
163. Dubey, J.P., et al., *High prevalence and abundant atypical genotypes of Toxoplasma gondii isolated from lambs destined for human consumption in the USA.* Int J Parasitol, 2008. **38**(8-9): p. 999-1006.
164. da Silva, R.C., et al., *Genotypic characterization of Toxoplasma gondii in sheep from Brazilian slaughterhouses: new atypical genotypes and the clonal type II strain identified.* Vet Parasitol, 2011. **175**(1-2): p. 173-7.
165. Dubey, J.P., et al., *Molecular and biological characterization of Toxoplasma gondii isolates from free-range chickens from Guyana, South America, identified several unique and common parasite genotypes.* Parasitology, 2007. **134**(Pt 11): p. 1559-65.
166. Zhou, P., et al., *Genetic characterization of Toxoplasma gondii isolates from pigs in China*. J Parasitol, 2010. **96**(5): p. 1027-9.
167. Huson, D.H. and D. Bryant, *Application of phylogenetic networks in evolutionary studies.* Mol Biol Evol, 2006. **23**(2): p. 254-67.
168. Excoffier, L. and H.E. Lischer, *Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows.* Mol Ecol Resour, 2010. **10**(3): p. 564-7.

169. Diamond, J. and P. Bellwood, *Farmers and their languages: the first expansions.* Science, 2003. **300**(5619): p. 597-603.
170. O.Bar-Yosef, *The transition to Agriculture in the old world.* Special issue of rev.Archaeol, 1998.
171. K.Gupta, A., *Origin of agriculture and domestication of plants and animals linked to early Holocene climate amelioration.* Current Science, 2004.
172. Armelagos, G., Jacobs, *The Origins of Agriculture: Population growth during a Period of Declining Health.* Population and Environment, 1991.
173. Rosenthal, B.M., *How has agriculture influenced the geography and genetics of animal parasites?* Trends Parasitol, 2009. **25**(2): p. 67-70.
174. Lehmann, T., et al., *Globalization and the population structure of Toxoplasma gondii.* Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11423-8.
175. Mercier, A., et al., *Human impact on genetic diversity of Toxoplasma gondii: example of the anthropized environment from French Guiana.* Infect Genet Evol, 2011. **11**(6): p. 1378-87.
176. Scallan, E., et al., *Foodborne illness acquired in the United States--major pathogens.* Emerg Infect Dis, 2011. **17**(1): p. 7-15.
177. Ballash, G.A., et al., *Seroprevalence of Toxoplasma gondii in White-Tailed Deer (Odocoileus virginianus) and Free-Roaming Cats (Felis catus) Across a Suburban to Urban Gradient in Northeastern Ohio.* Ecohealth, 2015. **12**(2): p. 359-67.
178. Dubey, J.P., et al., *Molecular and biologic characteristics of Toxoplasma gondii isolates from wildlife in the United States.* J Parasitol, 2004. **90**(1): p. 67-71.
179. Dubey, J.P., et al., *Epidemiology of toxoplasmosis in white tailed deer (Odocoileus virginianus): occurrence, congenital transmission, correlates of infection, isolation, and genetic characterization of Toxoplasma gondii.* Vet Parasitol, 2014. **202**(3-4): p. 270-5.
180. Dubey, J.P., et al., *Transplacental toxoplasmosis in naturally-infected white-tailed deer: Isolation and genetic characterisation of Toxoplasma gondii from foetuses of different gestational ages.* Int J Parasitol, 2008. **38**(8-9): p. 1057-63.
181. Humphreys, J.G., R.L. Stewart, and J.P. Dubey, *Prevalence of Toxoplasma gondii antibodies in sera of hunter-killed white-tailed deer in Pennsylvania.* Am J Vet Res, 1995. **56**(2): p. 172-3.

182. Vanek, J.A., et al., *Prevalence of Toxoplasma gondii antibodies in hunter-killed white-tailed deer (Odocoileus virginianus) in four regions of Minnesota.* J Parasitol, 1996. **82**(1): p. 41-4.

183. Dubey, J.P., et al., *Prevalence of Toxoplasma gondii infection in raccoons.* J Am Vet Med Assoc, 1992. **200**(4): p. 534-6.

184. Hill, R.E., Jr., et al., *Seroprevalence of antibodies against Toxoplasma gondii in free-ranging mammals in Iowa.* J Wildl Dis, 1998. **34**(4): p. 811-5.

185. Mitchell, M.A., et al., *Serologic survey for selected infectious disease agents in raccoons from Illinois.* J Wildl Dis, 1999. **35**(2): p. 347-55.

186. Walton, B.C. and K.W. Walls, *Prevalence of Toxoplasmosis in Wild Animals from Fort Stewart, Georgia, as Indicated by Serological Tests and Mouse Inoculation.* Am J Trop Med Hyg, 1964. **13**: p. 530-3.

187. Clark, R.K., et al., *Serologic survey of California wild hogs for antibodies against selected zoonotic disease agents.* J Am Vet Med Assoc, 1983. **183**(11): p. 1248-51.

188. Stieve, E., et al., *Neospora caninum and Toxoplasma gondii antibody prevalence in Alaska wildlife.* J Wildl Dis, 2010. **46**(2): p. 348-55.

189. Zarnke, R.L., et al., *Serologic survey for Toxoplasma gondii in selected wildlife species from Alaska.* J Wildl Dis, 2000. **36**(2): p. 219-24.

190. Dubey, J.P. and J.L. Jones, *Toxoplasma gondii infection in humans and animals in the United States.* Int J Parasitol, 2008. **38**(11): p. 1257-78.

191. Gerhold, R.W. and M.J. Yabsley, *Toxoplasmosis in a red-bellied woodpecker (Melanerpes carolinus).* Avian Dis, 2007. **51**(4): p. 992-4.

192. Demar, M., et al., *Acute toxoplasmoses in immunocompetent patients hospitalized in an intensive care unit in French Guiana.* Clin Microbiol Infect, 2012. **18**(7): p. E221-31.

193. Dubey, J.P., et al., *Genetic characterisation of Toxoplasma gondii in wildlife from North America revealed widespread and high prevalence of the fourth clonal type.* Int J Parasitol, 2011. **41**(11): p. 1139-47.

194. Khan, A., et al., *Genetic analyses of atypical Toxoplasma gondii strains reveal a fourth clonal lineage in North America.* Int J Parasitol, 2011. **41**(6): p. 645-55.

195. Smith, L.N., et al., *Disseminated toxoplasmosis Toxoplasma gondii in a wild Florida manatee Trichechus manatus latirostris and seroprevalence in two wild populations.* Dis Aquat Organ, 2016. **122**(1): p. 77-83.

196. Work, T.M., et al., *Fatal toxoplasmosis in free-ranging endangered 'Alala from Hawaii.* J Wildl Dis, 2000. **36**(2): p. 205-12.

197. Desmonts, G. and J.S. Remington, *Direct agglutination test for diagnosis of Toxoplasma infection: method for increasing sensitivity and specificity.* J Clin Microbiol, 1980. **11**(6): p. 562-8.

198. Su, C., et al., *Globally diverse Toxoplasma gondii isolates comprise six major clades originating from a small number of distinct ancestral lineages.* Proc Natl Acad Sci U S A, 2012. **109**(15): p. 5844-9.

199. Dubey, J.P., et al., *High prevalence and genotypes of Toxoplasma gondii isolated from goats, from a retail meat store, destined for human consumption in the USA.* Int J Parasitol, 2011. **41**(8): p. 827-33.

200. Chikweto, A., et al., *Isolation and RFLP Genotyping of Toxoplasma gondii in Free-Range Chickens (Gallus domesticus) in Grenada, West Indies, Revealed Widespread and Dominance of Clonal Type III Parasites.* J Parasitol, 2017. **103**(1): p. 52-55.

201. Dubey, J.P., et al., *Isolation and RFLP genotyping of Toxoplasma gondii from the domestic dogs (Canis familiaris) from Grenada, West Indies revealed high genetic variability.* Vet Parasitol, 2013. **197**(3-4): p. 623-6.

202. Dubey, J.P., et al., *Occurrence, isolation, and genetic characterization of Toxoplasma gondii from white-tailed deer (Odocoileus virginianus) in New Jersey.* J Parasitol, 2013. **99**(5): p. 763-9.

203. Dubey, J.P., et al., *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, 2009. **136**(6): p. 589-94.

204. Dubey, J.P., et al., *Isolation of viable Toxoplasma gondii from tissues and feces of cats from Addis Ababa, Ethiopia.* J Parasitol, 2013. **99**(1): p. 56-8.

205. Dubey, J.P., et al., *Characterization of Toxoplasma gondii isolates in free-range chickens from Chile, South America.* Vet Parasitol, 2006. **140**(1-2): p. 76-82.

206. Rajendran, C., C. Su, and J.P. Dubey, *Molecular genotyping of Toxoplasma gondii from Central and South America revealed high diversity within and between populations.* Infect Genet Evol, 2012. **12**(2): p. 359-68.

207. Dubey, J.P., et al., *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.* Vet Parasitol, 2007. **143**(2): p. 182-8.

208. Dubey, J.P., et al., *Toxoplasma gondii infections in cats from Parana, Brazil: seroprevalence, tissue distribution, and biologic and genetic characterization of isolates.* J Parasitol, 2004. **90**(4): p. 721-6.

209. Dubey, J.P., et al., *Prevalence of Toxoplasma gondii in dogs from Colombia, South America and genetic characterization of T. gondii isolates.* Vet Parasitol, 2007. **145**(1-2): p. 45-50.

210. Dubey, J.P., et al., *Biologic and genetic characteristics of Toxoplasma gondii isolates in free-range chickens from Nicaragua, Central America.* Vet Parasitol, 2006. **142**(1-2): p. 47-53.

211. Dubey, J.P. and J.K. Frenkel, *Feline toxoplasmosis from acutely infected mice and the development of Toxoplasma cysts.* J Protozool, 1976. **23**(4): p. 537-46.

212. Suzuki, Y., et al., *Interferon-gamma: the major mediator of resistance against Toxoplasma gondii.* Science, 1988. **240**(4851): p. 516-8.

213. Suzuki, Y., F.K. Conley, and J.S. Remington, *Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice.* J Immunol, 1989. **143**(6): p. 2045-50.

214. Soete, M., et al., *Toxoplasma gondii: kinetics of bradyzoite-tachyzoite interconversion in vitro.* Exp Parasitol, 1993. **76**(3): p. 259-64.

215. Oh, S., et al., *Time series expression analyses using RNA-seq: a statistical approach.* Biomed Res Int, 2013. **2013**: p. 203681.

216. Magombedze, G. and N. Mulder, *Understanding TB latency using computational and dynamic modelling procedures.* Infect Genet Evol, 2013. **13**: p. 267-83.

217. S. Oh, S.S., G. Grabowski, H. Zhao, J. P. Noonan, *Time series expression analyses using RNA-seq: a statistical approach.* BioMed research international, 2013.

218. Fux B., et al., *Toxoplasma gondii strains defective in oral transmission are also defective in developmental stage differentiation.* Infect Immun, 2007. **75**(5): p. 2580-90.

219. Conrad, P et al., *Transmission of Toxoplasma: Clues from the study of sea otters as sentinels of Toxoplasma gondii flow into the marine environment*. Int J Parasitol, 2005,**35**(12):p 1155-68.

220. Boyle, P et al., *Just one cross appears capable of dramatically altering the population biology of a eukaryotic pathogen like Toxoplasma gondii.* PNAS, 2006, 103**(27)**: p. 10514-59.

221. Khan, A et al., *Recent transcontinental sweep of Toxoplasma gondii driven by a single monomorphic chromosome*. PNAS, 2007. 104**(27)**: p. 14872-77.

222. Felix Yarovinsky, *Innate immunity to Toxoplasma gondii infection*. Nature Immunol, 2014, 14: p.109-21.

223. Darde, M., Ajzenberg, D., Su, C., *Toxoplasma gondii. The model apicomplexan: Perspectives and Methods* In: Weiss, L. and Kim. editor. Academic Press publications, 2014, **61**-93.

APPENDICES

APPENDIX A: MODERN DAY *T. GONDII* GENETIC DIVERSITY IS DERIVED FROM RECOMBINATION OF FIVE ANCESTRAL GENOMES

1. Introduction

T. gondii is one of the most widespread pathogens and is responsible for causing disease across the globe [1]. The most recent knowledge on the diversity structure of *T. gondii* indicates that population structure is much more complex than the previous notion of three clonal lineages [11]. The accumulation of information from genotyping studies has also revealed previously unknown genotypes of *T. gondii*. Previous study with 46 *T. gondii* samples has shown that the mixing of four ancestral groups can define the population structure of *T. gondii* [221]. However, this study was based on limited *T. gondii* strains, which may miss important ancestors. Thus, to better understand the composition of current population structure and to correctly identify the number of ancestral genomes, we utilized sequence data for 160 strains collected worldwide. Our study is an update on the previous studies utilizing few strains representing the population of *T. gondii.*

2. Materials and Methods

DNA sequencing

DNA sequencing was performed by our collaborators in China. A hundred and sixty DNA samples were collected from previous studies in our laboratory [36]. Location and host information for these isolates were obtained from previously published papers **(Table A.1).** DNA for all the isolates was either obtained from the heart or brain tissues of experimentally infected mice or cell culture. Fifty genetic loci across the 65 Mb *T. gondii* genome were pre-amplified by multiplex PCR in three different groups.

SNP identification and STRUCTURE analysis

Sequence data available for 47 loci for a total of 160 strains of *T. gondii* was aligned and SNPs was identified using *Molecular Evolutionary Genetics Analysis (MEGA)* software *v 6.0*. The number of population clusters (k) were predicted individually for each marker as well as for concatenated data for all SNPs, using STRUCTURE v2.3.4 ADMIXTURE and Linkage model

respectively, with ancestral clusters set from K=1 to 10 **(Table A.1).** The results are averages across three independent simulations with 10^3 burn-in iterations, followed by 10^3 MCMC iterations.

Chromosome painting

Based on the population clusters identified for 37 loci using STRUCTURE v2.3.4, a chromosome map was constructed using Microsoft Excel (2010).

Phylogenetic analysis

A neighbor joining tree was also constructed using sequence data of 43 loci for 160 (type I, II and III) strains of *T. gondii* using SplitsTree 4.8 [167]

3. Results

To reveal the shared ancestry among isolates, local admixture analysis was conducted on SNPs identified for 37 loci located on 14 different chromosomes for a total of 160 strains **(Figure A.1)**. Each strain was assigned a particular ancestral population based on admixture analysis and was represented by a colored block. A chromosome painting map was constructed for the identified population clusters for all 37 loci of 160 strains. The color block patterns were compared horizontally among different populations to reveal the shared ancestry across 160 strains of *T. gondii* **(Figure A.1)**. When strains were aligned by clades identified using sequence data for 160 strains of *T. gondii* **(Attachment 1),** recombination was observed for the population as a whole. Divison of haploblocks being shared across strains of specific lineages was not observed **(Figure A.1).**

Although chromosome painting revealed the recombination events among the current population, it failed to report the admixture pattern for the population as a whole. Thus, to illustrate this more directly, STRUCTURE analysis was conducted for SNPs across all the 160 strains, using Linkage model (Figure A.2). This analysis revealed that the population is derived from five ancestral genomes similar to strains; type I (BOF, GPHT, etc.), type II (PTG, DEG etc.), type III (P89, TgCatBr3 etc.), MAS and TgCkGh1. Additionally, the modern day type I appeared to be a result of inheritance of haploblock from the ancestral PTG (type II) and BOF or GPHT (ancestral type I) strains **(Figure A.2a).** Whereas, the modern day type III strains was a

result of a cross between ancestral PTG (type II) and P89 (ancestral type III) strains. These findings were in agreement with a 2006 study [220]. Similar to the results from chromosome painting, irrespective of the region, the population showed recombination **(Figure A.2b).** As expected, the majority of isolates from North America (TgWtdUs8, TgSoUs1, TgShUs32 etc.) showed genetic makeup similar to type II reference PTG strain **(Figure A.2b).** South America population as a whole showed a lot of recombination and could be virtually divided into three major clusters. A third of the population had the genetic makeup similar to GT1 (type I) strain, one-third similar to MAS (atypical) and one-third similar to CTG (type III) **(Figure A.2b).**

4. Conclusions

The mosaic genomic patterns based on chromosome painting shows ongoing recombination in the current population where large chromosomal haploblock are shared among members of different clades **(Figure A.1).** Remarkably, our findings based on genome–wide polymorphic data of 160 strains of *T. gondii* is in strong agreement with the previous reports [220, 221]. Our data suggests that the current population (based on the assortment of 160 strains) is inherited from five ancestral population clusters **(Figure A.2a).** Furthermore, based on our data the proposed genealogy of three modern day reference strains representing the major clonal lineages (GT1, PTG, and CTG) is in accordance with previous reports. The distribution of the colored haploblock strongly suggests that a cross between type II (PTG/green) and type III (P89, TgCatBr3 etc./blue) strain gave rise to modern day type III lineage represented by CTG **(Figure A.2a).** The P89 population was previously referred as β strain in a 2006 study (Boyle et al. 2006). Similarly, modern day type I strain (GT1) is a result of a cross between type II (PTG/green) and type I (BOF, GPHT, FOU etc. /red) strain, hypothetically referred as α strain in a previous study [221]. Genome–wide SNPs STRUCTURE analysis for 160 strains, revealed that the current population structure is a mixture of five ancestral groups. The number of ancestral groups (five) suggested through our analysis is close to previously reported four ancestral

Position of marker on the chromosome

Strains



Figure A.1: Chromosome painting of 160 *T. gondii* strains.

Figure A.2: Analysis of present day *T. gondii* population structure using genome-wide SNPs data for 160 strains using STRUCTURE.

FigureA.2a: Grouped by ancestor.

Figure A.2b: Grouped by geographical region.

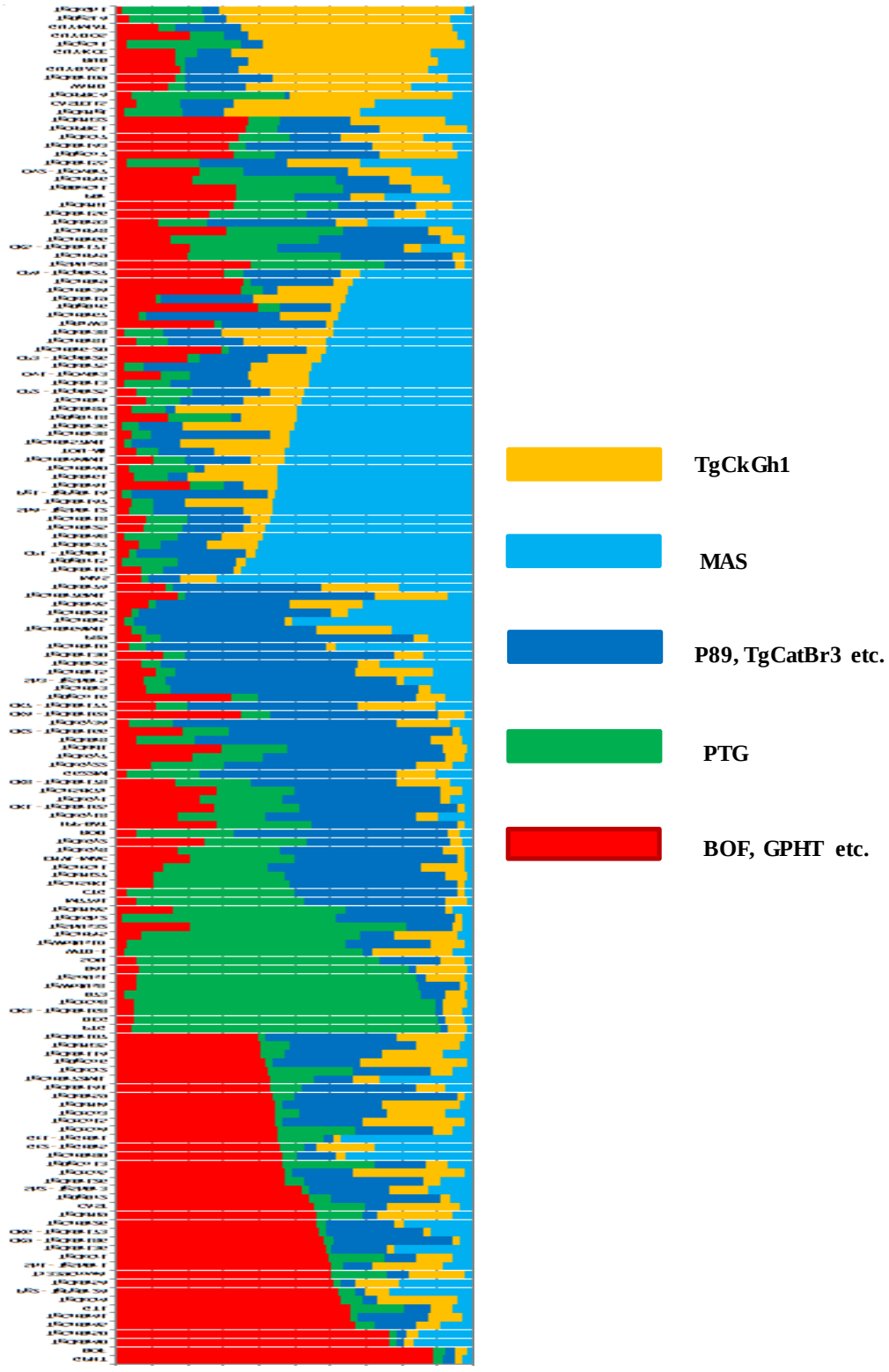


Figure A.2 (continued)

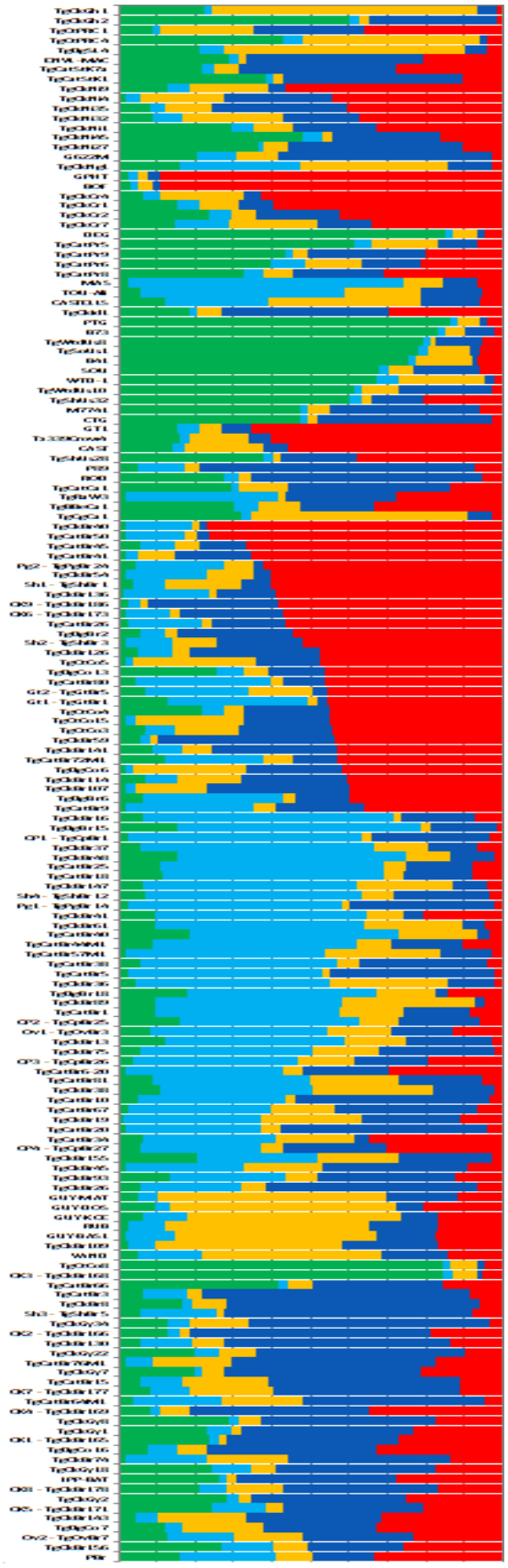


Figure A.2 (continued)

groups, however, this 2007 study was based on intron sequences of a limited number of *T. gondii* strains (n=46) [220].

Based on the genome-wide SNPs analysis, South American strains also showed the dominance of strains with haploblock similar to type I and atypical strains. This observation also reflects on the severity of disease due to infection with *T. gondii* in South America. AIDS patients are often found to be infected with type I strain and atypical strains are often associated with the severe forms of diseases. Together, this study suggests that the current day population structure of *T. gondii* is a result of recombination of five ancestral groups.

5. Future perspectives

Even though limited variability exists among different lineages of *T. gondii*, studies have reported that certain genotypes are associated with severe forms of toxoplasmosis. Our study has shown that strains from South America show similar haploblock pattern to type I like and atypical strains, often associated with extreme cases of toxoplasmosis. Thus, in future, it will be interesting to study the oral infectivity and transmissibility of representative strains from this study. This will aid in exposing the admixture of lineages best adapted to domestic transmission.

APPENDIX B: TO ESTABLISH AN EXPERIMENTAL PROTOCOL TO STUDY PHASE INTERCONVERSION IN *TOXOPLASMA GONDII*

1. Introduction

The apicomplexan life cycle requires switching between distinct developmental stages. The life cycle of *T. gondii* can be divided into three different stages: tachyzoite, bradyzoite and sporozoite [1]. Tachyzoites represent the rapidly dividing stage whereas; bradyzoites are the slow replicating form of the parasite responsible for chronic infection in the host. Following infection, *T. gondii* enters into a rapidly dividing tachyzoite form that enters the lymph nodes and can disseminate by infecting migratory cells like dendritic cells and macrophages. Eventually, on encountering the host immune system stress responses, the tachyzoite form is converted to a more slowly replicating dormant bradyzoite form. Bradyzoites enclosed in the cyst walls are resistant to immune responses and can be retained primarily in brain and muscle neurons for the entire lifespan of the infected host [1, 8]. Infections in immunocompetent individuals are mostly asymptomatic. However, the key to *T. gondii* pathogenesis is its ability to differentiate from a rapidly- replicating tachyzoites stage during acute infection to a relatively non-immunogenic, dormant bradyzoite stage contained in tissue cysts [211]. Although tissue cysts are considered dormant, they occasionally rupture and release bradyzoites that convert to faster-replicating tachyzoites which in turn disseminate and can cause severe pathology and death in the absence IFN-γ-dependent cell-mediated immune response [211-213]. Reactivation is thus a serious and life-threatening condition in immune-compromised individuals. Bradyzoite formation is also of concern during organ transplantation between individuals. The combination of drugs like sulfonamide and pyrimethamine can readily inhibit tachyzoites; however, no drug currently exists that inhibits the parasite in the bradyzoite form. Since tachyzoite to bradyzoite conversion is the key to the establishment of a chronic infection and the reverse phenomenon is responsible for life-threatening pathology, understanding of the molecular prerequisites for interconversion will aid in developing a strategy to coerce the parasite out from latency and treat it using existing drugs [93, 114, 116, 214]. In order to achieve this overarching aim, the goal of the study was to establish a methodology to successfully convert the tachyzoite form into a bradyzoite form and vice versa, *in vitro* using external stress factors.

Specific Aims

2.1 To establish a protocol to study stress induced tachyzoite to bradyzoite conversion.

Previous studies have shown that tachyzoite to bradyzoite differentiation can be induced *in vitro* in response to physiologically relevant exogenous stress like a NO donor (sodium nitroprusside). Infected human foreskin fibroblast (HFF) cells will be cultured in medium containing sodium nitroprusside (SNP) for three to four days at 37°C. Exposure of parasites to SNP will be used to enhance bradyzoite differentiation *in vitro* [114, 116].

2.2 Time course expression analysis using RNA-seq.

Time course expression profiles will be carried out on the stress induced tachyzoites using RNA-seq. RNA-seq technology should reveal a snapshot of RNA presence and quantity from a genome at a given moment in time[215].

2.3 Analysis of the profile using GeneNet Package.

GeneNet package will be used to analyze time-series data obtained from RNA-seq to identify the genes that are upregulated/downregulated [216, 217] **(Figure B.1).**

2.4 Gene knockout studies to confirm the role of the predicted gene.

Gene deletion assay will be performed for the predicted gene to confirm its role in regulating the tachyzoite to bradyzoite conversion.

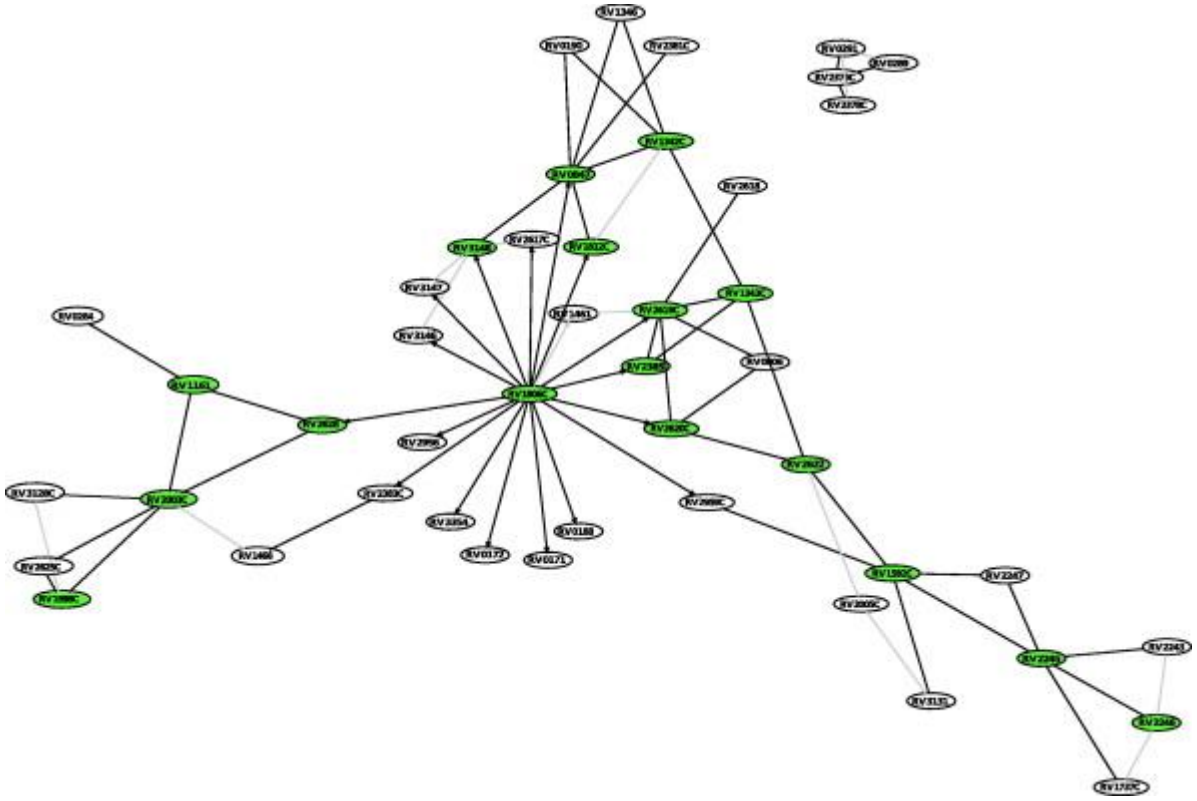


Figure B.1: Examples of network structure of predicted genes analyzed using Gene net analysis [216].

2. Materials and Methods

Parasites

The ME49 strain originally isolated from the diaphragm of sheep was selected for this study. The parasites were cultured in human foreskin fibroblast (HFF) cells grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), 1 mM glutamine without antibiotics at 37°C under 5% CO_2.

In vitro differentiation of *T. gondii*

In vitro bradyzoite differentiation was achieved using exogenous NO donor (sodium nitroprusside). A three-day confluent monolayer of HFF cells grown in 6-well plates was infected with tachyzoites of ME49 strain. Differentiation was induced by adding 70 μM SNP in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), 1 mM glutamine without antibiotics. Cultures were incubated for 2 to 7 days with medium changed daily. HFF cells infected with ME49 and cultured in medium without SNP were used as a control for the study.

Immunostaining

HFF cells grown on chamber slides (Labtek, Campbell, CA) were infected with ME49 parasites and monitored daily after infection. For immunostaining, cells were washed with PBS three times and fixed using 4% formaldehyde for 30 minutes. Cells were permeabilized using 0.5% Triton X-100 diluted in PBS for 20 min at RT and blocked in blocking buffer (0.1% Triton X-100, 10% BSA diluted in PBS) for 1 hour at 37°C. Cells were incubated in primary antibody, Rabbit anti-Bag1 antibody (1:100) overnight at 4°C.The cells were washed gently with PBS, and the secondary antibody, Goat-anti-rabbit IgG-FITC (Sigma-Aldrich)(1:80 dilution) was added to each well and incubated at 37°C for 1 hour. The cells were washed gently with PBS 3 times. Coverslips were removed from each well air-dried and mounted using VECTASHIELD anti-fading mounting medium with DAPI (Vector laboratories, Burlingame, CA). Immunofluorescence was observed under a confocal microscope.

Detection of Bag1 using Western blot

HFF cells cultured in 6 well plates were infected with ME49 and allowed to invade for 24 hours or 4-6 parasites per vacuole stage. Medium (with or without SNP) was changed daily for both the control and experimental sample. Respective 6 well plates were processed for the western blot. Cells on these 6 well plates were harvested using a scraper and transferred to Eppendorf tubes. Cells were lysed using a RIPA buffer containing 1% phosphatase inhibitor (Roche), and 1% protease inhibitor (Roche). Protein concentrations were measured using the BCA method. 20 µg of total protein for day 2 control, day 3 control, drug- treated day 2 –day 5 and revert day 1 and 2 was used for SDS-PAGE. Proteins in the gel were transferred to a LiCor Immobilon-FL PVDF membrane and allowed to dry overnight. The next day the membranes were sliced into two parts at 32 kDa and blocked in LiCor blocking buffer for 1 hour at 37°C. The upper part was incubated with anti-β-tubulin (1:2500) (Dr.Sibley lab) and the bottom part was incubated with anti-Bag1 (1:2500) at 37°C for 1 hour. The membranes were washed with TBST 4x5 times. Membranes were incubated with the secondary IR dye Goat anti-rabbit antibody at a 1:10000 dilution for 1 hour at room temperature followed by washing with TBST 4x5 and visualized using LiCor Odyssey instrument.

3. Results

Previously studies have shown that exogenous stress can successfully induce differentiation of tachyzoites *in vitro*. Sodium nitroprusside a NO donor has been reported as the most physiologically relevant stress and therefore chosen as the stress factor for this study. Studies have reported that ME49 strain of *T. gondii* is efficient in the formation of cysts *in vitro*. To induce the conversion of tachyzoite to bradyzoite, 70 uM concentration of SNP was used for our study. In order to confirm the interconversion process *in vitro*, Immunostaining and western blotting were performed.

In vitro cyst development

Fluorescent intensity of bradyzoite stage specific protein Bag1 was used as a marker for the stage conversion [218]. Cysts were stained with anti-Bag antibody and observed by confocal microscopy. Representative examples of staining with Bag1 and DAPI for each day are shown in the figure below **(Figure B.2 a-d)**. The intensity of Bag1 increased from day 2 to day 4 post drug

treatments. For the control, Bag1 intensity increases slightly in comparison to the drug treated parasites and lysed at day 2 post infection. These results reveal that SNP drug treatment can successfully retain the parasites as cysts *in vitro*. Additionally, on removing the stress, the intensity of Bag1 fluorescence decreased till day 2 post stress removal and is lysed at Day 2 **(Figure B.2 a-d)**. Together, these results show that the ME49 strain of *T. gondii* is converted to cyst form *in vitro* using a NO donor and successfully reverts back to tachyzoite form on the release of the exogenous stress.

Western Blot

To confirm the differentiation of tachyzoite to bradyzoite, Bag1 protein expression was quantified using western blot **(Figure B.3).** Western blot analysis showed that Bag1 protein expression increased from day 2 to day 5 for SNP treated plates. Whereas, the Bag1 protein was absent for day 2 and day 3 control post infection. This confirms that *in vitro,* tachyzoites are converted to bradyzoites post treatment with SNP. Interestingly, Bag1 protein expression appears to be more at revert day 1 and 2 (SNP stress removed). This could be explained by the possibility that Bag 1 protein is not immediately degraded on reversion to the tachyzoite form. However, bradyzoites are confirmed to be reverted to tachyzoite form as the PV is lysed, following parasite release on day 2 post stress removal.

4. Conclusion

So far we have successfully established a protocol to study the interconversion dynamics of *T. gondii*. These results are in agreement with previous studies reporting the use of SNP as the exogenous NO donor for conversion of tachyzoites to bradyzoites *in vitro*. Additionally, our study also shows that on removing the external stress factor (in this study SNP), bradyzoites can be converted back to tachyzoites form. However, this conversion seems to be really fast. Hence, the time points used for the RNA profile in future should be decided accordingly.

5. Future perspectives

The above established protocol can be used to study the most important/ master regulatory gene(s) involved in the conversion of tachyzoites into bradyzoite stage and vice versa for *T.*

Figure B.2 (a-d): **Expression of Bag1 (Green) antigen following the *in vitro* differentiation of ME49 strain of *T. gondii*.** On Day 2 and 3 in control plates, Bag1 is decreased in comparison to day 2 and day 3 SNP treated plates. Day 2 Control and SNP were not treated with DAPI. Hence, images shown are without DAPI. By day 4 and 5 Bag1 expression is increased. On release of exogenous stress, Bag1 expression decreases. Nuclei are stained with DAPI (Blue). All the pictures shown were recorded under similar optical conditions, imaged with the same exposure time, and processed identically.

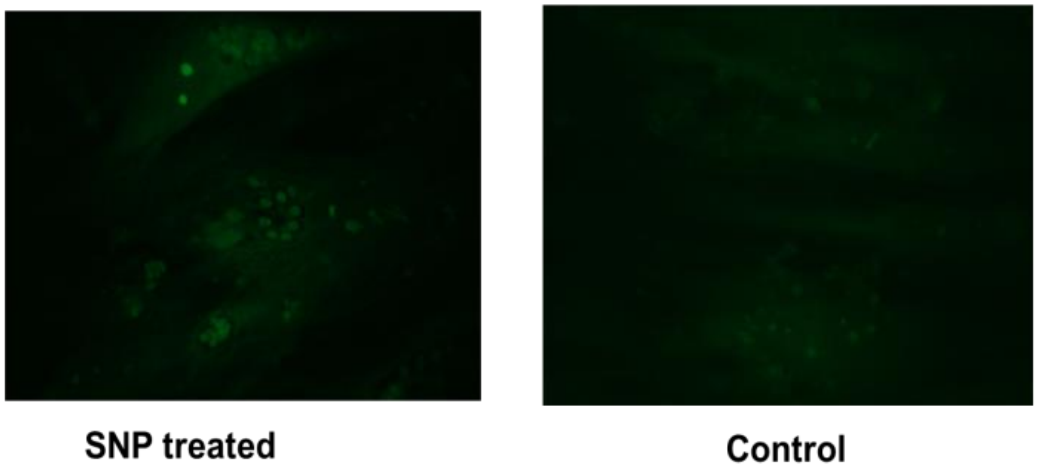


Figure B.2a: Day 2 (Green: Bag1, No DAPI).

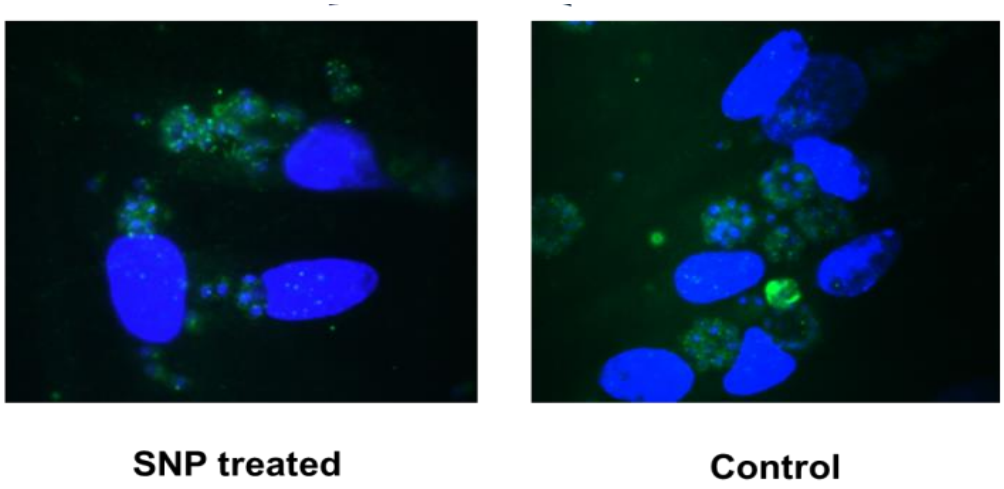


Figure B.2b: Day 3 (Green: Bag1 and Blue: DAPI).

Figure B.2 (continued)

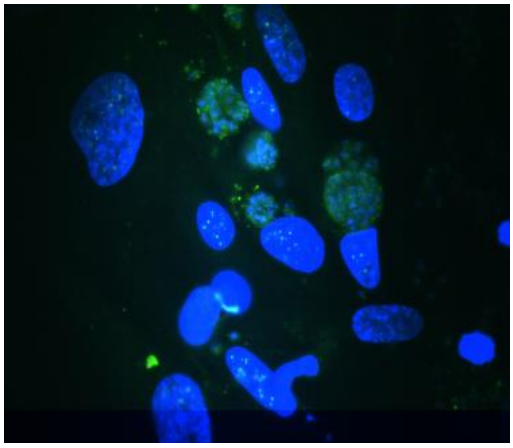
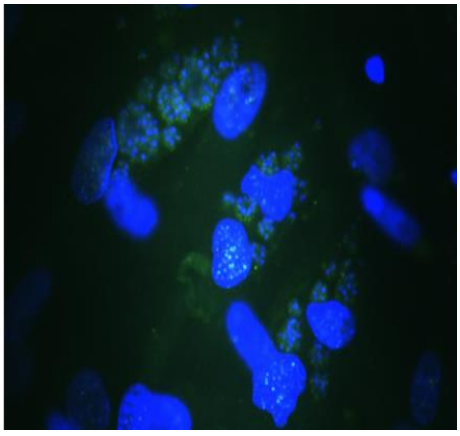


Figure B.2c: Day 3 SNP treated (Green: Bag1 and Blue: DAPI).



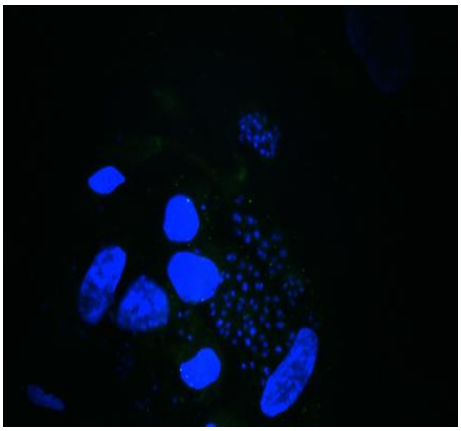
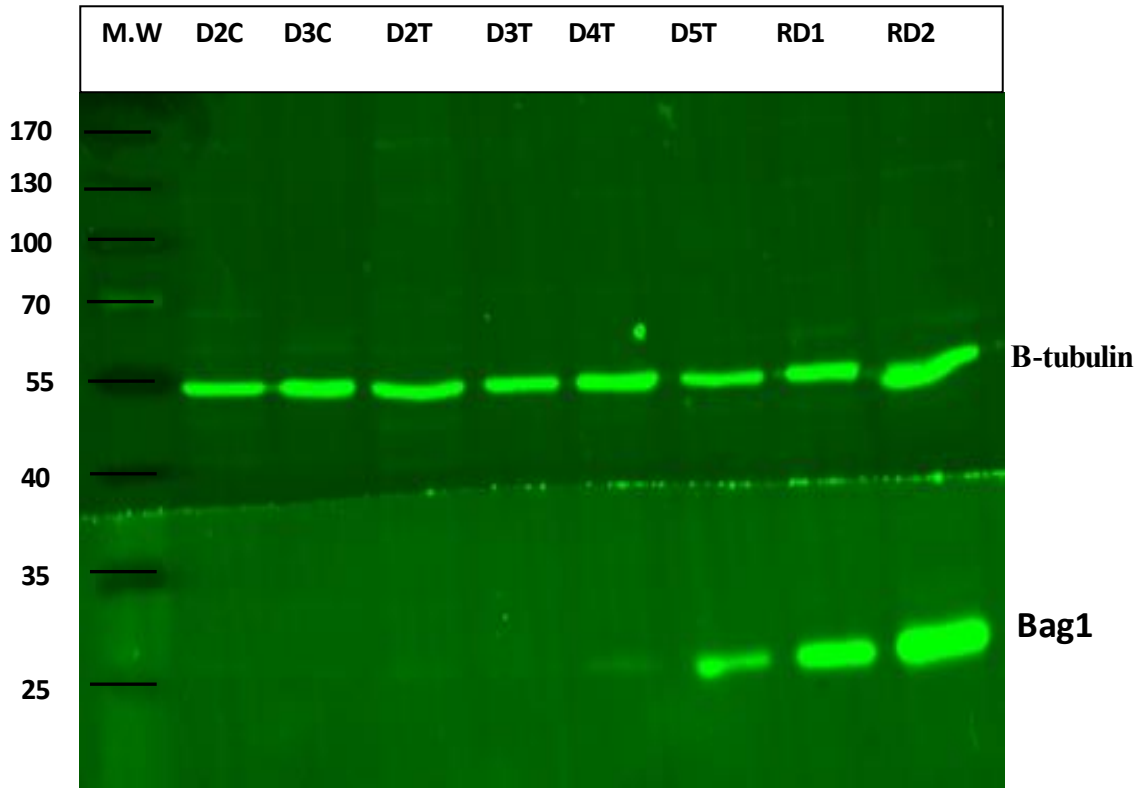


Figure B.2d: Revert day 1 and 2 SNP (Green: Bag1 and Blue: DAPI).

Figure B.2 (continued)



M.W	Molecular weight marker
D2C	Day 2 control flask
D3C	Day 3 Control flask
D2T	Day 2 SNP treated
D3T	Day 3 SNP treated
D4T	Day 4 SNP treated
D5T	Day 5 SNP treated
RD1	Revert day 1
RD2	Revert day 2

Figure B.3: Western blot analysis of *in vitro* Bag 1 protein expression of ME49 strain of *T. gondii*. Bag 1 is not expressed for control day 2 and 3. Bag1 expression increases at day 2, day 3, day 4 and day 5 for SNP treated plates.

T. gondii. To identify these genes, RNA will be isolated at day 0, 1, 2, 3 and 4 post infection as well as 8 hours, 20 hours and 30 hours after the removal of SNP drug. These RNA samples will then be processed for RNA seq and genes that appear to be down/upregulated during the switch will be sorted by comparing the time points associated with tachyzoites to bradyzoite and bradyzoite to tachyzoites interconversion. Genes identified using this method would then be analyzed using gene networking software's like GeneNet, which generates a network for the selected genes in order to identify the master regulatory gene(s).

VITA

Pooja Saraf was born on January 13th, 1988 in Nagpur, India. She attended Hislop College; RTM Nagpur University, India where she received her Bachelors of Science degree with triple majors in Botany, Chemistry and Biotechnology in 2008. She further received a Master's degree in Biotechnology from Hislop College; RTM Nagpur University, India in 2010. She started her Ph.D. at the University of Tennessee Knoxville in August 2012 in the lab of Dr. Chunlei Su. While her lab studies molecular parasitology and pathogenesis, work her research is focused on studying the transmission pattern of major clonal lineages of *Toxoplasma gondii*. She has also pursued research on establishing a protocol to identify the master regulatory genes for interconversion of *T. gondii*. She completed her studies and graduated with a Doctor of Philosophy in Microbiology in August 2017.

.